SOME EFFECTS OF SODIUM FLUORIDE ON THE METABOLISM AND STRUCTURE OF JACK PINE (PINUS BANKSIANA LAMB.) SEEDLINGS

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Janusz J. Zwiazek

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ABSTRACT

The effects of root-acquired sodium fluoride on the physiology and structure of Jack pine seedlings were studied. Presence of relatively low concentrations of fluoride in sand culture caused wilting and leaf necrosis. Increased leakage of electrolytes from seedlings was observed before necrotic lesions appeared on leaves, indicating damage to cell membranes. The increase in electrolyte leakage was accompanied by changes in the composition of plant lipids and their fatty acid components. Fluoride treatments resulted in a decrease in water content of seedlings, growth inhibition, and a reduction in rates of photosynthesis and respiration. Fluoride also altered the levels and composition of carbohydrates, organic acids, and amino acids, and the concentration of soluble proteins. It also inhibited acid phosphatase activity. Little change was observed in the activity of cytokinins.

In seedlings exposed to sodium fluoride and drought, structural alterations of guard and mesophyll cells were studied using light and electron microscope techniques. Many of the observed changes in mesophyll cell structure were similar in plants subjected to either stress. Guard cells were generally more resistant to both stresses than mesophyll cells. The collapse of cells adjacent to guard cells in fluoride-treated plants may be responsible for the opening of stomata during wilting.

Possible reasons for the observed metabolic and structural changes and their implications are discussed. A plausible mechanism for fluoride's action on Jack pine seedlings is presented.

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GENERAL INTRODUCTION

Our knowledge of the effects of anthropogenic pollutants on vegetation is very limited. Detrimental effects of fluoride on plants were recognized in the late 19th century (Weinstein & McCune, 1971). Presently, among the common air pollutants fluoride is ranked fifth with respect to the amount of plant damage in the United States, and first with respect to phytotoxicity (Weinstein, 1977). Fluoride can be absorbed either by the aerial parts of plants (gaseous and particulate forms), or by roots (mainly in the form of water soluble salts). The opportunity to investigate structural and physiological effects of fluoride on plants is greater when it is absorbed by roots from soil. In this case it can penetrate various plant tissues. For this reason sodium fluoride was selected for the present studies. The choice of Jack pine as a target species was influenced by its widespread occurrence in the southern boreal forest where many industrial sites are located. Additionally, Jack pine is known to be relatively sensitive to fluoride (Weinstein, 1977), and grows well in sandy, acidic soils where fluoride is more readily available to plants (Amundson & Weinstein, 1980). The importance of seedlings in forest regeneration and their fast growth made them particularly suitable for physiological and structural studies.

Most of the published references to phytotoxicity of fluoride are descriptive reports of vegetation damage. However, in order to deal with and to prevent the destruction caused by fluoride both the effects and mechanisms of its action on plants must be understood. Relatively few studies have attempted to investigate possible mechanisms of fluoride's detrimental action on plants. Additionally it is difficult to make general conclusions based on the existing reports because of different experimental conditions used. The effects of fluoride are influenced by many factors, such as: the concentration of fluoride, duration and frequency of exposure, age and stage of development of plants, plant species and variety, climatic and edaphic conditions, and interactions in the biotic environment (Weinstein, 1977). In this study a broad range of plant responses was studied after subjecting seedlings to various periods of sodium fluoride exposure. Treatments which resulted in a pronounced cytoplasmic leakage were emphasized. The study also compared similarities and differences between fluoride-induced stress and other environmental stresses.

The purpose of this study was to investigate the effects of fluoride on Jack pine (Pinus banksiana Lamb.) seedlings, and specifically:

1. To compare the effects of fluoride-induced stress and drought on the structure of mesophyll and guard cells.

2. To investigate loss of membrane selective permeability in fluoride-treated plants and its possible causes.
3. To determine the effects of fluoride on levels of metabolic constituents and basic physiological processes such as growth, photosynthesis, and respiration.

The main objective of the present study was to contribute to a better understanding of the mechanisms of fluoride's action on plants. The investigation of a wide range of processes using similar experimental conditions allowed a model of fluoride's action on plants to be developed. This may serve as a basis for future in-depth investigations.

LITERATURE REVIEW

Introduction

There are numerous reports of fluoride effects on plants in the literature. Unfortunately many of them were published in non-refereed journals and conference proceedings. Frequently they are 20 or 30 years old and have never been confirmed by other studies. In addition, some do not have a methods section, others do not fully describe procedures used or report single observations. However, these papers often dealt with important issues and they cannot be ignored in a comprehensive literature review. This chapter briefly reviews present knowledge of the effects of fluoride on plants with an emphasis on studies which deal with structural and physiological effects.

Sources of fluoride

Fluorine is a member of the halogen family of elements. It is the most electronegative and reactive of all elements (Weast, 1982), and due to its reactivity it does not occur in an elemental form in nature. Fluorine occurs chiefly as fluoride in fluorspar (calcium fluoride, CaF_2) and cryolite (sodium aluminum fluoride, Na_3AlF_6), but it is also widely distributed in other minerals (Weast, 1982). Uncontaminated

soils usually contain between 20 and 500 μg F g^{-1} soil (Robinson & Edgington, 1946), but levels as high as 3500 μg F g^{-1} soil (Kowda, 1984), and higher (MacIntire & Associates, 1949) may be also found. The common soil fluoride is insoluble in water, and therefore, is not readily available to plants. In most uncontaminated soils soluble fluoride constitutes only a small fraction of the total fluoride (Robinson & Edgington, 1946; Larsen & Widdowson, 1971; Kowda, 1984).

Fluorides are released to the atmosphere by high temperature reactions. Volcanoes and fumaroles are the natural sources of gaseous and particulate fluoride (Garrec et al., 1977; EPA, 1978). Considerably more fluoride is released to the atmosphere during industrial processes, including the manufacture of brick and tile products, steel, aluminum, elemental phosphorus, phosphoric acid, phosphate fertilizers, combustion of coal and welding operations. In the United States it was estimated that the total fluoride emissions from such industrial sources reached 118700 tons in 1968 (National Academy of Sciences, 1971). Many studies showed that soils in the vicinity of certain industrial sites may accumulate substantial quantities of fluoride (Israel, 1974; Johnson, 1976; McClenahen, 1976; Ares, 1978; Thompson et al., 1979).

Uptake and distribution in plants

Plants can absorb fluoride from the atmosphere or soil. Atmospheric fluoride reaches the plant in a gaseous or particulate form (EPA, 1978). The most phytotoxic and best studied form of atmospheric fluoride is gaseous hydrogen fluoride (HF). Gaseous fluoride is rapidly absorbed by the aerial parts of plants (Bennet & Hill, 1973). It enters the plant primarily through the stomata (Poovaiah & Wiebe, 1973). The epidermis is thought to be largely impermeable to fluoride ion (Pitman, 1977), but significant uptake of fluoride salts (Brewer et al., 1960c) and HF (Garrec & Plebin, 1986) through the epidermis was reported. Insoluble particulate fluoride cannot easily enter the plant. It is mostly adsorbed onto the plant surface, and it is thus less injurious (McCune et al., 1965; Davison & Blakemore, 1976).

Once fluoride has passed the stoma and entered the substomatal cavity, it is dissolved in water of the apoplast and carried in the transpiration stream to leaf tips and margins where it accumulates (Keller, 1974; Heath, 1980). From there it may be transported in the phloem to other parts of plant (Kronberger et al., 1978). Absorption of fluoride through the stomata can alter stomatal movements. Partial stomatal closure was observed in soybean plants subjected to 0.15 ppm HF (Poovaiah & Wiebe, 1973). Such stomatal response may reduce further pollutant uptake.

The significance of soil fluoride in inducing plant injuries has long been disputed (Weinstein, 1977, Amundson & Weinstein, 1980). Most fluoride in soils occurs in insoluble forms which are not readily available for plants. The uptake of soil fluoride depends on such factors as concentration of soluble fluoride in soil, the capability of the soil to replenish fluoride in the soil solution when the latter is depleted (Larsen & Widdowson, 1971), the pH, the clay and organic matter content, and other factors (Thomas & Alter, 1966). Soil fluoride is thought to enter roots by diffusion (Venkatesvarlu et al., 1965) and fluoride absorbed through roots tends to accumulate in roots and leaves (Daines et al., 1952; McCune, 1969; Cooke et al., 1978).

Little is known about the accumulation of fluoride at tissue and cell levels. There are indications in the literature that different patterns of leaf injury may be caused by soil and atmospheric fluoride (Woltz, 1964; McCune, 1969), suggesting different sites of fluoride accumulation. The sites for fluoride accumulation inside the leaf cells are not known with certainty. Vacuoles (Treshow, 1971), chloroplasts (Ledbetter et al., 1960; Chang & Thompson, 1966a), cell walls, soluble proteins, mitochondria, and microsomes (Ledbetter et al., 1960) have all been implicated.

Accumulation and plant response

Fluoride is generally considered not essential for normal plant development (Heggestad & Bennet, 1984). However, due to its widespread distribution in the environment and tendency to accumulate in plants, even plants growing in non-industrialized areas contain fluoride (Thomas & Alter, 1966). Some, such as certain species of the tea family and some poisonous plants of South America, Africa, and Australia can accumulate 50 to several hundred ppm fluoride without any apparent damage (Heggestad & Bennet, 1984). Most plants containing such high fluoride levels exhibit signs of foliar injury. Brandt (1971) correlated the fluoride level in plant tissues with fluoride injury and grouped plants into three categories of different sensitivity: very sensitive plants, responding to a fluoride level below 50 ppm F; sensitive plants, responding to 50-200 ppm; and resistant plants, injured when more than 200 ppm fluoride accumulates in their tissues. In reality it is difficult to predict injury based upon fluoride accumulation because plant response also depends on climatic, edaphic, biological and many other factors (Weinstein, 1977). According to Garsed (1984), the lack of sound mathematical correlation between the pollutant accumulation and injury is largely because the acute pollutant injury is more severe if the exposure concentration is increased and the time reduced, while greater accumulation is obtained by reducing the concentration and increasing the

time. Other factors listed by Garsed (1984) are: pollutant turnover within the plant, environmental factors, variations in individual metabolic systems within diferent species, and lack of general agreement as to what constitutes injury.

Visible injury

Fluoride-induced visible injury in plants has been discussed by Treshow & Pack (1970), Weinstein & McCune (1970), Weinstein (1977), the Environmental Protection Agency (1978), Malhotra & Blauel (1980), Halbwachs (1984), and others. Foliar damage is probably the best documented response to fluoride. Leaf injury occurs when the accumulated fluoride exceeds a threshold value (Treshow & Pack, 1970). Thus, the distribution pattern of fluoride generally determines the pattern of visible injury. This pattern is generally agreed upon. Broad-leafed trees and dicotyledonous herbaceous plants develop necrotic lesions at the tips and margins of leaves, later these extend downward toward the midrib. In monocotyledonous and coniferous plants leaf tip necrosis predominates. Usually a dark brown zone occurs between necrotic and healthy tissues. Many grasses develop characteristic injury symptoms which appear initially as scattered chlorotic flecks at the tips and upper margins of leaves. Later the flecking becomes more intense and extends downwards. Necrotic lesions in many plants may be preceded

by chlorosis. Fluoride can also induce wilting of plants and dessication of tissue at the tip or along the margins of the leaf (Leone et al., 1948; Brewer et al., 1959; MacLean et al., 1968).

Impact on forests and agriculture

There are numerous literature reports of fluoride damage to vegetation. The first reports of vegetation damage in the vicinity of industrial plants appeared in 19th century (Weinstein, 1977). Since then evidence has gathered pointing to fluoride as a cause of extensive damage to forests. Incidents of fluoride damage to European and North American forests were mostly attributed to fluoride emissions from aluminum, ore reduction, and phosphate fertilizer plants (Adams et al., 1952; Treshow et al., 1967; Jung, 1968; Carlson & Devey, 1971; Flueherl et al., 1981; Bosshard, 1984; Bunce, 1984). In many studies coniferous forests suffered most, due to their relative sensitivity to fluoride and other air pollutants, and the concentration of heavy industry in the northern hemisphere. But damage to tropical forests caused by fluoride emissions was also observed (Pandey, 1985).

Comparatively little is known about the effects of fluoride on agriculture. The subject was reviewed by Weinstein & McCune (1971) and more recently by Heggestad & Bennett

(1984). Fluoride can affect agricultural crops by decreasing growth and yield, injuring ornamental plants, fruits, and vegetables thus lowering their value, and by accumulating in crops, which makes them unsuitable for human and animal consumption.

Effects on growth, yield, and reproduction.

The effects of fluoride on growth and development are of great economic importance. Both field and laboratory studies proved that fluoride can alter growth with or without foliar damage. Plant growth can be influenced by any change in structure and metabolism. The destruction of part of the photosynthetic leaf area by necrosis reduces photosynthesis and thus, growth (Hill & Pack, 1983). A fluoride-induced decrease in photosynthetic pigments has similar effects (Woltz & Leonard, 1964). However, fluoride can also affect plant growth in the absence of visible injuries (Treshow et al., 1967; Treshow & Harner, 1968). Low concentrations of fluoride stimulated growth of Douglas fir needles (Treshow et al., 1967), tomato plants (MacLean et al., 1976), bean seedlings (Adams & Sulzbach, 1961), citrus (Brewer et al., 1960a), and roses (Brewer et al., 1967b). Other studies failed to show any growth response in numerous crop plants (Hill & Pack, 1983) including corn (Hitchcock et al., 1964; Amundson et al., 1982), tomatoes (Hill et al., 1958), and

beans (Pack & Wilson, 1967; MacLean et al., 1977). Most frequently damage resulted in a reduction in plant mass, volume, length, or area. Brewer et al. (1960b) sprayed citrus trees with 2-3 ppb HF (approximately 1.6 - 2.4 $\mu g\ F\ m^{-3)}$ and reported that leaf size was reduced by 25% to 35%. Reduction in leaf size was also reported in citrus by Leonard & Graves (1966, 1970), in birch and spruce by Halbwachs & Kisser (1967), and in apricot and apple by Hill & Pack (1983). Reduction of above ground mass by fluoride was reported in alfalfa, lettuce (Bennedict et al., 1964), common vetch, garden pea, onion (Guderian, 1971), roses (Brewer et al., 1967b), citrus (Brewer et al., 1960b), wheat (MacLean et al., 1984; Sharma, 1985), sorghum (MacLean et al., 1984), chickpea (Janardhan, 1985), Brassica juncea, and Phaseolus aureus (Sharma, 1985). Fluoride and sulphur dioxide fumes from a brickfield were found responsible for reduced height growth and branching patterns in several deciduous trees, including Salix alba, Populus sp., Alnus incana, Fraxinus excelsior, Acer pseudoplatanus, A. campestre, Quercus robur, and Ulmus procera. (Gilbert, 1983). Reduction in growth of roots was observed in HF fumigated alfalfa, orchard grass, spinach, and romain lettuce by Benedict et al. (1964), in Triticum aestivum, Brassica juncea, and Phaseolus aureus by Sharma (1985). Chang & Thompson (1966b), and Chang (1968) reported drastic reduction of root growth in NaF treated corn seedlings. The effect of fluoride on growth of trees

was frequently measured as a change in increment growth. Accumulation of fluoride in Douglas fir below injury threshold levels resulted in a reduction of radial growth by more than 40% (Taylor & Basabe, 1984). Reduction in radial growth of Douglas fir was also observed by Treshow et al. (1967), Carlson (1978), and Treshow & Anderson (1982); in citrus by Brewer et al. (1960a); and in western white pine and lodgepole pine by Carlson (1978).

Fluoride can affect flowering, fruiting, and seed production in various plants. When peach trees were fumigated with HF, fruits prematurely ripened and split (Benson, 1959). Brewer et al. (1960b, 1967a) reported a reduction in yield of oranges when trees were sprayed with HF and NaF. Fluoride may affect plant fruiting in the presence or absence of visible leaf damage. Pack (1966) noted that the number of tomatoes and their weight were reduced by HF in presence of leaf injury, but MacLean et al. (1977) reported no change in tomato fruit production of plants exposed to HF. The same fluoride treatment resulted in a reduction of fresh mass of bean pods in the absence of visible foliar injury, although tomato plants are generally regarded as more sensitive to fluoride than bean (Halbwachs, 1984). The effect of HF on fruiting of strawberry was also found to be independent of leaf damage (Pack, 1972).

Fruiting response of several crop plants to HF was tested by Pack & Sulzbach (1976). The most common response was the development of fewer seeds. Additionally, in pepper and corn plants fluoride inhibited flower development and this effect was independent of HF injury to the foliage. The effects of fluorides on reproductive characteristics of plants growing in natural environments were studied by Staniforth & Sidhu (1984), and Sidhu & Staniforth (1986). In the earlier paper the authors reported 89% and 78% flower mortality and 21- and 10-fold decreases in seed production as well as decreases in the size, number, and dry weight of fruits in blueberries and raspberries growing in the vicinity of a phosphorus plant. Fluorides also caused a reduction in seed size, seed and cone number, and seed germination of balsam fir, black spruce, and larch growing in the same area (Sidhu & Staniforth, 1986). The decrease in fruit and seed production could be caused by inhibition of flower development (Pack & Sulzbach, 1976) or high flower mortality (Staniforth & Sidhu, 1984), but according to Treshow (1970), flowers are highly resistant to fluoride. Similarly, The National Academy of Sciences (1971) reported that even fluoride sensitive plants, such as gladiolus, showed little floral damage when exposed to fluoride emissions. Low fertilization success can also be responsible for a decrease in fruit and seed production. Sulzbach & Pack (1972) observed reduced pollen germination and pollen tube elongation in fluoride treated toma-

toes. Similar effects were observed in sweet cherry by Facteau & Rowe (1977). In contrast, fluoride stimulated pollen tube growth in apple, pear, and sweet cherry (Lai Dinh et al., 1973).

Effects on structure

Histological studies

Studies of structural responses of plants to fluoride were pioneered by Solberg et al. (1955). The authors studied the influence of HF on the leaf anatomy of ponderosa pine and concluded that microscopic changes in green needle tissue were observed only in the tissue adjacent to necrotic areas. The changes included hypertrophy of epithelial cells, transfusion tissue cells, and phloem and xylem parenchyma. Solberg & Adams (1956) extended the studies to include leaves of apricot, apple, pinto bean, and tomato. They also compared patterns of HF and SO_2 induced leaf damage, and demonstrated that histological responses to these pollutants were indistinguishable. All leaves showing no macroscopic injury appeared normal under the microscope. In all plants disintegration of chloroplasts preceded collapse of the tissues. First signs of microscopic injury were observed in lower epidermal and mesophyll cells. Similar findings were reported by Stewart et al. (1973), who studied the patholog-

ical anatomy of pine needle necrosis caused by several major environmental stresses including HF. The authors examined the histology of transition zones between healthy and necrotic leaf tissues of Scots, white, lodgepole, and ponderosa pine, and Douglas fir. Early fluoride symptomatology included hypertrophy and hyperplasia of phloem cells, and occlusion of resin ducts with enlarged epithelial cells. The same symptoms were observed in naturally senescing leaves and those subjected to other environmental stresses including salt (NaCl), drought, suffocation, SO_2, O_3, and a combination of SO_2 or O_3 and HF. Structural changes in portions of leaves distant from the principal site of fluoride injury were observed in geranium by Poovaiah & Wiebe (1969). In plants fumigated with HF, tyloses were observed in xylem vessels of leaf blades and petioles in moderately and severely injured leaves. Tyloses are commonly induced by mechanical injury and diseases (Fahn, 1982).

Cytogenetic studies

Mohamed et al. (1966a,b) and Mohamed (1969) studied cytological reactions induced by NaF and HF in onion, tomato, and corn roots, and observed chromosomal abberations. Among other changes, fluoride-induced the formation of anaphase bridges and resulted in the appearance of tetraploid nuclei and multipolar anaphases. Using corn seedlings, Mohamed

(1977) further demonstrated that HF was able to reduce crossing over in certain chromosome segments. Similar findings were reported by Bale & Hart (1973) in barley seedling root tips. On the other hand, Temple & Weinstein (1978) did not observe mutations or chromosomal abberations in HF treated tomato seedlings.

Ultrastructural studies

Most light microscope studies were unable to discern structural changes prior to the appearance of visible leaf damage. The electron microscope proved to be a more powerful tool for the early detection of fluoride injury. Wei & Miller (1972) observed a sequence of events leading to leaf necrosis in mesophyll cells of HF fumigated soybean. The first change could be observed after 24 hours of HF treatment, before visible necrosis was detectable. It involved an increase in endoplasmic reticula (ER) and their aggregation. On the second day, small vacuoles appeared in the cytoplasm and phytoferritin accumulated in the chloroplasts. Later changes included the accumulation of lipid-like bodies in the cytoplasm, detachment of ribosomes from the ER, vesiculation of the ER and dictyosomes, swelling of mitochondria and loss of their matrix electron density, breakdown of tonoplasts, decrease in free ribosomes, change in morphology of chloroplasts, increase in plastoglobuli, and change in

the shape and content of nuclei. The morphology of plasmalemma and microbodies seemed to be affected only in very late stages of injury. Horvath et al. (1978) examined the ultrastructure of chloroplasts in Vicia faba leaves fumigated with HF. Destruction of the photosynthetic apparatus was apparent after 24 hours of fluoride treatment. At that time the chloroplasts showed reduction in grana and dilation of thylakoids. Damage to chloroplasts was also observed by Bligny et al. (1973) in fluoride treated young fir needles. The authors also noted that fluoride delayed the formation of epicuticular waxes on the needles. Soikkeli & Tuovinen (1979) and Soikkeli (1981) studied the cytology of pine and Norway spruce needles in relation to air pollution. The pollutants, composed mostly of SO_2 and fluorides, were emitted by wood pulp, fertilizer, and chemical factories. At the light microscope level the damaged cells were clearly distinguished by the abnormal appearance of tannin. Ultrastructural changes included a reduction in chloroplast lamellae, dilation and curling of thylakoids, and the appearance of lipid-like droplets in the cytoplasm. However the changes could be also caused by any of the unaccounted environmental stresses, such as frost or drought.

Effects on physiology and biochemistry

Respiration

Fluoride is a potent inhibitor of many respiratory enzymes. Warburg & Christian (1942) demonstrated that enolase prepared from yeast was weakly inhibited by fluoride, but in the presence of phosphate the inhibition of the Mg^{2+} activated enzyme was extremely potent. An inhibition of enolase extracted from pea seeds was dependent on the concentration of Mg^{2+}, phosphate, and fluoride (Miller, 1958). The kinetic investigation by Wang & Himoe (1974) revealed that the strength of fluoride inhibition depended on the type of the enzyme activating cation. The Mg^{2+} activated enzyme was most strongly inhibited, the Mn^{2+} activated enzyme was inhibited less readily, and the Zn^{2+} activated enzyme showed no signs of inhibition. The resultant formation of ligand complexes and their structural properties were investigated by Maurer & Novak (1981) and Novak & Maurer (1981).

Fluoride can also inhibit the activity of other respiratory enzymes. Phosphoglucomutase, sucrose synthetase (Yang & Miller, 1963b), hexokinase (Melchior & Melchior, 1956), succinic dehydrogenase (Lovelace & Miller, 1967a,b), ascorbic acid oxidase (Lee et al., 1966), and ATP-ase (Miller & Miller, 1974) have all been inhibited by fluoride. On the other hand, in vivo fluoride treatments frequently resulted in a stimulation of the activities of enzymes. When enolase and pyruvate kinase were extracted from corn and bean plants fumigated with HF, an increase in their activity was observed (McCune et al., 1964). Stimulatory effects of in vivo fluor-

ide treatments were also demonstrated for glucose-6-phosphate dehydrogenase (Lee et al., 1966), cytochrome oxidase (Lee et al., 1966; Poovaiah & Wiebe, 1971), ascorbic acid oxidase, mitochondrial oxidase, catalase McCune et al., 1964; Lee et al., 1966), and peroxidase (Lee et al. 1966; Mikhajlova, 1984).

Altered activity of respiratory enzymes by fluoride can be manifested as an inhibition or stimulation of respiration. An increase in respiratory rates was observed by numerous authors including Applegate & Adams (1960a), McNulty & Lords (1960a,b), Lustinec et al. (1962), Yu & Miller (1967), Miller & Miller (1974), Horvath et al. (1978), and Lorenc-Plucinska & Oleksyn (1982). Fluoride inhibition of respiration was also frequently reported (Younis, 1958; Yu & Miller, 1967; Pilet & Bejaoui, 1975; Holub, 1975). Hill et al. (1959) studied the effects of atmospheric fluoride and various types of injury on the respiration of leaf tissue. They found that the increase in respiration was greatest near the injured tissue and it was proportional to the amount of damage produced. McNulty (1959) reported that 1 and 5 mM concentrations of NaF significantly increased the respiratory rates in bean leaf discs without producing visible damage. Higher concentrations of fluoride resulted in a decrease in respiratory rates before the appearance of leaf injury. Many authors demonstrated that besides the fluoride concentration, the respiratory response of plants depends on

such factors as the species and the age of plant, the length of exposure (Miller & Miller, 1974), and the pH of the medium in which the plant tissues are cultured (Younis, 1958; Yu & Miller, 1967).

Decreased tissue respiration following exposure to fluoride can be easily explained by the inhibitory effects of fluoride on respiratory enzymes. The reasons for fluoride-induced respiratory stimulation are less obvious. Ross et al. (1962) demonstrated an increase in use of the pentose phosphate pathway by fluoride treated Chenopodium and Polygonum leaves. A similar increase was observed in oat coleoptiles (Ordin & Skoe, 1963), and in fluoride resistant varieties of gladiolus (Ross et al., 1968). In contrast, Lustinec et al. (1962) reported an activation of glycolysis during stimulation of wheat leaf respiration by fluoride. McNulty & Lords (1960) demonstrated that fluoride increased respiratory rates in Chlorella pyrenoidosa and at the same time increased levels of phosphorylated nucleotides. An increase in the ATP levels was also observed by Ballantyne (1984) in KF treated pea shoots. Yu & Miller (1967) showed a close similarity between the effects of fluoride and 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation. Subsequently, Miller & Miller (1974) investigated respiration of soybean leaves and the activity of mitochondrial ATP-ase extracted from fluoride treated tissue. An increase in leaf tissue respiration was always accompanied by an in-

crease in mitochondrial respiration and mitochondrial ATP-ase activity. The same pattern was found for fluoride inhibition. Fluoride also stimulated mitochondrial swelling and caused leakage of proteins which suggested the membranes as a site of fluoride action.

Very little is known about the effects of fluoride on photorespiration. Lorenc-Plucinska and Oleksyn (1982) studied the intensity of photorespiration in Scots pine in response to fluoride. The HF treatment caused only insignificant changes in the progenies of fluoride tolerant trees. The same treatment of susceptible progenies resulted in over 60% rise in the rates of photorespiration.

Photosynthesis

With the exception of cotton and fluoride resistant varieties of Scots pine, where high concentrations had no effect on apparent photosynthesis, all other studies demonstrated a decrease in photosynthetic rates in plants subjected to acute fluoride exposures. Thomas & Hendricks (1956) were among the first to correlate a decrease in the assimilation of carbon dioxide with the extent of leaf injury in HF treated gladiolus plants. Recovery was observed only in plants treated for a short period of time. Bennet & Hill (1973) exposed alfalfa and barley to HF and found that the rates of photosynthesis in the leaves dramatically de-

creased, but normal photosynthetic rates could be restored if cellular destruction had not occurred. Inhibition of photosynthesis was also demonstrated in the needles of three pine species and six species of broad-leaf trees after they had been treated with 0.1 to 10 mM NaF (McLaughlin & Barnes, 1975). Lorenc-Plucinska (1980) and Lorenc-Plucinska & Oleksyn (1982) studied the effects of HF on gas exchange in Scots pine and found that different progenies of pine trees reacted differently to HF treatments. Fluoride sensitive progenies showed a significant decrease in photosynthesis, while the photosynthetic rates of tolerant trees remained unchanged. Chronic fluoride exposures may inhibit or have no impact on the rates of photosynthesis depending on the amount of tissue damage. Hill et al. (1958), Thompson et al. (1967), and Hill (1969) observed no change in phosynthesis of plants in the absence of fluoride injuries. When leaf tissue showed signs of injury, photosynthesis was inhibited proportionally to the amount of leaf damage (Thomas & Hendricks, 1956; Thomas, 1958; Hill, 1969), or more than the leaf damage (Thomas, 1958; Woltz & Leonard, 1964; Hill & Pack, 1983).

The mechanisms of fluoride's interference with photosynthesis are unclear. There are indications that the interaction may occur at the light reaction stage. Fluoride inhibited the Hill reaction in isolated chloroplasts (Spikes et al., 1955; Ballantyne, 1972). Yang & Miller (1963c)

found that dark fixation of CO_2 and the activity of PEP carboxylase were enhanced in soybean treated with HF *in vivo*, and KF *in vitro*. Instead of being converted to carbohydrates most of the fixed CO_2 was retained as organic and amino acids. On the other hand, McCune et al. (1964) observed no change in the activity of PEP carboxylase in HF fumigated bean. An early symptom of fluoride damage is a loss of chlorophyll (Newman & McNulty, 1959; Gronebaum-Turck & Mahle, 1976; Mikhajlova, 1984). McNulty & Newman (1961) investigated the mechanism of fluoride induced chlorosis and observed that NaF prevented accumulation of chlorophyll a, chlorophyll b, and protochlorophyll in bean leaves etiolated at the begining of fluoride treatment. The authors also found that there was no inhibition of chlorophyll synthesis following attachment of the magnesium atom to the molecule ring structure. This indicated that fluoride affected pigment synthesis in the very early stages. Wallis et al. (1974) showed that the rate of incorporation of C^{14} aminolevulonic acid into the chlorophyll precursors and chlorophyll a of *Nicotiana tabacum* was reduced by fluoride. Damage to the photosynthetic apparatus structure also has been suggested as a cause of fluoride-induced reduction in photosynthetic rates. Chloroplasts were shown by various authors to be damaged during early stages of fluoride injury (Wei & Miller, 1972; Lhoste & Garrec, 1975; Horvath et al., 1978). Another line of evidence points to the inhibition of ribu-

lose-P_2-carboxylase. Parry et al. (1984) isolated this important photosynthetic enzyme from wheat leaves and demonstrated strong inhibition by fluoride.

Carbohydrates

Metabolism of carbohydrates in fluoride treated plants has been the subject of several studies. Fluoride can cause an increase, decrease, or no change in levels of various carbohydrates. The reasons for such inconsistences are not clear. Adams & Emerson (1961) reported a decrease in starch and an increase in ethanol soluble carbohydrates in HF treated needles of ponderosa pine. Yang & Miller (1963a) examined levels of free sugars in fluoride treated soybean leaves and reported a drastic decrease in sucrose with simultaneous increase in fructose and glucose. A ratio of non-reducing sugar to reducing sugar decreased, and starch levels remained unchanged. Barker & Mapson (1964) studied the effects of several enzymic poisons, including fluoride, on respiration and carbohydrate metabolism of strawberry and poplar leaves. According to the authors, iodoacetate and fluoride had similar effects on carbohydrates. When the leaves were exposed to high doses of iodoacetate or fluoride both sucrose and hexose levels declined. Smaller doses resulted in a decrease in sucrose and an increase in hexose. However, no experimental data were presented for fluoride to

support the authors' claims. Weinstein (1961) demonstrated a decrease in glucose, fructose, and sucrose in HF fumigated bean. Similar treatments did not alter carbohydrate levels in tomatoes. On the other hand, Pack (1971) observed a slight increase in total soluble and reducing sugars and a decrease in starch in bean plants exposed to chronic fluoride exposure. Carbohydrate levels in relation to fluoride accumulation in the needles of Abies alba were studied by Garrec et al. (1981). A decrease in the tissue levels of fructose, glucose, sucrose, and raffinose were correlated with an increase in fluoride levels in the needles. Galactose and starch levels did not change. Mejnartowicz & Lukasiak (1985) examined levels of sugars in Scots pine trees of different sensitivity to fluoride and noted that needles of trees relatively tolerant to fluoride contained lower levels of reducing sugars and higher levels of glucose and fructose compared with the sensitive trees.

The sizes of pools of phosphorylated sugars were investigated in fluoride treated bean plants by Pack & Wilson (1967), and in bean, corn, and tomato by McCune et al. (1970). Both studies reported no effect of fluoride on phosphorylated sugars.

As in the case of other metabolic processes fluoride is thought to interfere with carbohydrate metabolism by altering enzymic reactions. Amylase (Rockwood, 1919; Janardhan,

1985), hexokinase (Melchior & Melchior, 1956), phosphatase (Lorenc-Kubis & Morawiecka, 1978; Malhotra & Khan, 1980), phosphoglucomutase, sucrose synthetase (Yang & Miller, 1963b), and possibly cellulose synthetase (Ordin & Skoe, 1963) were demonstrated to be inhibited by fluoride.

Effect on nucleotides, amino acids, organic acids, and fatty acids.

Relatively few studies have investigated the effect of fluoride on metabolic constituents other than carbohydrates. Chang & Thompson (1966b) reported a decrease in RNA in fluoride treated roots of corn. Chang (1970) further determined the amount of ribosomal RNA, and found that it was reduced proportionately/ to fluoride concentration. Chang (1968) also reported that fluoride altered the base composition of RNA in corn seedling roots. Conner & Linden (1970) demonstrated changes in the catabolism of purine and pyrimidine 5' nucleotides in Tetrahymena pyriformis. McCune et al. (1970) did not observe any consistent effect of fluoride on the levels and composition of acid soluble nucleotide pools in leaves of bean, tomato, and corn. However, fluoride reduced the incorporation of P^{32} by the whole acid soluble nucleotide pool. Other studies reported an increase in ATP levels in response to fluoride (Chang & Thompson, 1966; Treshow & Harner, 1968; Ballantyne, 1984).

Fluoride can alter the metabolism of organic and amino acids. Weinstein (1961) reported an increase in concentration of organic acids and amino acids in the leaves of tomatoes and beans. The levels of organic acids and amino acids in HF treated leaves of necrotic soybeans increased by over 100% (Yang & Miller, 1963a). Among the organic acids, striking increases occurred in the concentrations of succinic, malonic, malic, and citric acids. Among the amino acids the most drastic increase was observed in the levels of aspartic, glutamic, and pipecolic acids and asparagine. Elevated levels of amino acids in plant tissues may indicate inhibition of protein synthesis. Kalinnikov & Tolokonnikov (1971) demonstrated inhibition of the incorporation of S^{35} - methionine into the albumin and globulin protein fractions of NaF treated maize.

Knowledge of the effects of fluoride on lipid metabolism is very limited. Simola & Koskimies-Soininen (1980) investigated the effects of KF on the composition of fatty acids in Sphagnum fimbriatum gametophytes and observed an increase in the proportion of palmitic acid and a decrease in linoleic and linolenic acids. The authors speculated that the observed change was due to the inhibition by fluoride of the fatty acid chain elongation.

Mineral composition and water economy

Fluoride forms insoluble salts with many cations. Formation of fluoride-metal complexes is thought to be responsible for fluoride inhibition of the activity of numerous enzymes. It is possible that the precipitation of physiologically active cations in plants can result in mineral deficiency. Ramagopal et al. (1969) studied fluoride injury of wheat roots in relation to calcium nutrition and suggested that major adverse effects of fluoride are due to precipitation and depletion of available calcium in cells. The suggestion was supported by Garrec et al. (1978) and Garrec & Chopin (1982), who demonstrated that calcium in plants migrates towards the sites of fluoride accumulation, and also that fluoride applied to leaves previously pretreated with calcium does not induce necrotic lesions. Garrec et al. (1978) also found reduced levels of magnesium and manganese in the leaves of Abies alba grown in a fluoride polluted environment.

Fluoride affects the water balance of plants. At high concentrations wilting has been reported in various species (De Ong, 1946; Leone et al., 1948; Brewer et al., 1959; MacLean et al., 1968), but little is known of how fluoride affects the plant water status. Reduced transpiration rates were observed in several plant species by Navara & Kozinka (1967). Poovaiach & Wiebe (1973) demonstrated reduced tran-

spiration rates due to stomatal closure in HF fumigated soybean leaves. Leaf water potential initially increased and later, when necrosis occurred, it drastically declined. Increased water potential in trees with fluoride-induced leaf injury was observed by Halbwachs (1970).

Fluoro-organic compounds

Numerous tropical plants are known to synthesize monofluoroacetic acid (Weinstein, 1977). Fluoroacetic acid is a potent inhibitor of the citrate cycle (Karlson, 1969). The biosynthesis of fluoroacetate in the presence of inorganic fluoride has been demonstrated in Acacia georginae in several studies (Peters & Shorthouse, 1964; Peters et al., 1965; Peters & Shorthouse, 1972), but the accuracy of analytical methods used in these studies for the detection of fluoro-organic compounds has been questioned (Hall, 1974). Organic forms of fluoride were found in various plants exposed to atmospheric fluoride (Reckendorfer, 1952; Cheng et al., 1968; Lovelace et al., 1968; Yu & Miller, 1970). On the other hand, Weinstein et al. (1972) were unable to detect the presence of fluoro-organic compounds in HF or NaF treated hay grass, soybean, crested wheat grass, corn, alfalfa, and tomato. The extent of organic transformations of fluoride in plants and their importance in plant metabolism require further investigations.

EXPERIMENTAL CONDITIONS AND GENERAL OBSERVATIONS

To study the effects of sodium fluoride on Jack pine seedlings it was important to maintain uniform experimental conditions. Seeds used for the experiments were generously provided by the Forestry Branch of the Manitoba Department of Natural Resources. They were all collected at the same site (seed zone #04.4, seed lot #387). Plants were grown in sand culture for all experiments except the cytokinin determination (details of the latter experiment are given in Chapter IV). Sand was cleaned by washing with 5% HCl followed by distilled and double distilled water until a constant pH was obtained. Two circles of filter paper were placed in each pot, and the pots filled with the sand and placed in a drying oven at 70 C. After three days pots were weighed on an analytical balance to 672 g (650 g sand + 22 g pot and filter paper), and 130 mL of 3/4 strength Hoagland's nutrient solution (Hoagland & Arnon, 1950) was added. Seeds were rinsed with distilled water and germinated on moist filter paper. Two to three days after germination, seedlings of uniform length were selected and transplanted into plastic pots (10 cm diameter) each containing 650 g of demineralized sand. Depending on the experiment 3 to 10 seedlings were planted in each pot. The seedlings were placed in a growth chamber maintained at a constant temperature of 22 C, 18 h light / 6 h dark regime (light intensity of 120 μE

$m^{-2}s^{-1}$). Humidity was not controlled during the experiments but ranged from 35% to 75%. Seedlings were watered daily with 30 mL of deionized water. For longer experiments 30 mL of Hoagland's solution was added every week instead of water. It was determined during the preliminary experiments that the conditions described above were satisfactory for healthy and rapid growth of seedlings.

The effects of two levels of sodium fluoride were studied: 3 µg F, and 15 µg F g^{-1}d. wt. sand (3 and 15 ppm). Similar levels of soluble fluoride may be expected in soils near industrial sites (Johnson, 1976; Thompson et al., 1979). Changes in levels of soluble fluoride in the sand during the experiments were determined with an Accumet 750 Selective Ion Analyser equipped with an Orion fluoride electrode (model 94-09-00), using the method of Larsen & Widdowson (1971). Levels of soluble fluoride in sand declined with time (Fig.1). After three weeks of the 3 µg treatment and four weeks of the 15 µg treatment soluble fluoride could no longer be detected in the sand.

The effects of drought on the structure of mesophyll and guard cells were studied in plants showing early signs of wilting. Drought conditions were achieved by withholding water for 4 days.

Figure 1. Levels of soluble fluoride in sand during four weeks of 3 µg (●), and 15 µg (○) sodium fluoride treatments. Error bars are the SD of replicates (n=3).

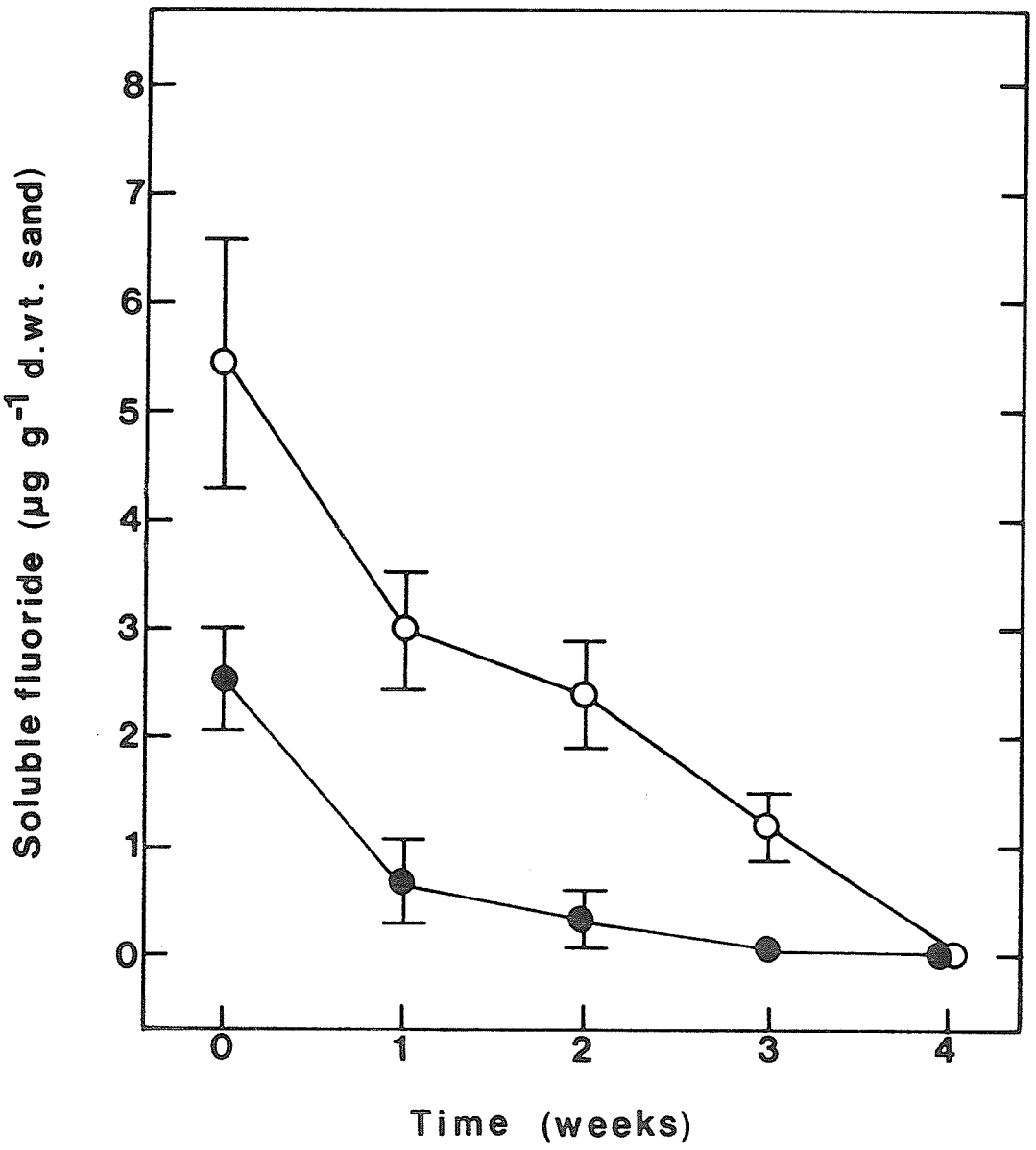
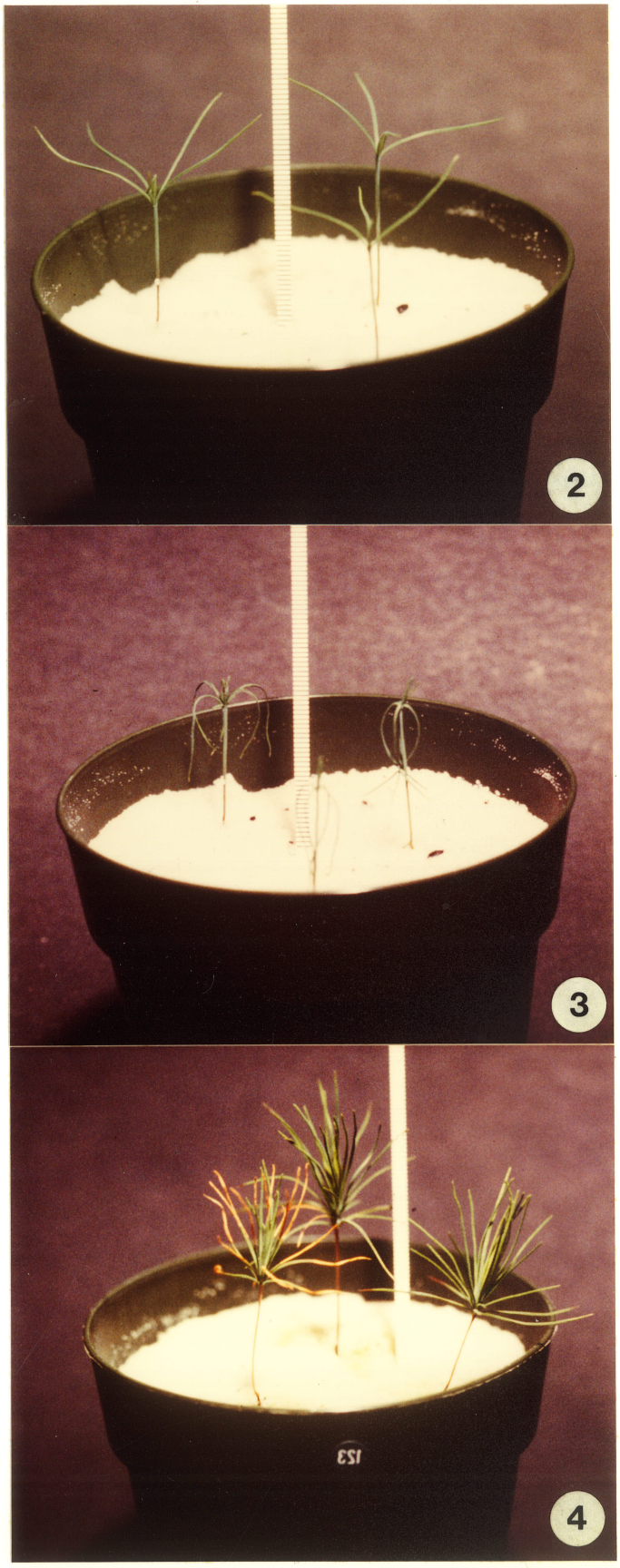

Soluble fluoride (µg g^{-1} d.wt. sand)
8
7
6
5
4
3
2
1
0
0
1
2
3
4
Time (weeks)

Figure 2. Two week old Jack pine seedlings shortly before fluoride treatments. Scale divisions are 2 mm.

Figure 3. Wilted Jack pine seedlings after a 24 hour exposure to sodium fluoride (15 µg treatment).

Figure 4. Jack pine seedlings after treatment with 3 µg fluoride. One of the seedlings shows signs of advanced leaf necrosis.


2
3
4

All treatments started when seedlings were two weeks old. At that time the first juvenile leaves were emerging (Fig.2). The first effects of fluoride treatments were observed as wilting, approximately 24 hours after the 15 µg treatment (Fig.3). Plants treated with the 3 µg level showed first signs of water imbalance 24 to 48 hours after commencement of fluoride treatment. Fewer seedlings wilted compared with the 15 µg treatment. Indications of leaf-tip necrosis were observed later than wilting, usually about 72 hours after either fluoride treatment. At those times tips of some leaves appeared desiccated and shrunk, initially without the loss of color. Shortly after, the affected leaf tips turned yellow and brown. Necrosis affected more new leaves with time. It always started at the leaf tips and progressed downward (Fig.4). The number of plants showing fluoride injury increased within the first 5 days after the 15 µg and 7 days after the 3 µg treatment (Fig.5). After that time the number of injured seedlings did not change. The extent of necrosis and number of seedlings with necrotic leaves did not appear to be significantly altered by high humidity and by pretreatments with abscisic acid. Wilting, however, was prevented by keeping seedlings in a water saturated atmosphere (100% humidity) during the treatments with sodium fluoride.

Distribution of fluoride in Jack pine seedlings was studied using plants grown for 7 months in a sandy loam soil

Figure 5. Cumulative percentage increase in injured seedlings with time after 3 µg (●), and 15 µg (○) fluoride treatments (70 seedlings per treatment).

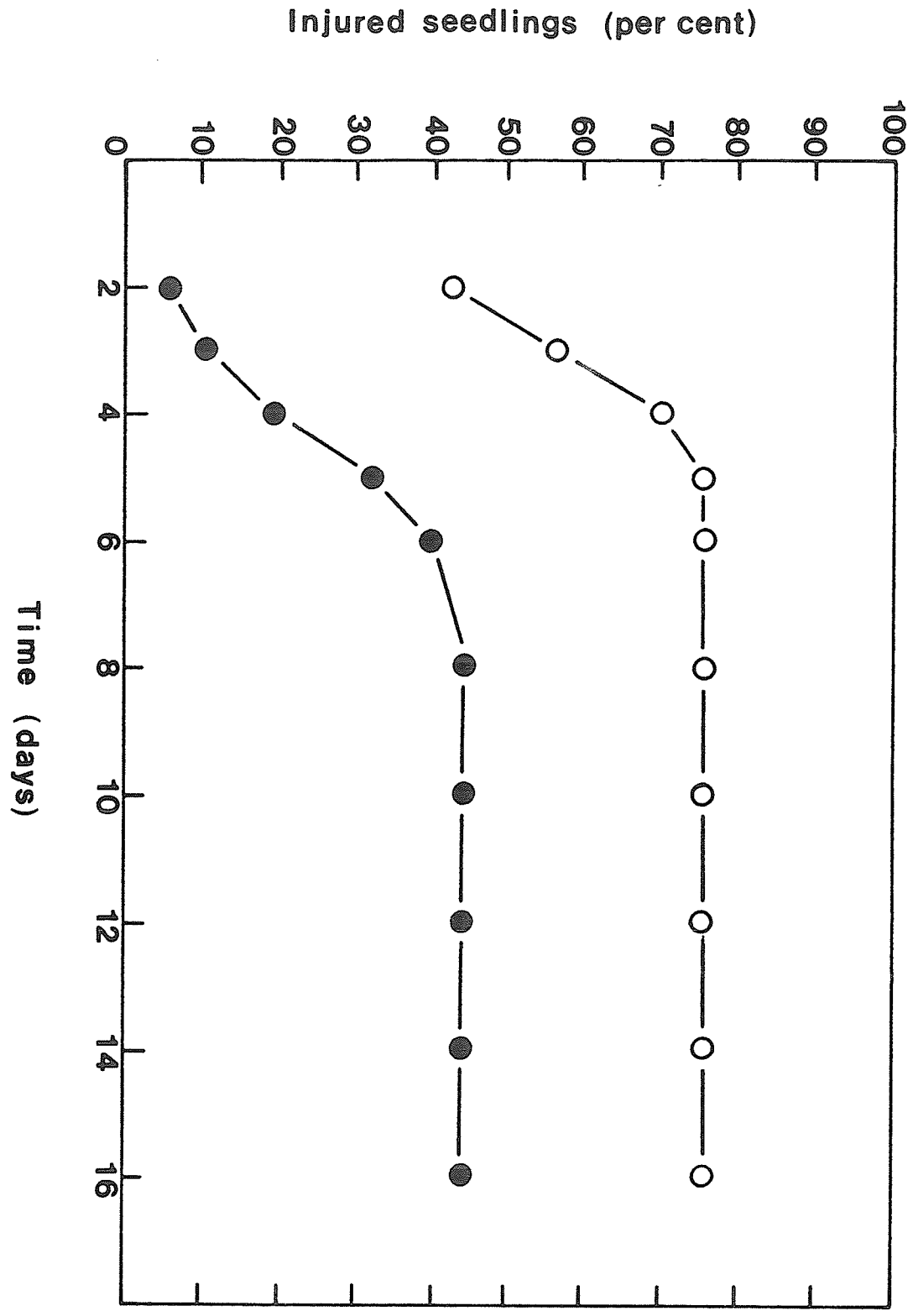

Injured seedlings (per cent)
0
10
20
30
40
50
60
70
80
90
100
Time (days)
2
4
6
8
10
12
14
16

Table 1. Distribution of fluoride in control and sodium fluoride treated Jack pine seedlings.

Part of plant	Fluoride ($\mu g\ g^{-1}$ d. wt. tissue)	
	Control	NaF*
New leaves	4.6±1.5**	8.9±2.2
Old leaves	9.4 ±2.7	54.3±6.0
Stems	2.7±2.4	21.6±4.9
Roots	6.3±1.7	39.01±3.1

*15 µg, 4 week treatment.
**±SD (n=3).

(horticultural soil/sand/peat moss, 1:1:1), transplanted to sand, and exposed to sodium fluoride (15 µg level) for 4 weeks. Total fluoride in plants was measured using an alkali fusion - selective ion electrode technique of McQuaker & Gurney (1977), and an Accumet 750 Selective Ion Analyser. Fluoride accumulated mostly in old leaves and roots (Table 1). Stems and young leaves contained considerably less fluoride, indeed the amount of fluoride in young leaves was only slightly higher than that of control plants.

Statistical analyses were performed only when the total number of samples exceeded 4. These included the analysis of variance procedure (Scheffe's test).

CHAPTER I

THE EFFECTS OF SODIUM FLUORIDE ON CYTOPLASMIC LEAKAGE AND THE LIPID AND FATTY ACID COMPOSITION OF JACK PINE (*PINUS BANKSIANA* LAMB.) SEEDLINGS

ABSTRACT

The effects of sodium fluoride on the lipid and fatty acid composition of Jack pine seedlings were studied using chromatographic techniques. Sodium fluoride markedly reduced levels of phospholipids and increased those of non-polar lipids. Fatty acid composition was less drastically affected. In several fluoride treatments, elevated levels of palmitic and stearic acid were found while those of behenic acid declined. All changes in lipid and fatty acid composition were accompanied by increased leakage of solutes from cells. Cellular membranes are implicated as possible sites of fluoride injury.

INTRODUCTION

The effects of fluoride on plants have been studied at all levels of biological organization. Relatively few studies, however, have examined biochemical and cytological alterations. Such research is essential in order to improve our understanding of how fluoride acts on plants. We know that fluoride affects processes such as respiration (Mikhajlova, 1984; Yu & Miller, 1967); photosynthesis (Lorenc-Plucinska, 1980; Lorenc-Plucinska & Oleksyn, 1982); and protein and carbohydrate metabolism (Jaeger & Grill, 1975; Yang & Miller, 1963b), but do not understand how it affects plant membranes. Cellular membranes have been implicated as the possible sites of fluoride damage (Miller et al., 1983). Signs of membrane damage were also noted during investigations of mesophyll cells in sodium fluoride-treated cotyledons of Pinus banksiana (Chapter II). In these cells early signs of fluoride injury were observed as deposits of lipid material which were most abundant near plasmalemma, tonoplast, chloroplast and mitochondrial membranes. Little is known about the effects of fluoride on fatty acid and lipid metabolism of plants. Among the few reports of fluoride-induced alterations which may influence lipid metabolism and, in turn, membrane performance, are those that deal with esterases and fatty acids. An increase in the activity of non-specific esterase was observed in leaves of Betula verrucosa and Picea abies by Yee-Miller (1975). Changes in the fatty

acid composition of KF treated Sphagnum fimbriatum gametophytes were demonstrated by Simola & Koskimies-Soininen (1980).

In the present study the effects of sodium fluoride on cell membrane permeability, and lipid and fatty acid composition in Jack pine (Pinus banksiana Lamb.) seedlings were investigated. Because lipids are major components of plant membranes it is suggested that any change in the lipid or fatty acid composition may result in altered membrane structure and function.

METHODS

Experimental conditions

Jack pine seedlings were grown in pots, each containing 650 g of demineralized sand. All seedlings were initially provided with 130 mL of 3/4 strength Hoagland's mineral solution (pH 5.5) and were watered every day. The pots were placed in a growth chamber at 22 C, 18 hour photoperiod (light intensity of 120 $\mu E\ m^{-2}\ s^{-1}$), with uncontrolled humidity. When the seedlings were two weeks old they were divided into three groups. One group served as a control; sodium fluoride solutions were added to the second and third groups to attain an initial concentration of either 3 or 15 $\mu g\ g^{-1}$ d. wt. of sand. At various intervals between 12 hours and 2 weeks, both treated and control plants were harvested. All treatments were always harvested on the same day.

Determination of solute leakage

Electrical conductance of a cell effusate (Prendeville & Warren, 1977) was used to determine changes in the permeability of cellular membranes in the seedlings. Changes in electrical conductance were taken as indicators of solute leakage from the plants. The seedlings were treated for 12, 24, 36, 48, 91, or 168 h with either 3 or 15 μg levels of sodium fluoride. Treated and control seedlings were removed

from pots, divided into groups, each containing four plants, and weighed. After brief washing they were placed in test tubes containing deionized water, incubated in a water bath at 25 C and gently shaken. After 30 min, the solutions were replaced with 10 mL of fresh deionized water. Specific conductance of the ambient solution was measured every hour for 5 h using an Electrometer conductivity bridge. Three consecutive readings of each sample were taken each time. The experiment was repeated, each time all treatments and controls had 3 replicates (n=6). The results were calculated as $\mu S\ h^{-1}\ g^{-1}$ f.wt. Solute leakage was regressed against incubation time (5 h) and slopes of these lines calculated using the least squares procedure (Remington & Schork, 1985). The slopes indicate the rate of solute leakage from plants and were used as indicators of membrane damage.

Isolation and separation of lipids

Jack pine seedlings were harvested for lipid analysis after 16 and 24 h of the 15 µg fluoride treatment, and after 91 h and 2 weeks of the 3 µg treatment. Samples of 2-4 g fresh weight were ground in a mortar, with 100 mL of cold iso-propanol to eliminate lipolytic activity (Hitchcock & Nichols, 1971) and filtered. The residue was homogenized with 40 mL of methanol, then 80 mL of chloroform was added and homogenization continued for several minutes. The mix-

ture was filtered and the residue re-extracted with chloroform-methanol (2:1, v/v) until all pigments were removed (Christie, 1982). Iso-propanol from the first fraction was evaporated in vacuo and the residue dissolved in chloroform-methanol (2:1, v/v). All filtrates were combined, reduced almost to dryness and dissolved in 200 mL of chloroform-methanol mixture. Non-lipid contaminants were removed from the mixture by washing it twice with 0.88% KCl solution (Folch et al., 1957). Chloroform and methanol were evaporated in vacuo, the residue containing lipids was resuspended in about 10 mL of chloroform and applied to a 2 X 12 cm silicic acid column (Sigma, 325 mesh). The column was successively washed with 10 column volumes of chloroform, 40 column volumes of acetone, and 10 column volumes of chloroform-methanol (1:1, v/v) and methanol (Rouser et al., 1967). The first chloroform fraction contained the less polar lipids, and will be further referred to in the text as neutral lipids (NL). The acetone eluate contained mostly glycolipids (GL) and sulphoquinovosyldiacylglycerols. The last chloroform-methanol and methanol fraction contained mostly phospholipids (PL). The purity of the fractions was determined using Thin-layer chromatography (TLC) methods. All solvents used for the extraction and chromatography contained 0.0075% butylated hydroxytoluene (BHT) as an antioxidant. The experiment was repeated three times (n=3), each time means of the 2 or 3 replicates were taken.

Lipid analysis

The composition of fatty acids was determined in all three lipid fractions. Samples containing lipids and known amounts of n-octacosane as an internal standard were transesterified with 0.5 M sodium methoxide for 20 min at 50 C. The reaction was stopped by addition of glacial acetic acid and water. Methyl esters of fatty acids were extracted three times with hexane, reduced to dryness under nitrogen, and dissolved in known amounts of hexane. Separation of fatty acids was achieved by use of a Carlo Erba Fractovap Gas Chromatograph, equipped with a 3 m long, 2 mm ID glass column containing 3%SP-2310/2%SP-2300 as a stationary phase on 100/120 Chromosorb W AW. The oven temperature during the runs was 196 C; the injector and detector temperatures were maintained at 240 C; carrier (nitrogen) flow was 30 mL min^{-1}. Integration was performed using a Hewlett Packard integrator model 3390A. To aid in identification, several samples were also analyzed on a column containing a low polarity stationary phase (1%SE-30 on Gas Chrom Q mesh 80-100), and on a 20 m DB-225 silica fused capillary column (0.25 mm ID) using a Perkin Elmer 8320 Gas Chromatograph and helium as a carrier. Equivalent chain length (ECL) values were calculated for all chromatogram peaks (Jamieson, 1975). Methyl esters of fatty acids were also separated according to the degree of unsaturation by argentation chromatography, using silver nitrate impregnated silica gel G plates. Hex-

ane-diethyl ether solvent systems (90:10 and 40:60, v/v) were used as a mobile phase. After separation the chromatograms were sprayed with a 0.1% solution of 2'7'-dichlorofluorescein solution in 95% methanol. Spots were viewed under UV light and scraped. Methyl esters of fatty acids were eluted from the absorbent with chloroform-methanol (9:1, v/v). The eluents were evaporated under nitrogen, the residues dissolved in hexane-ether (1:1, v/v) and washed with a sodium chloride solution and then with a dilute solution of ammonia. Washed extracts were reduced to small volumes and analysed by gas chromatography.

Phospholipids were analysed in Jack pine seedlings after 24 h of 15 µg treatment and compared with controls. Samples containing phospholipids were spotted on coated quartz rods (Chromarod-SII) and developed in saturated chambers in chloroform-methanol-acetic acid-water (60:30:9:3, v/v/v/v), or chloroform-methanol-35% ammonia solution (60:3:5, v/v/v). Phospholipids were detected and quantified with a flame ionization detector (Iatroscan TH-10, Iatron Laboratories, Inc.) Due to poor separation of phosphatidylglycerol (PG) and phosphatidylethanolamine (PE), these phospholipids were quantified jointly. Phospholipids were identified by co-chromatography with standards obtained through Sigma. The same samples were also separated on silica gel G TLC plates using similar solvent systems. Molecular species of phospholipids were identified using a modified Dittmer-Lester

reagent as a general stain for phospholipids (Ryu & MacCoss, 1979, periodate - Schiff's reagent (Shaw, 1968) for phospholipids with vicinal diol groups (PG) and phosphatidylinositol (PI); 0.2% ninhydrin in water saturated butanol (Krebs et al.,1969) for PE; and a modified Dragendorff reagent (Krebs et al.,1969) for phosphatidylcholine (PC).

Thiobarbituric acid assay

Thiobarbituric acid (TBA) assay was used to compare intensities of peroxidation processes in fluoride-treated and untreated plants. Malondialdehyde content was analysed in plants subjected to the following treatments: 3 µg for 91 h, 15 µg for 24 h and control. Plant samples weighing 600 mg were immersed in 5 mL of boiling methanol and ground in a mortar. After filtration, 1 mL samples were combined with 2 mL of TCA-TBA-HCl reagent (Beuge and Aust, 1978) and heated for 15 min in a boiling water bath. When cool, the precipitate was removed by centrifugation at 1000 G for 5 min. The absorbance was determined at 535 nm in a LKB Biochrom Ultrospec spectrophotometer against blank samples.

RESULTS

Cytoplasmic leakage

Sodium fluoride treatments of 3 µg and 15 µg resulted in an increased permeability of cell membranes to electrolytes (Table 1). The 15 µg treated plants were the first to show signs of membrane leakiness. After 24 h the rate of solute leakage from these plants was more than 50% higher than that from the controls. The rate gradually increased and reached maximum after 91 h. After 168 h (1 week) the rate of leakage was similar to that of plants treated for 36 hours. Leakage of solutes from the 3 µg treated plants started later and progressed more slowly. Like those of the 15 µg treated seedlings, the membranes of plants in this treatment were most damaged after 91 h of the treatment. The rate of electrolyte release from seedlings treated with 3 µg fluoride was similar after 1 week to that of the controls. Thus, the repair process must have taken place sometime between the 4th and 7th day after the plants had been treated with both fluoride levels.

The first signs of membrane leakage appeared before any visible injuries could be observed. The visible injuries in both treatments were either wilting followed by collapse and death of plants, or necrosis. Wilting occurred after approximately 26 hours of the 15 µg treatment, and 30 to 35

Table 1. Electrolyte leakage from sodium fluoride treated and untreated seedlings

Treatment (hours)	Control	3μg treatment	% of control	15μg treatment	% of control
12	15.5±1.4	15.3±1.0	98.8	16.2±2.0	104.6
24	15.2±2.0	17.7±1.9	116.6	22.8±1.4	150.7
36	19.9±1.3	22.6±1.6	113.3	30.5±3.5	153.2
48	19.3±2.2	27.3±3.3	141	37.0±5.4	191.4
91	10.5±0.6	16.8±2.5	160.5	29.4±3.1	280.8
168	17.8±1.3	16.5±1.1	92.5	29.2±5.0	163.9

Note: Presented as the slopes of regression lines of conductivity ($\mu S\ g^{-1}$ f.wt. $h^{-1} \pm$ SEM) against incubation time (hours). (n=6)

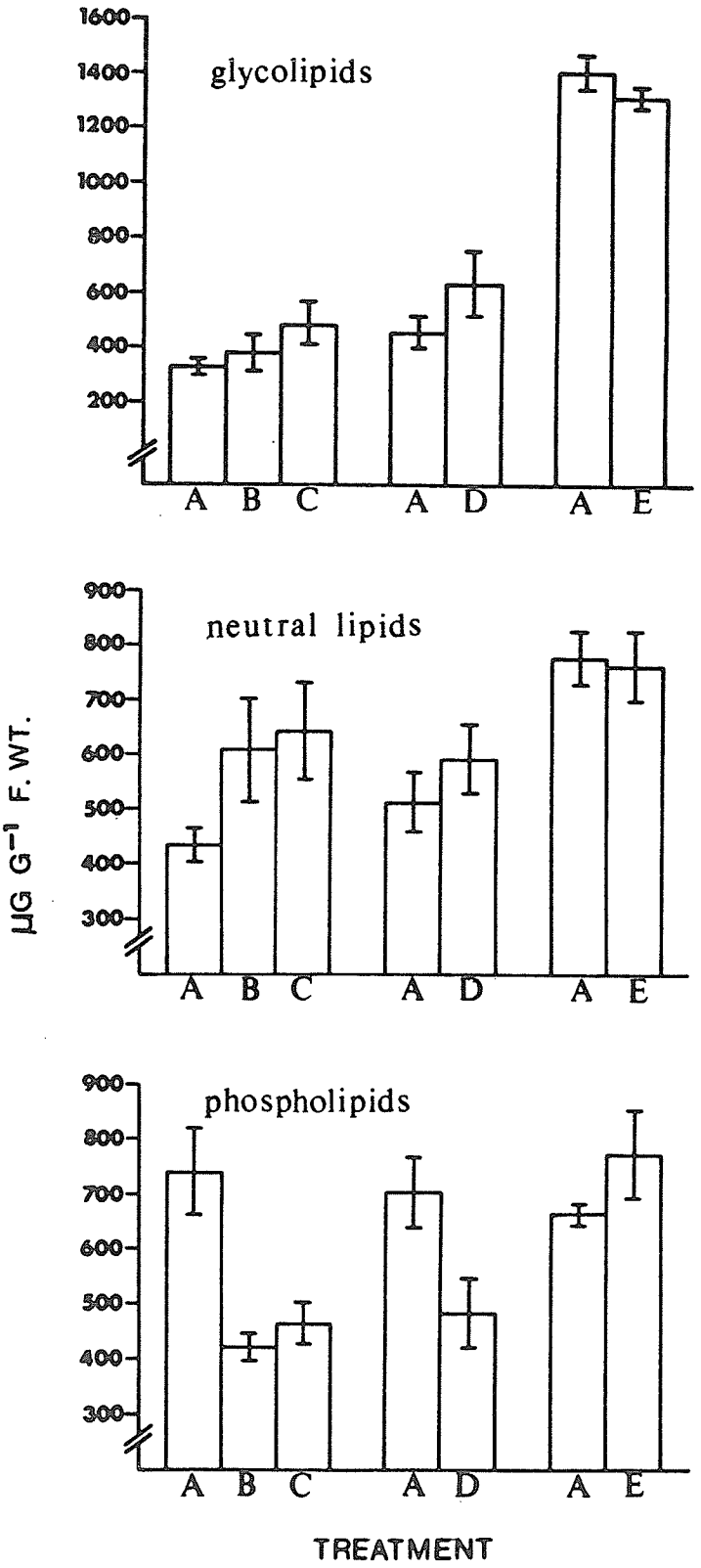
hours of the 3 µg treatment. Necrosis started at the tips of cotyledons and/or juvenile leaves and slowly progressed basipetally. The first signs of seedling leaf tip necrosis appeared on the third day of the treatments. Thus, prior to any visible injury, fluoride had some effect on leaf cell membranes.

Lipid composition

The quantities of lipids present in the three major fractions (NL, GL, and PL) were estimated as the amounts of fatty acids constituting these lipids (Christie, 1982). This gave a better estimation than weighing the fractions, particularly for the neutral lipid fraction which contained pigments and other non-lipid components.

Changes in the composition of lipids were noticed in Jack pine seedlings at various stages of development. This was particularly noticeable in glycolipids whose levels rapidly increased with the age of plants (Fig.1). Fluoride had little effect on GL levels in seedlings (Fig.1). Compared with the controls, amounts of GL slightly increased in plants of all fluoride treatments of a short duration. No increase was observed after prolonged, 2 week exposures to fluoride. More pronounced changes occurred in the NL fraction. The fluoride treatments (15 µg for 24 hours and 3 µg for 91 hours) which resulted in elevated rates of electrolyte leakage caused a large rise in levels of these

Figure 1. Quantity of fatty acids in phospholipid, neutral lipid and glycolipid fractions isolated from fluoride treated Jack pine seedlings and controls (±SEM, n=3). A=control; B,C=15µg F treatments for 16 hours (B) and 24 hours (C); D,E=3µg F treatments for 91 hours (D) and 2 weeks (E)


glycolipids
neutral lipids
phospholipids
1600
1400
1200
1000
800
600
400
200
900
800
700
600
500
400
300
A B C A D A E
μG G⁻¹ F. WT.
TREATMENT

lipids. No increase was discernible after 2 weeks of the 3 µg treatment. Phospholipids, the main components of most cellular membranes including plasmalemma (Mazliak, 1976), were found in smaller quantities in all fluoride-treated plants, except for the 2 week, 3 µg treatment. In this treatment there was a small increase in PL (Fig.1). Thus the fluoride treatments which induced solute leakage from plants also resulted in a decrease in PL, and in an increase in NL levels.

The effect of fluoride on the composition of phospholipids was studied in plants subjected to 24 h of the 15 µg treatment. Decreases in the amounts of PE+PG account for the greatest changes in the phospholipid composition, and for the general decrease in the phospholipid levels (Table 2). Only about half the amount of these phospholipids found in control plants was present in plants subjected to 24 h of the 15 µg treatment.

Fatty acid composition

The composition of fatty acids was studied in all three lipid fractions. Although many changes were found which were associated with fluoride treatments, they were often inconsistent and only a few trends could be seen. In all

Table 2. Effects of sodium fluoride on phospholipid composition of Jack pine seedlings

Phospholipid	Treatment*	µg lipid g^{-1} f.wt. ± SEM	% of control	% of total phospholipids
Phosphatidyl-glycerol+Phosphatidyl-ethanolamine	A	405.5±17.0		54.8
	C	232.5±10.2	57.3	45.9
Phosphatidylcholine	A	231.6±11.8		31.3
	C	205.1±7.0	88.6	40.5
Phosphatidylinositol	A	102.9±12.6		13.9
	C	69.3±16.7	67.3	13.6

*A, control; C, 15µg, 24h fluoride treatment; n= 3

fluoride treatments the amounts of palmitic acid (16:0) increased and those of behenic acid (22:0) decreased in NL (Table 3). Levels of stearic acid (18:0) also increased in several treatments. A large increase in stearic acid was noticed in PL of plants exposed to both fluoride treatments (Table 4). These treatments also resulted in a slight decrease in linoleic acid ($18:2\Delta^{9,12}$) levels. Fatty acids of GL seemed to be less altered by fluoride (Table 5) because only occasional, inconsistent changes were observed in some treatments.

Lipid oxidation

Increased levels of saturated fatty acids in plants treated with sodium fluoride could be caused by intensified lipid peroxidation. These findings and the fact that fluoride can stimulate the activity of peroxidase and catalase in plants (Lee et al., 1966; Mikhajlova, 1984) led us to examine levels of malondialdehyde in the fluoride-treated seedlings. Malondialdehyde is a secondary product in the oxidation of polyunsaturated fatty acids, and it is often used as an indicator of lipid peroxidation (Gray, 1978). Malondialdehyde levels were examined in plants treated with 15 µg fluoride levels for 24 h and 3 µg for 91 h, and compared with controls. No significant differences were found (Fig.2). Thus, the increase in palmitic and stearic acids in the fluoride-treated plants was probably caused by processes other than peroxidation.

Table 3. Fatty acid composition of neutral lipids in controls and fluoride treated Jack pine seedlings. Presented as percent weight of total fatty acids (±SEM), n=3

Fatty Acid	A*	B	%of A	C	%of A	A	D	%ofA	A	E	%of A
16:0**	8.5±1.5	8.7±0.2	102.3	14.7±2.1	172.9	6.7±1.3	11.5±3.7	171.6	8.0±1.1	9.4±1.9	117.5
18:0	9.1±0.5	18.2±4.5	200.0	9.6±1.2	105.5	8.0±1.4	14.3±1.1	178.7	12.9±2.6	12.5±2.3	96.9
18:1	3.3±0.5	3.9±0.2	118.2	3.5±0.5	106.1	3.7±0.3	2.5±0.3	67.6	4.2±0.8	3.9±0.2	92.9
18:2 (Δ9,12)***	11.5±0.9	10.9±0.4	94.8	12.4±1.4	107.8	11.8±1.0	8.7±0.2	73.7	14.4±0.9	14.4±0.4	100.0
18:3 (Δ5,9,12)	44.1±1.5	38.5±2.6	87.3	45.4±2.2	102.9	43.1±1.3	37.6±2.3	87.2	29.4±1.8	25.6±0.7	87.1
18:3 (Δ9,12,15)	5.1±0.7	5.1±0.5	100.0	6.1±1.3	119.6	7.5±0.3	7.0±1.3	93.3	6.3±0.2	7.2±0.1	114.3
18:4 (Δ5,9,12,15) +20:0	12.4±0.5	8.0±1.2	64.5	7.5±0.8	60.5	12.8±1.0	15.6±2.2	121.9	15.1±2.3	19.5±1.2	129.1
22:0	5.7±1.2	2.6±0.6	45.6	2.3±0.4	40.3	5.1±0.9	3.0±0.1	58.8	4.7±0.5	3.9±0.1	83.0

Note: Experimental conditions are described in the text.

* A=control; B,C=15μg F treatment for 16 hours (B), and 24 hours (C); D,E=3μg F treatment for 91 hours (D), and 2 weeks (E)
** Number of carbon atoms in the fatty acid : number of double bonds present.
*** Tentative position of double bonds.

Table 4. Fatty acid composition of phospholipids in controls and fluoride treated Jack pine seedlings. Presented as percent weight of total fatty acids (±SEM), n=3

Fatty Acid	A*	B	%of A	C	%of A	A	D	%ofA	A	E	%of A
16:0**	27.9±0.5	24.1±1.1	86.4	23.5±1.2	84.2	21.6±1.1	25.7±1.2	119.0	24.3±0.8	25.3±0.4	104.1
18:0	1.7±0.2	3.1±0.9	182.3	3.7±0.8	217.6	2.4±0.1	4.1±0.9	170.8	1.8±0.0	2.5±0.2	138.9
18:1	10.0±1.5	10.2±0.8	102.0	10.5±1.3	105.0	12.9±1.1	11.3±0.4	87.6	7.7±0.5	9.6±0.4	124.7
18:2 (Δ9,12)***	37.1±3.1	32.8±1.0	88.4	32.6±0.9	87.9	37.8±1.4	35.0±2.1	92.6	39.7±0.4	43.0±0.7	108.3
18:3 (Δ5,9,12)	4.6±0.5	3.9±0.2	84.8	4.5±0.5	97.8	4.3±0.6	3.7±0.1	86.0	5.3±1.1	3.2±0.2	60.4
18:3 (Δ9,12,15)	8.9±1.9	9.2±0.4	103.4	9.1±0.3	102.2	9.3±0.4	10.4±1.5	111.8	10.5±0.7	10.5±0.6	100.0
18:4 (Δ5,9,12,15) +20:0	2.0±0.6	4.8±1.9	165.5	4.8±1.3	165.5	3.6±1.0	3.9±0.8	108.3	3.3±0.6	2.3±0.4	69.7
20:3 (Δ5,11,14)	5.2±0.9	6.6±0.3	126.9	5.8±0.5	111.5	4.8±0.7	3.4±0.6	70.8	1.6±0.4	1.0±0.2	62.5

Note: Experimental conditions are described in the text.

* A=control; B,C=15µg F treatment for 16 hours (B), and 24 hours (C); D,E=3µg F treatment for 91 hours (D), and 2 weeks (E)
** Number of carbon atoms in the fatty acid : number of double bonds present.
*** Tentative position of double bonds.

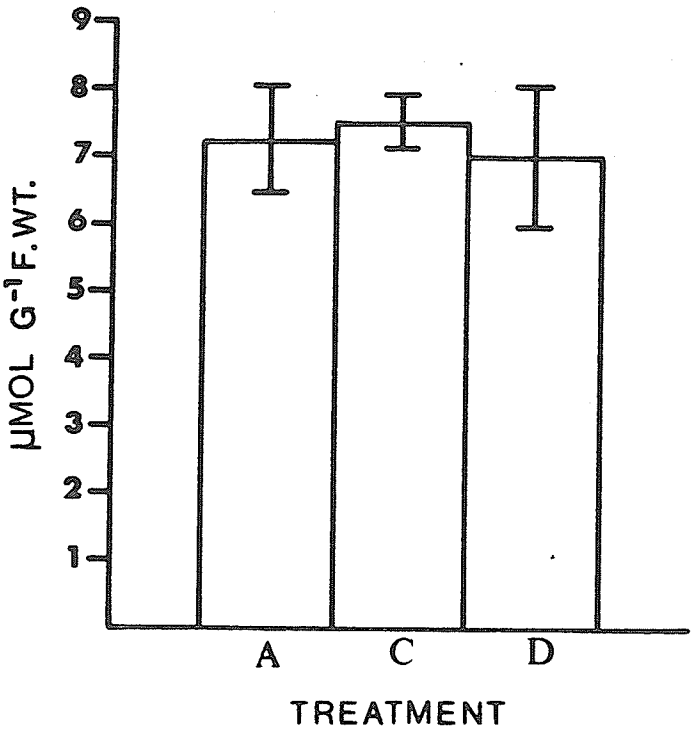
Table 5. Fatty acid composition of glycolipids in controls and fluoride treated Jack pine seedlings. Presented as percent weight of total fatty acids (±SEM), n=3

Fatty Acid	A*	B	%of A	C	%of A	A	D	%ofA	A	E	%of A
16:0**	11.5±1.2	15.3±2.5	133.0	13.4±1.1	116.5	12.7±2.7	11.8±1.4	92.9	10.1±1.2	11.0±0.5	108.9
16:3 (Δ7,10,13)***	2.4±0.5	2.2±0.5	91.7	2.1±0.5	87.5	2.2±0.4	1.9±0.3	86.4	2.2±0.1	2.3±0.2	104.5
18:0	1.7±0.4	1.4±0.3	82.3	1.4±0.1	82.3	1.5±0.2	1.4±0.2	93.3	1.8±0.1	1.8±0.1	100.0
18:1	2.1±0.1	2.5±0.3	119.0	1.7±0.1	80.9	2.6±0.5	2.3±0.2	88.5	1.8±0.1	1.6±0.1	88.9
18:2 (Δ9,12)	10.2±0.6	11.2±2.3	109.8	10.2±0.7	100.0	10.5±1.6	10.5±1.2	100.0	8.0±0.3	7.6±0.3	95.0
18:3 (Δ5,9,12)	1.9±0.4	2.4±0.5	126.3	2.4±0.4	126.3	1.9±0.1	1.8±0.1	94.7	0.9±0.2	0.7±0.1	77.8
18:3 (Δ9,12,15)	59.3±1.5	55.7±2.9	93.9	54.2±2.5	91.4	57.2±1.6	58.7±1.6	102.6	62.7±2.8	62.8±2.4	100.2
18:4 (Δ5,9,12,15) +20:0	10.1±2.4	7.5±0.5	74.3	9.4±0.9	93.1	8.6±1.6	6.8±1.9	79.1	8.7±0.8	10.0±1.0	114.9
22:0	1.8±0.3	2.0±0.3	111.1	2.2±0.3	122.2	2.3±0.6	2.9±0.4	126.1	1.7±0.4	1.3±0.2	76.5

Note: Experimental conditions are described in the text.

* A=control; B,C=15μg F treatment for 16 hours (B), and 24 hours (C); D,E=3μg F treatment for 91 hours (D), and 2 weeks (E)
** Number of carbon atoms in the fatty acid : number of double bonds present.
*** Tentative position of double bonds.

Figure 2. Malondialdehyde content of Jack pine seedlings treated with sodium fluoride and untreated (±SEM, n=4). A=control; C=15µg, 24h F treatment; D=3µg, 91h F treatment.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8
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TREATMENT

DISCUSSION

Most plant membranes contain phospholipids, glycolipids, and neutral lipids in various proportions (Harwood, 1980). Any change in the structure of the lipid bilayer can affect both the passive diffusion and the active transport of compounds through membranes (Raison, 1980), and result in membrane leakiness. In the present study, increased leakage of electrolytes from Jack pine seedlings was accompanied by pronounced changes in lipid composition. The levels of phospholipids in sodium fluoride-treated seedlings declined significantly, while those of neutral lipids increased. Seedlings recovered two weeks after the initiation of the 3 µg fluoride treatment. At that time neither increased leakage nor any change in lipid composition was detected. A decrease in phospholipids may indicate either intensified breakdown of membrane lipids or inhibition of phospholipid synthesis. Appearance of lipid bodies near cellular membranes was observed in mesophyll cells of Jack pine cotyledons treated with sodium fluoride (Chapter II). These bodies may have contained neutral lipids or a mixture of neutral lipids and glycolipids, both found in higher amounts in fluoride-treated plants.

Various environmental stresses can result in leakiness of plant membranes. Membrane permeability is also altered in senescing plants (Barber & Thompson, 1980), in plants ex-

posed to ozone (Beckerson & Hofstra, 1980; Evans & Ting, 1973), in plants under anaerobic conditions (Hiatt & Lowe, 1967), and when tissues are treated with inhibitors of respiration (Younis, 1969; Younis et al., 1969; McCullough & Simon, 1973). McCullough and Simon (1973) reported increased leakage of electrolytes from cucumber cotyledons treated with iodoacetate. The leakage was accompanied by a decline in the amounts of phospholipids. Phospholipids with the most rapid turnover disappeared first from iodoacetate treated cotyledons. The authors concluded that iodoacetate converted the normal turnover of lipids, in which the rates of synthesis and degradation are evenly balanced, into a net breakdown of phospholipids. In their opinion the rate of synthesis was reduced due to a restricted supply of energy from respiration. Fluoride is also a potent inhibitor of many respiratory enzymes, such as ascorbic acid oxidase (Lee et al., 1966), enolase (Miller, 1958; Maurer & Novak, 1981), hexokinase (Melchior & Melchior, 1956), phosphoglucomutase (Yang & Miller, 1963b), succinic, malic, and NADH dehydrogenases (Lovelace & Miller, 1967b). Fluoride can also inhibit the activity of ATP-ase (Miller & Miller, 1974), acid phosphatase (Malhotra & Khan, 1980), nitrate reductase (Kadam et al., 1980), phytase (Chang, 1967); and pyrophosphatase (Baykov et al., 1979). It can stimulate the activity of certain enzymes, such as non-specific esterase (Yee-Miller, 1975) and glucose-6-phosphate dehydrogenase (Lee et al., 1966).

The effect of fluoride on various metabolic enzymes may result in gross metabolic disruption and energy depletion. Plant membranes could be affected either because of insufficient energy for a rapid synthesis of membrane components, or because membrane lipids are hydrolyzed and used as an alternative source of energy. The composition of phospholipids in Jack pine changed after fluoride treatments. A decline in PG+PE may indicate that these lipids are more prone to hydrolysis than PC, or that their turnover is faster. A decrease in PG and PE was also observed in young cotyledons of iodoacetate treated cucumbers by McCullough & Simon (1973). The iodoacetate treatments resulted in a small decrease in PI and in an increase in PC. Similar alterations were observed in fluoride-treated Jack pine seedlings. Thus, the effects of iodoacetate and fluoride on plants may be in some ways similar.

More difficult to explain is a small increase in GL levels in fluoride-treated plants, if the hypothesis of metabolic inhibition is correct. A slightly lower water content (higher dry weights of the samples) in fluoride-treated plants resulting from fluoride-induced water stress (Chapter III) partly accounts for the increase. An other possible reason might be elevated substrate concentration for glycolipid production. Breakdown of phospholipids would likely cause a rise in levels of diacylglycerols and other neutral lipids. A concentration of monosaccharides is also known to

increase in fluoride-treated plants (Yang & Miller, 1963a). There was no evidence, however, of an increased presence in GL of major fatty acids characteristic of PL or NL, such as palmitic, linoleic, and 9,12,15-octadecatrienoic acids.

No sign of intensified peroxidation was observed in fluoride-treated plants. Thus, the increase in ratios of saturated to unsaturated fatty acids in NL and PL must have occurred as a result of other processes. Simola and Koskimies-Soininen (1980) observed an increase in the proportion of palmitic acid and a decrease in linoleic acid in all three lipid fractions of KF treated Sphagnum fimbriatum gametophytes. They also noted a decrease in linolenic acid in glyco- and neutral lipids. Based on this observation they hypothesized that fluoride ions inhibit lengthening of the fatty acid chain. The present results appear to support their hypothesis, although the changes were not as pronounced as those observed in Sphagnum moss. A higher proportion of shorter chain fatty acids was seen most clearly in NL of Jack pines after all fluoride treatments. Changes in the fatty acid composition of fluoride-treated plants may, however, merely reflect changes in the lipid composition. More fatty acids typical of PC would be expected in the PL fraction, because the proportions of PC increased in fluoride-treated seedlings. It is possible that the composition of other lipid fractions was also altered and resulted in different proportions of fatty acids.

The present results strongly imply membrane damage as one of the reasons for fluoride phytotoxicity. Lipid material was deposited near the cellular membranes of mesophyll cells in the early stages of fluoride injury (Chapter II). This material probably contained mainly non-polar lipids, formed either as a result of membrane breakdown, or due to an inhibition of phospholipid synthesis and the incorporation of lipids into membranes.

Fluoride can strongly influence the metabolism of plants. Both stimulatory and inhibitory effects on various physiological processes have been reported. The type of response depends on factors such as dosage, duration of exposure, type and age of plant and of tissue (Weinstein, 1977). Thus, in order to better understand the mechanisms of fluoride action it may be neccessary to study various metabolic and structural effects using the same plants and environmental conditions. Additional investigations of basic energy conversion processes in Jack pine seedlings, such as photosynthesis and respiration, are required to permit an explanation of some of the alterations reported in this chapter.

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CHAPTER II

SODIUM FLUORIDE- AND DROUGHT-INDUCED STRUCTURAL ALTERATIONS OF MESOPHYLL AND GUARD CELLS IN COTYLEDONS OF JACK PINE (*PINUS BANKSIANA* LAMB.)

ABSTRACT

Fluoride- and drought-induced injuries to mesophyll and guard cells were studied in Jack pine cotyledons using light and electron microscopy techniques. Most early structural alterations were similiar in cells of fluoride and water stressed seedlings. Both treatments resulted in an appearance of lipid material in the cytoplasm during early stages of injury, suggesting damage to the cell membranes. Treatment with sodium fluoride also resulted in deposition of starch in chloroplasts. Guard cells were more resistant to both stresses than mesophyll cells. Both metabolic injury and collapse of neighbouring cells may be responsible for the opening of stomates in wilting, fluoride-treated seedlings.

INTRODUCTION

Fluoride is recognized as one of the most phytotoxic common air pollutants (Weinstein, 1977). It is also present in many soils. Plants can readily absorb water soluble forms of soil fluoride (Brewer, 1966; Cooke & Johnson, 1978) and develop similar injury symptoms to those caused by the airborne pollutant. Low concentrations of fluoride can affect the growth and yield of plants even in the absence of visible injury (Stocks, 1960). Higher fluoride doses frequently cause wilting of plants (Leone et al.; 1948, Brewer et al., 1959; MacLean et al., 1968), chlorosis, and necrosis of leaf tips and margins (Weinstein & McCune, 1971), but the mechanisms of fluoride action are largely unknown. It was seen in Chapter I that wilting followed increased electrolyte leakage from fluoride-treated seedlings. This suggested osmotic imbalance, which may be a result or cause of biochemical alterations and membrane impairment leading to cell disorganization (Heath, 1980). Thus, the biochemical and structural alterations observed in plants after fluoride treatment may also result from water stress and not from the initial fluoride injury.

To understand the mechanisms of fluoride action on plants both biochemical and structural research is needed, but this has rarely been conducted on the same material. In the present study the effects of fluoride on mesophyll and guard

cell structure in Jack pine (Pinus banksiana Lamb.) cotyledons were determined and compared with the changes caused by drought. Both light and electron microscopy observations were conducted. The study focussed on the initial effects of both stresses rather than later stages of cell injury and disintegration.

METHODS

Experimental

Jack pine seeds were germinated on moist filter paper and after 2 days seedlings were transplanted to pots, each containing 650g of demineralized sand. All seedlings were provided with 130 ml of 3/4 strength Hoagland's mineral solution (pH 5.5) and were watered daily with deionized water. The pots were placed in a growth chamber maintained at a constant temperature of 22 C with an 18 h light / 6 h dark photoperiod and light intensity of 120 $\mu E\ m^{-2}\ s^{-1}$. Relative humidity varied from 35% to 60%.

When juvenile leaves appeared at the shoot apex (seedlings 2 weeks old) the seedlings were divided into four groups. Two groups were treated with a sodium fluoride solution which was added to the sand, to attain an initial concentration of either 3 or 15 $\mu g\ g^{-1}$ d. wt. of sand. The plants were examined 24 h after the 15 μg treatments and 91 h after the 3 μg treatments. At these times, signs of increased solute leakage from fluoride-treated seedlings were observed (Chapter I). Solute leakage was detected by measuring the electrical conductance of an effusate (Prendeville and Warren, 1977). Only intact cotyledons without visible signs of injury were selected for microscopy. In the third group water stress was induced by witholding water for 4 days. At the first signs of wilting, plants were collected

for microscopic examination. The fourth group consisted of control plants sampled with drought- and fluoride-stressed plants.

Intact seedlings were immersed in absolute ethanol (Willmer, 1983) and the stomates immediately observed either in reflected light using whole plants or in transmitted light after preparing epidermal strips. For cytological studies distal parts of the cotyledons were examined. Each cotyledon was cut approximately 5 mm below the tip. The tip was discarded and a 1 mm terminal segment excised. At least ten cotyledons from different plants of every group (five pots per group) were examined. The entire experiment was repeated.

Conventional fixing and staining for light and electron microscopy

Segments were fixed at room temperature overnight in 3% glutaraldehyde in 25 mM cacodylate buffer, pH 6.9, and post-fixed in 1% osmium tetroxide in cacodylate buffer for 4 hours at 4 C. After fixation the blocks were dehydrated in an ethanol series and transferred to propylene oxide and then to mixtures of propylene oxide and Spurr resin (1:2,1:1,2:1). Propylene oxide was then allowed to evaporate under a fume hood. After the tissue segments had been infiltrated for 7 days in Spurr resin, the resin was polymerized overnight at 70 C. Transverse sections 1 µm in thickness

were cut with glass knives, stained with 2% crystal violet (Gerhardt et al., 1981) and viewed with a Carl Zeiss universal microscope equipped with a Nikon M-35 S camera. Ultrathin sections for electron microscopy were cut with a diamond knife and mounted on copper grids. They were stained for 30 min with a saturated solution of uranyl acetate in 50% methanol and poststained for 10 min with lead citrate (Reynolds, 1963). Sections for light and electron microscopy were interchangeably cut from the same blocks. Many sections were viewed to obtain a good representation of cellular structures. All electron microscopic examinations were carried out on an AEI 801 microscope at an accelerating voltage of 60 kV.

Solvent extraction

To extract lipid materials, glutaraldehyde-fixed tissue was placed at room temperature in chloroform-methanol (3:1) for 2 h followed by 1 h in chloroform (Hickey and Coffey, 1978). The tissue was postfixed with buffered osmium tetroxide and processed for electron microscopy as above.

Cytochemical staining

The periodic acid - thiocarbohydrazide - silver proteinate technique (Thiery, 1967) was used to stain starch grains. Ultrathin sections were mounted on nickel grids and immersed in 1% periodic acid for 20 min in a high humidity

chamber, washed in distilled water and immersed for 4 h in 0.2% solution of thiocarbohydrazide in 20% acetic acid. The sections were washed in 10%, 5% and 1% ice cold acetic acid (10 min in each change) and in distilled water, and immersed in 1% aqueous silver proteinate for 30 min in the dark. For controls, either periodic acid, thiocarbohydrazide, or silver proteinate was omitted (Courtoy & Simar 1974). To enhance the contrast of thylakoids some sections were poststained with uranyl acetate and lead citrate.

OBSERVATIONS

Wilting was the first observed sign of fluoride injury in Jack pine seedlings. This occurred in approximately 50% of the plants after 25 to 26 hours of the 15 µg treatments and 2 to 6 hours later in 7% of the 3 µg treated seedlings. Examination of stomates in the wilted plants revealed that over 60% of them were open at the time of wilting (Fig.1). All the wilted plants soon died. The first signs of leaf tip necrosis appeared on many surviving plants after approximately 72 h of the fluoride treatments. Wilting was never observed among control plants. At the time of examination, most stomates of these plants were open.

The anatomy of fully grown cotyledons of Jack pine (Fig.2) is simpler than that of mature needles. The cotyledon epidermis is uniseriate with a thin cuticle and contains slightly sunken stomates. Cotyledons lack a hypodermis, endodermis, and transfusion tissue. Resin ducts are also absent from the apical parts of cotyledons. Mesophyll cells in transverse sections have a typically round or oval appearance. They contain large central vacuoles. Narrow strips of cytoplasm and nuclei are close to the cell walls in fully turgid cells (Fig.3).

Abbrevations used: E-epidermis, L-lipid body, M-mesophyll, N-nucleus, P-plastid, PL-plastoglobule, S-stoma, ST-starch, V-vacuole, VB-vascular bundle, Ag-stained with silver proteinate, U/Pb-stained with uranyl acetate and poststained with lead citrate.

Figures 1-4. Light and electron micrographs of control and fluoride treated cotyledons.

Figure 1. Open stomata in the epidermal strip of a wilting, fluoride treated seedling. X130

Figure 2. Cross section of a distal part of a Jack pine cotyledon. X90

Figure 3. Typical mesophyll cells of a control plant. X360

Figure 4. Fluoride treated mesophyll cells (15 µg treatment) with signs of injury and desiccation. Note clumps of chromatin in the nucleus (arrow) and advanced plasmolysis. X360

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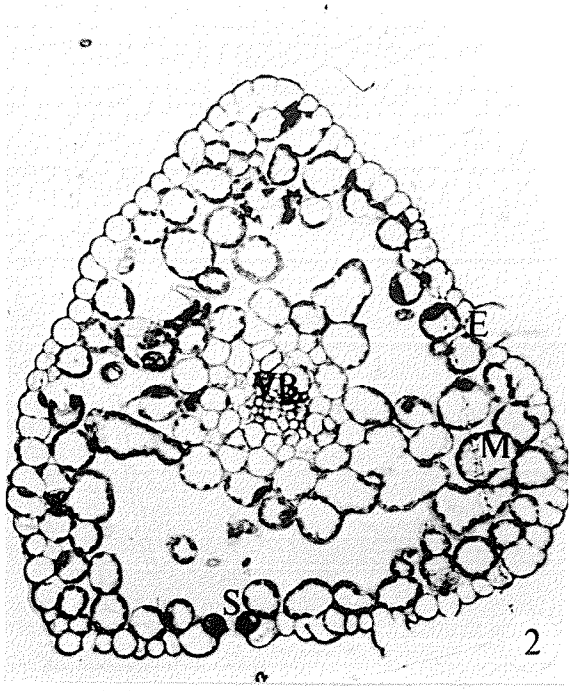

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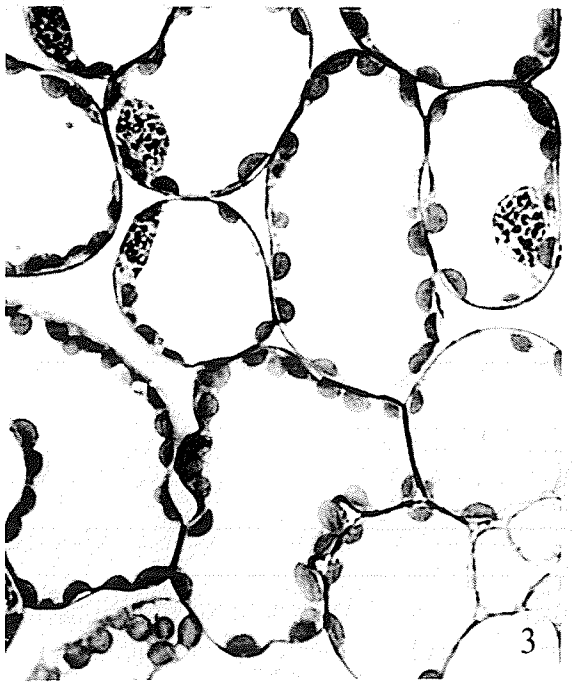

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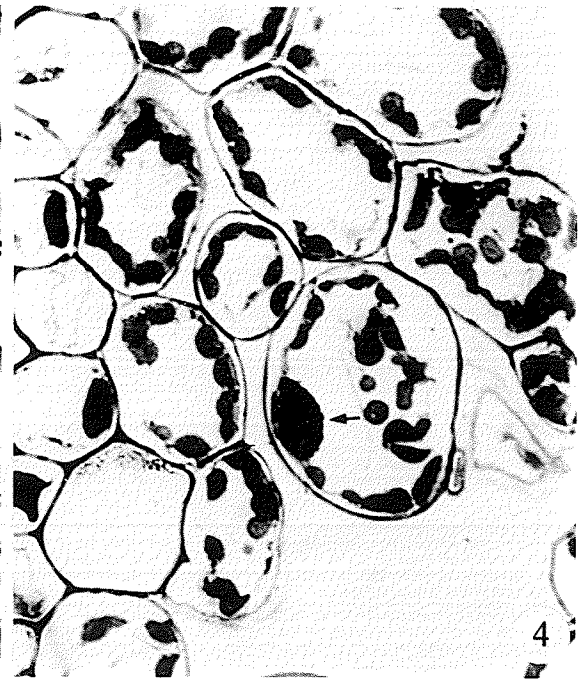

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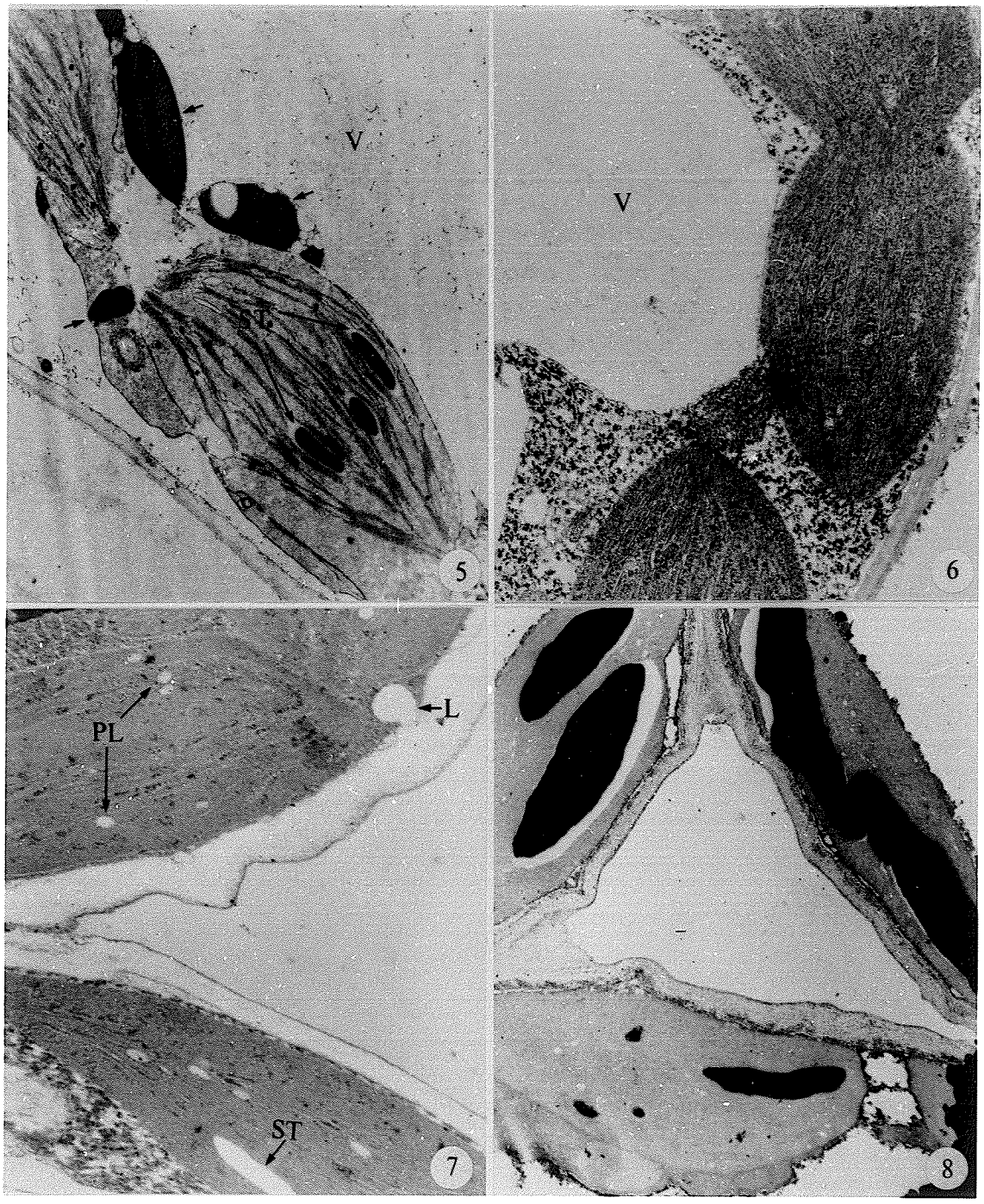
Fluoride injuries

Mesophyll cells:

Both levels of fluoride resulted in similar patterns of cell injuries. The extent and degree of damage, however, was greater in the 15 µg treatment. Plasmolysis of mesophyll cells was often seen in plants which had been treated with fluoride. (Fig.4). The most frequently noted change in both treatments was the appearance, in the cytoplasm, of a large number of electron opaque bodies (Fig.5). These were normally absent from, or present in very small numbers in mesophyll cells of control plants (Fig.6). These bodies were often seen in seemingly intact cells and were frequently attached to vacuolar, plasmalemma, outer chloroplast, and outer mitochondrial membranes. They were completely extracted using a mixture of chloroform-methanol (Fig.7), suggesting their lipid nature. All stages of fluoride-induced cell injuries were also characterized by an abundance of starch in the chloroplasts (Fig.8). Only occasionally were small starch granules seen in the chloroplasts of control plants. Thylakoids in control plants were distributed rather evenly in the chloroplasts. Many plastoglobuli, which were rarely seen when chloroplasts were stained with uranyl acetate and lead citrate, were clearly visible when stained for the Thiery reaction (Fig.9). Fluoride treatments often resulted in dilation and curling of thylakoids (Fig.10). The structure of mitochondria and the endoplasmic reticulum (ER) did not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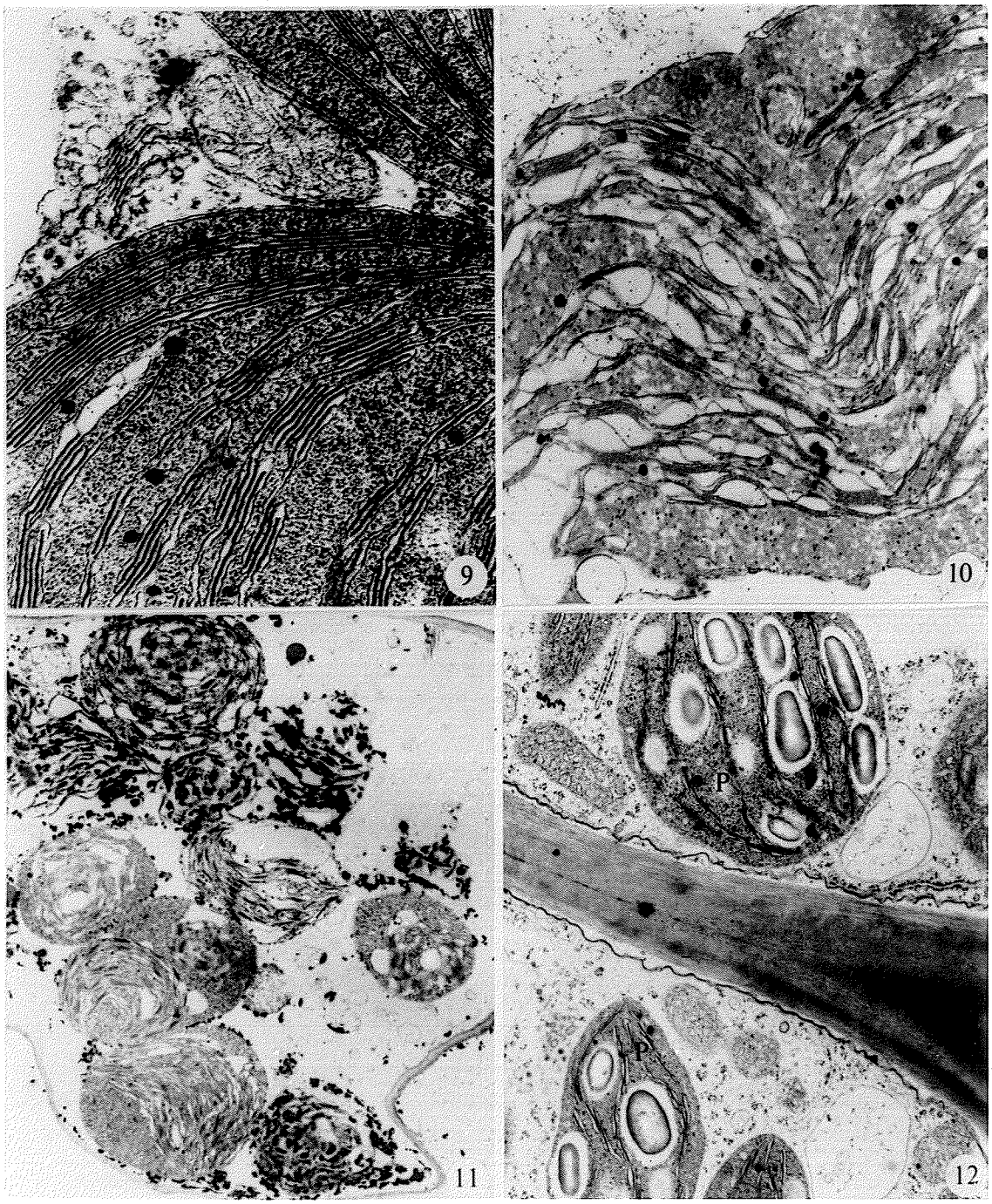
Figures 9-12. Electron micrographs of control and fluoride treated guard and mesophyll cells.

Figure 9. A chloroplast in a mesophyll cell of a control plant. A mitochondrion and dictyosome are also visible. Ag, poststained with U/Pb, X44220

Figure 10. A chloroplast in a fluoride treated (15 µg) seedling. Note dilation and undulation of thylakoids. Ag, X29130

Figure 11. Collapsed mesophyll cell after 15 µg treatment. U/Pb, X6400

Figure 12. Guard cell of a control plant. Note plastids filled with starch grains and an undulating appearance of the plasmalemma. U/Pb, X16750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appear to be affected by fluoride in early stages of injury. At later stages of injury the appearance of chromatin clumps in the nuclei was observed. Finally, cellular integrity was lost and the cells collapsed (Fig.11).

Guard cells:

All guard cells examined in control plants contained many plastids with numerous starch grains (Fig.12). The plasmalemma had an undulating appearance with granular material deposited between the membrane and cell wall. Both fluoride-treated and control plants contained numerous mitochondria, dictyosomes, and rough ER (Figs.12,13,14). The guard cells always contained several lipid bodies, usually not attached to any membranes.

The structure of the guard cells of 3 µg fluoride-treated seedlings did not differ from those of controls. Guard cells of 15 µg fluoride-treated plants were frequently found adjacent to collapsed mesophyll cells (Fig.13). Their plastids contained, at most, only a few small starch grains. The plasmalemma appeared smooth with a few or no invaginations, cup-shaped dictyosomes were frequently observed, and aggregations of chromatin were common (Fig.14). No increase in numbers of lipid bodies like those in mesophyll cells was observed.

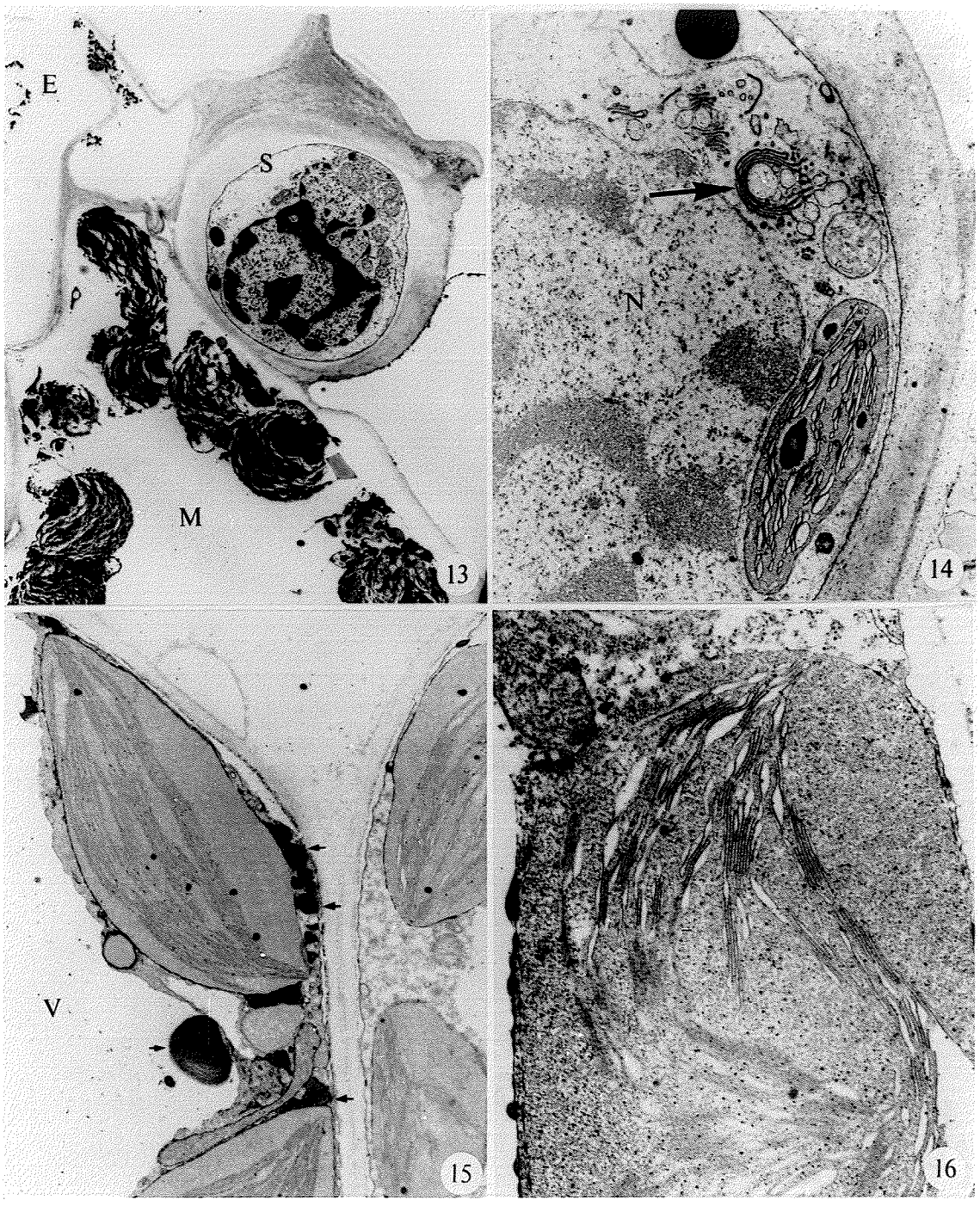
Figures 13-16. Electron micrographs of cotyledons in fluoride and drought stressed seedlings.

Figure 13. Guard cell and adjacent collapsed mesophyll cell in a 15 µg treated seedling. Note aggregation of chromatin. U/Pb, X4270

Figure 14. Guard cell of a 15 µg treated seedling. Note cup shaped dictyosome (arrow), smooth plasmalemma, and plastid with a few small starch grains. Ag, X16750

Figure 15. Lipid bodies (arrows) in a wilting, drought stressed seedling. Note lack of starch in chloroplasts. Ag, X16750

Figure 16. Twisted and dilated thylakoids in a chloroplast of a water stressed seedling. U/Pb, X29130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Water stress injuries

Plants subjected to drought suffered various injuries to their mesophyll cells. The general pattern of damage was similar to that caused by fluoride. One of the earliest noticeable changes was plasmolysis and the appearance of lipid bodies in the cytoplasm (Fig.15). These bodies, like those in the fluoride-treated seedlings, were often attached to cytoplasmic membranes. Many chloroplasts were found with twisted and dilated thylakoids (Fig.16). Water stress, unlike the fluoride-induced stress, did not result in an accumulation of starch in the chloroplasts.

No alterations of guard cell ultrastructure were seen, and as expected, all guard cells contained numerous starch grains.

DISCUSSION

Fluoride affects plants at various levels of their organization. At present, it is believed that the initial injury takes place at the biochemical level and results in structural alterations (Malhotra & Khan, 1984). Both biochemical and structural alterations can result in reduction of growth and yield. Plant membranes have been implicated as sites of fluoride damage (Miller & Miller, 1974; Ramagopal et al.,1969; Wei, 1972). The appearance of lipids in the cytoplasm of mesophyll cells after fluoride treatments in the present study could be a result of membrane breakdown. The appearance of this lipid material was correlated with a decrease in phospholipids and an increase in neutral lipid levels in fluoride-treated seedlings (Chapter I). Increased solute leakage from these plants was also observed at that time. Structures similar to the lipid bodies observed in this study were seen in needles of Norway spruce grown in the vicinity of a pulp mill in Finland (Soikkeli & Tuovinen, 1979). Lipid material was also detected in the cytoplasm of HF-fumigated soybeans during later stages of cell injury (Wei, 1972). Although the lipid bodies could be seen in the early stages of fluoride injury they may be a result of water stress. During the early stages of drought injury, similar lipid material was also deposited in the cytoplasm of stressed Jack pine seedlings. Similar findings were reported by Giles et al. (1974), and Nir et al. (1969). Degrada-

tion of membrane phospholipids occurs in senescing and stressed tissues. There is both electron microscopic and biochemical evidence to confirm extensive membrane degradation immediately upon wounding in storage tissues (Jackman & Van Steveninck, 1967; Theologis & Laties, 1981). This degradation has been associated with a change in respiratory substrate from glycolytic and TCA cycle intermediates to lipid (Robinson, 1985). It is possible that a similar process occurs in fluoride treated plants. Fluoride is a potent inhibitor of many enzyme systems. Enzymes of the glycolytic pathway such as enolase (Miller, 1958; Maurer & Nowak, 1981), hexokinase (Melchior & Melchior, 1956), and phosphoglucomutase (Yang & Miller, 1963b) have been shown to be strongly inhibited by fluoride. The inhibition of enolase or other glycolytic enzymes could force the seedlings to use alternative pathways and substrates other than carbohydrates. The observations of starch accumulation in chloroplasts of fluoride-treated Jack pine seedlings (Figs.5,8) indicate altered carbohydrate metabolism. Disturbances of carbohydrate metabolism in fluoride-treated plants were also reported by Adams & Emerson (1961), Weinstein (1961), Yang & Miller (1963b), and Garrec et al. (1981). There is no general agreement, however, concerning the cause of these changes.

Drought and fluoride-induced stresses resulted in damage to chloroplasts. Similar patterns were observed after sub-

jecting the seedlings to either of the above stresses. Displaced, undulating and dilated thylakoids were often observed. Such injuries are commonly found in plants subjected to various stresses. Curled thylakoids were attributed by Soikkeli & Touvinen (1979) to fluoride and/or frost. Similar injuries occurred in Jack pine seedlings subjected to drought. Thus the cause of this type of injury is uncertain.

Guard cells were more resistant to fluoride than mesophyll cells. After 15 µg treatments, however, signs of senescence and dehydration such as plasmolysis and aggreagation of chromatin were frequently observed. At no time was there any increase in the number of lipid bodies. Several factors may have contributed to the unusual opening of stomates in fluoride-treated plants, such as the frequently observed collapse of neighbouring mesophyll cells. This might result in turgor changes between guard and adjacent cells and thereby affect stomatal movements. Such collapse might also make fewer assimilates available for the guard cells. All direct and indirect fluoride injuries to guard cells could result in metabolic changes expressed as a depletion of starch in guard cell plastids and the appearance of cup-shaped dictyosomes. According to Robinson and Kristen (1982) cup-shaped dictyosomes may be indicative of energy depletion in cells treated with various metabolic inhibitors.

The mode of action of fluoride on plants is complex. Conclusions must be carefully made so that initial and secondary events can be separated. Osmotic imbalance is without doubt a major contributory factor in fluoride-induced plant injuries. However, more biochemical and physiological studies must follow in order to understand the step by step cause-effect interactions. Investigations of respiration and possible changes in the carbohydrate and organic acid metabolism are necessary to explain the cytological alterations observed in fluoride-treated plants.

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CHAPTER III

SODIUM FLUORIDE-INDUCED METABOLIC CHANGES IN JACK PINE (*PINUS BANKSIANA* LAMB.) SEEDLINGS. I. THE EFFECT ON RESPIRATION, PHOTOSYNTHESIS, WATER CONTENT, AND CARBOHYDRATE POOLS

ABSTRACT

Dark respiration, photosynthesis, water content, and carbohydrate pools were examined in Jack pine seedlings treated for one to seven days with sodium fluoride. When compared with controls, both respiratory rates and the respiratory quotient decreased in plants treated for one day. Photosynthetic rates declined in plants exposed to fluoride for one, two, and four days. Recovery was observed in seedlings treated for seven days. A decrease in water content was noted as early as one day after the exposure of plants to fluoride and seedlings treated for longer periods continued to contain less water than control plants. Various effects of sodium fluoride on carbohydrates were observed. Inositol accumulated in plants treated for one day. Glucose and fructose increased in plants exposed to fluoride for one, two, and four days; and decreased in seven day treated plants. Levels of starch declined in all treated plants with the exception of the two day treatment. Sucrose was also initially present in lower amounts in fluoride treated seedlings. Plants treated for seven days and controls contained similar levels of sucrose. The possibility of water stress participation in the metabolic disturbances exhibited by fluoride-treated Jack pine seedlings is discussed.

INTRODUCTION

Contradictory reports of the effects of fluoride on plant metabolism are found in the literature. Some authors report that fluoride affects growth and yield of plants adversely (Pack, 1966; Garrec & Audigier, 1981; Taylor & Basabe, 1984). Other studies showed that fluoride had no effect on growth (Hitchcock et al., 1964; Pack & Wilson, 1967), or that growth was stimulated (Brewer et al., 1967; MacLean et al., 1976). Fluoride is a potent inhibitor of many respiratory enzymes. Both stimulatory and inhibitory effects of fluoride on the rates of respiration in plants have been reported (Applegate & Adams, 1960a; McNulty & Lords, 1960; McLaughlin & Barnes, 1975). There is no general agreement concerning the involvement of fluoride in carbohydrate metabolism. Accumulation of starch in chloroplasts of mesophyll cells of Jack pine seedlings was observed when plants were subjected to sodium fluoride treatments (Chapter II). This suggested that either a delicate balance between the synthetic and degradative processes was altered by fluoride, or that metabolites were not transported out of mesophyll cells. An increase, decrease, and no changes in levels of various carbohydrates have been reported (Adams & Emerson, 1961; Weinstein, 1971; Yang & Miller, 1963a; Pack, 1971; Garrec et al., 1981).

The effect of fluoride on the water status of plants has not been thoroughly studied, but fluoride is capable of altering plant water potential (Poovaiach & Wiebe, 1973). Wilting in response to fluoride treatments has been frequently observed (Leone et al., 1948; Brewer et al., 1959; MacLean et al., 1968). Wilting of Jack pine seedlings subjected to sodium fluoride was the first observed sign of fluoride injury (Chapter II). If fluoride treated plants suffer from water stress it is possible that some of the metabolic alterations observed in them may be caused by low plant water potential rather than by a direct involvement of fluoride in plant metabolism. This study examines how different durations of sodium fluoride exposure influence respiration, photosynthesis, carbohydrate pools, and water content of Jack pine seedlings. It also discusses the hypothesis that the conditions of water stress created in plants by fluoride are co-responsible for some of the observed metabolic alterations.

METHODS

Growth conditions

Jack pine seeds were germinated on moist filter paper. Two days after germination, seedlings uniform in length were selected and planted in pots (10 per pot) containing 650 g d. wt. of demineralized sand. The seedlings were initially provided with 130 mL of 3/4 strength Hoagland's mineral solution. The pots were placed in a growth cabinet maintained at 22 C, and an 18 h light / 6 h dark regime (light intensity of 120 $\mu E\ m^{-2}s^{-1}$). Seedlings were watered daily with deionized water. When two weeks old they were divided into a control group and an experimental group. Plants of the latter group were treated with sodium fluoride which was added in solution to the sand to attain an initial concentration of 3 $\mu g\ F\ g^{-1}$ d.wt. of sand. Seedlings were harvested 24, 48, 91, and 168 hours after treatment. Control and fluoride treated seedlings were always collected at the same time.

Gas exchange

Photosynthesis and dark respiration were measured in a Warburg respirometer (B.Braun Helsungen AG.). The uptake of oxygen and release of respiratory CO_2 were measured using the "direct method" (Umbreit et al., 1964). Harvested see-

dlings were divided into groups of four or five and weighed. Each group was placed into a respirometer vessel containing 50 mM phosphate buffer (pH 5.5). Either 10% KOH or water was added to the center well, the vessels were wrapped in aluminum foil and placed in a water bath at 25 C for 20 min. After that time manometer readings were taken every 15 min for 1 hour, means calculated, and converted to µL O_2 $h^{-1}g^{-1}$ d.wt. tissue. Each treatment had four replicates, and the entire experiment was repeated to give a sample size of 8.

The evolution of photosynthetic O_2 was measured using the "one vessel method" (Umbreit et al., 1964). Sodium fluoride-treated and control seedlings were again divided into groups of 4 or 5, weighed, and placed into the respirometer vessels containing 100 mM carbonate-bicarbonate mixture of pH 8.7. The vessels were immersed in a water bath maintained at 25 C during the experiment and illuminated with incandescent lamps (light intensity of 475 µE m^{-1} s^{-1} at the vessel level). Manometer readings were taken every 10 min for 30 min. Corrections for the respiratory uptake of O_2 were made by wrapping the vessels in aluminum foil and taking three more readings every 10 min. The means of all readings were converted to µl O_2 $h^{-1}g^{-1}$ d.wt. tissue. The experiment was repeated to give a sample size of 8.

The gas exchange data were analysed using Scheffe's test, an analysis of variance procedure.

Carbohydrate analysis

a) Starch

Starch content in fluoride-treated and untreated seedlings was examined using the anthrone method (Yemm and Willis, 1954). Jack pine seedlings were oven dried and pulverized. Samples weighing 100 mg were dispensed into conical flasks and stirred overnight at room temperature with 30 mL of deionized water. The mixtures were filtered and the residues were washed three times with deionized water. The residues containing starch were returned to the flasks and placed in an ice bath. Starch was hydrolysed for 40 min with 20 mL of 35% perchloric acid (Madsen, 1968). After hydrolysis the samples were filtered and the residues washed 5 times with deionized water. Each filtrate was made up to 100 mL with deionized water and well shaken before 1 mL was added to 5 mL of anthrone reagent. The mixture was shaken and placed in a boiling water bath for 12 min. After cooling, absorbance was measured at 625 nm against blank samples (5 mL of anthrone reagent + 1 mL of water) in a LKB Biochrom Ultrospec Spectrophotometer. The concentrations of hydrolysed starch were read from a standard curve prepared from the solutions of α-glucose. At least three readings of each sample were taken, each sample was replicated twice, and the entire experiment was repeated once (n=4).

b) Ethanol-soluble carbohydrates

Sucrose, glucose, fructose, and inositol were analysed by means of gas chromatography. Fresh plants were divided into groups (each weighing 1.5g), cut with scissors into small fragments, and placed in beakers. Sorbitol, which served as an internal standard, was added to each beaker followed by 40 ml of hot 95% ethanol, and the mixtures were boiled for 5 min. Five mL of deionized water was added to each cooled extract and the tissue was ground in a mortar with sand. Extracts were filtered and the residues extracted three more times with 80% ethanol. Filtrates were combined, condensed in vacuo to about 10 mL, and passed through a 2.5 X 10 cm cation exchange column containing Dowex 50W-X8 (100-200 mesh) in a hydrogen form. The column was washed with 250 mL of deionized water and the collected effluent was reduced to a small volume and applied to a 2.5 X 10 cm Bio-Rad AG1-X8 (200-400 mesh) anion exchange column in a formate form. The column was washed with 250 mL of water, the effluent condensed and transferred to small vials where water was evaporated under nitrogen. The residue containing carbohydrates was azeotropically dried with iso-propanol and left overnight in a dessicator over silica gel. Trimethylsilyl (TMS) derivatives of carbohydrates were prepared by dissolving the residues in 1 mL of pyridine, and adding 0.2 mL of trimethylchlorosilane followed by 0.4 mL of hexamethyldisilazane. After 20 to 26 hours TMS ethers of carbohydrates were analysed using a Carlo Erba Fractovap Gas Chromatograph equipped

with a Hewlett Packard 3390A Electronic Integrator. Samples of 0.5 to 2.0 µL were injected into a 1.8 m long, 2 mm internal diameter glass column packed with 3% OV-3 on 80/100 mesh Chromosorb W. Conditions during the runs were: oven temperature, 180 C held for 25 min, rapidly increased to 240 C and held at this temperature for 20 min; injector temperature, 250 C; carrier (nitrogen) flow, 40 mL min^{-1}. Carbohydrates were identified by co-chromatography with original carbohydrate standards. To aid identification and eliminate unknown contaminants obscuring fructose the samples were also analysed on a 3 m long, 2 mm ID column containing a higher polarity stationary phase (GP3% SP-2310/2%SP-2300 on 100/120 mesh Chromosorb W AW). Oven temperature during the runs was 145 C; injector and detector temperature, 200 C; nitrogen flow, 30 mL min^{-1}. Estimated detector response factors (Holligan & Drew, 1971) for equivalent weights of carbohydrates were used to quantify carbohydrates. Stereoisomers of inositol and inositol methyl ethers (such as pinitol and sequoitol) could not be quantitatively separated and will be referred to as inositol. Three samples of fluoride-treated and control plants were analysed in three repeated experiments. All reagents and standards for carbohydrate analysis except pinitol and sequoitol were purchased from Sigma. Samples of pinitol and sequoitol were generously provided by R.Binder, USDA, Albany, California.

Water content

For the determination of water content, sodium fluoride-treated and control seedlings were divided into groups of four or five, weighed, placed in a drying oven at 80 C for 48 hours, and weighed again. The experiment was repeated four times, each sample had four replicates (n=16). The results are expressed as percentages of fresh weight of seedlings. Analysis of variance procedure (Scheffe's test) was used to determine statistically significant differences.

RESULTS

Visible injuries

Visible signs of fluoride injury appeared in Jack pine seedlings about 27 hours after sodium fluoride treatment when approximately 5% of the seedlings began to wilt. All wilting plants collapsed shortly thereatfer. Many other seedlings showed the first signs of leaf tip necrosis on the third day of the treatment. Cotyledons were usually affected earlier than protruding juvenile leaves but on many occasions tip necrosis was observed only on juvenile leaves.

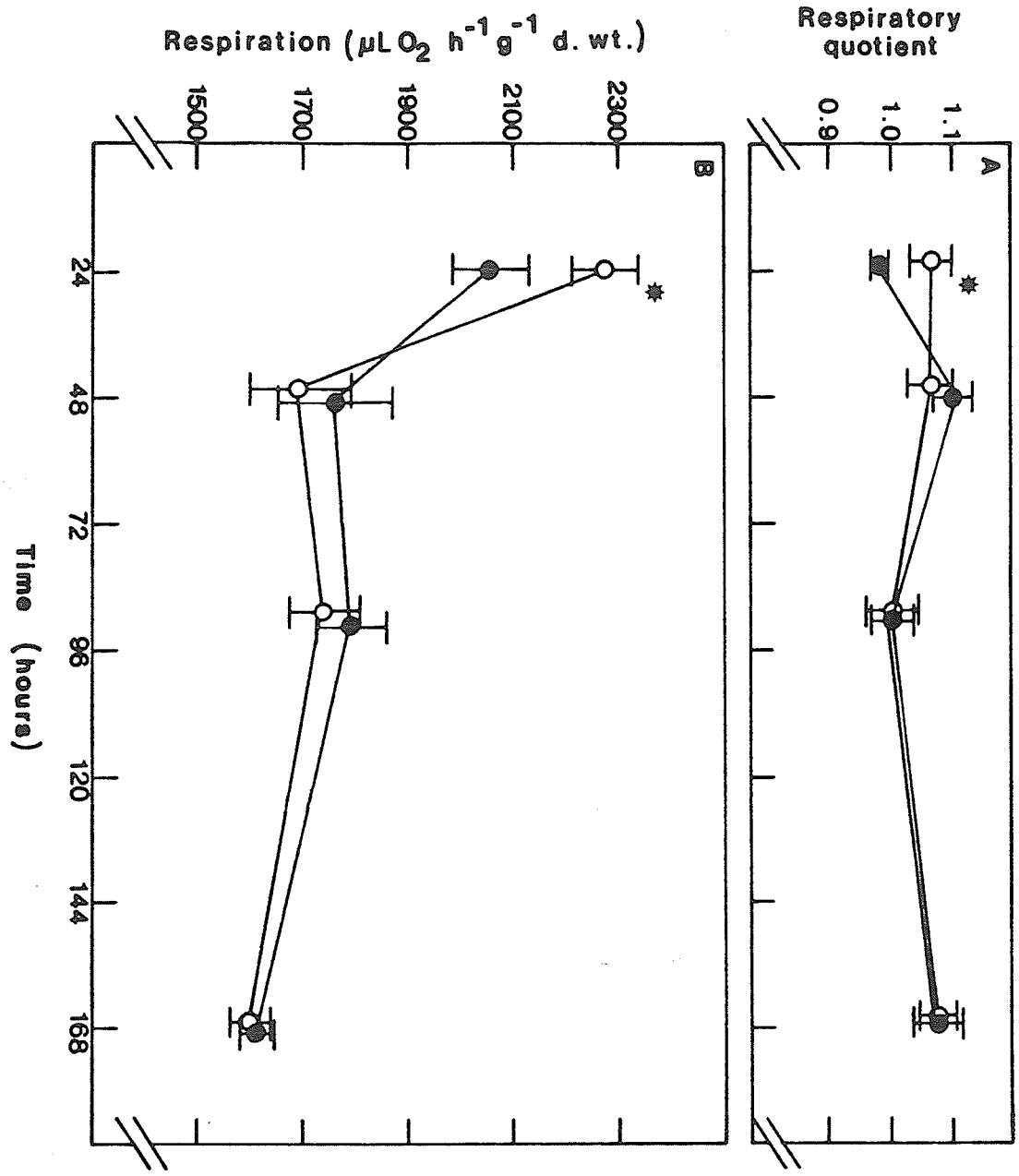
Gas exchange

Dark respiration rates and the respiratory quotient (RQ) values of fluoride-treated and control seedlings (Fig.1) show a decrease in the respiratory rates in plants with time. This could be attributed to the developmental changes taking place in seedlings. When the respiratory rates and the RQ values of control and fluoride-treated seedlings were compared at different times, only the 24-hour fluoride treatment significantly lowered both parameters. In these seedlings respiration was inhibited by approximately 10% compared with the controls. The RQ value of 24-hour treated seedlings was also lower than that of control plants by almost 10%. Treatments of longer duration than 24 hours did

Figure 1. The effects of sodium fluoride treatments on the respiratory quotients (A) and respiratory rates (B) of Jack pine seedlings. Each value is the mean of 8 replicates (±SE). Asterisks indicate significant difference at the 0.05 level.

(○) - Control

(●) - 3 μg fluoride treatment


Respiratory quotient
0.9
1.0
1.1
A
Respiration (μL O2 h-1 g-1 d. wt.)
1500
1700
1900
2100
2300
B
24
48
72
96
120
144
168
Time (hours)

not substantially alter the rates of oxygen uptake even though many plants at this stage showed signs of necrotic lesions.

The rates of photosynthetic release of oxygen seemed to be affected by sodium fluoride more than the process of respiration (Fig.2). The lowest rates of photosynthesis were observed in plants treated with sodium fluoride for 48 hours. Compared with controls the rate of photosynthetic oxygen evolution by these plants was reduced by about 20%. Slight recovery was noticed in plants treated for 91 hours. At that time, however, the rates of photosynthetic oxygen release from the fluoride-treated seedlings were still significantly lower than those of control plants. Full recovery was observed after 168 hours. Despite the tissue damage (leaf tip necrosis) seen in these plants the rates of photosynthesis were not altered. Thus, the reduction in the photosynthetic activity in Jack pine seedlings treated with fluoride can probably be attributed to factors other than leaf necrosis.

Water stress

The decrease in photosynthetic activity was observed in fluoride treated plants at the time when rapid loss of water occurred (Fig.3). Fluoride treated Jack pine seedlings contained less water than control plants after only 24 fours of the sodium fluoride treatment. However, plants treated for 48 hours were more affected. They contained 77% water

Figure 2. Photosynthetic rates in Jack pine seedlings exposed to 3 µg fluoride treatment (●) and in control plants (○). The means of 8 replicates (±SE) are indicated. Asterisks indicate significant difference at the 0.05 level.

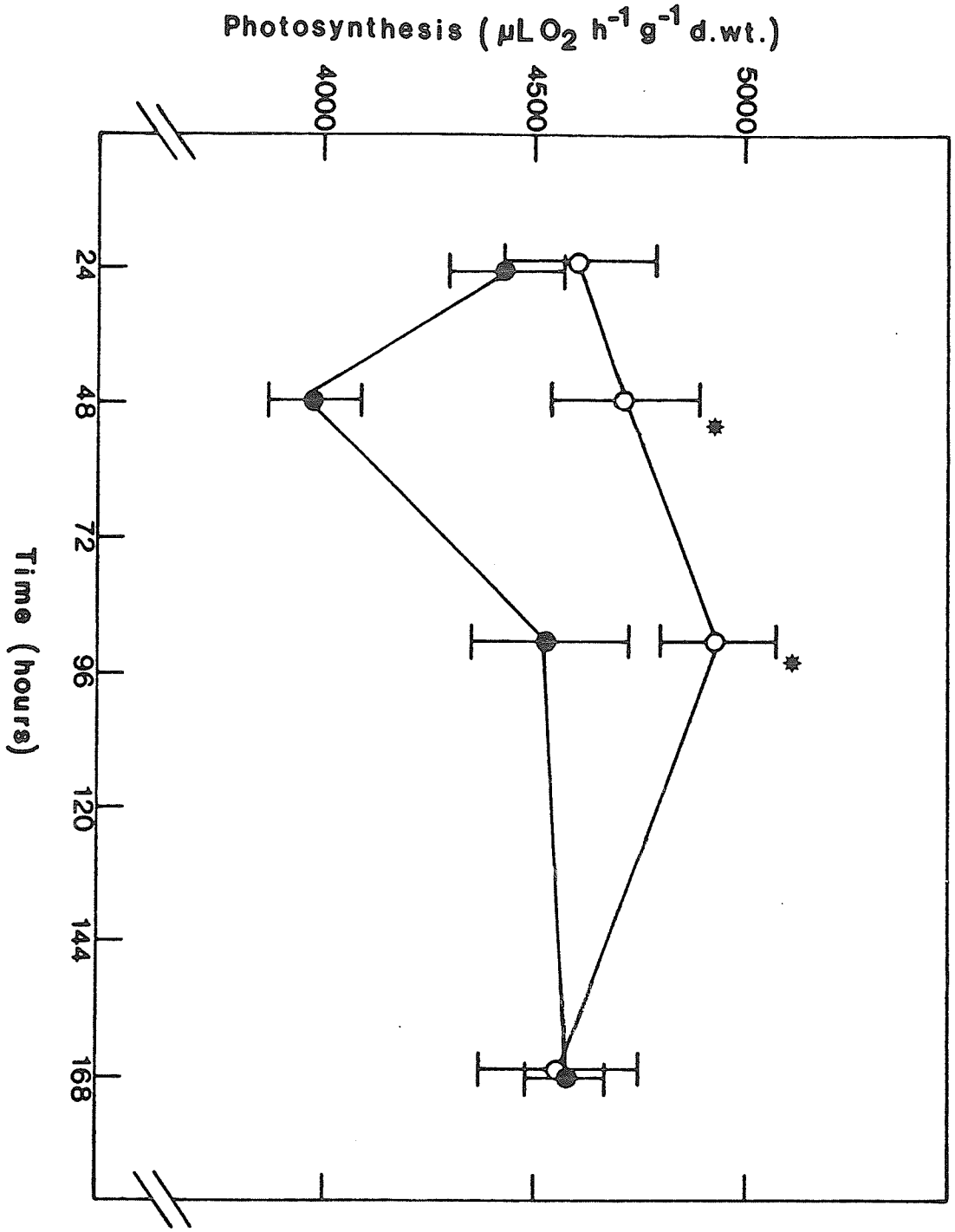
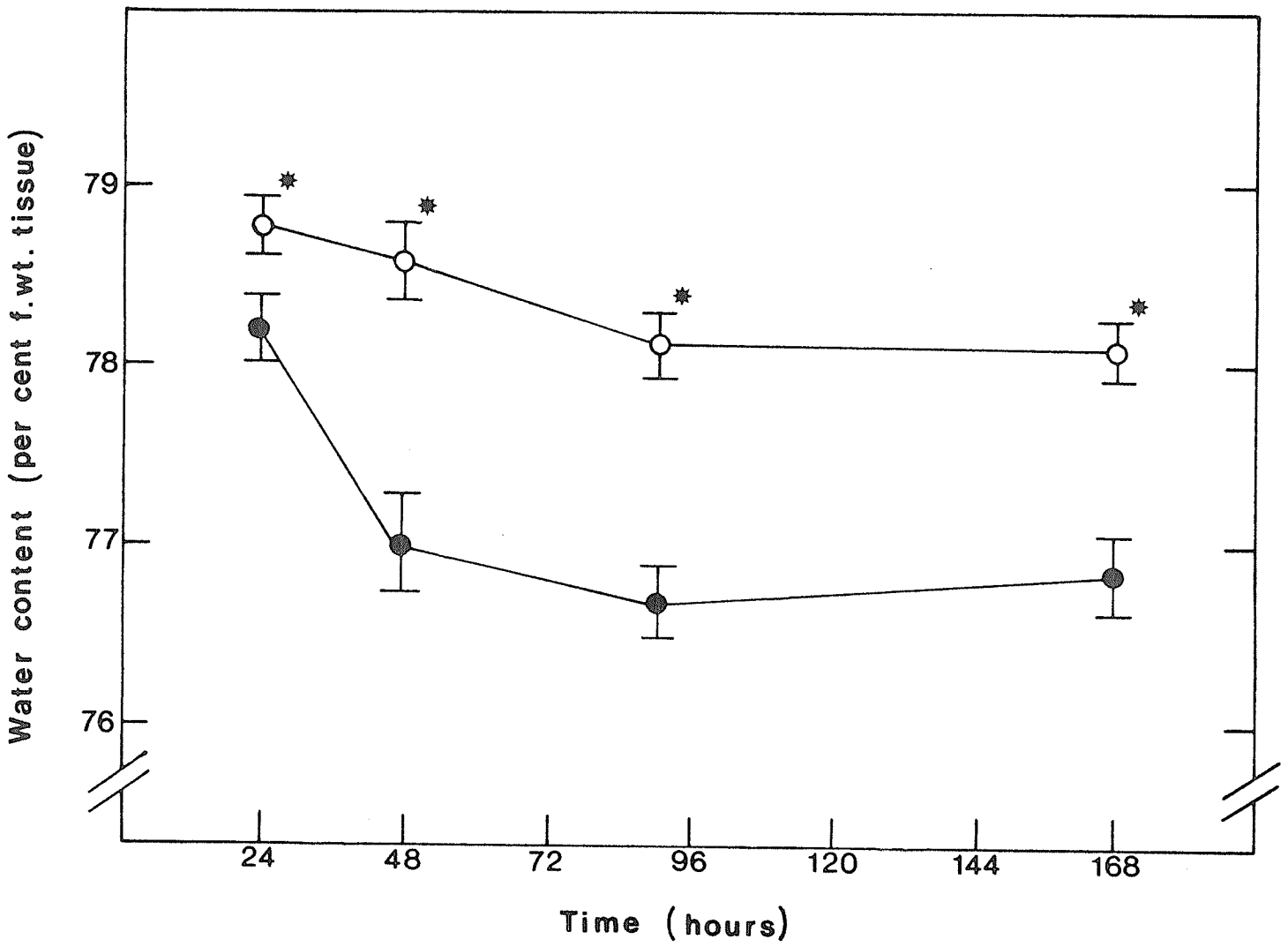

Photosynthesis (μL O_2 h^{-1} g^{-1} d.wt.)
4000
4500
5000
Time (hours)
24
48
72
96
120
144
168

Figure 3. Water content in control seedlings (○) and in plants exposed to 3 µg fluoride treatment (●). The means (±SE) of 16 replicates are shown. Asterisks indicate significant difference at the 0.05 level.


Water content (per cent f.wt. tissue)
79
78
77
76
24
48
72
96
120
144
168
Time (hours)

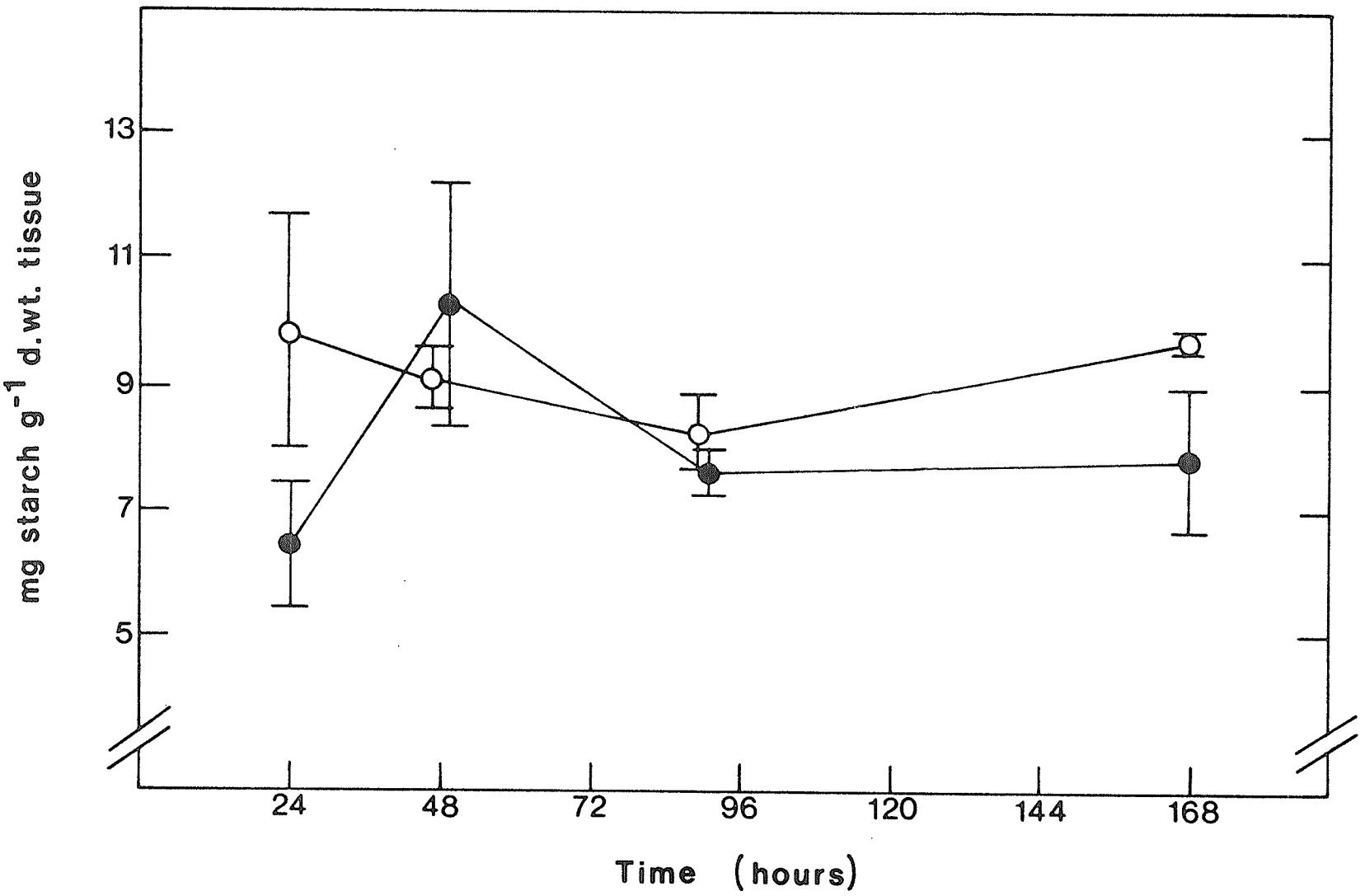
compared with 78.6% of fresh weight measured in control seedlings. Slightly smaller changes were recorded 91 hours and 168 hours after treatment. The decrease in water content could be partly explained by the presence of necrotic, desiccated tissues in plants treated for 91 hours or longer. However, leaf tip necrosis was not very extensive and it probably did not fully account for the decrease in water content of plants (Fig.3). Leaf tip necrosis progressed with time but water content did not decline. Thus, the decrease in water content must have been reflected by lower water potential of living tissues.

Carbohydrates

Changes in carbohydrate levels with time were observed in control plants. These changes were probably due to developmental changes taking place in the seedlings. In addition, interconversions of carbohydrates may have occurred. Therefore, the levels of carbohydrates in fluoride-treated seedlings were always compared with those of control plants harvested at the same time.

Sodium fluoride altered the composition of carbohydrates in Jack pine seedlings. Plants treated for 24 hours (Fig.4) contained an average of 35% less starch than controls. Treatments of a longer duration had less impact on starch

Figure 4. The content of starch in 3 µg fluoride treated (●) and control (○) Jack pine seedlings. The means (±SE) of 4 replicates (2 repeated experiments) are shown.


mg starch g^{-1} d.wt. tissue
13
11
9
7
5
24
48
72
96
120
144
168
Time (hours)

levels. An increase in starch above the control level was observed in plants treated for 48 hours. Starch levels declined again in plants treated for 91 and 168 hours.

More definite changes were observed in sucrose levels of plants treated with sodium fluoride for 24, 48, and 91 hours (Fig.5). All these treatments resulted in a 25% to 30% lower level of sucrose. Controls and seedlings subjected to the 1 week fluoride treatment did not differ in the amount of sucrose.

Glucose metabolism was also altered by fluoride (Fig.6). An almost two-fold increase in glucose was observed in plants treated with sodium fluoride for 24 fours compared with the controls. This difference gradually declined with time. Fluoride treatments of 1 week duration resulted in similar glucose levels to those found in control plants.

Changes in fructose levels in treated plants were less pronounced (Fig.7), but small increases were observed in seedlings treated for 24, 48, and 91 hours. Fluoride treatment of a longer duration resulted in a more definite change. Plants treated for one week contained only 70% of the amount of fructose present in control seedlings.

Inositol levels in control seedlings declined with time.

Figure 5. The effect of 3 µg fluoride treatment on sucrose levels in Jack pine seedlings. Data points are means of 3 repeated experiments (±SE).

(○) - Control

(●) - 3 µg fluoride treatment

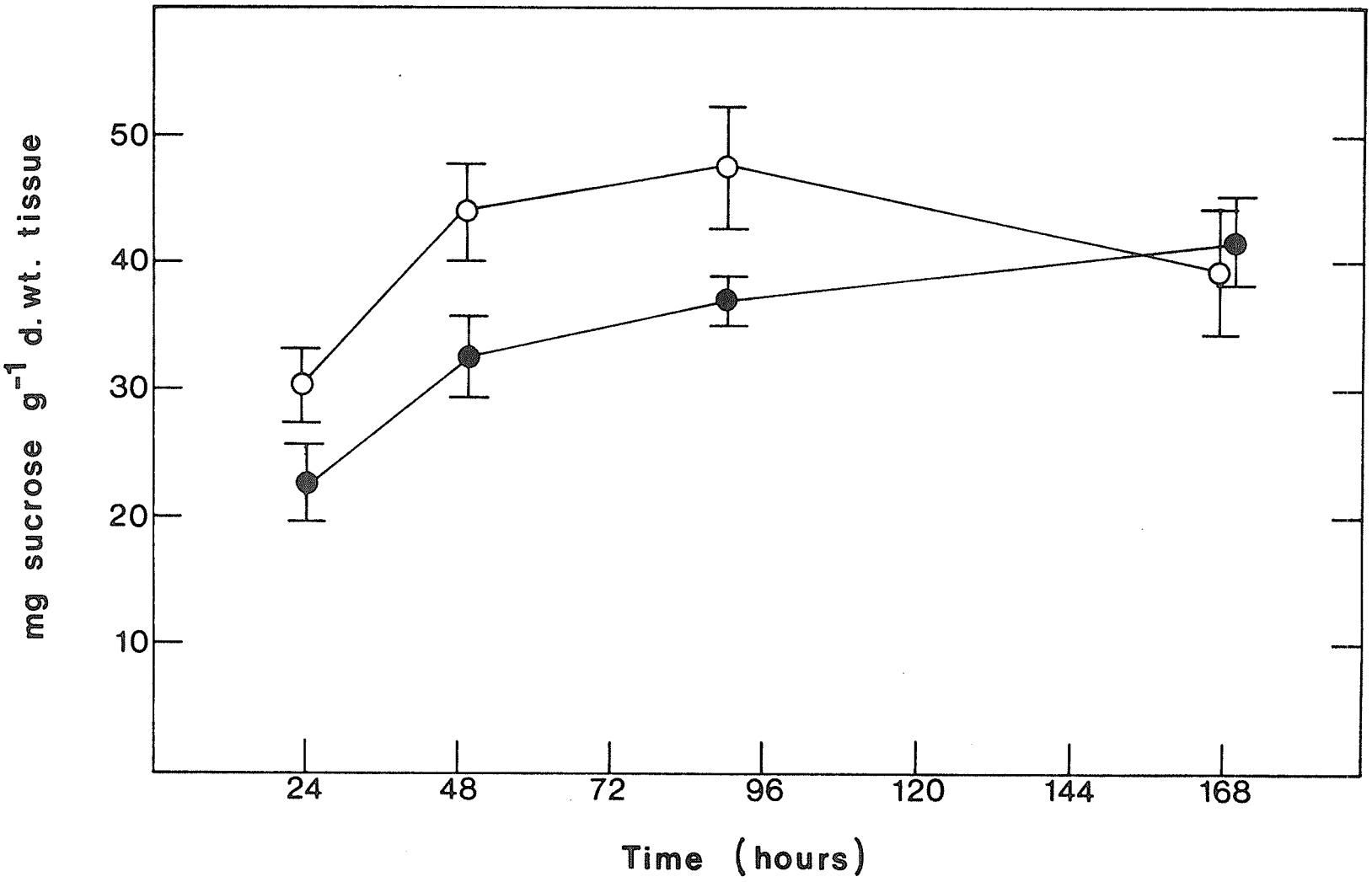

mg sucrose g^{-1} d. wt. tissue
50
40
30
20
10
24
48
72
96
120
144
168
Time (hours)

Figure 6. Glucose levels in Jack pine seedlings exposed to 3 µg fluoride treatment (●) and controls (○). Data points are means (±SE) of 3 repeated experiments.

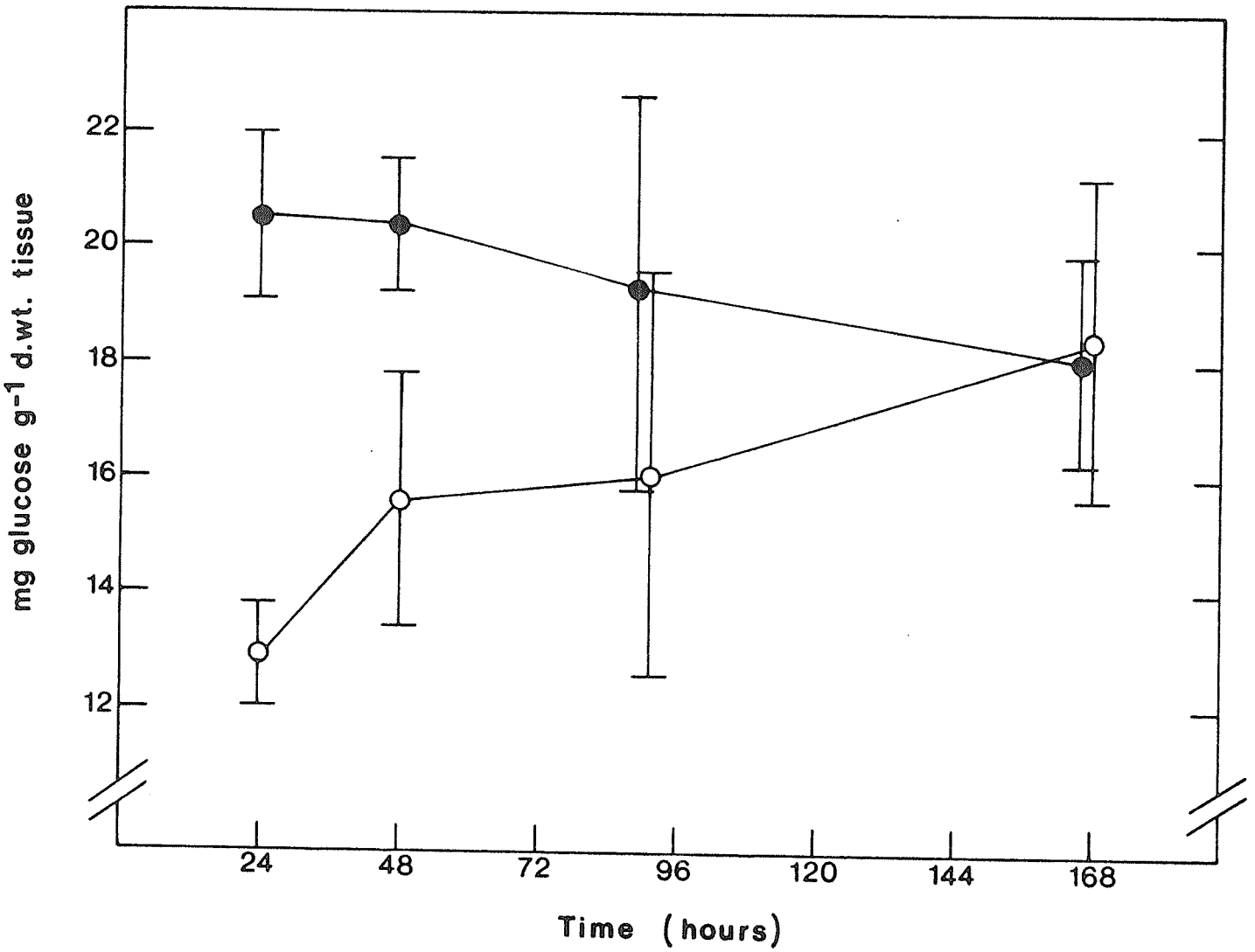
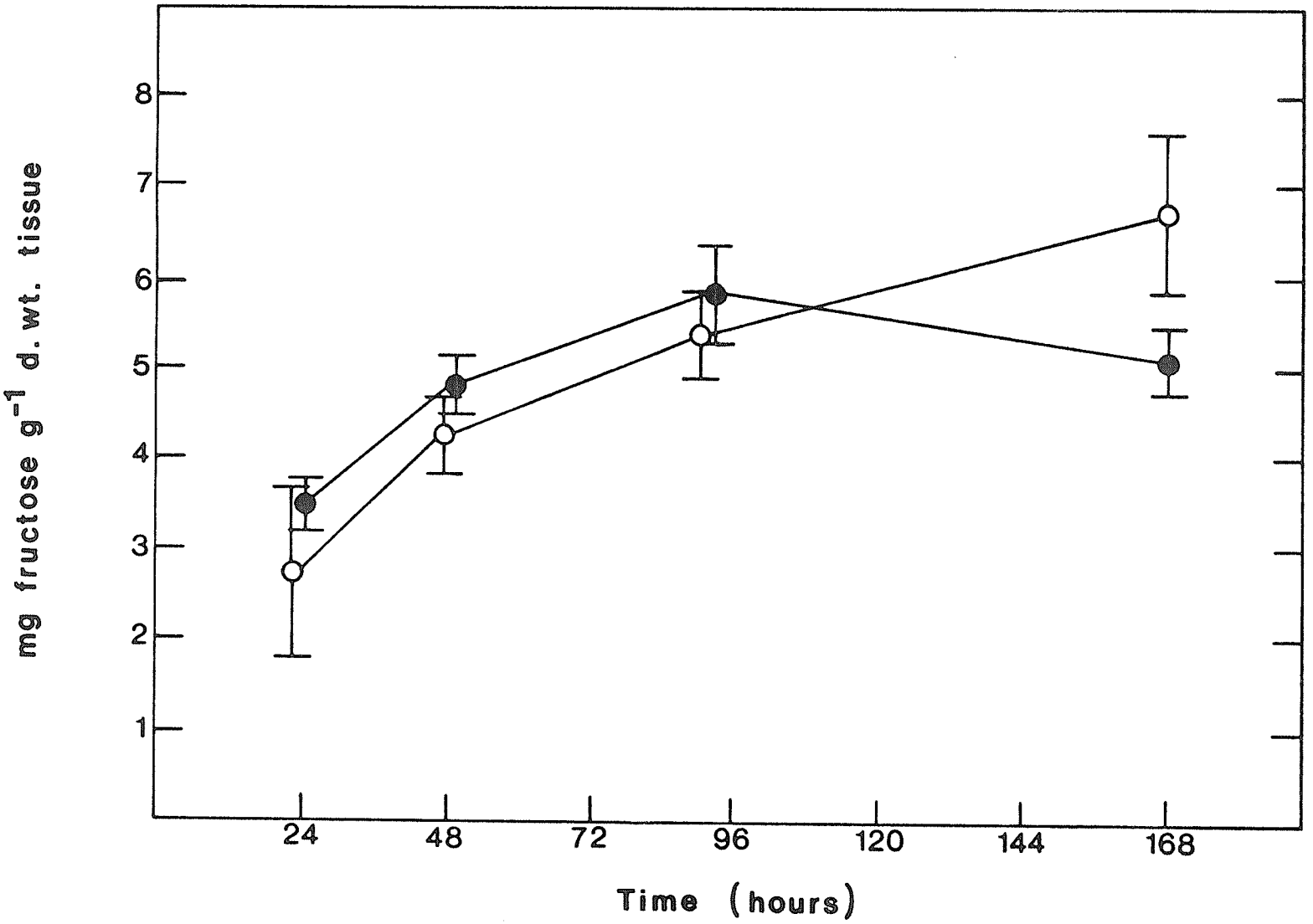

mg glucose g-1 d.wt. tissue
22
20
18
16
14
12
24
48
72
96
120
144
168
Time (hours)

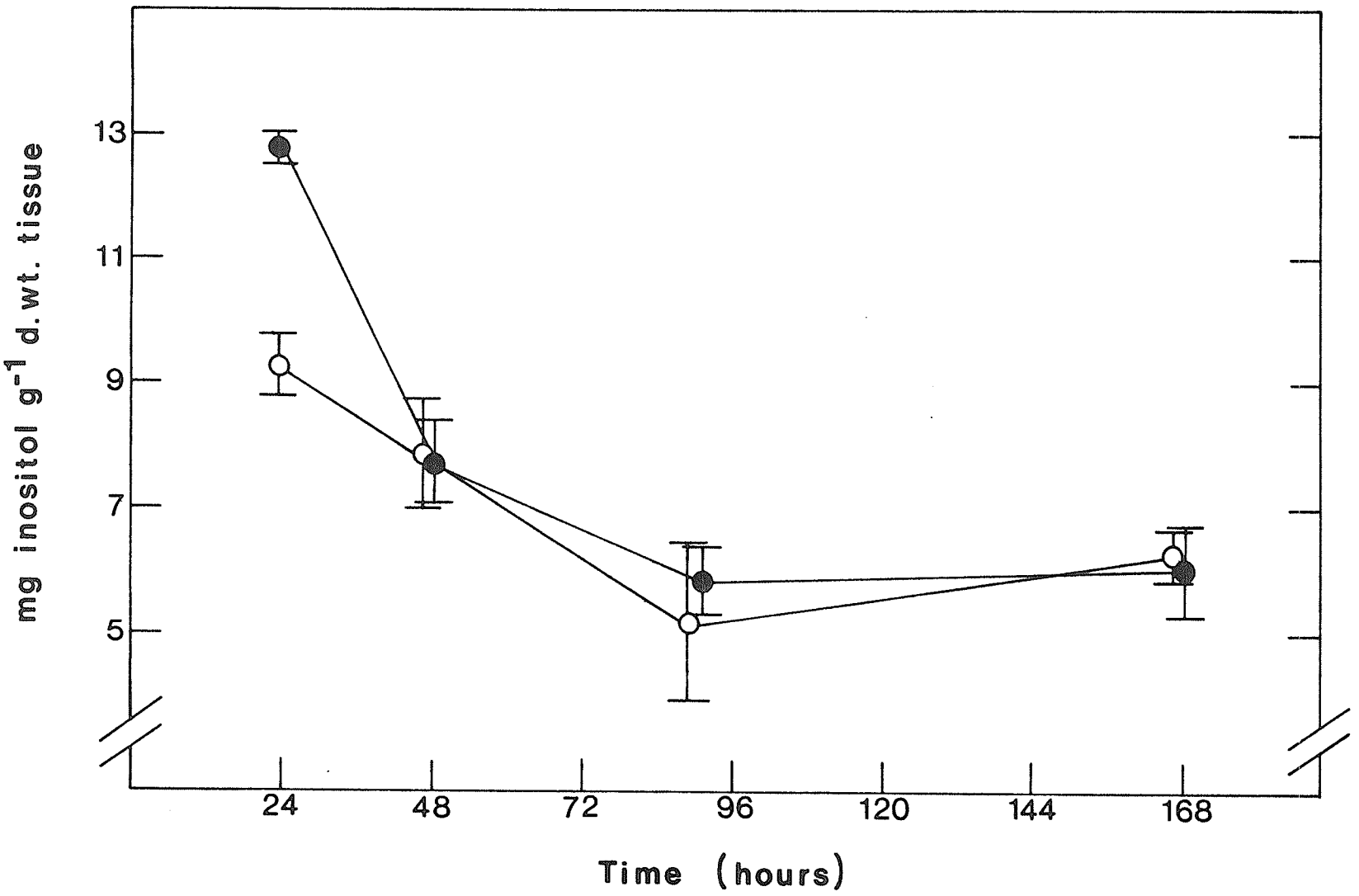
Figure 7. Fructose in Jack pines subjected to 3 µg fluoride treatment (●) and in control plants (○). Data points are means of 3 repeated experiments (±SE).


mg fructose g^{-1} d. wt. tissue
8
7
6
5
4
3
2
1
24
48
72
96
120
144
168
Time (hours)

This was probably due to the conversion of cyclitols into other carbohydrates, such as glucuronic acid, required for the formation of cell wall polysaccharides and into phosphatidyl inositol, needed for the formation of cell membranes by rapidly growing plants (Loewus & Dickinson, 1982).

Sodium fluoride had a transient effect on levels of inositol. Short duration, 24-hour sodium fluoride treatments resulted in an almost 50% increase in the content of inositol (Fig.8). Treatments of a longer duration did not seem to have any effect on levels of these carbohydrates.

Figure 8. Inositol content (including methyl ethers of inositol) in 3 µg fluoride-treated (●) and control (○) Jack pines. Data points are means of 3 repeated experiments (±SE).


mg inositol g-1 d.wt. tissue
13
11
9
7
5
24
48
72
96
120
144
168
Time (hours)

DISCUSSION

Many contradictory reports of the effects of fluoride on plant metabolism are found in the literature. Fluoride induced changes in respiration are among the best documented physiological effects. At the same time there is no general agreement as to the mechanism of fluoride's involvement in respiration. Both stimulatory and inhibitory effects have been reported (Treshow, 1971). The concentration of fluoride and plant species are thought to be factors determining the type of response (Yu & Miller, 1967). The response may also depend on other factors, such as age of tissue, duration of exposure, pH of culture medium (Yu & Miller, 1967), interactions between various mineral elements and fluoride (Applegate & Adams, 1960b; Pilet & Bejaoui, 1975), and the extent of tissue damage (Mikhajlova, 1984). Low respiratory values have often been attributed to an inhibition of the activity of oxidative enzymes, such as enolase (Miller, 1958), hexokinase (Melchior & Melchior, 1956), phosphoglucomutase (Yang & Miller, 1963b), succinic dehydrogenase (Lovelace & Miller, 1967a), and others. Few studies, however, were able to demonstrate <u>in vivo</u> inhibition of the respiratory enzymes by fluoride. The reasons for fluoride induced respiratory stimulation are even less understood. One possible explanation is an increased use of the pentose phosphate pathway (Ross et al., 1962). In the present study respiration rates of Jack pine seedlings were early affected by fluoride

treatments. The initial response was a transient decrease in dark respiration and the respiratory quotient. A lower RQ value was observed before any visible signs of fluoride damage such as wilting or necrosis. Yang & Miller (1963a) also reported a marked decrease in the RQ of fluoride fumigated soya bean leaves. This effect was seen only in intact plants before the appearance of the first signs of leaf necrosis. A decrease in the RQ suggests that plants are respiring higher proportions of lipids or proteins. It may also indicate partial oxidation of respiratory substrates, or partial retention of carbon dioxide by plant tissues (Bidwell, 1979). Accumulation of lipid material near cell membranes was among the initial changes in the mesophyll cell structure of both sodium fluoride-treated and drought stressed Jack pine seedlings (Chapter II). The appearance of these lipid bodies was correlated with a decrease in phospholipids and an increase in solute leakage from cells of sodium fluoride stressed plants (Chapter I). It is possible that these lipids may serve as temporary respiratory substrates for stressed plants before such resistance mechanisms as the pentose phosphate pathway are functional.

Mechanisms by which fluoride affects photosynthesis are not clear (Thomas & Hendricks, 1956; Bennet & Hill, 1974; Horvath, 1980; Lorenc-Plucinska & Oleksyn, 1982). Reduction in the amount of chlorophyll was observed by Gronebaum-Turck & Mahle (1976), and Mikhajlova (1984). Other studies point

to inhibition of the Hill reaction (Ballantyne, 1972) and ribulose-P_2 carboxylase/oxygenase (Parry et al., 1984) as possible reasons for photosynthetic inhibition. The rates of photosynthesis of sodium fluoride-treated Jack pine seedlings were markedly reduced before the appearance of visible fluoride damage. It is possible that fluoride caused the inhibition indirectly by affecting the water status of plants. Maximum inhibition of photosynthesis was observed when the largest decrease in plant water content occurred. Water stress can be responsible for gross metabolic disturbance in plants. Like fluoride, water stress may produce either no change, an increase, or a decline in dark respiration depending on the species (Brix, 1962), and stress severity and duration (Kaul, 1966; Slatyer, 1967; Hsiao, 1973). Photosynthesis is more sensitive than dark respiration to low leaf water potentials (Brix, 1962; Boyer, 1970). Factors such as stomatal closure (Barrs,1968; Pasternak & Wilson, 1974), leaf senescence (Clarke & Durley, 1981), inhibition of the Hill reaction (Fry, 1970; Keck & Boyer, 1974), electron transport (Keck & Boyer, 1974), and the activity of ribulose-1,5-diphosphate carboxylase (Huffaker et al., 1970; Jones, 1973) have been implicated in photosynthetic inhibition of drought stressed plants. Thus, neither fluoride nor drought appears to have a single, specific effect on photosynthetic and respiratory processes.

Accumulation of solutes is another common result of fluoride and drought stress. Levels of glucose, fructose, and inositol were elevated in sodium fluoride-treated Jack pines. The increase in monosaccharides was accompanied by a decrease in sucrose and starch, suggesting either activation of sucrose and starch hydrolysis, or inhibition of their synthesis. An increase in glucose and fructose with a simultaneous decrease in sucrose was seen in fluoride fumigated soya bean leaves (Yang & Miller, 1963a). An increase in monosaccharides and a decrease in starch in plants subjected to fluoride were also reported by Adams & Emerson (1961) and Pack (1971). Other studies indicated that fluoride induced a decrease in glucose, fructose, and sucrose (Garrec et al., 1981; Weinstein, 1961), or had no effect (Weinstein, 1961). The mechanism of interaction of fluoride with carbohydrate metabolism is not understood. Inhibition of phosphoglucomutase was demonstrated in vitro by Yang & Miller (1963b) and Ordin & Altman (1965). Other in vitro studies, however, showed the enzyme to be insensitive to fluoride (De Moura et al., 1973).

Starch degrading processes may be also altered by fluoride (Rockwood, 1919; Garrec et al., 1981). An accumulation of starch was observed in chloroplasts of sodium fluoride-treated Jack pines (Chapter II). The same, 91 hour fluoride treatment, however, resulted in only a slight decrease in the gross starch levels in seedlings (Fig.4). This suggests

that the transport of assimilates from chloroplasts may be affected by fluoride.

Accumulation of carbohydrates and inhibition of their transport is known to occur in plants subjected to water stress (Ackerson, 1981; Clarke & Durley, 1981; Jones, 1984). Monosaccharides, disaccharides, and polyhydric alcohols act as osmoregulators in dessicated tissues (Parker, 1972; Borovitzka, 1981). They may also directly protect DNA (Borovitzka, 1981) and proteins (Parker, 1972) by mimicking water molecules during the dehydration.

Accumulation of monosaccharides and inositol in fluoride-treated Jack pine seedlings indicates disruption of normal metabolism. Accumulation of carbohydrates in fluoride-treated seedlings could have a role in preventing the damage caused by tissue dehydration. However, inositol levels were elevated only in plants treated for 24 hours, thus, its role as the osmoregulator is uncertain. Reduced synthesis of cell walls and membranes is a more probable cause for the increase in inositol. It is shown in Chapter IV that initial growth reduction in fluoride-treated seedlings takes place within the first 24 hours following treatment. Levels of phosphatidyl inositol also declined in fluoride-treated seedlings (Chapter I). Fluoride treated seedlings exhibit many signs of metabolic disturbance similar to water stressed plants. It is possible that a water deficit in fluoride-

treated plants may create water stress conditions, and certain plant injuries develop not because of the initial fluoride action, but because of the secondary effects caused by tissue dehydration. Heath (1980) presented a hypothetical model of the initial events of fluoride pollution injury to plants. He suggested that osmotic imbalance is the main initial reaction in plants exposed to fluoride. When the effects of environmental stresses on plants are studied initial and secondary events can rarely be separated. There is no doubt that a water deficit develops in sodium fluoride-treated Jack pine seedlings, and it is well documented in the literature that water stress can cause many of the alterations exhibited by fluoride-treated plants.

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CHAPTER IV

SODIUM FLUORIDE-INDUCED METABOLIC CHANGES IN JACK PINE (*PINUS BANKSIANA* LAMB.) SEEDLINGS. II. THE EFFECT ON GROWTH, ACID PHOSPHATASE ACTIVITY, CYTOKININ ACTIVITY, AND POOLS OF SOLUBLE PROTEINS, AMINO ACIDS, AND ORGANIC ACIDS

ABSTRACT

The effects of sodium fluoride on growth, cytokinin activity, acid phosphatase activity, levels of soluble proteins, free amino acids, and organic acids in Jack pine seedlings were studied. After 24 hours of sodium fluoride treatment both fresh and dry weight of seedlings were reduced suggesting metabolic changes as the cause. No significant change in cytokinin levels was found in seedlings treated with 1 mM NaF. A higher (10 mM) concentration of fluoride resulted in some decrease in cytokinin activity insufficient, however, to account for the complete growth inhibition of seedlings. Marked reduction in cytokinin(s) with similar chromatographic behaviour to isopentenyl adenine and isopentenyl adenosine was observed in plants subjected to the 10 mM NaF treatment. Activity of acid phosphatase extracted from seedlings was drastically reduced by sodium fluoride *in vitro*. When the enzyme was isolated from fluoride-treated plants its activity was also lower, but the inhibition was less pronounced. Both organic acid and amino acid levels increased in fluoride treated seedlings. Depending on the level of fluoride treatment the amount of soluble proteins decreased or showed no significant change. Possible implications of the observed changes are discussed.

INTRODUCTION

Harmful effects of fluoride on vegetation are well recognized with natural ecosystems (Materna, 1984), and agricultural lands (Heck et al., 1973) affected. Growth and yield of plants may be reduced by fluoride even in the absence of visible injury (Treshow et al., 1967; MacLean et al., 1977). It is presently believed that air pollutants affect plant growth by inducing biochemical, physiological, and structural changes (Heck et al., 1973). Little is known about the processes which lead to a reduction of growth by plants exposed to fluoride. Earlier chapters in this thesis showed that fluoride affects the water status of Jack pine seedlings, induces cytoplasmic leakage, and alters the metabolism of lipids and carbohydrates. The effects of fluoride on the activity of plant phosphatases and its interference with organic acid and nitrogen metabolism are unclear. Several authors isolated acid phosphatase from various organisms and demonstrated inhibition of its activity by fluoride (Reiner et al., 1955; Lorenc-Kubis & Morawiecka, 1978; Malhotra & Khan, 1980). But Yee-Miller (1975) did not find changes in the activity of acid phosphatase isolated from spruce and birch leaves exposed to fluoride pollution. Reiner et al. (1955) showed that organic acids protect acid phosphatase activity from fluoride. An accumulation of organic acids in fluoride-treated plants was reported by Weinstein (1961), and Yang & Miller (1963a). Organic acids and

other organic molecules such as amino acids and sugars accumulate in water-stressed plants which can use them as osmoregulators (Clarke & Durley, 1981). The disruption of normal protein and organic acid metabolism in water-stressed plants largely accounts for the inhibition of plant growth. Water stress can also alter the hormonal status of plants. The activity of cytokinins - plant hormones promoting cell division (Letham, 1978) - was shown to decrease in drought-stressesd plants (Itai et al., 1968; Itai & Vaadia, 1971). Fluoride affected the water status of Jack pine seedlings. It is also known to influence growth processes, metabolism of nucleotides (Chang, 1968; Conner & Linden, 1970), and decrease RNA levels (Chang, 1968; Chang, 1973). These effects suggest that fluoride may produce changes in cytokinin metabolism and its activity in plants.

The present study investigates the effects of sodium fluoride on the growth of Jack pine seedlings. It examines the levels of cytokinins, soluble proteins, free amino acids, and organic acids as well as the activity of acid phosphatase *in vitro* and *in vivo*.

METHODS

Growth conditions

Jack pine seedlings for all the experiments except for cytokinin determination were grown in pots (10 seedlings per pot) containing 650g of demineralized sand. The seedlings were provided with 130 mL of 3/4 strength Hoagland's mineral solution and placed in a growth cabinet at 22 C and 18 h light, 6 h dark regime (light intensity of 120 $\mu E\ m^{-2}\ s^{-1}$). Plants were watered daily with deionized water. Two-week old seedlings were treated with sodium fluoride which was added in a solution to the sand to reach an initial concentration of either 3 μg F (3 μg treatment) or 15 μg F (15 μg treatment) per gram dry weight of sand. The treatments lasted for 24 to 168 h depending on the experiment. Control and fluoride-treated seedlings were always collected at the same time.

Due to the large amount of tissue required for the determination of cytokinin activity in seedlings, seeds for this experiment were sown on moist filter paper in large plastic trays. The trays were covered with a plastic film and placed in a growth cabinet in the conditions described above. One day after germination all water was drained from the trays, the filter paper was blotted dry, and 25 mL of 1 mM and 10 mM sodium fluoride solutions, or deionized water

were pipetted into each tray. After 48 h, 50 seedlings were chosen at random and their lengths measured.

Cytokinins

For the determination of cytokinin activity in plants 10 g samples were taken after 48 h of 1 mM and 10 mM NaF treatments, and compared with the controls. Cytokinins were extracted and purified according to Taylor et al. (1982). The seedlings were frozen in liquid nitrogen and homogenized in a Waring blender with cold (-25 C) 80% methanol (20 mL methanol g^{-1} tissue) for 3 min. The extract was filtered and the residue extracted twice more with methanol. All filtrates were combined and reduced in vacuo at 35 C to the aqueous phase. This phase was adjusted to pH 8.0 and centrifuged for 1 h at 5000 G. The supernatant was adjusted to pH 3.0 with acetic acid and applied to a 2 cm X 15 cm cellulose phosphate column, in NH_4^+ form, which was washed with 3 column volumes of distilled water adjusted to pH 3.0 with acetic acid, and 3 volumes of distilled water of pH 7.0. The column was then washed with 8 volumes of 0.3 N ammonium hydroxide. The ammoniacal effluent was de-gassed under vacuum, adjusted to pH 8.0, and partitioned four times against equal volumes of water saturated n-butanol. The butanol phase, containing cytokinins, was evaporated in vacuo at 35 C, and the residue strip-loaded at two different concentrations onto Whatman 3

MM chromatography paper (10 cm X 30 cm). Chromatograms were developed to 20 cm in iso-butanol : 30% ammonium hydroxide : water (10:1:1, v/v/v). Ten 2 cm strips were cut from each chromatogram, placed into flasks containing 10 mL of medium for cytokinin bioassay (Miller, 1968), and autoclaved for 15 min at 1.1 X 10^5 Pa. The content of cytokinins in each chromatogram strip was determined in comparison with a standard zeatin curve using the soybean section assay (Manos & Goldthwaite, 1976). Soybeans (Maple amber variety) for the bioassay were surface-sterilized in a 1.3% solution of sodium hypochlorate and rinsed with sterile distilled water. Two soybeans were placed into each of a number of sterile test tubes containing a small piece of cotton wool saturated with a dilute solution of sucrose. The test tubes were wrapped in aluminum foil and placed in a growth cabinet at 30 C. After 1 week soybean hypocotyls were inspected for signs of microbia and fungi. Uncontaminated hypocotyls were cut with a sterile razor blade into 1 mm segments. Four hypocotyl segments were placed in Petri dishes (3.5 cm X 1 cm) containing the growth medium with cytokinins eluted from the chromatogram strips. The dishes were sealed and placed in the dark at 30 C. After 9 days the individual hypocotyl sections were weighed on an analytical balance. A standard zeatin curve was prepared by adding solutions of zeatin in several different concentrations to the appropriately concentrated callus medium, autoclaving, and performing a

bioassay as described above. Tentative identification of cytokinins was achieved by chromatography of zeatin, zeatin riboside, zeatin-9-glucoside, isopentenyl adenine, and isopentenyl adenosine standards using the same conditions as for plant extracts and locating their activity on chromatogram strips using a soybean section assay. Cytokinin activity was determined in seedlings in two repeated experiments.

Acid phosphatase

Homogenate preparation

Jack pine seedlings were treated with sodium fluoride for 24 hours (15 µg treatment), or 91 hours (3 µg treatment). Fluoride treated and control plants were harvested, weighed, and divided into 1 g samples (3 samples per treatment). Seedlings were homogenized in a mortar containing clean sand and 15 mL of ice-cold solution containing 50 mM Tris-HCl buffer (pH 7.4), 1% polyvinyl pyrrolidone (PVP-10), and 1 mM dithioerythritol (Malhotra & Khan, 1980). The homogenate was filtered through two layers of cheesecloth and the filtrate immediately used for the enzyme assay and soluble protein determination.

Enzyme assay

Acid phosphatase activity in the homogenates of fluoride treated and control seedlings was determined using p-nitro-

phenyl phosphate (P-NPP) as a substrate. The reaction mixtures contained 50 µL of the enzyme extract (diluted with a homogenization medium when required), or 50 µL of the homogenization medium (blank samples), and 2 mL of 0.6 mM P-NPP in 50 mM acetate buffer (pH 5.0). Five replicates of each extract were made. Test tubes containing the reaction mixtures were placed in a water bath at 30 C. The reaction was stopped by adding 2 mL of 10% sodium carbonate. The rate of the reaction was linear for 30 min. The enzyme activity was determined by measuring the optical density of the reaction mixture in a LKB Biochrom Ultrospec Spectrophotometer at 410 nm against blank samples. Spectrophotometer readings were converted to units of enzyme activity, defined as the amount of enzyme that resulted in a spectral change of 0.1 optical density unit min^{-1}. The entire experiment was repeated once to give a sample size of 6 for each fluoride treatment and 12 for the control.

The in vitro effect of sodium fluoride on the activity of acid phosphatase from Jack pine seedlings was examined by adding sodium fluoride solutions to the reaction mixtures containing enzyme extracts obtained from control plants. The final concentrations of fluoride in the reaction mixtures were 1, 5, and 10 mM. The reaction was stopped after 20 min. The activity of acid phosphatase was measured as described above.

Protein determination

Protein content, in the extracts used for the acid phosphatase activity assay, was determined using the Bio-Rad standard protein assay procedure. Three samples, 50 µL each, were taken from each extract and pipetted into a test tube containing 5 mL of the diluted dye reagent. Three readings of the optical density were taken for each sample at 595 nm against a blank sample (dye reagent). Protein concentrations were read from a standard curve prepared from a series of concentrations of bovine albumin. Statistical significance was tested using the analysis of variance procedure (Scheffe's test).

Fresh and dry weights

Changes in fresh and dry weights of seedlings were examined in plants treated for 24, 48, 91, and 168 hours with a 3 µg fluoride treatment, and compared with controls. Fresh weights were determined by weighing groups of 4 to 5 seedlings on an analytical balance and estimating an average weight of a single seedling. For dry weight determinations seedlings were placed in a drying oven at 80 C for 48 hours and weighed again. Each treatment had 16 samples in two repeated experiments. Statistical analyses were performed as for the protein determinations.

Organic acids and amino acids

Organic and amino acid levels were determined in control plants and in seedlings subjected to the 3 μg fluoride treatment for 91 hours. Boiling 95% ethanol (40 mL) and 1 mg of maleic acid (internal standard) were added to plant samples, each containing 3 g of Jack pine seedlings, and the mixture boiled for 5 min. After cooling, 5 mL of distilled water was added to the mixture and the tissue ground in a mortar and filtered. The extraction was repeated three more times with 80% ethanol. All filtrates were combined and reduced *in vacuo* to the aqueous phase. The extracts were passed through a 2.5 cm X 10 cm Bio-Rad AG1-X8 (200-400 mesh) anion exchange column in a formate form which was washed with 250 mL of distilled water. The effluent was reduced under vacuum to a small volume and passed through a 2.5 cm X 10 cm Dowex 50W-X8 (100-200 mesh) cation exchange resin column in a hydrogen form which was also washed with 250 mL of water. Amino acids were removed from the cation column by washing with 300 mL of 2 M ammonium hydroxide. Organic acids, which were retained by an anion column, were obtained by washing the column with 300 mL of of 1N formic acid. The effluent containing organic acids was reduced *in vacuo* to a small volume, transferred to vials, and evaporated under nitrogen. The residue was azeotropically dried with iso-propanol and left overnight in a vacuum dessicator over silica gel. Next day trimethylsilyl (TMS) ethers of organic

acids were prepared by dissolving the residues in 1 mL of pyridine and adding 0.2 mL of trimethylchlorosilane following 0.4 mL of hexamethyldisilazane. After 16 to 24 hours TMS ethers of organic acids were analysed using a Carlo Erba Gas Chromatograph equipped with a 1.8 m long, 2 mm internal diameter glass column containing 3% OV-3 as a stationary phase on 80/100 mesh Chromosorb W. Conditions during the runs were: injector and detector temperature, 220 C; oven temperature, 45 C, raised to 180 C (2 C min^{-1}); carrier (nitrogen) flow, 40 mL min^{-1}. Integration was performed using a Hewlett Packard 3390A Electronic Integrator. Organic acids were identified by co-chromatography with original organic acid standards purchased from Sigma.

The effluent containing amino acids was reduced to a small volume, freeze-dried and stored for two weeks in a freezer prior to analysis. Amino acids were separated using a LKB 4151 Alpha Plus Amino Acid Analyser equipped with a 0.6 cm X 20 cm LKB Ultropack 8 resin column. Sodium citrate "three buffer system" was used. Integration was performed with a Hewlett Packard 3390A integrator. Amino acids were identified by co-chromatography with amino acid standards obtained from LKB Biochrom.

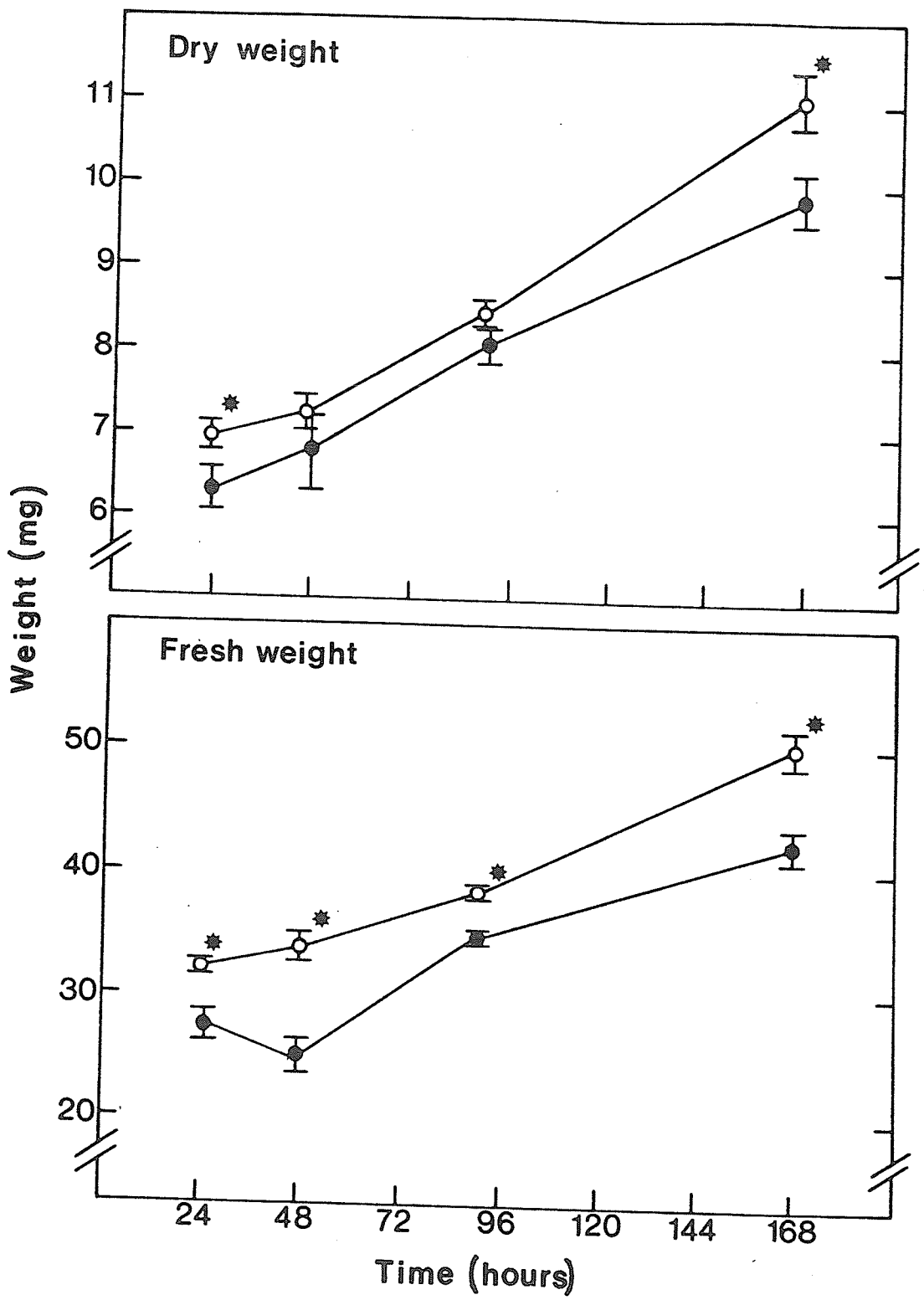
Three organic acid and amino acid samples of each extract from three repeated experiments were analysed.

RESULTS

Growth

Changes in dry and fresh weights of sodium fluoride treated and control Jack pine seedlings were monitored for one week. During this time fresh weight of control plants increased by 56% and dry weight by 60% (Fig.1). The percentage increase in fresh weight of treated seedlings was slower than that of dry weight. Fresh and dry weight of seedlings subjected to the 3 µg treatment for one week increased by 45% and 55% respectively. The adverse effect of sodium fluoride on growth of Jack pine seedlings was apparent as early as 24 hours after commencement of the treatment. Compared with controls the mean dry weight of an individual seedling treated with fluoride was lower by almost 9% (Fig.1). Seedlings treated for 48, 91, and 168 hours weighed respectively 7%, 5%, and 12% less than control plants. Thus the inhibition of growth occurred mostly within the first 24 hours and 7 days after the initial fluoride treatment. Fresh weight of fluoride treated seedlings was affected by a decline in growth and by fluoride-induced changes in water content. Fresh weight of plants treated with sodium fluoride for 24 hours was about 91% of that of control seedlings. Next day the fresh weight of fluoride treated seedlings further declined. At that time the mean weight of an individual seedling was lower than that of 24 hour treated seedling. Some recovery was noticeable in 91 hour treated plants

Figure 1. Changes in dry and fresh weight of an individual control Jack pine seedling (○), and seedling subjected to the 3 µg fluoride treatment (●). The means of 16 samples (±SE) are shown. Asterisks indicate significant difference at the 0.05 level.


Dry weight
11
10
9
8
7
6
Fresh weight
50
40
30
20
Weight (mg)
24
48
72
96
120
144
168
Time (hours)

followed by another decline 7 days after the initial fluoride treatment.

Cytokinins

The activity of cytokinins was studied in plants treated with 1 mM and 10 mM sodium fluoride for 48 hours, and compared to that of control seedlings. Both levels of sodium fluoride significantly altered seedling growth. Linear growth of plants treated with 1 mM NaF was reduced by 17.5%. The higher, 10 mM concentration resulted in almost total inhibition of growth. Compared with the controls, total cytokinin levels of 1 mM treated seedlings remained almost unchanged (control plants contained 17.3 ng zeatin equiv. g^{-1}d.wt.; 1mM treated plants, 18.7 ng zeatin equiv. g^{-1}d.wt.). The activity of cytokinins in 10 mM treated seedlings was reduced by approximately 21% (plants treated with 10 mM NaF contained 14.7 ng zeatin equiv. g^{-1}d.wt. tissue). A poor separation of various cytokinins on paper chromatograms prevented accurate quantification of individual cytokinins. When the original standards of naturally occurring cytokinins were co-chromatographed, zeatin glucoside was found on paper chromatogram strips corresponding to the Rf of 0.1-0.4, zeatin riboside to 0.2-0.4, zeatin to 0.3-0.6, and isopentenyl adenine and isopentenyl adenosine to 0.6-0.9. Three main peaks of cytokinin activity were

Figure 2. Cytokinin levels in control Jack pine seedlings and in plants treated for 48 hours with 1 mM NaF and 10 mM NaF. Measured by the soybean hypocotyl section assay at 2 extract concentrations following separation by paper chromatography. The experiment was replicated twice (——— and ·····).

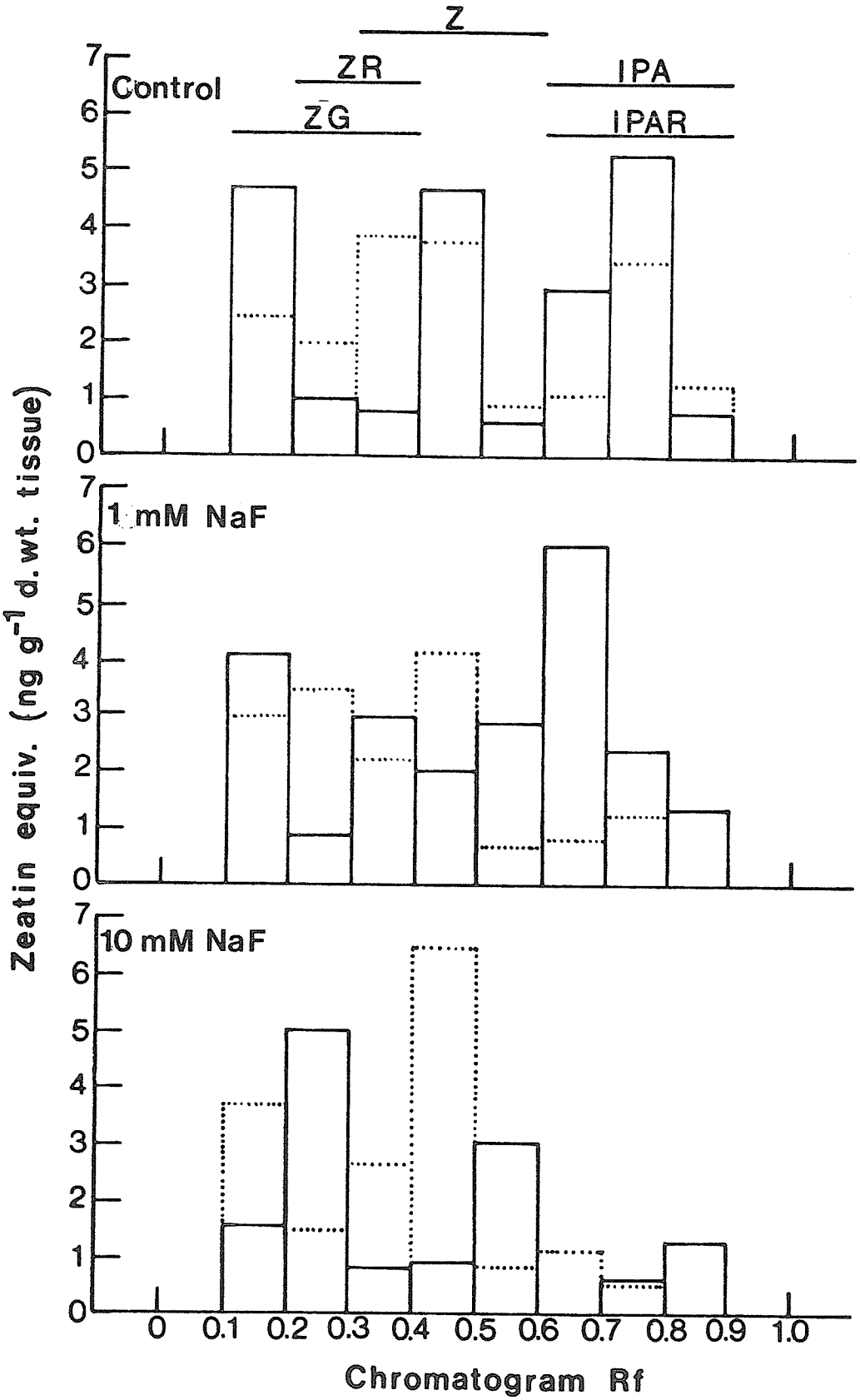
Z - zeatin

ZR - zeatin riboside

ZG - zeatin-9-glucoside

IPA - isopentenyl adenine

IPAR - isopentenyl adenosine


Z
ZR
ZG
IPA
IPAR
Control
1 mM NaF
10 mM NaF
Zeatin equiv. (ng g⁻¹ d.wt. tissue)
Chromatogram Rf
0
1
2
3
4
5
6
7
0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0

noticed in plant extracts (Fig.2). They corresponded to the chromatogram Rf of 0.1-0.3, 0.3-0.6, and 0.6-0.9. However, the last peak was markedly smaller in plants treated with 10 mM NaF. This peak corresponded to isopentenyl adenine and isopentenyl adenosine standards. The reduction in this peak accounted for the overall decrease in cytokinin activity in 10 mM treated seedlings.

Acid phosphatase and soluble proteins

Sodium fluoride drastically inhibited the activity of acid phosphatase in vitro (Table 1). The lowest, 1 mM concentration of fluoride had the greatest effect. Only 12% of the enzyme activity remained compared with acid phosphatase activity in a fluoride-free system. In the presence of 5 mM and 10 mM NaF, acid phosphatase activity was reduced respectively to 17% and 28% of the control. A significant decrease in acid phosphatase activity was also observed in extracts of plants treated with sodium fluoride (Table 2). However, the inhibition was less pronounced than in vitro. Acid phosphatase activity of plants subjected to 3 µg fluoride treatment for 91 hours was reduced to 65% of the activity in control plants, and that of plants exposed to the 15 µg, 24 hour treatment was reduced to 73%.

The content of soluble protein in Jack pine seedlings was also altered by sodium fluoride (Table 2). Plants subjected

Table 1. Effect of sodium fluoride on acid phosphatase from Jack pine seedlings (*in vitro*)

Concentration of sodium fluoride (mM)	Enzyme activity* (units mg^{-1} protein)	% of control
0(control)	33.3±3.7**	
1	4.1±0.3	12.3
5	5.5±0.7	16.5
10	9.2±0.6	27.6

*A unit of enzyme activity is the amount of enzyme that resulted in a spectral change of 0.1 O.D. min^{-1} at 410 nm

**The mean of 3 replicates ± standard error

Table 2. Acid phosphatase activity and soluble protein content in control and sodium fluoride treated Jack pine seedlings

Treatment*	Enzyme activity** (units mg^{-1} protein)	% of control	protein (mg g^{-1} d.wt.)	% of control
Control	23.4±2.0†		44.3±4.0††	
A	17.2±2.7	73.2†††	49.0±4.0	115
B	15.1±0.9	64.5†††	29.9±1.1	70.2†††

*A-15µg, 24 hour treatment; B-3µg, 91 hour treatment

**A unit of enzyme activity is the amount of enzyme that resulted in a spectral change of 0.1 O.D. unit min^{-1} at 410 nm

†The mean of 12 replicates (6 for both F treatments) ± SE

††The mean of 6 replicates ± SE

†††Significantly different from the control at the 0.05 level

to the 3 µg, 91 hour treatment contained significantly less soluble protein than control seedlings. Seedlings responded differently to the 15 µg, 24 hour treatment. They contained 15% more soluble protein than control plants, but this was not statistically significant.

Organic acids and amino acids

Quinic and shikimic acids, identified in Jack pine seedlings, together accounted for over 80% of the total major organic acids. A component representing about 10% of the total peak area was not identified. An unidentified compound of similar chromatographic behaviour was also reported by Sarkar and Malhotra (1979) for Jack pine needles. Syringic and succinic acids were present in smaller quantities (Table 3). All other organic acids were present in amounts too small for accurate quantification.

Total organic acid content of seedlings subjected to the 3 µg fluoride treatment for 91 hours increased by over 55% compared with control plants (Table 3). Levels of all analysed organic acids were elevated in fluoride treated seedlings. However, the accumulation of quinic and shikimic acids contributed most to the increase in acidity of seedlings. Fluoride treated plants accumulated over 1780 µmoles of these two acids per g d.wt. tissue, compared with 1154 umoles found in control plants.

Table 3. Content of major organic acids in sodium fluoride treated and control Jack pine seedlings

Organic acid	Amount, μmole g^{-1} d. wt.		
	Control	NaF*	% of control
Quinic acid	618.2±111.6**	956.7±135.6	154.8
Shikimic acid	535.9±74.4	823.8±57.1	153.7
Succinic acid	1.2±0.7	2.3±0.3	191.7
Syringic acid	48.1±7.9	86.9±36.9	180.7
Total	1203.4	1869.7	155.4

*3μg, 91 hour treatment
**The mean of 3 replicates ± standard error

Table 4. Content of major amino acids in sodium fluoride treated and control Jack pine seedlings

Amino acid	Amount nmole g^{-1} d.wt.		
	Control	NaF*	% of control
Alanine	213.2±7.0**	296.6±26.6	139.1
Arginine	3626.5±117.7	1724.5±117.6	65.7
Aspartic acid	1339.8±69.5	1800.8±306.1	134.4
Glutamic acid	2046.8±44.7	2520.4±87.1	123.1
Glycine	110.8±15.9	105.2±9.7	94.9
Histidine	229.4±17.1	129.8±19.5	56.6
Isoleucine	162.1±28.5	120.7±34.0	74.5
Leucine	144.9±20.1	103.4±25.6	71.4
Lysine	166.8±24.5	117.9±16.8	70.7
Phenylalanine	56.7±5.4	65.7±9.8	115.9
Proline	276.8±54.1	386.3±45.8	139.6
Serine	2488.8±366.8	3817.1±551.6	153.4
Tyrosine	93.6±6.5	58.4±9.1	62.4
Valine	90.4±3.1	185.6±24.4	205.3
Total	10046.6	11432.4	113.8

*3µg, 91 hour treatment
**The mean of 3 replicates ± SE

The most abundant amino acid in control seedlings was arginine which accounted for almost 35% of total amino acids. (Table 4). Serine, glutamic acid, and aspartic acid combined constituted almost 57% of all free amino acids. The remaining 8% was accounted for by other amino acids. The composition of amino acids was altered by the 3 μg, 91 hour treatment. Although the total amount of free amino acids in these plants increased by over 15%, levels of individual amino acids either increased or declined. An increase was observed in alanine, aspartic acid, glutamic acid, phenylalanine, proline, serine, and valine. Decreases were recorded in arginine, histidine, isoleucine, leucine, lysine, and tyrosine. Little change was observed in the amount of glycine. All the acidic amino acids are among those whose levels increased, and all the basic amino acids are present in the group whose levels declined.

DISCUSSION

The effects of fluoride on plant growth have been extensively studied (Weinstein, 1977). Depending on such factors as dose, environmental conditions, plant species, and stage of development, fluoride inhibits, has no effect, or stimulates growth (Weinstein, 1977). The most common observation in plants exposed to fluoride is a decrease in their fresh and dry weight (Benedict et al., 1964; Brewer et al., 1967b), and a reduction in dimensions or weight of roots (Benedict et al., 1964; Chang & Thompson, 1966b; Chang, 1968), shoots (Benedict et al., 1964; Keller, 1973), and leaves (Benedict et al., 1964; Leonard & Graves, 1970; Ivinskis & Murray, 1984). Many studies also reported decreases in radial growth of trees (Treshow et al., 1967; Carlson, 1978; Bunce, 1984), and in flower and seed production (Pack & Sulzbach, 1976; MacLean et al., 1977; Staniforth & Sidhu, 1984), but little is known about the mechanisms of fluoride's interference with growth processes. Reduction in photosynthesis, due to destruction of photosynthetic tissues, may be the direct cause of growth reduction. However, there have been reports of growth and yield reduction in the absence of visible leaf injury (Treshow et al., 1967; MacLean et al., 1977). Growth of Jack pine seedlings was inhibited within the first 24 hours of sodium fluoride treatment (Fig.1), long before the appearance of necrotic lesions on leaves. This indicates that fluoride, upon its entry to

plants, altered certain metabolic processes which resulted in growth inhibition.

Water stress is known to reduce plant growth (Kramer, 1983). It was shown in Chapter III that fluoride altered the water balance in Jack pine seedlings. However, decreased water content was probably not a major cause of growth inhibition, because growth was reduced before most of the water loss occurred. Many metabolic processes appear to be rapidly affected by fluoride. Decrease in dark respiration, respiratory quotient, decline of starch and sucrose levels, and accumulation of monosaccharides and cyclitols were observed in seedlings treated for 24 hours with sodium fluoride (Chapter III). Thus, the growth inhibition observed in fluoride-treated Jack pine seedlings is probably caused by fluoride's direct interference with metabolism. The possibility of fluoride's involvement in the metabolism of plant hormones has not been previously investigated. Cytokinins are known to promote growth of leaves and cotyledons and to delay their senescence (Koshimizu & Iwamura, 1986). Cytokinins stimulate growth by inducing cell divisions (Letham, 1976). Fluoride was shown to be capable of altering nucleotide metabolism and to induce changes in ratios of nucleotides and the base composition of RNA in corn seedling roots (Chang, 1968). It decreased the content of total and ribosomal RNA (Chang, 1973), inhibited deamination and dephosphorylation of purine and pyrimidine 5'-nucleotides (Corner &

Linden, 1970), reduced incorporation of P^{32} by the acid-soluble nucleotide pool (McCune et al., 1970), and increased ATP levels (Chang, 1968; Ballantyne, 1984). All of these changes could result in altered metabolism of cytokinins. However, total cytokinin levels of fluoride-treated Jack pine seedlings did not appear to be substantially altered, even by the 10 mM sodium fluoride treatment which completely inhibited seedling growth. A large decrease in the substance(s) which had similar chromatographic behaviour to isopentenyl adenine and isopentenyl adenosine was seen in seedlings treated with 10 mM NaF. No such change was observed in 1 mM treated plants. Isopentenyl adenine and isopentenyl adenosine are the initial products in the synthesis of zeatin-type cytokinins (Koshimizu & Iwamura, 1986) and their depletion might inhibit the synthesis of other cytokinins.

Activity of various enzymes has been often shown to be inhibited by fluoride. Acid phosphatase is widely distributed in the plant kingdom and it is implicated in key metabolic functions of plants such as growth (Hall, 1971), mineral nutrition (Hasegawa et al., 1976), and transportation of stored metabolites (Flinn & Smith, 1967). Acid phosphatase was isolated from Jack pine seedlings by Malhotra & Khan (1980). The enzyme was specific for many substrates including P-NPP, phenyl phosphate, phosphocreatine, ATP, ADP, AMP, cAMP, and glycerophosphate. The activity of the enzyme was drastically inhibited *in vitro* by ammonium fluoride. Al-

though many other in vitro studies also reported the inhibition of acid phosphatase by fluoride, Yee-Miller (1975) found no correlation between exposure to fluoride and the activity of acid phosphatase in Norway spruce needles and birch leaves. In the present study both in vitro and in vivo inhibition of acid phosphatase were observed in fluoride-treated Jack pine seedlings. This inhibition could affect many important metabolic processes in the seedlings. The largest in vitro inhibition occurred in the presence of 1 mM sodium fluoride. Higher concentrations were less effective. Similar observations were made by Reiner et al. (1955), who hypothesized that there are two forms of fluoride, of which the second [$(HF_2)_2^{--}$] is formed at higher concentrations and combines with the enzyme protecting it against the first (HF_2^-). The in vivo inhibition of acid phosphatase activity in Jack pine seedlings was less pronounced. Reiner et al. (1955) showed that prostatic acid phosphatase can be protected from fluoride's inhibitory action by a number of multivalent organic anions such as citrate and oxalate. It is possible that the accumulation of organic acids observed in fluoride-treated Jack pine seedlings may serve as protection against inhibition of acid phosphatase and perhaps other enzymes.

Accumulation of organic acids in plants exposed to fluoride was also reported by Yang & Miller (1963a), and Weinstein (1961). Synechococcus leopoliensis cells excreted an

unknown anionic substance into the growth medium which protected the cells from the growth inhibition by fluoride (Nichol & Budd, 1984). Organic acids are also known to accumulate in drought-stressed plants, where they are thought to act as osmoregulators (Clarke & Durley, 1981). Shikimic acid and quinic acid, which accumulated in fluoride stressed Jack pine seedlings, are intermediates in biosynthesis of aromatic acids and lignin (Davis, 1955; Neisch, 1968). Thus, any impairment of normal metabolism of these acids might cause some physiological disorder that could influence plant growth.

Change in the composition of amino acids in fluoride-treated Jack pine seedlings is another indication that plants suffered from water stress. Free amino acids, particularly proline (Borowitzka, 1981), are known to accumulate in drought-stressed plants. Total levels of free amino acids increased in fluoride-treated Jack pine seedlings. Proline, all acidic amino acids, and several others increased, while the levels of basic amino acids in treated plants declined. Aromatic amino acids either increased (phenylalanine), or decreased (tyrosine). Thus, the accumulation of shikimic and quinic acid may not have had a specific, immediate effect on the synthesis of these amino acids. There was no indication of changes in any specific family of amino acids which could imply alterations in biosynthetic pathways of amino acids. Change in free amino acid levels in fluoride-treated see-

dlings implies an inhibition of protein synthesis, intensified proteolysis, or both. Inhibition of protein synthesis in plants by fluoride was reported by Kalinnikov & Tolokonnikov (1971). A significant decrease in soluble protein content of Jack pine seedlings exposed to 3 µg fluoride treatment was observed. A small increase in soluble proteins in the 15 ug treatment can probably be explained by the extensive damage to cells (Chapter II). This may have resulted in a release and degradation of membrane proteins. The increase in levels of all acidic amino acids and the decrease in basic amino acids are intriguing observations. Acton & Gupta (1979), and Cooke & Davies (1980) showed that soluble acidic proteins degrade more rapidly than neutral proteins. This might explain the increase in acidic amino acids in fluoride-treated seedlings. However, it is also possible that the synthesis of acidic proteins is inhibited in fluoride-treated plants. One of the possible reasons for the depletion of basic amino acids could be their transformation into polyamines which are known to accumulate in plants exposed to such air pollutants as sulphur dioxide.

In order to explain the reasons for the changes in nitrogen metabolism observed in fluoride-treated plants more studies are required. These should include examination of the polyamine status of fluoride-treated plants, and protein and nucleotide turn-over.

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SUMMARY

Relatively low levels of sodium fluoride in sand culture adversely affected the structure and metabolism of Jack pine seedlings. The visible signs of fluoride injury included wilting, desiccation, and necrosis of leaves. More plants were injured and plants responded sooner when treated with 15 µg F g^{-1}d.wt. sand than 3 µg F g^{-1}d.wt. sand. To study the mechanisms involved, permeability of cell membranes was examined in plants subjected to 3 µg and 15 µg F treatments for various periods of time between 12 and 168 hours. Increased electrolyte leakage from seedlings was observed after 24 hours of 15 µg, and 48 hours of 3 µg F treatments, in both cases before foliar injury was noticeable. The rates of electrolyte leakage continued to rise, and reached a maximum after 91 hours of both fluoride treatments. After that time a reversal of the process was observed, indicating that membranes started to regain their property of selective permeability to electrolytes.

Studies of the chemical composition of plant lipids revealed that when electrolyte leakage increased, phospholipids drastically declined, while neutral lipids increased. Changes also occurred in the composition of the molecular species of phospholipids. The composition of fatty acids was examined in phospholipid, glycolipid, and neutral lipids extracted from plants subjected to various durations of 3 µg

and 15 µg F treatments, and compared with controls. In nearly all treatments the levels of palmitic acid increased in the neutral fraction, and phospholipids contained less linoleic acid and more stearic acid than controls. Because fluoride did not affect the malondialdehyde level in seedlings, changes in fatty acid composition were probably caused by processes other than peroxidation.

The second part of the study compared structural alterations induced in mesophyll and guard cells of seedlings by fluoride and those subjected to drought. It was noticed that many stomata in fluoride treated plants remained open during wilting. Cytological investigations revealed that cells adjacent to guard cells frequently collapsed, particularly in plants subjected to the 15 µg F treatment, and that in comparison with both controls and water-stressed plants, plastids in guard cells of fluoride treated plants contained only a few small starch granules. In contrast, the chloroplasts of mesophyll cells in fluoride treated plants accumulated large amounts of starch, rarely found in the chloroplasts of control and drought-stressed seedlings. All other structural alterations observed in fluoride treated mesophyll cells were also frequently seen in cells of drought-stressed plants. They included plasmolysis, accumulation of lipid bodies near cell membranes, dilation and curling of thylakoids, and changes in the morphology of nuclei.

The last series of experiments focussed on the effects of fluoride on seedling growth, water content, gas exchange, the activity of acid phosphatase, and levels of carbohydrates, organic acids, proteins, amino acids, and cytokinins. The seedlings subjected to the 3 µg F treatment for one to seven days contained less water than control plants, and their fresh and dry weights were reduced. The rates of respiration and respiratory quotient were low in plants treated for as little as one day, while a significant decrease in photosynthetic rates was observed in plants treated for two and four days. Fluoride affected the carbohydrate content of seedlings in various ways. The concentration of inositol increased in plants treated for one day, glucose and fructose levels were elevated, and those of sucrose declined in plants treated for one, two, and four days. The amount of starch was below the control levels in one, four, and seven day treated seedlings. Changes in the composition of organic acids and amino acids were examined in plants subjected to the 3 µg F treatment for 91 hours. The levels of all examined organic acids increased by 50% - 90%. Total levels of amino acids also increased by approximately 15%, but the concentration of individual amino acids either increased, decreased, or showed little change. A significant decrease in the levels of soluble proteins was observed in seedlings subjected to the same F treatment. Acid phosphatase activity was drastically reduced by the <u>in vitro</u> fluor-

ide treatments. The presence of 1 mM NaF in the incubation medium reduced the activity of the enzyme to 12% of the control level. The in vivo treatments with 3 µg and 15 µg F levels lowered the activity of acid phosphatase by 35% and 27% respectively. Cytokinin activity was examined in seedlings treated with 1 mM and 10 mM NaF, and compared with that of control plants. Only small changes in the total cytokinin levels were observed in fluoride treated plants. However, seedlings treated with 10 mM NaF contained markedly less cytokinin-like compound(s) of similar chromatographic behaviour to isopentenyl adenine and isopentenyl adenosine, which are intermediates in the synthesis of zeatin.

The mechanism of fluoride's action on plants is complex. Sodium fluoride induced conditions of water deficit in Jack pine seedlings, altered membrane permeability, and accelerated processes of senescence. Thus, the structural and metabolic changes observed in sodium fluoride treated plants might be combined plant responses to all the above conditions. On the basis of the present studies and with the support of other reports a model is proposed to explain possible mechanisms of fluoride's action on Jack pine seedlings. The following is a sequence of events proposed for the model:

1. Uptake of fluoride and transport in the transpiration stream to the places of most rapid transpiration (open stomata).

2. Cells lining the stomatal chambers accumulate more fluoride than other cells and, therefore are injured sooner.
3. Injury and collapse of cells adjacent to guard cells impairs stomatal movements.
4. Cells in most affected areas lose water, and if the damage is extensive plants wilt and collapse.
5. Uptake of fluoride continues, affecting more cells. In addition to inducing water imbalance, fluoride affects cells directly because it acts as a cytoplasmic poison. Respiration and dephosphorylation processes are inhibited, which together with the conditions of water stress results in a temporary depletion of energy. Consequently, there is a decrease in the rates of synthesis of macromolecules, and metabolic intermediates such as neutral lipids, hexoses, organic acids, and amino acids accumulate. These intermediates may play an important role as osmoregulators in cells suffering from water stress. Lipid intermediates in cell membrane synthesis and/or the products of membrane breakdown accumulate near the membranes. Together with amino acids they are available for plants as alternative respiratory substrates. Transport processes are inhibited, with the result that starch accumulates in the chloroplasts of photosynthetic cells.
6. Metabolic imbalance due to combined fluoride and water stress results in the inhibition of plant growth.

7. Plant membranes are unable to replace all the phospholipids lost during normal turnover. This results in a loss of membrane selective permeability and a leakage of solutes.
8. The loss of normal membrane function creates further metabolic imbalance and results in accelerated senescence and the appearance of necrotic lesions on leaves.
9. The extensive damage to cells and conditions of water stress reduce photosynthetic rates in plants which result in slower plant growth.
10. Fluoride is then deposited in plants in physiologically inactive forms (such as calcium fluoride), and isolated in dead parts of plants, plants recover.

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APPENDIX A

EFFECT OF SODIUM FLUORIDE TREATMENTS ON THE MITOTIC CYCLE OF ROOT TIP CELLS IN JACK PINE SEEDLINGS

INTRODUCTION

A number of effects of fluoride on plants have been reported in the literature. Growth retardation of plants exposed to fluoride was frequently observed (Brewer et al., 1960b; Treshow et al., 1967; Hill & Pack, 1983), but only a few studies investigated the mechanisms of growth retardation. Growth inhibition can occur due to inhibition of cell multiplication and/or elongation. Lengthening of any of the phases of the cell cycle will result in plant growth reduction. Chang & Thompson (1966b) demonstrated that rates of both cell elongation and multiplication of corn seedling roots were inhibited by short duration of sodium fluoride treatments. Whether and how longer fluoride exposures affect the cell cycle in plants are not known.

This study investigates the effects of two levels of sodium fluoride on the duration of the mitotic cycle, and mitotic phases of Jack pine seedlings root meristem cells using the metaphase accumulation method of Evans et al. (1957).

METHODS

Jack pine seeds were germinated on moist filter paper. Two days after germination, seedlings of uniform length were selected and transplanted to pots containing 650 g of demin-

eralized sand. Seedlings were provided with 130 mL of 3/4 strength Hoagland's mineral solution and placed in a growth cabinet at 22 C, 18 h light / 6 h dark regime (120 µE $m^{-2}s^{-1}$). The pots with seedlings were weighed daily and supplemented with deionized water to the original weight. Every week, instead of water, 30 mL of fresh Hoagland's solution was added. Two-week old seedlings were treated with sodium fluoride which was added in a solution to the sand to reach a concentration of either 3 µg or 15 µg F g^{-1} d. wt. sand. Water, instead of sodium fluoride was added to control plants. Four weeks after commencement of fluoride treatments several pots of each treatment were randomly selected and a solution of colchicine added to the sand to reach the final concentration of 0.05% of sand solution (Evans et al., 1957). After 4 hours all plants were harvested and root tips excised. The root tips were fixed overnight at room temperature with 3% glutaraldehyde in 0.025 M phosphate buffer (pH 6.9). They were dehydrated in an ethanol series and infiltrated with glycol methacrylate for 2 weeks. Glycol methacrylate was polymerized overnight at 60 C under nitrogen (O'Brien & McCully, 1981). Six root tips of every treatment were randomly selected and sectioned with glass knives on a Sorvall JB-4 microtome (sections 2 µm thick). Every 10th - 15th section was attached to a microscope slide and stained with 0.05% toluidine blue O in benzoate buffer, pH 4.4 (O'Brien & McCully, 1981); the remaining sections

were discarded. Two thousand meristematic cells were counted in each root tip and the stage of the mitotic cycle recorded for each of them. Duration of the mitotic cycle was estimated for roots treated with colchicine according to Evans et al. (1957). Because the duration of each of the mitotic phases is proportional to the number of cells found in a phase compared with total number in mitosis (Clowes & Juniper, 1967), the duration of each of the phases could be determined by taking mitotic counts of root meristem cells of the seedlings which were not treated with colchicine. Statistical analyses were performed using the analysis of variance procedure, Scheffe's test (n=6).

RESULTS AND DISCUSSION

Mitosis occupies a variable fraction of the cell cycle, and for this reason the mitotic index could not indicate the rate of mitosis, particularly if fluoride equally affected all phases of the cell cycle (Clowes & Juniper, 1968). Sodium fluoride lengthened the mitotic cycle (Table 1). Four-week, 15 µg treatments increased the duration of the cycle by almost 33%. A less significant increase was observed in seedlings subjected to the 3 µg treatment. The lengthening of the mitotic cycle was due to an increase in interphase. Both fluoride treatments significantly increased

Table 1. Duration (in hours) of the mitotic cycle (T), mitosis (M), and of the phases of mitosis in root tips of sodium fluoride treated and control Jack pine seedlings, determined by the metaphase accumulation method.

Treatment*	T	M	Interphase	Prophase	Metaphase	Anaphase	Telophase
Control	54.83 A**	2.81 A	52.02 A	1.28 A	0.20 A	0.23 A	1.10 A
1	60.59 A	3.09 A	57.50 B	1.41 A	0.18 A	0.32 A	1.18 A
2	72.74 B	3.36 A	69.38 C	1.82 A	0.23 A	0.25 A	1.06 A

* 1 - 3 µg, 4 week treatment; 2 - 15 µg, 4 week treatment.
**Means (n=6) with the same letter are not significantly different.

the duration of this phase. Thus, both fluoride treatments did not affect cell divisions directly but increased the time required by cells to prepare for mitotic divisions. It is interesting that such pronounced changes took place in plants 4 weeks after adding sodium fluoride to the sand, when soluble fluoride was no longer present in the sand. This indicates that the metabolism of seedlings was not able to recover quickly after fluoride treatments. It is also possible that a necrotic, a fluoride-induced reduction in the leaf photosynthetic area might bear partial responsibility for the alterations in the cell cycle.

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APPENDIX B

EXAMPLES OF CHROMATOGRAMS AND STANDARD CURVES

Figure 1. Gas chromatogram of fatty acid methyl esters separated on a Carlo Erba Fractovap Gas Chromatograph equipped with a 3 m long, 2 mm ID glass column packed with 3% SP-2310/2% SP-2300 on 100/120 Chromosorb W AW. Oven temp., 196 C; injector and detector temp., 240 C; N_2 flow, 30 mL min^{-1}.

Sample: phospholipid fraction of fluoride treated Jack pine seedlings (3 µg, 91 h treatment).

Identification of major peaks:

2.33 - butylated hydroxytoluene (BHT), 3.49 - unknown, 4.73 - 16:0*, 5.36 - 16:1, 8.26 - 18:0, 9.25 - 18:1, 9.91 - $18:2\Delta^{5,9}$, 11.04 - $18:2\Delta^{9,12}$, 11.82 - $18:3\Delta^{5,9,12}$**, 13.50 - $18:3\Delta^{9,12,15}$, 15.10 - $18:4\Delta^{5,9,12,15}$+20:0, 18.21 - $20:3\Delta^{5,11,14}$, 21.05 - n-octacosane (internal standard).

*number of carbon atoms in the fatty acid : number of double bonds present.

**tentative positions of double bonds.

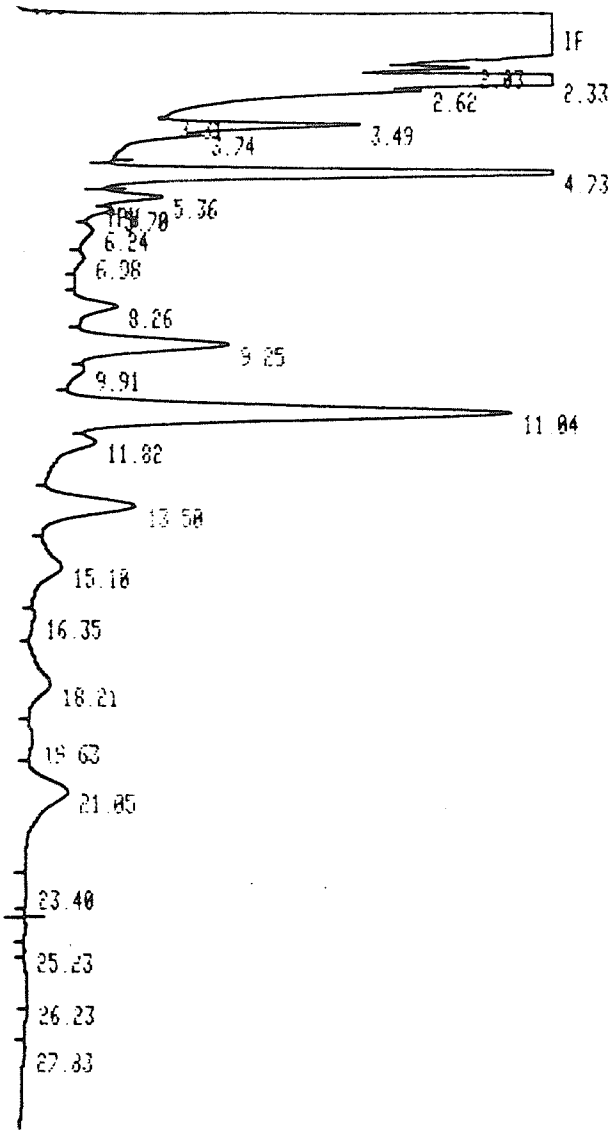

IF
2.33
2.62
3.49
3.74
4.23
5.36
6.24
6.38
8.26
9.85
9.91
11.04
11.82
13.50
15.10
16.35
18.21
19.63
21.05
23.40
25.23
26.23
27.83

Figure 2. Gas chromatogram of fatty acid methyl esters separated using a Parkin Elmer 8320 Gas Chromatograph equipped with a 20 m long DB-225 silica fused capillary column (0.25 mm ID). Oven temp., 195 C held for 3 min, raised to 235 C (5 C $min^{-1)}$ and held at this temp. for 10 min; injector and detector temp., 255 C; Linear velocity, 28 cm s^{-1}(He).

Sample: phospholipid fraction from fluoride treated Jack pine seedlings (15 µg, 24 h treatment).

Major chromatogram peaks were identified as: 3.273 - BHT, 3.784 - 14:0*, 5.356 - 16:0, 5.569 - 16:1, 6.007 - 17:0 (branched), 7.44 - 18:0, 7.72 - 18:1, 7.86 - $18:2\Delta^{5,9}$**, 8.37 - $18:2\Delta^{9,12}$, 8.37 - $18:3\Delta^{5,9,12}$, 8.85 - $18:3\Delta^{9,12,15}$, 9.96 - 20:0, 10.24 - $18:4\Delta^{5,9,12,15}$, 10.39 - unknown, 10.97 - $20:3\Delta^{5,11,14}$, 12.78 - 22:0, 13.17 - 22:1, 14.52 - unknown, 16.64 - unknown.

*number of carbon atoms in the fatty acid : number of double bonds.

**tentative position of double bonds.

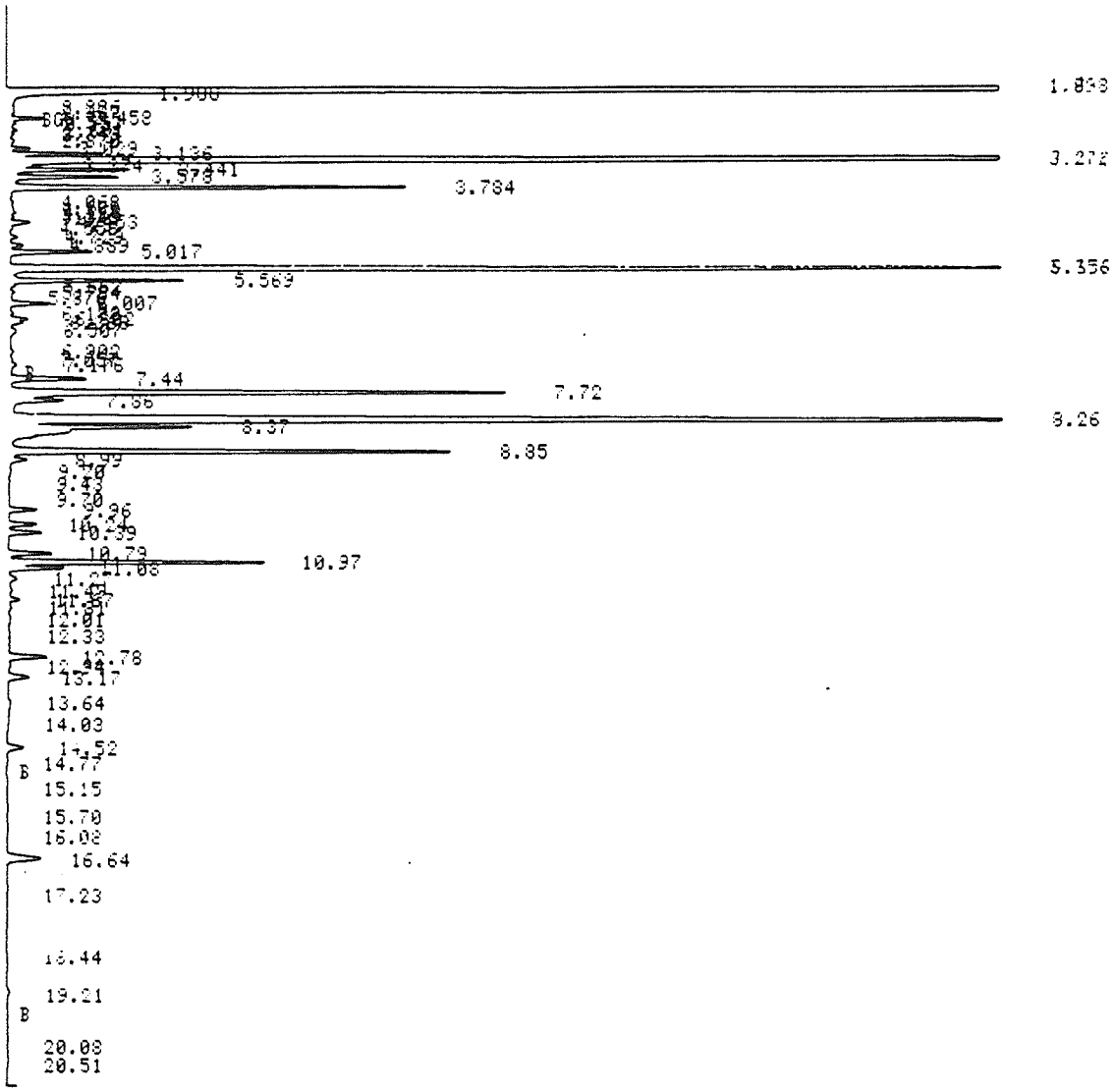

1.893
3.272
5.356
8.26
1.900
3.136
3.578
3.784
5.017
5.569
7.44
7.72
8.37
8.85
10.37
11.08
12.78
13.64
14.03
14.77
15.15
15.70
16.08
16.64
17.23
18.44
19.21
20.08
20.51
B

Figure 3. Thin-layer chromatogram of fatty acid methyl esters. Silica G plate developed in hexane - diethyl ether (40:60, v/v), sprayed with 50% sulphuric acid and charred.

1-8 - lipids from fluoride treated (15 µg, 24 h treatment) and control Jack pine seedlings.

9-10 - standards.

1 - total lipids, control; 2 - total lipids, F; 3 - neutral lipids, control; 4 - neutral lipids, F; 5 - glycolipids, control; 6 - glycolipids, F; 7 - phospholipids, control; 8 - phospholipids, F; 9 - fatty acid methyl ester standards (palmitate + stearate + oleate + linoleate + linolenate); 10 - BHT.

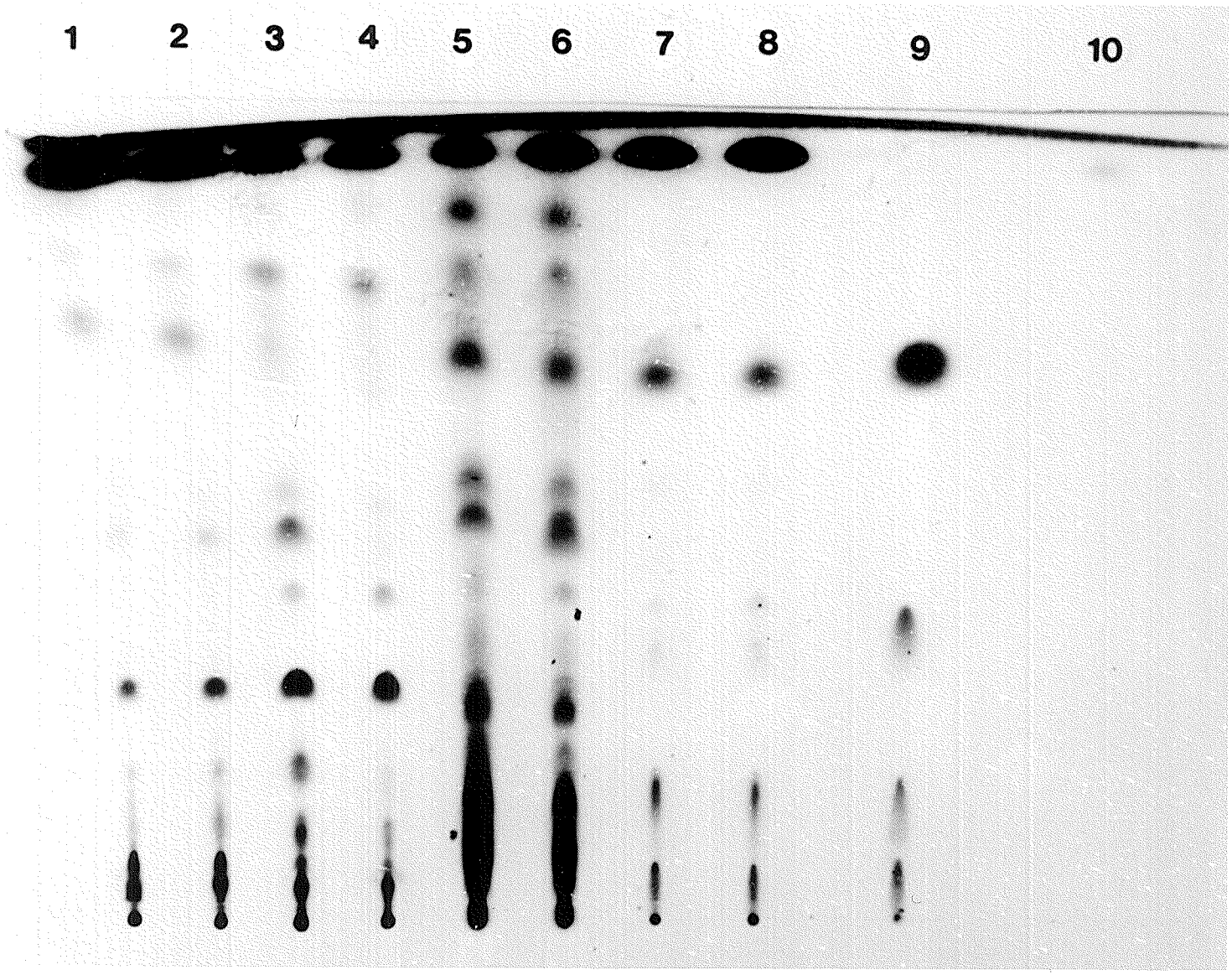
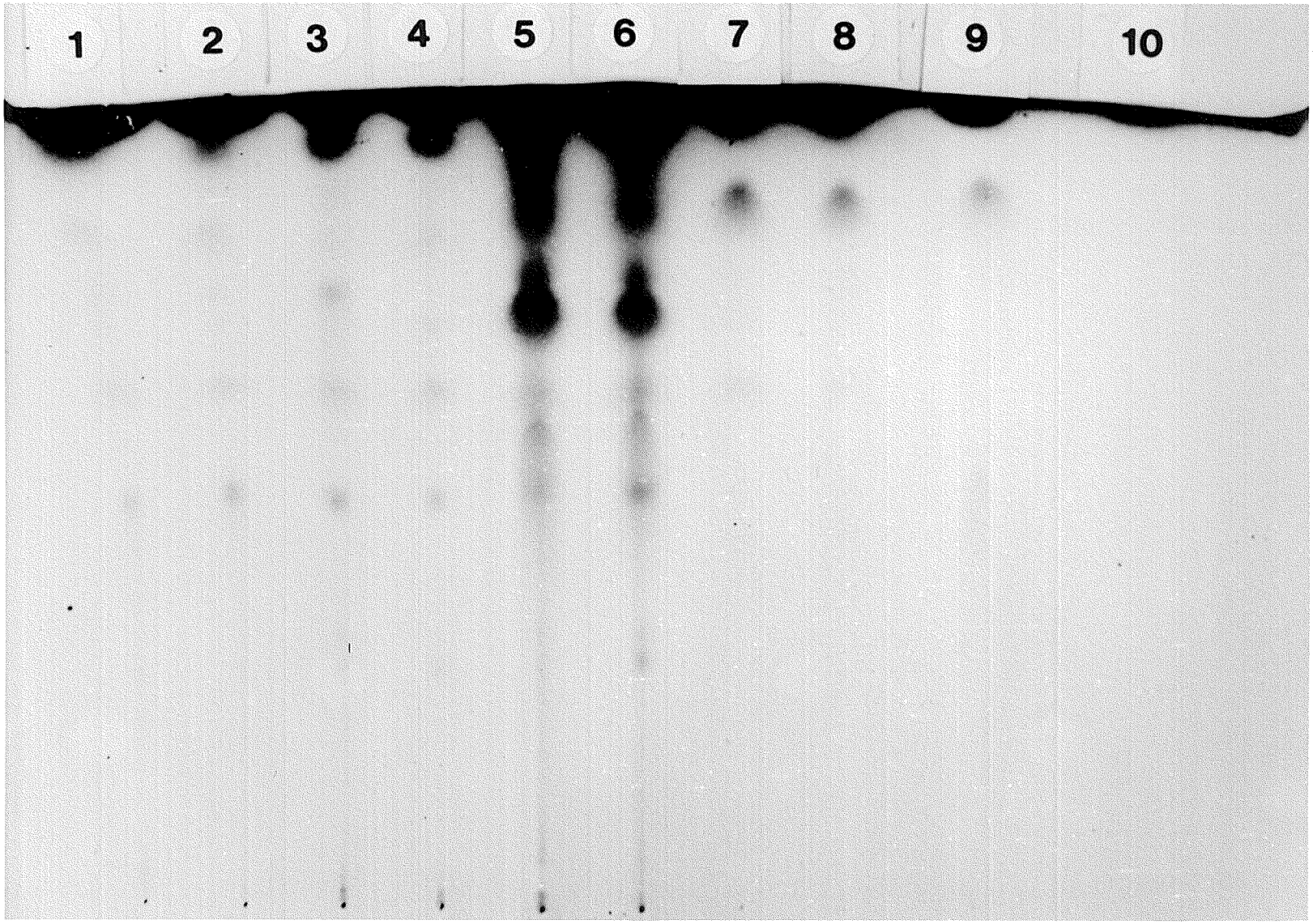

1
2
3
4
5
6
7
8
9
10

Figure 4. Thin-layer chromatogram of fatty acid methyl esters. Silica gel G plate developed in hexane - diethyl ether (90:10, v/v), sprayed with 50% sulphuric acid and charred.

1-8 - lipids from fluoride treated (15 µg, 24 h treatment) and control Jack pine seedlings.

9-10 - standards.

1 - total lipids, control; 2 - total lipids, F; 3 - neutral lipids, control; 4 - neutral lipids, F; 5 - glycolipids, control; 6 - glycolipids, F; 7 - phospholipids, control; 8 - phospholipids, F; 9 - fatty acid methyl ester standards (palmitate + stearate + oleate + linoleate + linolenate); 10 - BHT.


1
2
3
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5
6
7
8
9
10

Figure 5. Iatroscan separation of molecular species of phospolipids extracted from Jack pine seedling subjected to 15 µg, 24 h F treatment. The chromatogram (Chromarod-SII) was developped in chloroform - methanol - 35% ammonium hydroxide (60:30:5, v/v/v) and analysed using an Iatroscan TH-10 (Iatron Laboratories, Inc.).

Major chromatogram peaks were identified as: 0.15, 0.17 - phosphatidylethanolamine + phosphatidylglycerol; 0.22 - phosphatidylcholine; 0.39 - phosphatidylinositol.

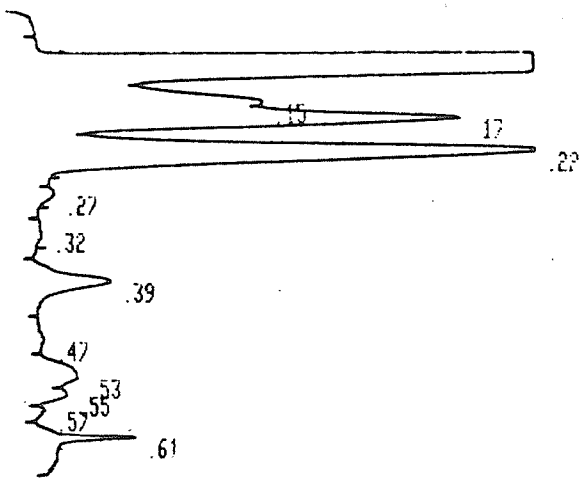

.15
17
.22
.27
.32
.39
47
53
55
57
.61

Figure 6. Thin-layer chromatogram of molecular species of phospholipids. Silica G plate developed in chloroform - methanol - ammonium hydroxide (30%) - water (65:35:5:2.5, v/v/v/v) and sprayed with a modified Dittmer-Lester reagent (Ryu & McCoss, 1979).

1 - 7 - standards

8, 9 - phospholipids extracted from Jack pine seedlings

1 - phosphatidic acid, 2 - phosphatidylcholine (dipalmitoyl), 3 - phosphatidylethanolamine, 4 - phosphatidylglycerol, 5 - phosphatidylcholine (dioleoyl), 6 - phosphatidylinositol, 7 - phosphatidylserine, 8 - control, 9 - 15 µg, 24 h F treatment.

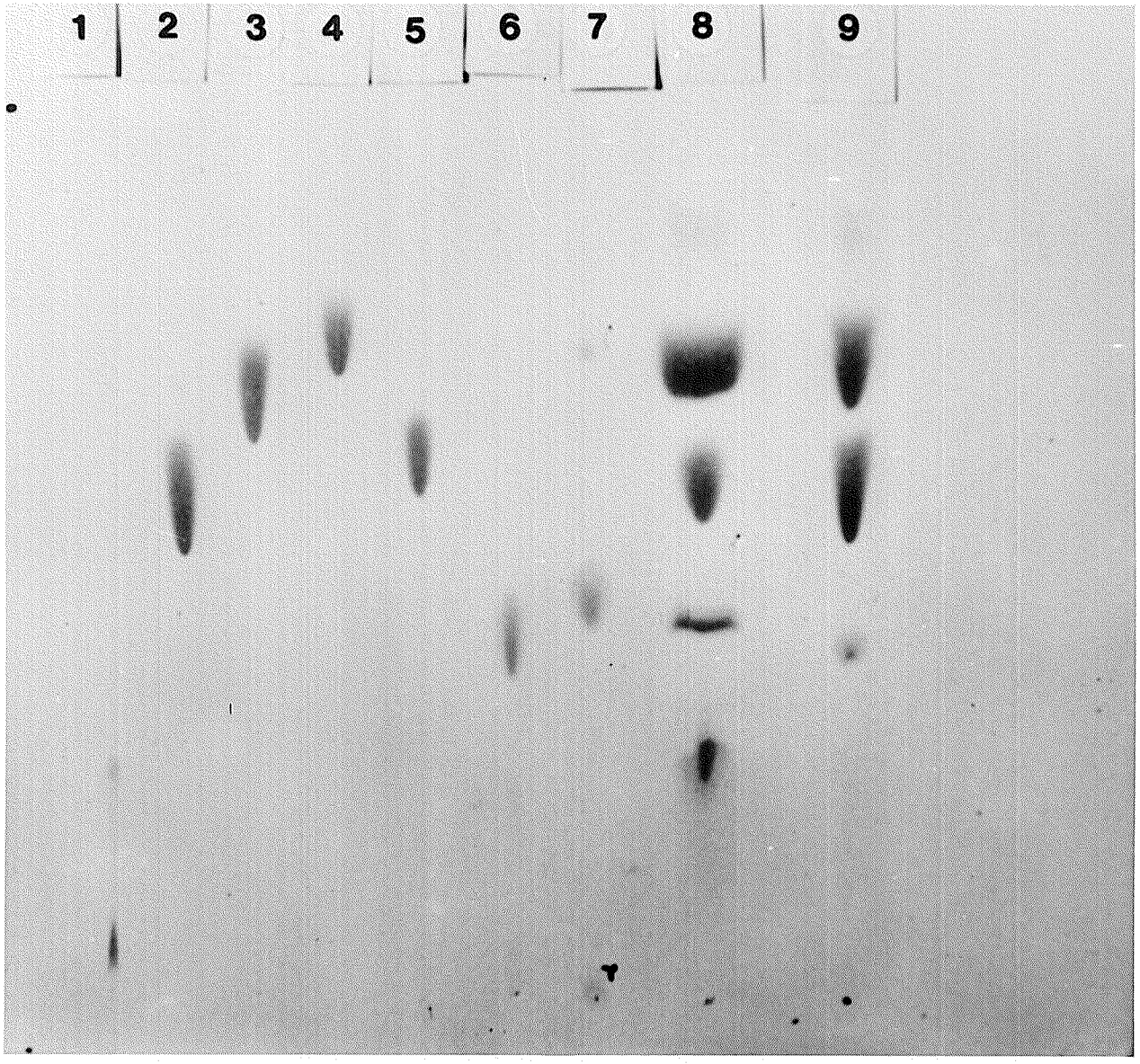

1
2
3
4
5
6
7
8
9

Figure 7. Gas chromatogram of trimethylsilyl derivatives of carbohydrates separated using a Carlo Erba Fractovap Gas Chromatograph equipped with a 1.8 m long, 2 mm ID glass column containing 3% OV-3 on 80/100 mesh Chromosorb W. Oven temp., 180 C for 25 min, rapidly increased to 240 C and held for 20 min; injector and detector temp., 250 C; N_2 flow, 40 mL min^{-1}

Sample: Jack pine seedlings; 3 µg, 91 h fluoride treatment.

Identification of major peaks: 6.24 - fructose + mannose, 6.65 - unknown, 8.82 - α-glucose, 10.51 - sorbitol (internal standard), 11.54 - unknown, 13.51 - ß-glucose, 19.73 - cyclitols, 36.14 - sucrose.

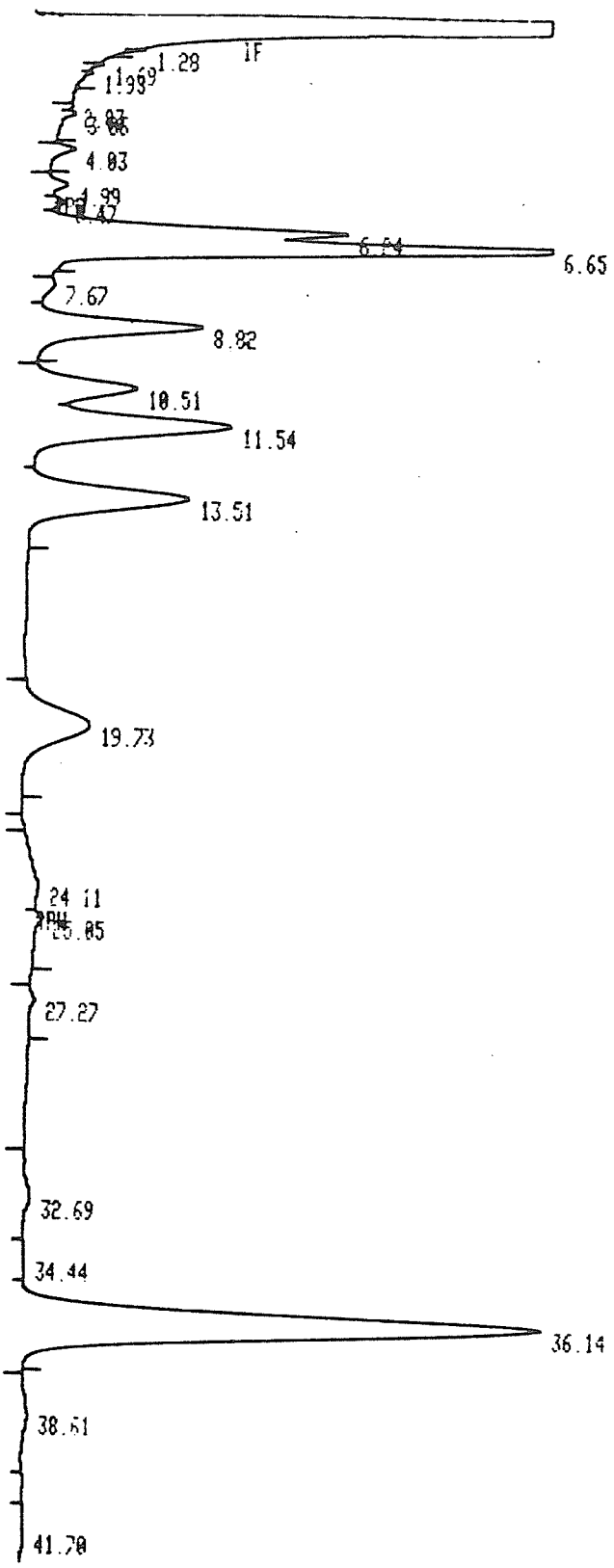

1.28
4.03
6.65
7.67
8.82
10.51
11.54
13.51
19.73
24.11
27.27
32.69
34.44
36.14
38.61
41.70

Figure 8. Gas chromatogram showing separation of fructose from mannose and an unknown compound, both with similar retention times when analysed on a column containing 3% OV-3.

Carlo Erba Fractovap GC equipped with a 3 m long, 2mm ID glass column containing 3% SP2310/2% SP2300 on 100/120 Chromosorb W AW. Oven temp., 145 C; injector and detector temp., 200 C; N_2 flow, 30 ml min^{-1}.

Sample: fluoride treated Jack pine seedlings (3 µg, 7 day treatment).

Identification of major peaks: 12.21 - α-mannose, 12.99 - unknown, 14.17 - fructose, 19.28 - unknown.

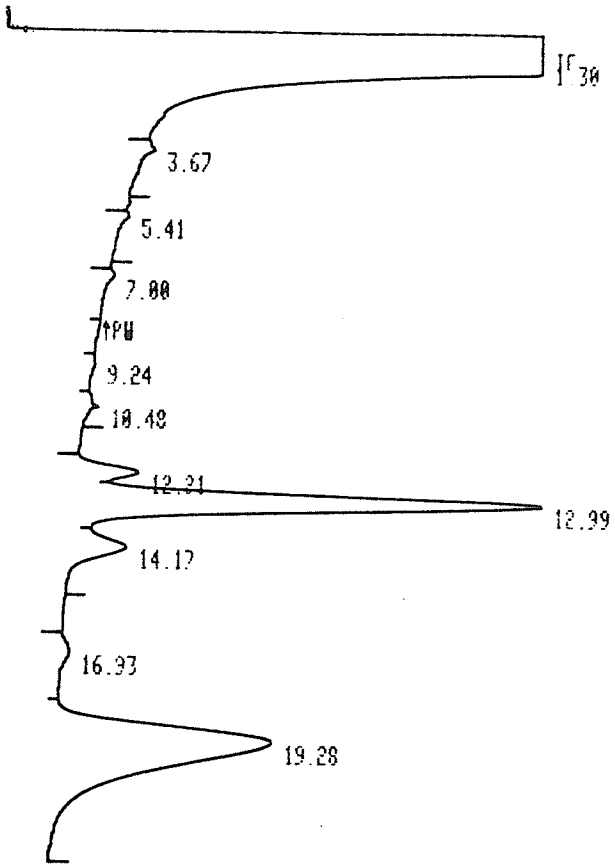

3.67
5.41
7.00
TPW
9.24
10.48
12.21
12.99
14.17
16.93
19.28

Figure 9. Gas chromatogram of trimethylsilyl derivatives of organic acids separated on a Carlo Erba Fractovap GC equipped with a 1.8 m long, 2 mm ID glass column containing 3% OV-3 on 80/100 mesh Chromosorb W. Oven temp., 45 C - 180 C (2 C min^{-1}); injector and detector temp., 220 C; N_2 flow, 40 mL min^{-1}.
Sample: fluoride treated Jack pine seedlings, (3 µg, 91 h F treatment).
Identification of major peaks: 14.12 - succinic acid, 20.10 - maleic acid (internal standard), 38.68 - unknown, 43.20 - shikimic acid, 46.17 - quinic acid, 47.63 - syringic acid.

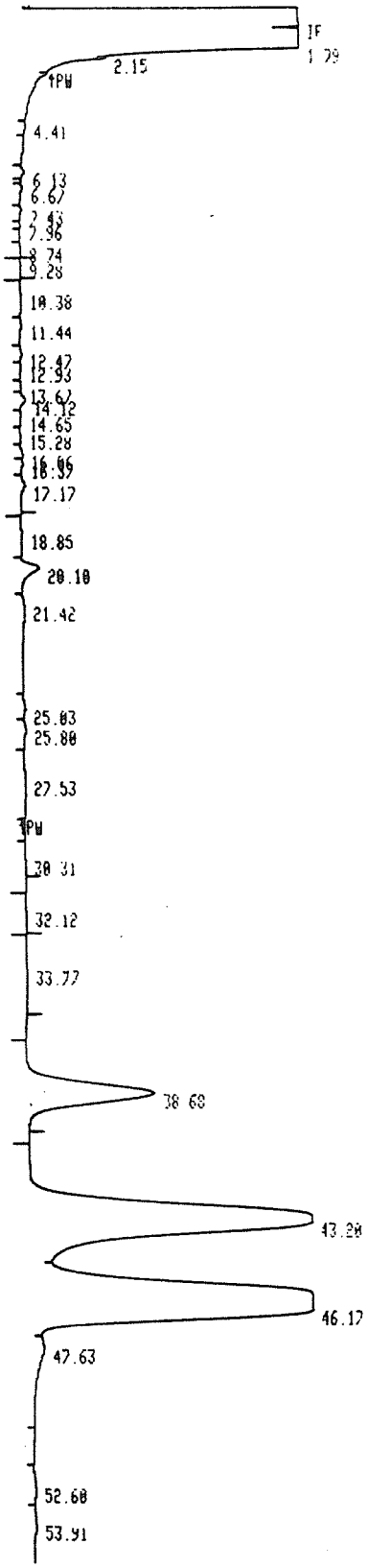

1F
1.79
2.15
PW
4.41
6.13
6.67
7.96
8.74
9.28
10.38
11.44
12.47
12.93
13.62
14.12
14.65
15.28
17.17
18.85
20.10
21.42
25.03
25.80
27.53
PW
30.31
32.12
33.77
38.68
43.20
46.17
47.63
52.68
53.91

Figure 10. Separation of amino acids using a LKB Amino Acid Analyser equipped with a 20 cm X 0.6 cm LKB Ultrapack 8 resin column.
Sample: Jack pine seedlings, control.
Identification of major peaks:
10.521 - aspartic acid, 12.251 - serine, 14.486 - glutamic acid, 15.949 - proline, 19.732 - glycine, 21.065 - alanine, 22.497 - valine, 25.707 - isoleucine, 26.446 - leucine, 28.807 - tyrosine, 30.173 - phenylalanine, 33.605 - unknown, 38.180 - histidine, 39.307 - lysine, 40.673 - unknown, 42.621 - ammonia, 44.362 - unknown, 46.770 - arginine, 48.722 - unknown.

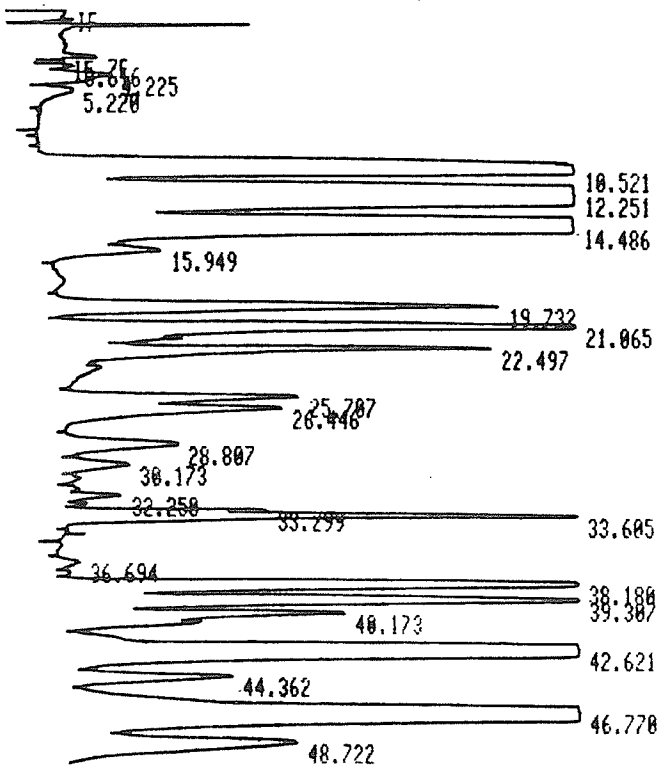

5.220
10.521
12.251
14.486
15.949
19.732
21.865
22.497
26.446
28.807
30.173
32.258
33.605
36.694
39.307
40.173
42.621
44.362
46.770
48.722

Figure 11. Quantitative separation of major amino acids using a LKB Amino Acid Analyser equipped with a 20 cm X 0.6 cm LKB Ultrapack 8 resin column.

Sample: fluoride treated Jack pine seedlings (3 μg, 91 h treatment).

Identification of major peaks:

7.830 - aspartic acid, 9.850 - serine, 11.754 - glutamic acid, 17.166 - glycine, 18.500 - alanine, 20.606 - valine, 36.377 - histidine, 37.570 - lysine, 40.880 - ammonia, 44.510 - arginine.

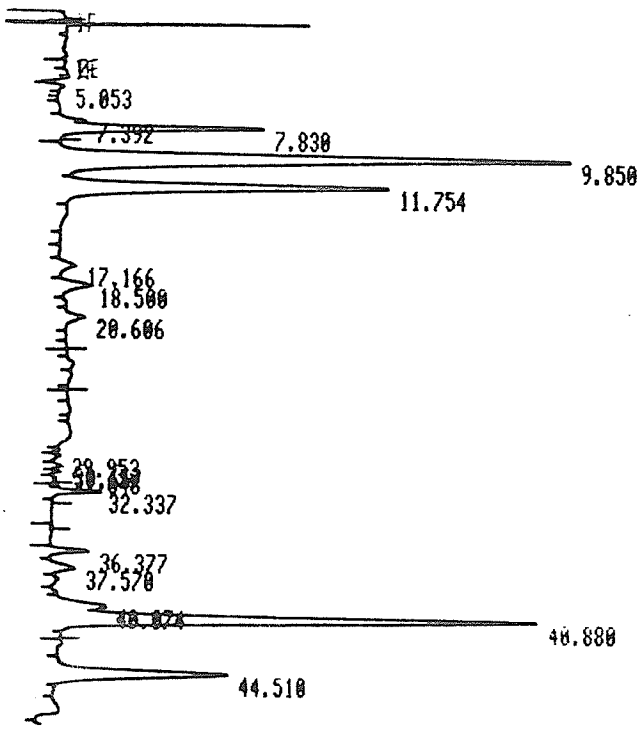

5.053
7.830
9.850
11.754
17.166
18.500
20.606
32.337
36.377
37.570
44.510

Figure 12. Typical standard protein curve for the Bio-Rad protein assay. Black dots indicate spectrophotometer readings of individual samples.

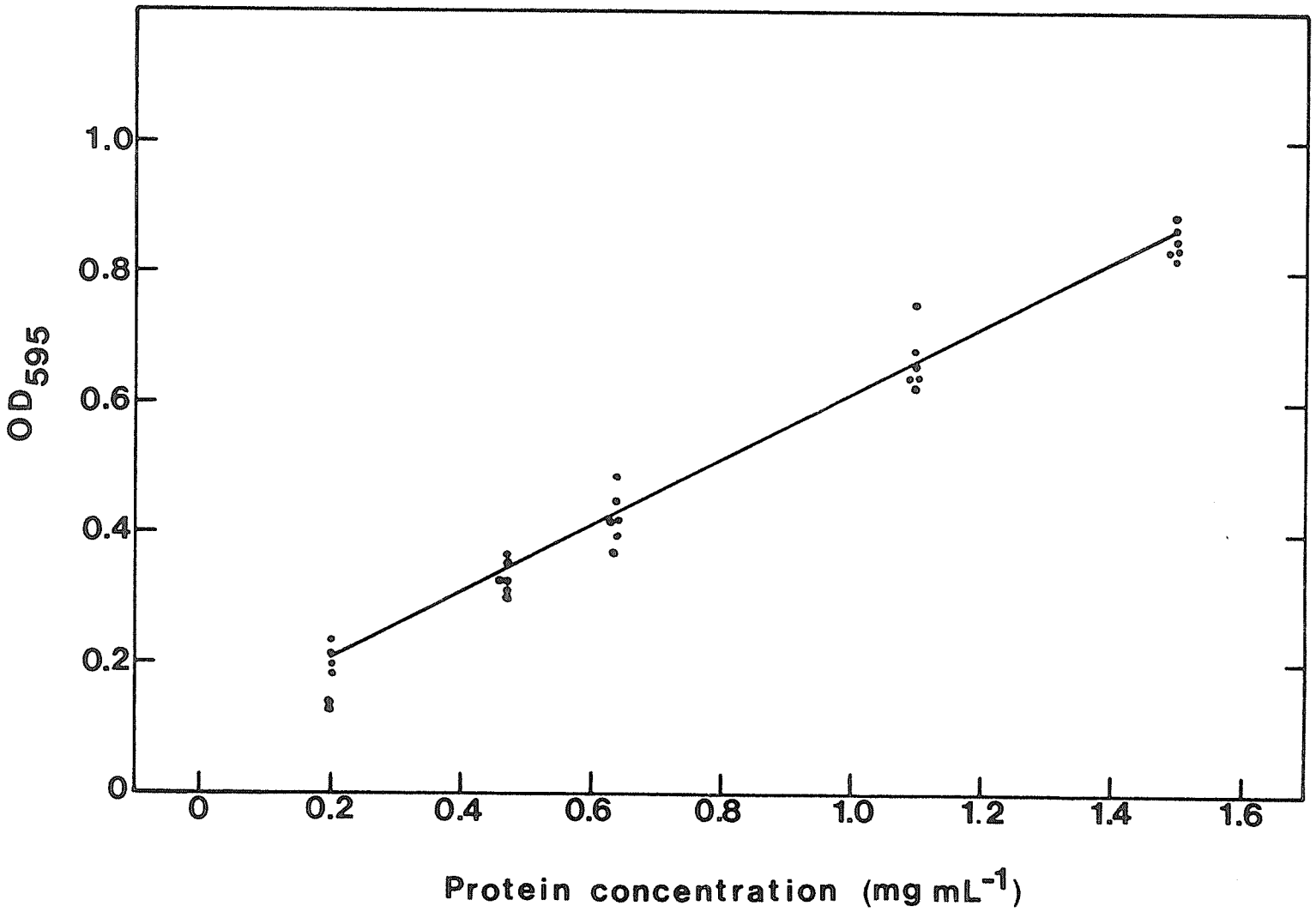
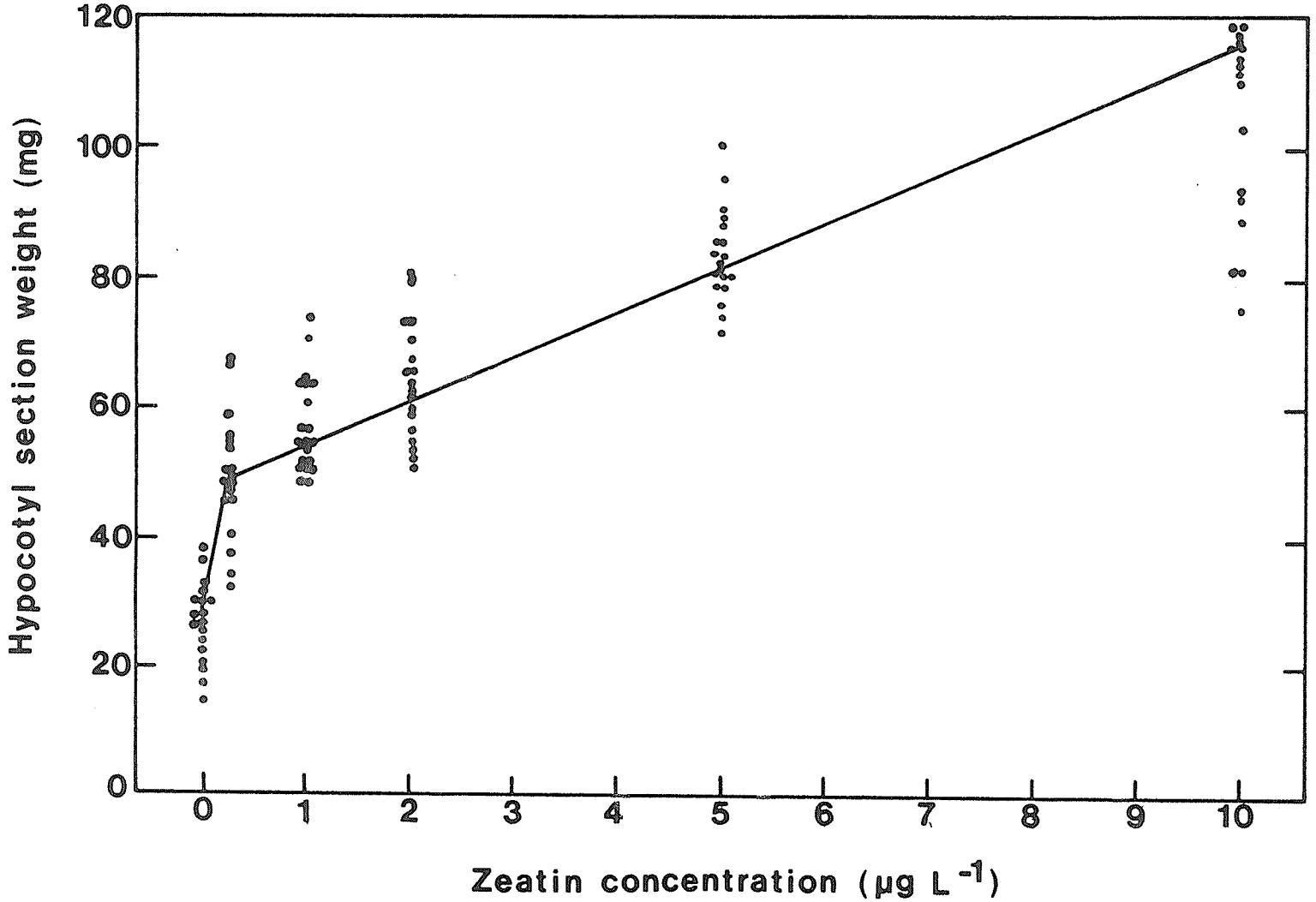

1.0
0.8
0.6
0.4
0.2
0
OD_{595}
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
Protein concentration (mg mL^{-1})

Figure 13. Standard zeatin curve used to quantify cytokinin-like compounds in extracts obtained from fluoride treated and control Jack pine seedlings. Black dots indicate weights of individual hypocotyl sections.


Hypocotyl section weight (mg)
120
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
9
10
Zeatin concentration (µg L -1)