Spring 1968

SEASONAL VARIATION OF ALIPHATIC AND PHENOLIC ACIDS AND THE RELATIONSHIP BETWEEN TITRATABLE ACIDITY AND PERCENTAGES OF CALCIUM, MAGNESIUM AND POTASSIUM IN THE LEAVES OF SELECTED MCINTOSH APPLE CLONES

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Dip. Agric. Makerere University College
of East Africa, 1956

M. S. University of New Hampshire, 1964

A Dissertation

Submitted to the University of New Hampshire

In Partial Fulfillment of

The Requirements for the Degree of

Doctor of Philosophy

Graduate School

Department of Plant Science

June, 1968

This dissertation has been examined and approved.

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Apr. 30, 1968

Date

ACKNOWLEDGEMENTS

The author wishes to express his sincere appreciation to:

Prof. R. Eggert for guidance, encouragement, and interest during all phases leading to the completion of this dissertation.

Dr. D. G. Routley for guidance in conducting this investigation and criticism of the manuscript.

Dr. L. C. Peirce for suggestions, comments, and criticism of the manuscript.

Dr. L. A. McFadden and

Dr. S. Dunn for criticism of the manuscript.

Dr. L. C. Grant and

Miss Dorothy Josselyn for mineral analyses of leaves.

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ABSTRACT

SEASONAL VARIATION OF ALIPHATIC AND PHENOLIC ACIDS AND THE RELATIONSHIP BETWEEN TITRATABLE ACIDITY AND PERCENTAGES OF CALCIUM, MAGNESIUM AND POTASSIUM IN THE LEAVES OF SELECTED MCINTOSH APPLE CLONES

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To investigate whether calcium and magnesium accumulation was correlated with titratable acidity in the leaves of decidous fruit trees late in the season, water extracts of dry leaf tissues from selected McIntosh apple clones were analyzed by titration with sodium hydroxide, paper and ion exchange-resin chromatography, and by atomic absorption spectrophotometry. A useful solvent system for paper chromatographic separation of organic acids in apple leaf extracts was developed. Citric, malic and succinic were the principal acids detected. It was shown that titratable acidity decreased during the period July 4 - August 1. This decline corresponded to the disappearance of citric and malic acids. Titratable acidity increased as the season advanced beyond August 1. This increase was correlated with the re-appearance of citric and malic acid spots on chromatograms. Succinic, caffeic and chlorogenic acids increased as the season advanced. An unidentified phenolic acid having similar R_f values as those of γ-indole-3-n-butyric and indole propionic acids, appeared in significant amounts toward the end of the growing season. The data suggest

that calcium and magnesium, which accumulate in leaves as the season advances, may be the principal cations associated with bases involved in the buffering systems which maintain pH relatively constant. The appearance of deficiency symptoms late in the season is attributed to diversion of the available magnesium toward buffering acidity at the expense of other functions. Potassium which accumulates only under conditions of deficient calcium and/or magnesium may not ordinarily be associated with pH buffering systems late in the season. It is theorized that change in pH may be a major factor in the characteristic damage resulting from certain mineral deficiencies in apple leaves.

INTRODUCTION

Since John Woodward's mint experiment in 1699, and Liebig's work in 1840, when it became known that mineral elements were essential for the healthy growth of plants, various scientists have been greatly interested in the field of plant nutrition (Reed 1907; Wallace 1928; Blake, Nightingale and Davidson 1937; Hoagland 1944; Cain 1948; Gilbert 1949; Boynton and Embleton 1950; Jones and Parker 1951; Mulder 1952; Titus and Lott 1956; Hewitt 1958; Ward 1959; Steward 1963; Hiatt 1964; Ford 1966). Researchers have observed that the calcium content increased in the leaves of some perennial and decidous fruit trees as the season advanced (Boynton, Cain and Compton 1944; Cain and Boynton 1948; Emmert 1954; Rogers, Batjer and Billingsley 1955; Batjer and Westwood 1958; Embleton, Jones and Allen 1958; Bingham 1961; Ward and Kenworthy 1963; Simons 1965). Rogers, Batjer and Thompson (1953) who expressed their results on a unit area basis, found that the amount of calcium in apple leaves at leaf fall was almost double that at the beginning of the season. There appears, however, to be no satisfactory explanation for the phenomenon.

Unlike calcium, the potassium content of leaves decreases with time if there is an ample amount of available magnesium in the growing medium (Lilleland and Brown 1938; Ruther and Boynton 1939; Wallace 1939; Boynton, Cain and Compton 1944; Cain and Boynton 1948; Emmert 1954). If the amount of magnesium is insufficient, or if there is excess potassium, the potassium content on a percentage basis increases as the season advances and induces magnesium deficiency symptoms by what has been termed "antagonism"

(Wallace 1939; Kidson, Askew and Chittenden 1940; Jacob 1958; Ward 1958; Semb and Tragethon 1959; Stembridge, Gambrell, Seffic and Van Blaricom 1962; Salmon 1963). Many papers have been published describing how the visible symptoms of magnesium deficiency usually develop in apple leaves later in the season after most normal terminal growth has been made (Hill and Johnson 1940; Boynton and Burrel 1944; Ford 1964; Ford 1966), but no convincing explanation has yet been given. Cain (1959) suggested that the so-called "antagonism" between magnesium and potassium associated with increases in N and K fertilization could largely be explained by growth dilution.

The purpose of this study was to find out if seasonal trends exist in titratable acidity which could be associated with the observed increase in calcium content of leaves and to determine if any of the changes in acidity could be related to the appearance of magnesium deficiency symptoms and to variations in the potassium content.

REVIEW OF LITERATURE

INTRODUCTION

Plants are still the ultimate source of organic carbon for both man and beast. The demand for agricultural products is serious enough when it is considered only in terms of increasing human population, with a fixed amount of cultivable land. The problem becomes more complicated with the increasing demand for higher quality products. In attempting to improve quality through hybridization, the plant breeder may change the physiological characteristics of the plant making it more susceptible to certain nutritional deficiencies (Eisenmenger and Kucinski 1947). It is the duty of scientists in related fields to try unceasingly to find causes of deficiencies and ways to correct them. It is also their duty to determine the various roles played by minerals in the metabolism of plants so that sufficient high quality products may be produced to meet man's needs.

CALCIUM

Calcium is widespread and abundant in most flowering plant tissues averaging around 1.5 per cent of the dry weight of leaves, 1.2 in buds, and 0.2 per cent in seeds (Brewbaker and Kwack 1963). According to Reed (1907) the first demonstration concerning the necessity of calcium for the growth of higher plants were carried out by Stohman in 1862 "by cultivating corn plants in solutions lacking calcium. At the end of five weeks, the plants were dead at the tips; after the addition of a small amount of calcium salts, the plants quickly took on a new life and began forming new leaves and shoots."

Although it took some years before any of the roles played by calcium in plants could be ascertained (Groom 1896; Hartwell 1916; Troug and Mecham 1919; True 1922; Nightingale, Addoms, Robbins and Schermerhorn 1931), recent research is providing much information on the subject.

A. FUNCTIONS

1. Cell structural function

In his search for the origin of the middle lamellae of cells, Allen (1901) made cytological and histochemical studies of many different species of plants. He observed that cambial cells were the origin of the middle lamella and described the sequence of the whole process. He stated that a non-pectic layer was formed within the first formed pectic stratum which continued to thicken after the appearance of the non-pectic layer. At that stage the whole 'substance' was elastic. The plastic nature allowed modification of its shape. After the cell had attained its final form, pectic acid was changed into insoluble calcium pectate. This was in principle confirmed more recently by other workers (Reed 1907; Sorokin and Sommer 1929; Cormack, Lemay and Maclachlan 1963; Brewbaker and Kwack 1963). It was True (1922) who, after a long series of experiments, came to the conclusion that no other cation could replace calcium in the calcium pectate of the middle lamella without injury to the cell.

2. Root and Root-hair development

Since plant cell walls are not only made of cellulose that requires calcium for its synthesis (Reed 1907) but also are cemented together by compounds of calcium (True 1922), it becomes obvious that the size of the root system developed will depend, among other things, on the amount of available

calcium in the growing medium. Nightingale (1937) found that the root system of calcium deficient apple trees was drastically suppressed. Frequently, the roots failed to develop and those that did appear were characteristically short, bulbous, brown at the tips, with sloughing-off of cells further back. The sloughing-off of cells had previously been observed by Nightingale, Addoms, Robbins and Schermerhorn (1931) in the roots of calcium deficient tomato plants. They explained that it was probably due, in part, to the failure of the middle lamella of calcium pectate to develop under such extremely deficient conditions. Studying the development of root-hairs in angiosperms, Cormack (1949) found that root-hair cell walls were comprised of an inner layer of cellulose surrounded by a harder layer of calcium pectate except at the softer tip area where either pectic acid or a modified form of pectate was present. When adequate calcium was present, the softer area was enlarged and a root-hair tip swelled to a bulbous state. It was later concluded that calcium was required for the formation of root-hair initials. The mechanism was thought to depend on internal water pressure forcing out the cell wall at the weakest points left during the hardening of the remaining area by calcium pectate. In a later publication, Cormack (1965) reported that calcium ions as well as pH of the growing medium affected the development of roots and root-hairs.

3. Absorption and translocation of nutrients

When it was observed that on replacing calcium of the calcium pectate in the middle lamella with magnesium, there was a "fatal change in permeability relations" of the cell, True (1922) suggested that calcium ions make "physiologically

available" other equally indispensable nutrient ions. Since Nightingale, Addoms, Robbins and Schemerhorn (1931) noticed that tomato plants which lacked calcium accumulated carbohydrates in large quantities, they concluded that it was a result of calcium deficiency that the plants were unable to absorb and assimilate nitrate. Burstrom (1954) produced experimental evidence to show that calcium was necessary for the absorption of nitrate from external medium.

A number of other investigators have found that calcium is involved in selective absorption and transport of cations. In the experiment of Epstein (1961) excised barley roots washed free of diffusible calcium were made to absorb ions from solutions of KCl or RbCl or NaCl or combinations of these in the presence of calcium or other divalent ions for a period of 60 minutes. It was found that in the absence of calcium, Na interfered with K absorption and K with Na at all concentration ratios examined. In the presence of calcium, Na interfered slightly with K absorption at low Na concentrations but at progressively higher concentrations of Na there was no further inhibition of K absorption. It was concluded that calcium was required in maintaining the integrity of the selective absorption mechanism and Mg was largely ineffective for this purpose. In an experiment by Waisel (1962), it was discovered that while calcium stimulated the uptake of K, Rb, and Cs, it depressed the permeation of the highly hydrated cations such as Li and Na. In a split-root experiment on apple trees, Fucik and Titus (1965) found and reported that there were two calcium-associated processes involved in the nutrition of manganese. One of the processes located near or at the root surface controlled manganese absorption while the other located within the roots and possibly in other tissues as well,

helped regulate manganese translocation within the plant.

4. Enzymatic activity

According to Hewitt (1951) since calcium maintains cell organization through permeability and hydration, it therefore, directly or indirectly, influences enzyme systems. McElroy and Nason (1953) were able to specify that some adenosine triphosphatases required calcium ions rather than Mg^{++} for activity. This has been confirmed by Johnson and Jackson (1966) who, using mitochondria extracted from wheat roots found that the specific activity associated with the particulate fraction was sharply increased by calcium salts.

5. Carbohydrate translocation

Groom (1896) showed experimentally that, in the absence of calcium, there was an accumulation of oxalic acid which retarded the action of diastase on starch so that there was a stoppage in the conduction of carbohydrates. According to Reed (1907) a deficiency of calcium was believed to cause a disturbance in the translocation of not only carbohydrates but also proteins. Working with tomatoes, Nightingale, Addoms, Robbins and Schermerhorn (1931) observed that plants which lacked calcium, accumulated carbohydrates in large quantities. Joham (1957) observed the same in cotton. However, in view of the work by Hartwell (1916), in which he showed that any factor that resulted in retarded growth influenced the accumulation of starch, it would appear that still more research is needed to definitely establish the direct involvement of calcium in carbohydrate translocation.

6. Effect on pollen germination

In many field and horticultural crops, pollination, fertilization and fruit set are processes of great importance. Pollen must germinate before fertilization can take place. In recent years numerous investigations have been carried out to find what might be the ideal environment for pollen germination and the results so far, at the nutritional level at least, have been very encouraging. Brewbaker and Majumder (1961) working with pollen from a large number of species observed that when a small population of pollen grains was grown in vitro, germination and growth, if any at all, were very poor. But if the population was enlarged, germination and growth improved progressively under the same conditions. Water extracts of pollen and other plant parts contained a factor or factors which could overcome fully the population effect. The factor contained in the water extracts was called the pollen growth factor (PGF). It was dialyzeable, insoluble in ether, heat-stable and was not replaceable by either kinetin or auxin. This factor was finally shown to be the calcium ion, and its action has been confirmed in 86 species representing 39 families. Other cations as K^{+}, Mg^{++} and Na^{+}, singly or together could act to enhance calcium activity but could not replace it. In a later experiment, Kwack (1965) described how the importance of calcium in pollen growth was confirmed in 30 families comprising 46 genera and 46 species. The '+Ca' resulted in higher germination percentages in most species and much greater tube growth in all species than the '-Ca' treatments. Pollen from Mimosa pudica did not germinate at all unless soluble calcium was present in the media. From Ca^{45} autoradiographs it was suggested that the improvement in germination and growth of pollen due to calcium might be explained in terms

of the binding of calcium to the carboxyl groups of pectic acid along the pollen wall. This increases wall rigidity and stability and prevents bursting.

7. Seed germination

From the experiments of Albrecht (1941) it was learned that calcium was essential for seed germination. With tomato seed planted in media supplied with different amounts of calcium, it was shown that there were significant correlations between calcium supply and percentages of seed germination. Its effects appeared to be related to its nutritional value and could not be ascribed to changes in soil reaction.

8. Neutralizing effect

It is recognized that calcium salts act beneficially upon the soil through their ability to bring about the proper physical and chemical conditions required for growth without serving directly as nutrients. This is brought about principally by neutralizing the H ion in the soil and thereby altering the charge on the clay-colloid complex. For a long time it was thought that possibly bases associated with calcium would react with the H^+ ion inside a plant. Reed (1907) for example, stated that "by precipitating the poisonous oxalic acid in an insoluble form, the juices of the plant are maintained in a proper acidity for effective work." Truog and Meacham (1919) observed that in the life processes of plants, acids were formed, some of which were by-products, and that lime and other bases were needed to neutralize those acids. Moser (1933) who did not find any significant correlation between calcium-magnesium ratio and crop yields, concluded that the addition of lime to the soil was beneficial not because it changed the calcium-magnesium

ratio but because it increased the amount of replaceable calcium some of which, when taken up by the plant, was used for the neutralization and precipitation of the organic acids in the plant sap. Drosdoff, Barrows, Lagasse and Shear (1955) discovered that calcium deficiency symptoms on Tung trees growing on Lakeland fine sandy soil were accentuated by nitrate fertilization. Leaf analysis data showed that trees which did not receive calcium applications had a comparatively low calcium content and an excessive amount of oxalic acid. They explained that under these conditions, all nitrate supplies resulted in the formation of oxalic acid which took up all the available calcium to precipitate calcium oxalate so that there was none left for other functions. When enumerating the functions of calcium, Wallace (1961) stated that calcium provided a base for the neutralization of organic acids by detoxification of hydrogen ions. Working with excised roots, Rains, Schmid and Epstein (1963) observed that the presence of Ca was essential to minimize the injury caused by H ions to cation absorption mechanisms.

B. MOBILITY

Although direct quantitative evidence of calcium redistribution in plants is scanty (Williams 1955), the few earlier reports on the subject have not yet been disproved. In ringing experiments with cotton plants, Mason and Maskell (1931) showed that nutrients moved downward from the leaves to the roots through the phloem. They found calcium oxalate crystals in the ray cells of the phloem but none in the sieve tubes. They postulated that calcium gained access to all cells from the roots by transpiration stream mechanisms but once present within the cell it was either precipitated

or combined with tissue material in such a way that very few calcium ions were left in solution. On analyzing expressed sap, however, they found as many milligram-equivalents of calcium as those of potassium indicating that there was a considerable concentration of free calcium ions within the living cells. They were obliged to revise their theory and restated that it might be because of impermeability of cell membranes to outward passage that calcium remained within the cell. This was later confirmed experimentally by Mason, Maskell and Phillips (1936), who categorically stated that calcium was immobile within the plant tissues. In further support, Wallace (1961) reported that although a large proportion of calcium contained in the plant might be soluble in water, calcium did not appear to move freely from the older to younger parts of the plant and hence young tissues contained lower proportions of calcium than older ones.

C. SEASONAL VARIATION

When an element serves a particular function in the metabolism of a plant it is only logical to expect that it would be in greatest demand at the time when the reactions it is involved in are going on most actively. With calcium it has also been noted that its content in leaves fluctuates throughout the season.

Boynton, Cain and Compton (1944) found that calcium percentages in apple leaves increased from 0.73 to 2.43 during the period May 27 - November 5. Cain and Boynton (1948) collected data indicating that calcium increased progressively as the season advanced. To disprove that the observed increase in the percentages of calcium was an artifact due to loss in dry matter, Rogers, Batjer and Thompson (1953) expressed their results on a unit area basis

and found that the amount of calcium at leaf fall was double that observed at the beginning of the season. Emmert (1954), who was interested in soluble cations, noted that soluble calcium increased as the season advanced but declined toward the end. Rogers, Batjer and Billingsley (1955) observed the same trend in peaches. Batjer and Westwood (1958) also working with peaches, collected leaves at 15 day intervals beginning 33 days after full bloom. Analytical results showed that on the 180th day, the latest sample date, the amount of calcium was double that on the earliest date. In avocado leaves, Embleton, Jones and Allen (1958) observed that the amount of calcium sharply increased and kept climbing steadily until the first of October. Thereafter the increases were very slight. Analyses by Mason (1958) throughout the season showed progressive increases in the concentrations of calcium in the leaves collected from shoots of M. VII apple rootstocks. Awad and Kenworthy (1963) in their investigations on apples, noted that there was a continuous increase in leaf calcium from the 30th of June to the last sampling date of August 11. Oland (1963) reported that calcium content in apple foliage increased by 18 per cent at the onset of senescence. There is, apparently, a continuous build-up of calcium in the leaves of fruit trees as the growing season advances.

POTASSIUM

According to Reed (1907) it was Birner and Lucanus in 1866 who, working with oat plants in water cultures, gave experimental evidence that potassium was absolutely essential for plant growth and could not be substituted for by any other metal. But Reed (1907) himself after a series of experiments with speciments of a moss Attrichum sp. came to

the conclusion that there were some instances where sodium or rubidium could participate in metabolic functions provided there was a minimum amount of potassium.

A. FUNCTIONS

Although the essentiality of the element has been confirmed by several workers, some of the exact functions of potassium have not yet been elucidated (Meyer, Anderson and Bohning, 1960; Steward, 1963). What has generally been agreed upon, however, is the fact that potassium has not been found anywhere as a structural component in plants (Nightingale, 1943; Sprague, 1963). To many researchers, the fundamental roles of this element in plant metabolism appear to be catalytic or regulatory.

1. Protein synthesis

By studying many different species of plants under normal and potassium deficient conditions some facts suggesting that the element is involved in nitrogen metabolism have been obtained. Investigating dicarboxylic acids and their amides, Richard and Berner (1954) found that under conditions of potassium deficiency, the free acids diminished and the amides increased. They interpreted this as a process of proteolysis. This was confirmed later by Mulder and Bakema (1956), who generally found considerably more soluble non-protein nitrogen in deficient potato plants than in those that had been well supplied with potassium. Investigating the effects of monovalent cations in peptide synthesis, Webster and Varner (1956) used mitochondria extracts from 3-day-old pea roots in 0.5 M sucrose buffered to pH 7.5. This was incubated with 0.01 M glutamate-2-C^{14} and 0.001 M of each of sixteen amino acids for one hour at 38 C.

Potassium, sodium, lithium, ammonia or rubidium were also added. It was found that only potassium markedly enhanced amino acid incorporation into protein. Rubidium ions were highly inhibitory. They postulated that the promotion of amino acid incorporation into protein by potassium ions was due, in part, to the necessity of the ion for the formation of the peptide bonds in the protein molecule. It was Spyrides (1964) who localized the action of the element in the process of protein synthesis when he discovered that potassium ions were necessary for the binding of sRNA to the template ribosome complex. He could readily detect the association of phenyl sRNA to the ribosome in the presence of polyuridilylic acid. When the ions were removed from the incubation fluid the sRNA detached itself from the ribosomes. Many investigators have observed that, regardless of plant species, potassium deficiency is always associated with low protein but with high content of soluble non-protein nitrogenous compounds (Hoffer, 1938; Wall, 1940; Nightingale, 1943; Hoagland, 1944; Richard and Coleman, 1952; Coleman and Richard, 1956; Mulder and Bakema, 1956; Kwong and Fisher, 1962; Lipke, Joham and Bird, 1967).

2. Pyruvate kinase

Miller and Evans (1957) seem to have been the first to produce evidence of the presence of pyruvate kinase in different species of higher plants. They demonstrated that the enzyme activity of extracts, regardless of source, was strikingly stimulated by the addition of KCl. McCollum, Hageman and Tyner (1958) working with enzyme extracts from acetone powder of corn seedlings, found that the activity lost through dialysis overnight against cold deionized water, was regained on addition of potassium salts. This was also

confirmed by Evan (1963) who used enzyme extracts from roots and leaves of peas. In a later experiment by Jones (1966) in which $^{14}CO_2$ was used, it was observed that there was reduced pyruvate kinase activity in potassium deficient tomato plants.

3. Acetic thiokinase

Although Millerd and Bonner (1954) received credit for having been first to show experimentally that plants had the enzyme acetic thiokinase catalyzing the reaction, adenyl acetate + Co A to produce acetyl Co A, which could be condensed with oxalacetate to form citrate, it was Hiatt (1964) who demonstrated the absolute requirement of potassium ions for the activation of the enzyme. Hiatt incubated adenyl acetate and Co A in the presence of various salts for 10 minutes at 30°C. By coupling acetyl Co A synthesis with the condensing enzyme, he found colorimetrically, that maximum amount of citrate was formed in the presence of potassium chloride or other potassium salts.

4. Carbohydrate metabolism

A number of experiments have indicated that potassium is involved not only in starch synthesis but also in the processes of carbohydrate transformations. Hartwell (1916) in a sand culture nutritional experiment, observed that lack of available potassium resulted in an accumulation of starch in potato vines. Wall (1940) found in tomato plants, that the first stage of potassium deficiency was associated with a considerable accumulation of carbohydrates which diminished greatly with increase in the severity of the deficiency. In another experiment where 14-day-old wheat seedlings were supplied with different levels of KNO_3, Ward (1960) made some rather interesting discoveries. His analyses showed

that there was a significant relationship between rate of potassium application and level of reducing sugars both in whole plants and in the second leaf of dissected plants. Reducing sugars accumulated with decreasing potassium supply. As regards the enzyme, invertase, the results showed that invertase activity increased with decreasing potassium application. This could be reconciled with the observed levels of reducing sugars. When he realized that the net amount of sucrose always remained the same, all attempts to offer an explanation were given up. Recently, by use of radioactive $^{14}CO_2$ on excised leaves of beans, Ozbun, Volk and Jackson (1965) also showed that potassium-deficient leaves of beans always contained more soluble carbohydrates than potassium-rich leaves of comparable age.

5. Growth regulation

Continued research is beginning to uncover some of the subtle functions of potassium. Cooil (1951) incubated Avena coleoptiles with some selected organic acids adjusted to a specific pH with a hydroxide of the desired cation. He found that in the presence and absence of iodoacetate, potassium salts always resulted in marked increases in growth of the coleoptiles. Sodium salts gave no comparable results and it was concluded that the principal response in the coleoptiles resulted from potassium. This was confirmed later by Purves (1966) who, working with etiolated cucumber (Cucumis sativus L.) hypocotyl segments found that maximum elongation was stimulated by potassium chloride and a number of other potassium salts. He suggested that the nutritional requirement for potassium in higher plants resulted from a specific involvement in certain enzyme systems and from a relatively non-specific role related to the elongation response.

6. Buffering function

It is known that the major part of the potassium in plant tissues is located in cell sap as salts of organic acids and is of significance to the plant buffer systems (Nightingale, 1943; Hoagland, 1944; Cooil, 1948). In his experiments with prune trees, Hoagland (1944) found that the concentration of potassium was very low in the expressed sap of the leaves of potassium deficient trees and that its pH was significantly lower than that in the leaves of potassium-rich trees. He speculated that changes in the chemical constitution of the leaf were the cause of the subsequently manifest injury. According to Miller (1957), potassium and magnesium are the cationic elements principally involved in the buffering mechanisms. In the studies of orchard factors which influence chemical composition of apples, Wilkinson (1958) found that under normal conditions, the pH of the fruit did not vary much. However, increasing potassium concentration in the foliage was closely correlated with increasing titratable acidity in the fruit and there was a parallelism between potassium and magnesium. He suggested that both ions were involved in the buffer system.

B. MOBILITY

In a leaching experiment designed to simulate what might happen to plants in the field during an exceptionally wet season, Wallace (1930) found that initially healthy apple leaves in cold water lost about a quarter of their potassium in a period of 24 hours and practically all of the potassium could be removed within 72 hours. With a doubly girdled cotton plant in which the potassium content had initially been determined for both basal and apical portions, Mason, Maskell and Phyillis (1936) discovered that,

in the apical region, there was a marked increase in the content of potassium which had been translocated from the regions below. Goodall (1942), studying the distribution of certain cations in apple foliage, observed an exceptionally high rate of mobility of potassium within the plant to the extent that some potassium from older leaves was translocated to new leaves formed at the end of the season. Another demonstration of the mobility of potassium was made by Edgerton (1948). He supplied high potassium to apple trees during the first season and withdrew the supply in the next. He found that the potassium stored in the trunk and the root system the first season was sufficient to promote fair growth of shoots the second season when the trees were supplied with minus potassium solution. Analyses of the leaves from various locations on the shoots indicated that potassium was being translocated from the lower leaves to the apical growing regions. An interesting report was made by Weevers (1949) who found that in annual plants, potassium ions were not only translocated back to the roots at the end of the vegetative period, but also excreted into the soil.

C. SEASONAL VARIATION

An examination of the various papers on the potassium content of plants on a percentage dry basis reveals that there exists a wide range of potassium content in the leaves of fruit trees. In a survey of orchards in New York state, Boynton, Cain and Compton (1944) found the potassium content ranging from 0.24 per cent in very deficient trees to 1.53 per cent in good orchards, and they observed that potassium deficiency symptoms were prevalent in orchards with a mean potassium content of 1.36 per cent. Under glass house

conditions, Edgerton (1948) recorded a potassium range of 0.24-3.35 per cent. There is considerable evidence, however, to show that when the content falls below 1.00 per cent, trees usually show deficiency symptoms and that between 1.25 and 1.50 per cent lies what might be termed an optimum level for growth (Wallace, 1931; Reuther and Boynton, 1939; Boynton and Burrel, 1944; Batjer and Westwood, 1958; Awad and Kenworthy, 1963). Nevertheless, there is considerable variation in potassium content throughout the season. Lilleland and Boynton (1938) noted that there was a general trend toward a lower percentage of potassium as the season advanced, and this has been widely confirmed (Reuther and Boynton, 1939; Boynton, Cain and Compton, 1944; Cain and Boynton, 1948; Emmert, 1954; Barden and Thompson, 1962).

MAGNESIUM

Although, by analytical methods, Liebig (1840) had already shown that magnesium was one of the mineral constituents essential for growth, it was not until Boem, according to Reed (1907), that any specific role could be assigned to the element in the metabolism of the plant. Boem discovered in 1875 that magnesium was toxic to plants growing in solution cultures. But Loew in 1877 observed that magnesium salts caused injury only in the absence of calcium. Since then, numerous investigations have been carried out to determine the role of magnesium in plants.

A. FUNCTIONS

1. Structural cell components

According to Garner, McMurtrey, Bacon and Moss (1923), it was Willstatter in 1909 who discovered that magnesium is

an essential part of chlorophyll. It occupies a central location in the molecule, being attached to each of the four pyrrole rings either by direct covalent bonds or by 'secondary valences' (Steward, 1963; Steward, 1964). In its role as a chlorophyll nucleus, it is unique in that it cannot be substituted for by any other metal (Gauch and Krauss, 1959; Jacob, 1959; Steward, 1963). There are inconsistent reports regarding the amount of magnesium in chlorophyll. According to Willstatter and Stoll (1928) the amount of magnesium calculated as magnesium oxide is 4.5 per cent. Terrien, Truffaut and Carles (1957) stated that leaves contain 2.7 per cent dry weight of magnesia. In a treatise on Inorganic Nutrition of plants edited by Steward (1963), it is reported that magnesium is the only metal contained in chlorophyll comprising 2.7 per cent of the total molecule. Chlorophyll, however, represents only approximately 10 per cent of the total leaf magnesium. According to Cain (1959) who was working with apples, it is reported that of the 10 per cent magnesium contained in the leaf, only 1 per cent is contained in chlorophyll.

It should be emphasized that the magnesium content of different plants and different plant parts varies considerably (Salmon, 1963). The magnesium which exists as part of protoplasm (Lutman and Walbridge, 1929; Steward, 1963) is believed also to be concerned with cell turgor and may be involved in cell membrane and permeability relationships.

2. Effects on phosphorus absorption and mobilization

According to Reed (1942), it was Loew, who after prolonged investigations, came to the conclusion that magnesium served as a carrier of phosphate used by plants.

This work has been subsequently supported. Working with Sudan grass, which had been selected as a test plant because of its poor feeding for phosphorus, Bartholomew (1933) noted that plants required increased amounts of phosphorus when additional magnesium was supplied. To further investigate the relationship between magnesium and phosphorus, Truog, Goates, Gerloff and Berger (1947) used the Alaska variety of peas for field and water culture experiments in which the supplies of available magnesium and phosphorus were varied. On the basis of chemical analyses of seed, they found that an appreciable and consistent increase in phosphorus content was correlated with increasing amounts of available magnesium. Increasing supplies of available magnesium raised the phosphorus content of the peas much more than did increasing amounts of available phosphorus. Their results supported the theory that magnesium functions as a phosphorus carrier. The theory was strengthened later by the experiments of Webb, Ohlrogge, and Barber (1954) with soybeans in sand cultures. They observed that omission of magnesium from the nutrient solution not only delayed translocation but also altered the final distribution of phosphate in the plant. Under complete nutrition with sufficient magnesium, the phosphorus content of the seed was increased progressively as the plants matured and there was less phosphate in the vegetative parts. In contrast, there was less phosphate in the seed and more in the vegetative parts of magnesium deficient plants. Jacobs (1958) summarizing reports of various authors, stated that besides facilitating the transport of phosphate within the plant, magnesium promoted uptake of phosphorus. He cited the research by J. Roschdjeswensky on sugar beets in which it was found that uptake of phosphate was consistently enhanced by magnesium salts.

3. Activation of enzymes

Apart from being a structural component of molecules and organelles, magnesium is required in a wide spectrum of enzymatic systems. The reactions requiring kinases, synthetases, peptidases and phosphatases have been more widely investigated than others.

It was Ingraham and Green (1958) who, after numerous investigations theorized that all reactions requiring Co enzyme A, thiamine pyrophosphate, diphosphopyridine nucleotide, triphosphopyridine nucleotide and adenosine triphosphate as reactants also require a metal chelator with a pyrophosphate specificity and a chelating strength which is intermediate between high and low. The chelating strength is necessary so that the products may dissociate from the enzyme. He concluded that the magnesium ion fulfilled all those requirements and that it was universally associated with ATP reactions. This was confirmed by Hiatt (1965) who, while studying the activation of the enzyme acetic thiokinase, proved that while magnesium ions were required in the reaction ATP + acetate $\rightleftharpoons$ adenyl acetate + PPi, they were not necessary in the reaction adenyl acetate + Co A $\rightleftharpoons$ acetyl Co A + AMP. He concluded that where the ions were required in such reactions, the 'reactive species' was a magnesium complex of ATP. Yamamoto (1966) working with NAD kinase extracts from different plants found that the concentration of Mg^{++} required to produce maximum activity was 10^{-2} M; and that Co^{++} or Mn^{++} could not replace Mg^{++} in the system.

Magnesium has been associated with amino acid and protein synthesis. Sheldon, Blue and Albrecht (1956) studying the influence of magnesium fertilization on the formation of amino acids in lucern found that 1.80 mg of tryptophan

per gram of dry matter were obtained without magnesium, but with magnesium the yield was 3.10 mg of tryptophan per gram of dry matter. Previously, Webster (1955) investigating the effect of different cations including Mg, Mn, Ca, Co, Zn, Cu, and Ba on the incorporation of radioactive amino acids into the proteins of cell-free extracts from pea seedlings, found that only magnesium ions produced a significant increase in the rate of glutamate-C^{14} incorporation. Ions such as Ba, Cu, Co and Zn were highly inhibitory.

4. Protective function

Since magnesium is a component of chlorophyll and directly involved in the production of carbohydrate, it could be assumed that a plant deficient in magnesium would be weak and susceptible to adverse conditions. Eisenmenger and Kucinski (1947) made some observations on the subject. They noted that in the presence of disease, plants on low magnesium soils, regardless of the stage of evolutionary development, manifested disease symptoms before plants grown on soils to which magnesium had been applied. Asters manifested yellows disease symptoms earlier on the low magnesium plots than any other. Sunflower plants carried heavier infection of leaf rust when grown on magnesium-deficient soil. Blight on cucumbers due to downy mildew was more severe on the low-magnesium area than on the magnesium fertilized plots. Plant lice infestation on Nasturtiums intensified with magnesium deficiency.

5. Fat synthesis

Although Loeb (1903) is credited for having reported the observation that there was a high concentration of magnesium in the seeds of oil plants (e.g. flax and cotton) it was Reed (1907) who discovered in a classical experiment

with Vaucheria gemata that cells failed to form oil globules in the absence of magnesium. Since then, the general thesis that magnesium may influence the formation of fatty storage material in plants has been widely supported (Jacob, 1958; Gauch, and Krauss, 1959).

6. Buffering action

Biological systems have an optimum pH range for proper functioning. Recently Rains, Schmid and Epstein (1963) found that the presence of hydrogen ions in the system of plants may cause a general derangement of the ion adsorption or translocation mechanisms. With few exceptions, plant sap is slightly acid in reaction. Both aliphatic and aromatic acids have been identified. The aliphatic acids do not ordinarily occur exclusively in the undissociated state. A certain portion of total acidity is combined with mineral cations to form salts which contribute towards the buffering systems in plant tissues (Miller, 1957). Pierce and Appleman (1943) were among the early scientists to record that magnesium participated in the neutralization of oxalic acid to form magnesium oxalate in plants. This was supported by Wilkinson (1958) who investigated the relationship between certain cations and titratable acidity in apple fruit. He found a parallelism between potassium and magnesium and suggested that ionic magnesium might be involved in the buffer system and that it might be attracted to regions of high acidity.

B. SEASONAL VARIATION IN THE FOLIAGE

Since magnesium became recognized as a major element in tree fruit culture, along with the development of foliar analysis as a tool in nutritional research, seasonal inves-

tigations have been carried out not only on apples, peaches, and olives, but also on citrus, avocados and many other plants mainly for the purpose of diagnosing incipient deficient levels so that corrective measures might be applied.

Cain and Boynton (1948) did research on seasonal variation in the mineral content of McIntosh apple leaves. Expressing their analytical data as milliequivalents per 100 grams of dry weight, they observed that the magnesium content was low at the beginning of June but continued to increase steadily, reaching a maximum around the 25th of July. Thereafter, it slowly declined, later climbing until there was an appreciable rise by the 23rd of October. Rogers, Batjer and Thompson (1953) on the other hand, did not find any significant seasonal fluctuations in the magnesium content of Delicious apple leaves; the amount remained more or less constant throughout the season. Likewise, Oland (1963) did not observe any change in the content of magnesium in apple leaves up to senescence. Smith and Reuther (1950) at the U. S. Subtropical Field Station, Orlando, Florida reported that the amount of magnesium in Valencia orange leaves of the summer flush built up to a maximum within about 90 days and declined very slowly as the leaf aged. This was confirmed later by Jones and Parker (1951). Studying changes in macro-nutrients elements of Souri olive leaves in alternate bearing years, Fahmy and Nasrallah (1959) found that magnesium increased as the season advanced regardless of fruiting or vegetative condition of the tree. Embleton, Jones, Kirkpatrick and Allen (1958) also observed increased magnesium content in Fuerte Avocado leaves with time. It would seem, therefore, that the trend in the magnesium content of leaves is to increase as the season advances or in some instances to remain fairly constant throughout the season.

C. MOBILITY AND REUTILIZATION

The frequent appearance of magnesium deficiency symptoms in mature leaves seems to have been the basis for a thesis that magnesium is mobile within the plant and is readily redistributed from old to young tissues (Wallace, 1953). Blake, Nightingale and Davidson (1937) associated failure of the magnesium deficient apple trees to attain a large stem diameter with translocation of magnesium from old stem tissues to the developing leaves and stem tips. They argued that with the disappearance of magnesium from the older tissues of the stem, there ceased to exist an active cambium in that region, and there was therefore, only little increase in diameter. They added that magnesium was likewise translocated from older leaves. Using a 5 per cent foliar spray of magnesium sulphate under various conditions, Oland and Opland (1956) found that young leaves absorbed magnesium more readily than old ones during the day. But when sprayed late in the evening, old leaves absorbed large amounts of magnesium, which remained there. There was no indication from this work that the magnesium absorbed by the leaves was carried away to other parts of the plant. Bukovac and Wittwer (1957), using a radioactive isotope, showed that when a solution of a salt of active Mg^{28} was applied to the leaf of a bean plant, magnesium was readily taken up and appreciable amounts were transported from the site of application to adjacent tissue but export from the treated leaf was negligible. In a later experiment on absorption and mobility of the element, Bukovac and Wittwer (1960) concluded that foliar - applied Mg^{28} was readily absorbed but was not translocated out of the treated primary leaves of seedling or fruiting bean plants within the 24 hours. Mg^{28} applied to the root medium was absorbed and

distributed throughout the plant. Allen (1960), experimenting with M. III rootstock shoots, each of which was bent and dipped in a polyethylene bag containing magnesium sulphate solution with added Triton X-100 as a wetting agent, found on analyzing treated and untreated leaves, that there was no evidence of redistribution of magnesium within that part of the shoot considered in the experiment. It seemed that movement was into or out of the entire shoot. In his glasshouse nutrition experiment, Koukkari (1962) observed that although plants in the treatment without magnesium developed severe deficiency symptoms to the extent that some leaves fell off the shoot, the small basal leaves on the same plants, appeared to be free of deficiency symptoms. This had also been observed by Delap and Ford (1958). Ford (1967) studying the effect on growth and mineral composition of applying magnesium through leaves by dipping, found that the amount of magnesium in the roots of plants receiving no Mg dipped and those receiving no Mg but undipped was similar. He concluded that there was little translocation of magnesium from leaves to roots.

From all these reports, it appears that magnesium is readily taken up from foliar application and is readily translocated to the entire plant from the roots but does not appear to be redistributed within the plant.

D. THE TIME-DEFICIENCY SYMPTOMS APPEAR

The description of magnesium deficiency symptoms has been cited in many reports (Wallace, 1953; Jacob, 1958; Cain, 1959) and it is beyond the scope of this study to review them. The time these deficiency symptoms appear is, however, highly pertinent for future correlations.

Wallace (1939) noticed deficiency symptoms on apple leaves during the period mid-August and late September. This was confirmed by later reports (Hill and Johnson, 1940; Southwick, 1943; Boynton and Burrell, 1944; Chucka, Waring and Wyman, 1945; Boynton, 1947; Boynton and Embleton, 1950).

They all agree that the visible deficiency symptoms on McIntosh apples and other fruit trees usually develop after a considerable amount of normal shoot growth has been made. There seems to be only one report (Moon, Harley and Regeimbal, 1952) in which magnesium deficiency symptoms were observed early in the season specifically 28 days after bloom. They could not attribute the early appearance to fertilizer practice or to excessive rainfall. By way of speculation, they suggested that certain trees, because of their location in the orchard in relation to pollination may have set an abnormally large number of seeds per fruit and thereby increased the demand for the magnesium which the soil could not supply. Delap and Ford (1958) at the East Malling Research Station in England, reported an incident where magnesium deficiency symptoms became apparent by the 28th of June, which was also rather early compared with subsequent observations by Koukkari (1962). Ford (1964) and Ford (1966) also observed that affected leaves began to drop early in September, and, in severe cases, trees became almost completely defoliated by late September. From all the reports, it is abundantly clear that magnesium deficiency symptoms usually appear late in the season after normal shoot growth has been completed.

INTERRELATIONSHIP AMONG CALCIUM, MAGNESIUM AND POTASSIUM

Some of the elements that have been demonstrated to be essential for the proper growth and reproduction of plants also have been found to serve specific functions in the life processes of plants. These processes cannot proceed at optimum rates if all of the elements are not present in proper proportion. It is important to examine effects due to the interrelationships among some of these elements.

Various workers used the term 'antagonism' for the reciprocal relationship between any two metals or nutrient elements irrespective of cause. In this review, the definition of antagonism is that of Jacob (1958), who stated "the frequently observed phenomenon that the uptake of an ion by the plant is inhibited by the increased supply of other ions, whether as a result of soil processes or the influence of the plant itself".

1. Calcium and magnesium

As regards ratios, Moser (1933) did not find any significant correlations between calcium-magnesium ratio and crop yields. He concluded that the beneficial effect of liming was not due to change in the magnesium-calcium ratio but instead it increased the amount of replaceable calcium ions in the soil. Lilleland and Brown (1935) working with French prunes observed a linear relationship between calcium and magnesium in the leaves. Wallace (1940) found that application of either chalk or magnesium sulphate was beneficial to apple trees, black currants, plum trees and gooseberries, whether calcium or magnesium or both simultaneously were lacking. In a fertilizer experiment by Cain and Boynton (1948), the results of analyses indicated that calcium and

magnesium increased while potassium decreased. The reason given was that Ca^{++} and Mg^{++} increased to satisfy the cation requirement due to reduced K^{+}. In contrast, the results of a sand culture experiment designed by Forshey (1963a) to study the effects of nitrogen on magnesium absorption, showed that absorption of calcium, magnesium, and potassium was increased by nitrogen fertilization but increases in absorption were more than could be explained in terms of differences in growth. Under California conditions, Jones and Parker (1951) confirmed the results of Smith and Reuther (1950) who found in the leaves of Valencia orange trees a direct linear relationship between calcium and magnesium contents at any time during the season. This was also verified by Martin and Page (1965) who, besides leaf correlations, found that the percentages of calcium and magnesium absorbed were directly related to exchangeable amounts of these elements in the soil. They did not observe any antagonistic efforts.

The relationship between calcium and magnesium, seems to be principally synergistic both in the soil and within the plant. The only exception appears to be the research reported by Jacoby (1961) who, by using a split-pot culture technique showed that excess amounts of calcium in the medium impaired magnesium uptake.

2. Calcium and potassium

The data obtained by Lilleland and Brown (1938) showing that increased potassium depressed the percentage of calcium in the leaves of French prunes were later verified by Smith and Reuther (1960) working with Valencia orange trees. The latter workers, however, observed one case in which calcium from liming the preceding season depressed

uptake of potassium at the start of the second season. It was Kahn and Hanson (1957) who pointed out that the effects of interaction between nutrient elements were also dependent upon plant species, when they observed that corn plants accumulated more potassium and less calcium than soybeans growing in the same solution. But, at moderate levels of potassium, calcium promoted potassium uptake in corn and inhibited it in soybeans. The depressing effect of calcium on the accumulation of potassium reported by Wenkataraman and Tejwani (1961) in tobacco leaves was of the same nature as the one observed in Souri olive leaves by Fahmy and Nasrallah (1959). The results of Embleton and Jones (1958) in Avocado leaves served simply to confirm what had previously been demonstrated, that increased potassium depressed the calcium content in the leaves of those trees.

3. Potassium and magnesium

The relationship between potassium and magnesium has attracted wider interest than the others mostly through increased use of potassium fertilizers in orchard culture and the subsequent demands for magnesium.

In fertilizer trials to overcome potassium deficiency which was so prevalent in apples growing on East Malling soils, Hoblyn (1940) discovered that with the disappearance of marginal scorch associated with the deficiency, leaves showed interveinal chlorosis characteristic of magnesium deficiency. Boynton and Burrell (1944) noticed that magnesium deficiency symptoms were induced in McIntosh apple trees under field conditions through potassium fertilization for three or more years, and in the glasshouse within a period of about 6 weeks. Leaf analyses indicated that not only was potassium content directly correlated with levels

of fertilization but also increased potassium was accompanied by appearance of chlorosis due to insufficient magnesium. Soil analysis data showed that potassium decreased exchangeable magnesium in the upper horizons of the soil under trees. Cain (1948), after observing that increased potassium fertilization reduced the magnesium content only in the leaves without appreciably reducing the magnesium in the stem, postulated that leaf symptoms appeared as an injury effect. Apple trees on a slightly acid soil which was relatively low in exchangeable bases were selected by Cain (1953) for another fertilizer experiment in which nitrate and potash were applied factorially at four levels. Although increasing potassium fertilization resulted in a decrease in total leaf magnesium, growth was not adversely affected. In later research to determine the response of 4-year-old Elberta peach trees to fertilization, Rogers, Batjer and Billingsley (1955) showed that yearly additions of potassium over a five year period did not significantly affect magnesium accumulation in the leaves. In another ingenious experiment equipped with automatic sub-irrigation pumps and bottles for collecting drainage liquid, Cain (1955) compared leaf mineral contents with minerals remaining in the nutrient solution. His data for potassium and magnesium showed that a high supply of one resulted in a lower concentration of the other in the leaves. On the basis of the data, he concluded that the 'antagonism' between the two elements was associated within the plant and was in no way related to external supply except as the external supply of one element influenced its own absorption. The conclusions from this experiment did not confirm those reached by Omar and Kobbia (1966). In their experiment they used lucern (Medicago sativa L.) plants fed with different concentrations of

potassium and magnesium in sand culture solutions. The analytical results revealed only a one-way interaction in which the uptake of magnesium was significantly reduced by increasing the amount of nutrient potassium. But potassium absorption was hardly affected by the presence of increasing amounts of magnesium. The depressing effect of potassium on magnesium uptake was interpreted in terms of competition for metabolically produced binding compounds.

In a series of pot experiments in sand and soil cultures, Welte and Werner (1963) found a two-way interaction in which high potassium in the substrate decreased the magnesium content in leaves, and lower potassium levels were caused by high applications of magnesium to potato plants. This was contrary to the findings of Omar and Kobbia (1966). On heavier soils, however, Welte and Werner (1963) noted that higher magnesium contents were found in plants at high potassium treatments. In order to explain their findings satisfactorily they assumed that "a high magnesium saturation was particularly strongly bound on the sorption complex", and that it had to be exchanged by potassium to become available. This assumption was supported later by the work of Ghoneim and Maier (1964) who, in a short-term nutrient absorption experiment with barley seedlings, found that addition of potassium to the substrate did not suppress but increased magnesium uptake. They also explained the phenomenon on the basis that the added K^+ ions displaced Mg^{++} ions into solution making them available to the plants.

4. Cation balance

Most of the papers on the interrelationships among the elements under review show clearly that the presence of an excess of one element in proportion to other elements in

the substrate tends to upset not only availability but also translocation and functioning of other elements within plants.

Carolus (1935), after a series of experiments with potatoes, observed that plants which showed magnesium deficiency symptoms sometimes contained more magnesium than the healthy controls. He remarked, therefore, that a condition which had been designated as "magnesium deficiency" was probably not always associated with an extremely low magnesium content in the plant, but might be a result of disproportional absorption of other cations in relation to magnesium. On deficiency symptoms noted in apples, Cain (1948) stated: "The typical leaf deficiency symptoms (marginal scorch, interveinal chlorosis and necrosis) which are normally associated with deficiencies of K and Mg, appeared only in cases of high content of the other cation in the tissue. These symptoms did not appear when both K and Mg were increased, a leaf injury pattern typical of commonly described deficiency symptoms of the other element resulted. Since the leaf symptoms usually appear after the leaf has made approximately normal growth, and the leaf symptoms appear as an injury effect ultimately resulting in death of apparently healthy tissue, it seems to be a logical conclusion that the leaf injury or symptom, normally attributed to a deficiency of one element (Mg) might actually be a toxic effect produced by an excess of the other element (K)."

On cation balance, Hoagland (1944) said that the quantitative relationships among calcium, potassium, magnesium and sodium were of considerable physiological importance, and that it was not unusual to find that the decrease in absorption of one base would be compensated for roughly by an increase in the absorption of other bases so that the total

equivalents of bases present in the plant tissues might remain approximately constant. Calmes (1959), who grew two maize plants side by side, one rich in potassium and the other poor in magnesium, found, on analysis of leaves, that although uptake of individual elements differed in each of the cases, the sum of $Mg^{++} + K^{+}$ remained fairly constant. He suggested that potassium and magnesium might be important in maintaining ionic equilibrium.

Since these cations serve specific functions, vital processes cannot proceed at optimum rates if the cationic elements are not present in a balanced state within the plant. According to Shear, Crane and Myers (1948) the main thesis involved in the concept of nutrient balance was described as follows: "All other factors being constant, plant growth and symptoms expression are functions of two variables of nutrition, intensity and balance as they are reflected in the composition of comparable leaves sampled when the plants are in the same stages of growth or development." They observed that at any level of nutritional intensity there existed an optimum balance or proportion among the elements at which maximum growth for that intensity level would result. The maximum potential growth and yield for any given plant species would be obtained only when the proper balance between all of the nutrient elements occurred in combination with their optimum intensity.

THE RELATIONSHIP BETWEEN CATIONS AND ORGANIC ACIDS

Although the role of some organic acids in plant metabolism has been well established, the involvement of cations in the production of organic acids in tissues is rather complex.

Working with excised barley roots Ulrich (1941) found that absorption of cations in excess of anions resulted in a tendency to shift the pH within the root cells toward the alkaline side, which in turn was counteracted by the formation of organic acids provided that there was an ample supply of sugar within the root cells. Conversely, when anions were in excess of cations, organic acid production decreased and the bases neutralized the increase in inorganic anions. In all of the twelve different plant species studied under glasshouse conditions, Pierce and Appleman (1943) found that excess cation uptake was correlated with total ether-soluble organic acids, except in cantaloupe which contained the largest amount of calcium and the lowest content of organic acids. Cooil (1948) found that the total acidity associated with high potassium in expanding guayule leaves was largely due to citric acid, whereas in mature leaves high malic acid was correlated with high calcium. In Valencia orange leaves, on the other hand, Rasmussen and Smith (1961) correlated increasing calcium in the substrates with high leaf contents of both oxalic and malic acids; increasing potassium resulted in an increase of only oxalic acid, and malic acid content remained unaffected. In a study of lime-induced chlorosis in two-year-old New Jersey blueberries, Cain (1954) observed that the pH of expressed sap from chlorotic leaves was consistently higher, and the sap higher in Ca, + Mg + K bases than the sap from healthy

leaves. It was thought that this resulted from organic acid production, the extra bases being absorbed to neutralize the acids.

In young excised barley roots supplied with $^{14}CO_2$ for 3 hours, Jacobson (1955) discovered that $^{14}CO_2$ fixation occurred greatest during excess cation uptake when malate increased, and least during excess anion uptake when malate decreased. He had evidence to suggest that carboxylation played an important role in the synthesis of malic acid under conditions of the experiment. The conclusions reached by Eaves and Leefe (1955) that potassium fertilization resulted in increased leaf potassium content, which was directly correlated with levels of titratable acidity in the apple fruit, have been verified by Wilkinson (1958); Kwong and Fisher (1962). The researches of Jackson and Adams (1963) and that of Hiatt (1967) in which excised barley roots were used, and that of Torii and Laties (1966) who experimented with barley and corn root segments, have all confirmed the previously discovered phenomenon that the response of sap pH to excess cations or anions was immediate and sensitive to small differences in uptake. Increase in cation uptake was followed by production of organic acids, and, when anions were absorbed in excess, organic acid production stopped or decreased.

It is obvious, therefore, that upon balance among nutrient elements at any intensity-level of feeding, is superimposed the phenomenon of interactions between cations and organic anions. But, cation and anion interaction studies cannot be made on the basis of mineral analysis of leaves alone, because the ions available to the plant through the nutrient medium do not alone control the charge and quantity of ions available for exchange phenomena within the

plant. Hydrogen, hydroxyl and carbonate ions as well as complex organic ions resulting from metabolic processes enter into cation: anion balance and the relationships of these to the mineral composition of leaves may be determined through biochemical studies (Shear, Crane and Myers, 1948).

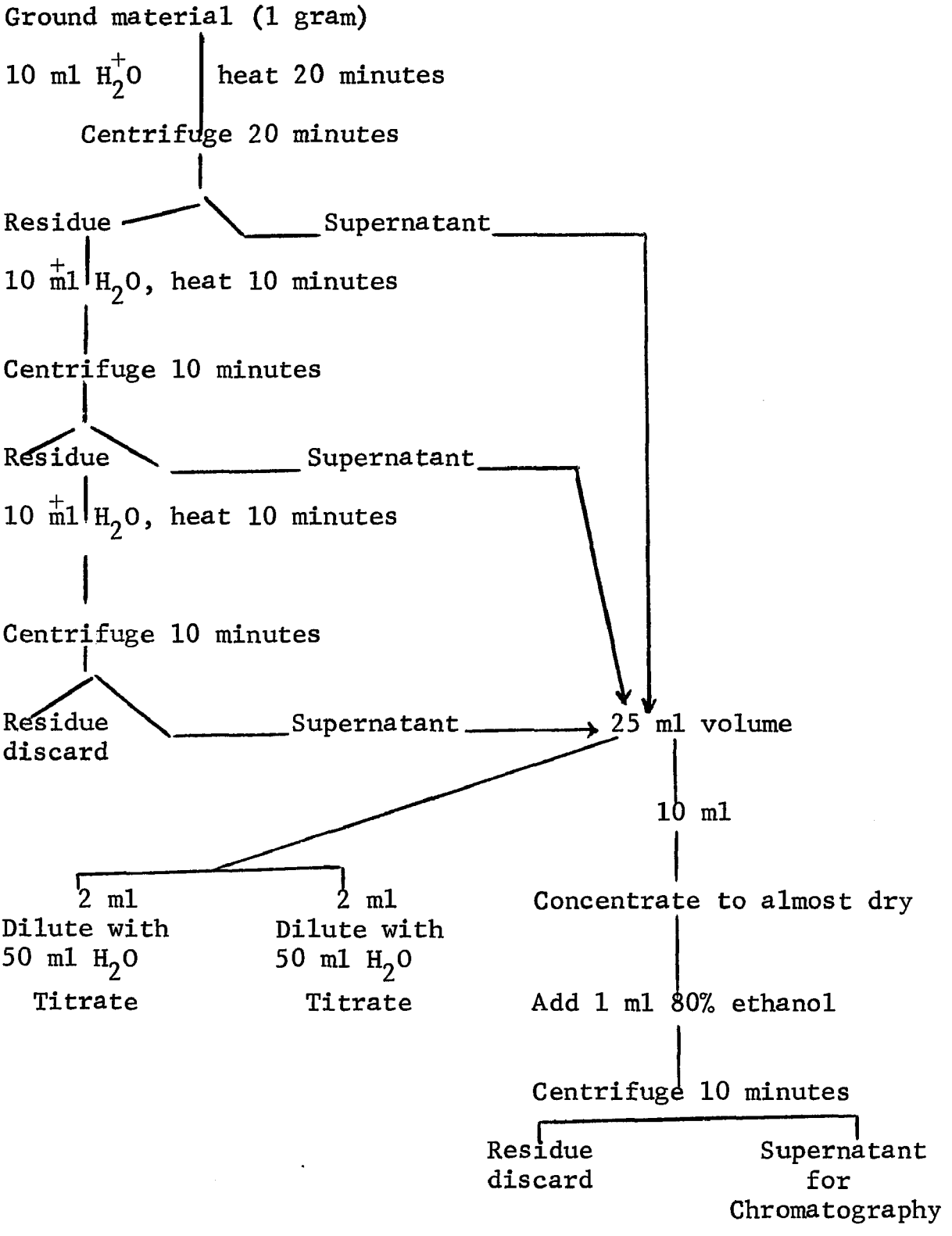
MATERIALS AND METHODS

Twelve bearing Rogers McIntosh apple trees on M. Robusta No. 5 clonally propagated rootstock were selected for this study. Samples of 100 leaves from the middle of growing shoots, at shoulder height, were collected at random from each tree. These samples were taken between 1:00 and 3:00 P.M. at 14 day intervals starting on June 20, 1966. The bagged leaves were stored at 0 C until they were washed. Each sample was washed in 2 liters of 0.1% Alconox detergent and rinsed with 2 liters of distilled water. The washed leaves were dried at 80 C for 72 hours, ground in a Wiley mill to pass through a 40 mesh stainless steel screen, and stored in glass bottles with plastic screw caps.

Extraction of organic acids

Water extraction of organic acids was carried out according to a modified method of Palmer (1955). Duplicate 1 g samples of ground material were placed in 50 ml centrifuge tubes, and 10 ml of distilled water was added slowly to each tube. The tubes were placed in a bath of boiling water for 20 minutes and the contents stirred with a glass rod every 5 minutes. After the rod and walls were rinsed with distilled water, each tube was finally spun in an International clinical centrifuged at 3,600 rpm for 20 minutes. From each tube the supernatant liquid was transferred to a 25 ml volumetric flask. The residue was washed twice with 5 ml portions of distilled water, heated for 10 minutes and centrifuges for 10 minutes after each washing. The washings were added to the volumetric flask and the contents made to volume. Such extracts were slightly cloudy, dark yellow-brown with a pH range of 5.5-5.8.

Table 1. A flow chart summarizing the procedure for preparation of leaf extracts for quantitative and qualitative determination of organic acids



Measurement of titratable acidity

Duplicate 2 ml aliquots of each extract were put into 125 ml Erlenmeyer flasks and diluted with 50 ml of distilled water. Two drops of 1% alcoholic phenolphthalein indicator were added. The extract in the flask, while agitated with a magnetic stirrer, was titrated with 0.0213 N sodium hydroxide from a micro-burette. The final figure was an average of four titrations.

Preparation of samples for chromatography

To reduce the volume of analytical work, 0.5 g of ground material from each of the twelve samples was weighed and combined into one composite sample per sampling date. Water extraction of organic acids from the composite samples was made as above. Ten ml of the water extract were transferred into a 25 ml test tube and evaporated almost to dryness in a bath of boiling water under a jet of air. The residue was dissolved in 1 ml of 80% ethanol, transferred to a 12 ml conical tube and centrifuged at 3,600 rpm for 10 minutes. The supernatant liquid was transferred to a 4 ml glass vial with a plastic screw cap and stored at 5°C until required for chromatographic analysis.

Paper chromatographic purification and separation of water extracts

Ten ml of the water extract were concentrated to 2 ml by evaporation as above and put into a 4 ml glass vial. The concentrated extract was applied in streaks along a line drawn 2 cm from the edge of 23 x 23 cm Whatman No. 1 paper. An additional quantity of extract was streaked on the paper in small portions allowing complete drying between applications. Drying was hastened by means of a jet of cold air

from an electric hand drier. One gallon wide-mouth fruit jars were used as chromatographic chambers. The inside of the jar was lined with blotting paper which dipped into a liquid that barely covered the bottom of the jar. The liquid used for this purpose was usually the lower phase of two-phase solvent systems. When sufficient extract had been applied and dried, the paper was stapled into the form of a cylinder so that the edges did not meet. The cylinder, with the extract end at the bottom, was placed in an empty petri dish inside the jar which was then closed with a tightly fitting plastic cap. The paper was left to equilibrate inside the jar for one hour. After equilibration the jar was opened. By means of a long pipet, 20 ml of solvent A[1] were carefully but quickly placed in the petri dish and the jar re-closed immediately. The operations after equilibration had to be done rapidly so that humidity losses inside the jar were minimal. After 5 hours, the solvent front was usually 1.5 cm below the top edge of the paper. The chromatogram was then removed from the jar and hung to dry at room temperature under an exhaust hood. It took nearly 72 hours for most of the formic acid to evaporate from the chromatograms. Residual formic acid on paper interferred with reactions of subsequent locating reagents. The dry chromatogram was then unstapled and spread out. Both sides were covered with a piece of paper towel held in place with adhesive tape leaving 4 cm of uncovered margin on either side. The covered chromatogram was hung on a line prepared for the purpose. Both sides of the uncovered portions were

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[1]Solvent A comprised of the upper phase of a mixture of n-butanol and 3N formic acid (50:50, v/v).

sprayed with 0.04% alcoholic solution of bromophenol blue adjusted to pH 6.8 with 1 N sodium hydroxide. The yellow spots which developed were immediately marked and numbered consecutively from bottom to top. The paper towels were removed and the chromatogram scanned with the aid of a short wave UV lamp. There were mainly two fluorescent spots which were marked as F_1 and F_2.

Spots which developed in the sprayed portions of the chromatogram served as points between which two parallel lines were drawn to define undeveloped spots in the same location on the chromatograms. Strips containing individual spots were dissected out and cut to an arrow-hear point at one end. The eluting apparatus shown in Fig. 1 was that described by Dimler, Shaeffer and Rist (1952). It consisted of a shallow rectangular copper trough, 10 x 20 cm, separated in the middle of the longer axis by a metal strip 1 cm high. The wide end of the paper strip was inserted between two pieces of glass similar to microscope slides-(and henceforth referred to as slides). The slides with strips of spots from the same location on the same chromatogram were placed on either side of the trough. The trough was raised on a wood block so that a 15 ml test tube, 12.5 cm high placed in a rack under every strip could receive the eluting liquid. The trough was then filtered with water and a 1 liter beaker of boiling water was placed at one end of the block. Both beaker and trough were covered with a 47 x 30 x 25.5 cm plastic chamber to maintain a saturated atmosphere around the strips. Water moved through the paper strips by capillarity and by gravity carrying the material chromatographed into the test tubes. When approximately 1 ml had collected in each test tube, all solutions from spots between the same parallel lines on the chromato-

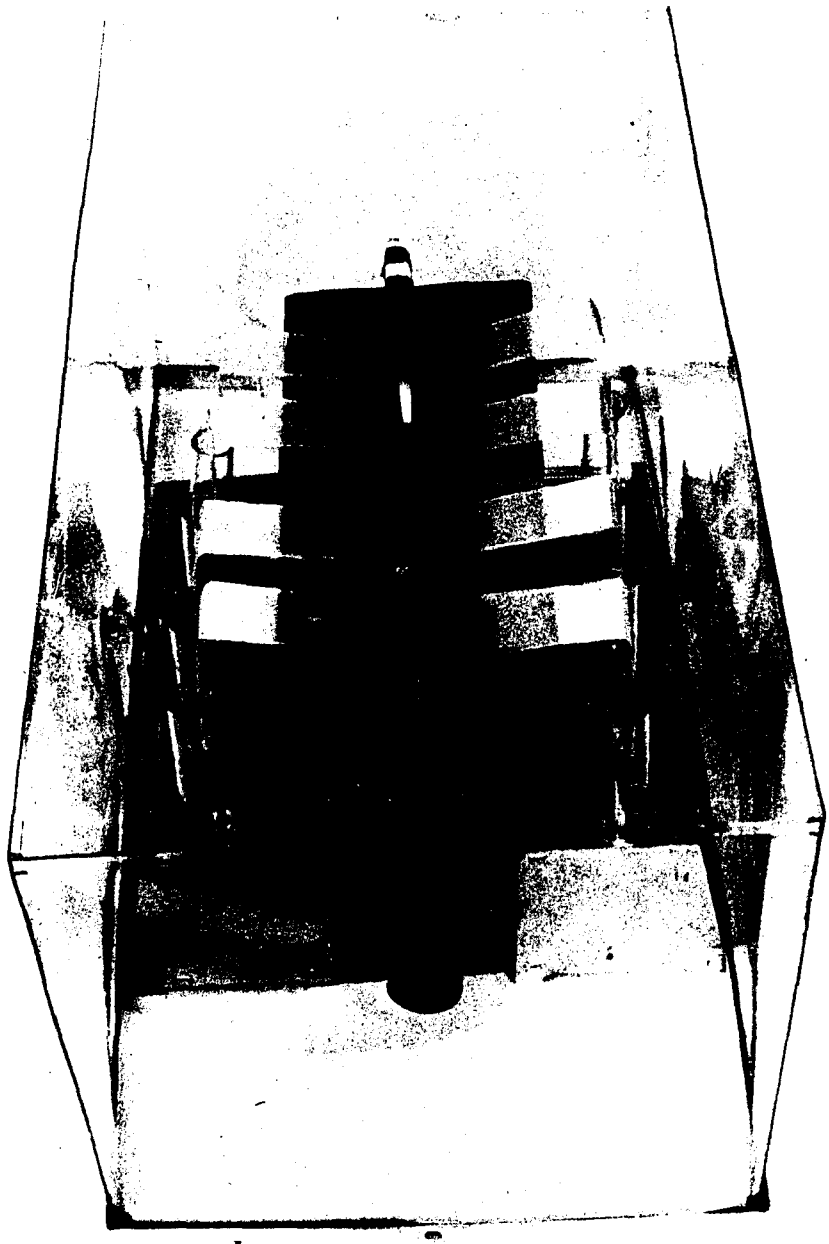


Fig. 1. The apparatus used to elute compounds from chromatogram strips.

gram were poured into one test tube. The combined volume of eluant was reduced to 0.5 ml by evaporation as above, transferred to a 4 ml glass vial and given a number corresponding to that on the chromatogram.

Exchange resin separation of extracts

a) Ion Exchange Resin

Dowex 1 x 10 - a styrene benzene-resin of 200 to 400 mesh size supplied in the chloride form was used. The finer particles were removed according to the method of Palmer (1955) by suspending the resin in distilled water, allowing the coarser particles to settle for 30 minutes, and decanting the cloudy supernatant liquid. This operation was repeated several times. The resin was then packed into a glass tube column 3 cm in diameter and 15 cm high. It was converted to a formate form by passing through it a 1 M solution of sodium formate until the effluent gave a negative test with silver nitrate. The resin was then washed with several bed volumes of distilled water and stored as a thick suspension in a dark brown bottle.

b) Preparation of chromatographic resin columns

The glass tubing consisted of two disposable non-volumetric pipets of 0.4 cm internal diameter. The upper one, 11.0 cm long, contained a piece of glass wool placed just above the constriction to support the resin. A second pipet, 4.0 cm long was connected to the lower end of the first by means of a 5.0 cm piece of rubber tubing. An adjustable clamp was attached between the two pipets and served as a control valve. The upper pipet was fitted through a piece of cork by means of which the apparatus was clamped on a stand.

A little distilled water was added to the bottle of the resin previously converted to the formate form. The bottle was thoroughly shaken to produce a uniform slurry. A portion of this slurry was poured into the upper pipet and the control valve partially opened. Gentle pressure from an air pump was applied to obtain uniform packing. The slurry was added in several portions allowing the resin to settle between additions. This was continued until a column of resin 6.0 cm long was obtained. Great care was taken not to introduce air bubbles into the column. This was achieved by keeping the water level above that of the resin at all times. After the column of desired height was obtained, the particles that adhered to the sides of the pipet were carefully washed down to the surface of the resin. A small piece of glass wool was placed on top of the resin and 4 bed volumes of distilled water under pressure were forced through the column at the rate of 0.75 ml per minute.

c) Elution of extract from the resin bed

One half ml of the concentrated ethanolic extract was placed on the glass wool and forced into the resin. It was then washed with 4 bed volumes of distilled water to remove neutral substances. The pipet containing the resin was connected to the capillary glass tube leading from the eluting apparatus connected to an air pump as shown in Fig. 2. The organic acids were eluted from the resin by forcing through it 6 N formic acid at the rate of 0.75 ml per minute. The effluent was collected in graduated jars in 120 ml portions each of which was called a fraction. When seven fractions were collected, elution was stopped. Each fraction was poured into a large test tube and the formic acid evaporated over a bath of boiling water under an air

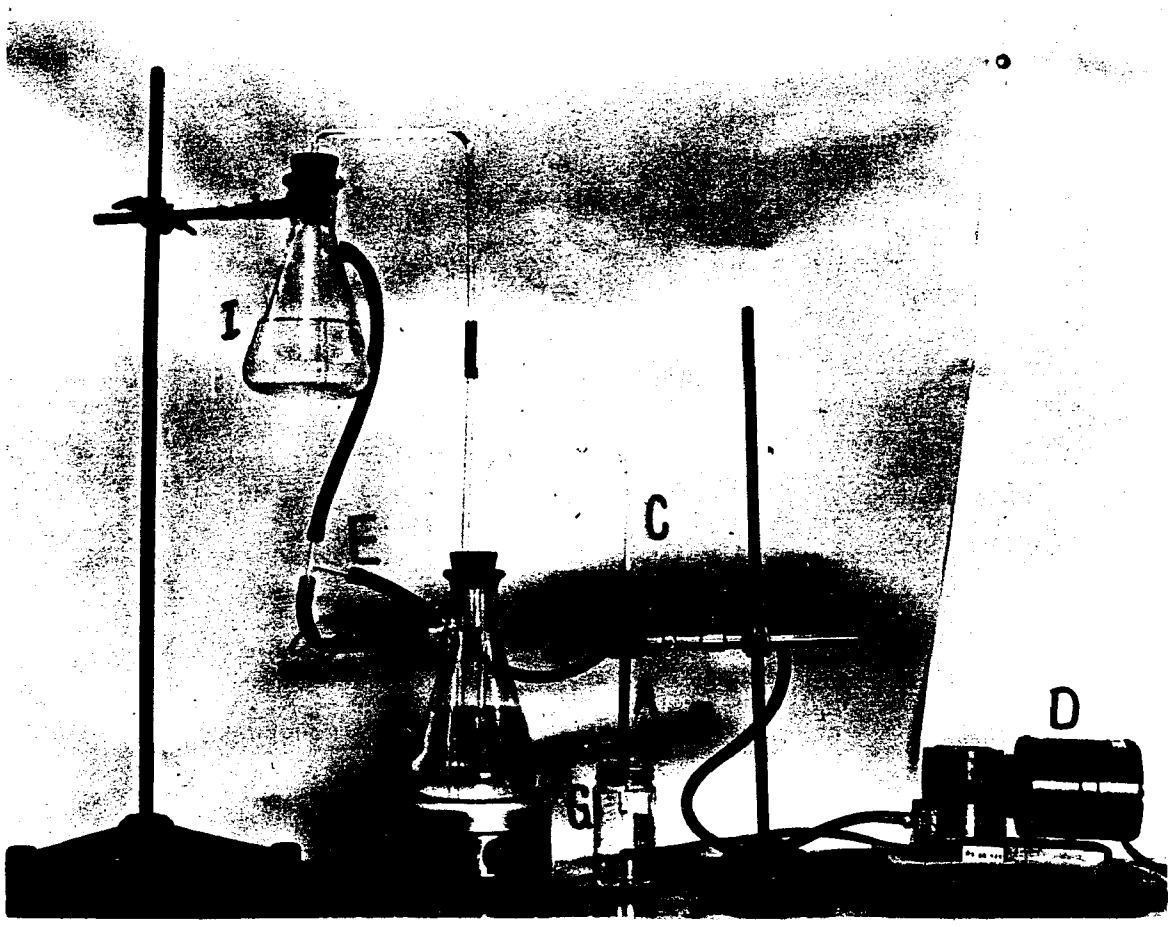


Fig. 2. Resin column eluting apparatus. A - upper pipet containing resin column; B - adjustable clamp; C - capillary glass tube; D - air pump; E - joint to air pump; F - magnetic stirrer; G - collecting jar; H,I - formic acid reservoirs

jet. The residue was dissolved in 1 ml of 80% ethanol, transferred to a 4 ml glass vial and stored at 5°C.

Ethyl acetate extraction

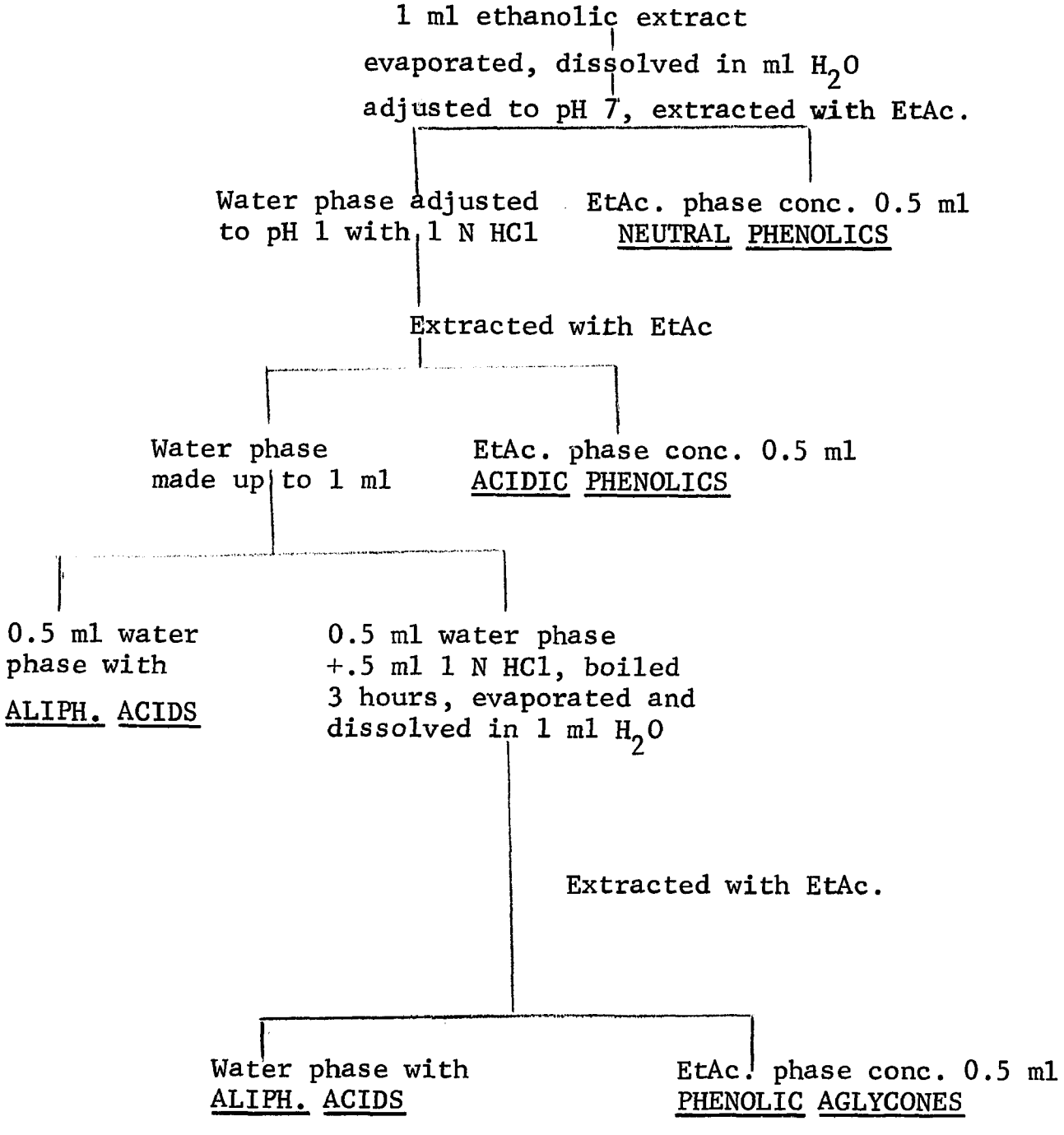
Fractionation of the extract was carried out by a modified method of Cambie and Mander (1962). Approximately 0.5 ml of the concentrated ethanolic extract was poured into a 15 ml test tube and the alcohol evaporated under a jet of air. The residue was dissolved in 1 ml of distilled water and transferred to a 15 ml centrifuge tube. The pH was adjusted to 7.0 with 1 N sodium hydroxide. The extract was shaken with 4 ml of ethyl acetate and centrifuged for 5 minutes. The supernatant ethyl acetate was pipetted into a 25 ml test tube. The process was repeated three times. The combined ethyl acetate extract was concentrated to .5 ml by evaporation and poured into a glass vial as the neutral phenolic fraction. The aqueous layer was acidified to pH 1 with 1N HCl, and ethyl acetate extraction continued as described previously. The remaining aqueous material was made up to 1 ml volume. Half of it was mixed with 0.5 ml of 1 N HCl and heated in a bath of boiling water for 3 hours to hydrolyze the glycosides. The HCl was finally evaporated and the residue taken up with 0.5 ml of water. It was transferred into 15 ml conical tube and centrifuged for 5 minutes. The supernatant liquid was poured into a glass vial as the phenolic aglycones.

Paper Chromatography

1. Spotting, developing and locating

The prepared extracts were dispensed with 10 lambda disposable micropipets to 23 x 23 cm Whatman No. 3 MM paper at the spacing of 2.5 cm between spots. The spots were kept

Table 2. A flow chart summarizing the procedure for ethyl acetate fractionation of phenolic compounds in leaf extracts



from spreading with the aid of a jet of cold air from an electric drier. Authentic samples of suspected compounds were spotted on the same paper as the extracts. To analyze for seasonal variation, extracts from composite material on the different sampling dates were spotted on the same chromatograms were run by an ascending technique in one gallon, wide-mouth fruit jars as described previously. Similar chromatograms were run in duplicate in different solvent systems shown in Table 3. Two dimensional chromatograms were usually run in solvent system B for the first dimension and in 2% acetic acid in the second-dimension. When the solvent front reached 1.5 cm below the top edge of the paper, the chromatogram was removed from the jar and dried under a hood at room temperature.

2. Locating phenolic compounds

a) UV fluorescence

Dry chromatograms were unstapled and scanned with the aid of both short wave and long wave UV lamps. The wave lengths were 2537 Å and 3660 Å, respectively. The positions and colours of the fluorescent spots were marked and recorded. The spots were then exposed to strong ammonia vapours and re-examined under UV. Some chromatograms were sprayed with Benedict solution, 1% methanolic aluminum chloride or with 10% aqueous sodium carbonate solution and re-examined under UV after drying.

Chromatograms of extracts from composite samples from the seven different sampling dates were analyzed as follows: Every extract on a specific date was examined under UV separately and the outline of the fluorescent spots marked with a lead pencil. They were numbered consecutively from bottom to top of the chromatogram. Similar spots on

Table 3. Solvent systems used and their respective developing time.

Solvent System	Composition	Time
A	n-Butanol: 3N formic acid (50:50 v/v upper Phase)	5 hours
B	n-Butanol: acetic acid: water (6:1:2)	6 hours
C	n-Butanol: 80% formic acid (50:50	3 hours 25 mins
D	Ethyl ether: 88% formic acid: water (5:2:1)	5 hours
E	n-Porpanol 50 ml (upper phase) Eucalyptol 50 ml (48 hours) Formic acid 90% 20 ml (old) Water 110 ml ( )	5 hours 20 mins
F	n-Butanol: xylene: acetic acid: water (6:4:2:8 upper phase)	3 hours

the entire chromatogram were given the same number. Any spots that appeared later in the season were given higher numbers.

b) Diazotized salts

Fast Red Salt GL (4-amino-3-nitrotoluene) was used according to the procedure of Pearl and McCoy (1960). The dry chromatogram was exposed to strong ammonia vapours and immediately sprayed with a 0.05% freshly prepared aqueous solution of the salt. The chromatogram was dried in air at room temperature. After 30 minutes, it was sprayed with a saturated solution of sodium carbonate in water and allowed to dry. The colour of the spots was recorded.

c) Sulphanilic acid

The method used with this reagent was similar to that by Smith (1960).

Reagent Formula: (i) Sulphanilic acid - 0.9 g was dissolved in 9.0 ml of concentrated HCl and diluted with 90 ml of distilled water. (1 vol.).
Sodium nitrite 5% solution in water. (1 vol.).
(ii) Sodium carbonate (anhydrous) - 10% aqueous solution. (2 vol.).

When required, the stock solutions (i) were mixed and allowed to stand for 5 minutes in a beaker of ice cubes. Then solution (ii) was added carefully to avoid excessive effervescence. The dry chromatogram was heated in an oven at 100°C for 5 minutes to remove residual acid vapours from the chromatogram. The oven-dry chromatogram was dipped through the reagent and placed flat on a clean glass pane. Dark brown spots developed against a yellowish background.

3. Locating aliphatic acids

a) Bromophenol blue

The dry chromatograms were sprayed with a 0.04% alcoholic solution of bromophenol blue adjusted to pH 6.8. Yellow spots developed against a blue background.

b) The dimethylglyoxime-nickel biuret reaction

This test was carried out by the procedure of Savory (1964) as indicated below:

(i) A 1% (w/v) solution of dimethylglyoxime in 95% ethanol was prepared.

(ii) To prepare an alkaline nickel-biuret solution, 1 g of nickel sulphate heptahydrate was dissolved in 50 ml of distilled water and 1 g of biuret added. The mixture was warmed to dissolve the biuret. Ten ml of 1 N sodium hydroxide was added and the mixture allowed to stand for 30 minutes before being filtered to remove the precipitated nickel hydroxide. A clear amber-colored solution was obtained.

(iii) An ethanol-ammonia solution was prepared by adding 5 ml of strong ammonium hydroxide (sp. gr. 0.88) to 1 liter of 50% ethanol.

The dry chromatogram free from acid was dipped through a trough of the dimethylglyoxime solution and left to dry partially. It was then sprayed with a freshly prepared alkaline-biuret solution. The acids appeared as red spots on a white background in 2 minutes. After 2 minutes, the chromatogram was washed twice in 400 ml of the ethanol-ammonia solution. It was then hung to dry under a hood at room temperature.

c) Ninhydrin

Amino acids were located by dipping air-dried chromatograms in a 0.2% solution of ninhydrin in acetone. The chromatograms were left to dry at room temperature.

The identity of some of the unknown acids in the extracts was ascertained by measuring and comparing R_f values and co-chromatogramming unknown materials with authentic compounds.

Photography

The SR-1 Minolta single lense reflex camera with which pictures of chromatograms were taken in UV light had a 28 mm T-mount wide angle lens system fitted with a 1A Vivitar UV filter. The lens diaphragm was pre-set at an f stop of 11. The time exposure was 45 seconds for Kodachrome II and 1 second for Tri-X films.

Mineral analysis

Analyses for calcium, magnesium, and potassium was done by the Engineering Experiment Station personnel according to A.O.A.C. methods (1960) as modified by Kenworthy and Miller (1956) for atomic absorption spectrophotometry.

Statistical methods

The analysis of variance according to Steel and Torrie (1960) and Duncan's (1955) multiple range tests were used in the statistical analyses of the data.

RESULTS

Acidic composition of leaf extracts

Some of the acids that could be identified by their R_f values on paper chromatograms developed in different solvent systems are shown in Table 4. Acids of low R_f values could scarcely be detected on chromatograms run in solvent systems C and D. Solvent system C was developed during the course of this study. Its performance in separating raw leaf tissue extracts is compared with solvent system B in Fig. 3 A and B. Fig. 4 A and B shows chromatograms of crude leaf extracts of samples taken from the same six trees on June 20 and on September 26. The white spots in 4 B represent the fluorescent material that accumulated as the season advanced. The chromatograms represented in Fig. 5 A and B show spots of fluorescent compounds in leaf extracts from composite samples on different dates. A chromatogram of phenolic compounds found in the acidic fraction of the ethyl acetate extracts on the different dates is shown in Fig. 6. The qualitative seasonal variation of acids in the aqueous phase after ethyl acetate extraction is represented in Fig. 7. Dowex-resin-purified-extracts from samples of June 20 and September 26 are compared in Fig. 8. The various Dowex fractions of the June 20 extracts are shown also.

Titratable acidity

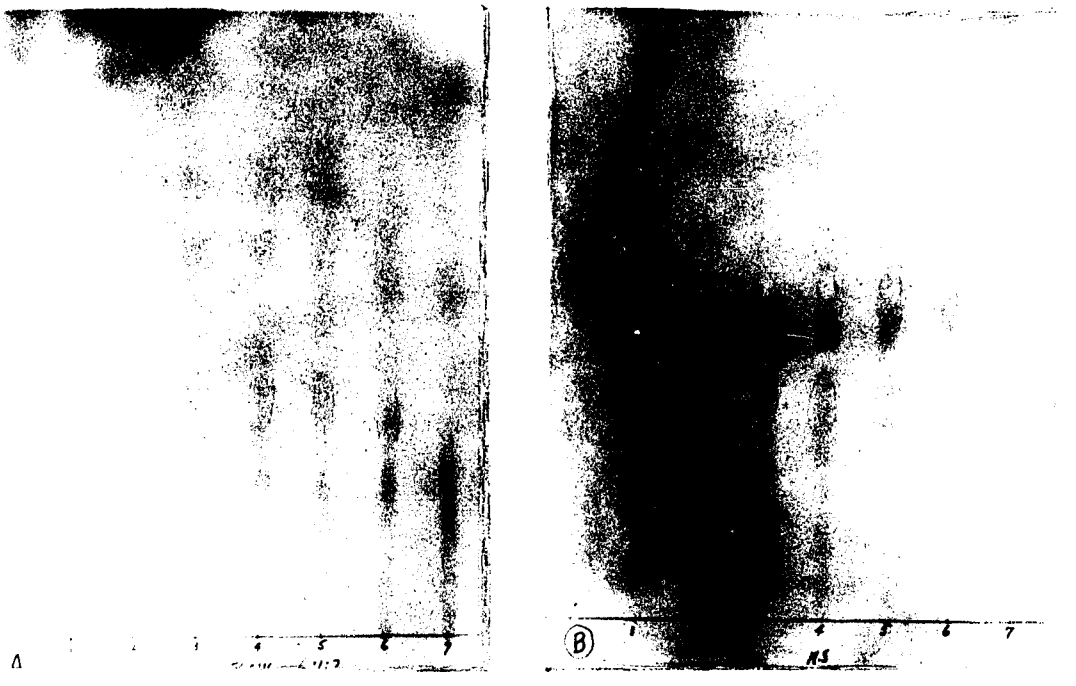
Table 5 shows the seasonal variation of titratable acidity per tree in milliequivalents per gram of oven dry tissue. Levels of acidity in different trees on the same sampling date were very consistent. The means for the 12

Table 4. R_f x 100 values of known acids and of some acids found in leaf extracts.

	Solvent A		Solvent B		Solvent C	
Compound	known	extract	known	extract	known	extract
Aspartic	18	17	--	--	--	--
Citric	40	40	41	40	47	47
Fumaric	85	84	81	79	83	82
Galacturonic	9	9	13	--	--	--
Glutamic	13	13	21	21	--	--
Malic	52	52	49	49	58	57
Malonic	62	61	58	58	67	67
Succinic	72	71	72	72	72	72
Caffeic	77	76	86	77	77	75
Chlorogenic	57	57	53	52	57	57
Ferulic	86	--	86	--	86	--
γ-indole-3-n-butyric	88	--	93	--	89	--
Indolepropionic	87	--	89	--	89	--
Sinapic	78	--	81	--	80	--
Unidentified phenolic	--	91	--	80	--	89
Phosphoric	32	32	34	34	35	--

Table 4. Continued

	Solvent D		Solvent E		Solvent F	
Compound	Known	extract	Known	extract	Known	extract
Aspartic	51	51	3	3	3	3
Citric	58	57	23	23	20	20
Fumaric	87	87	--	--	73	72
Galacturonic	--	--	3	3	3	3
Glutamic	--	--	4	4	3	--
Malic	61	60	33	33	28	28
Malonic	74	--	56	55	41	40
Succinic	79	79	65	65	62	61
Caffeic	88	--	76	--	68	--
Chlorogenic	80	80	34	34	30	--
Ferulic	94	--	85	--	81	--
γ-indole-3-n-butyric	98	--	86	--	76	--
Indolepropionic	98	--	89	--	84	--
Sinapic	91	--	71	--	88	--
Unidentified phenolic	--	90	--	--	--	--
Phosphoric	60	60	13	--	11	11

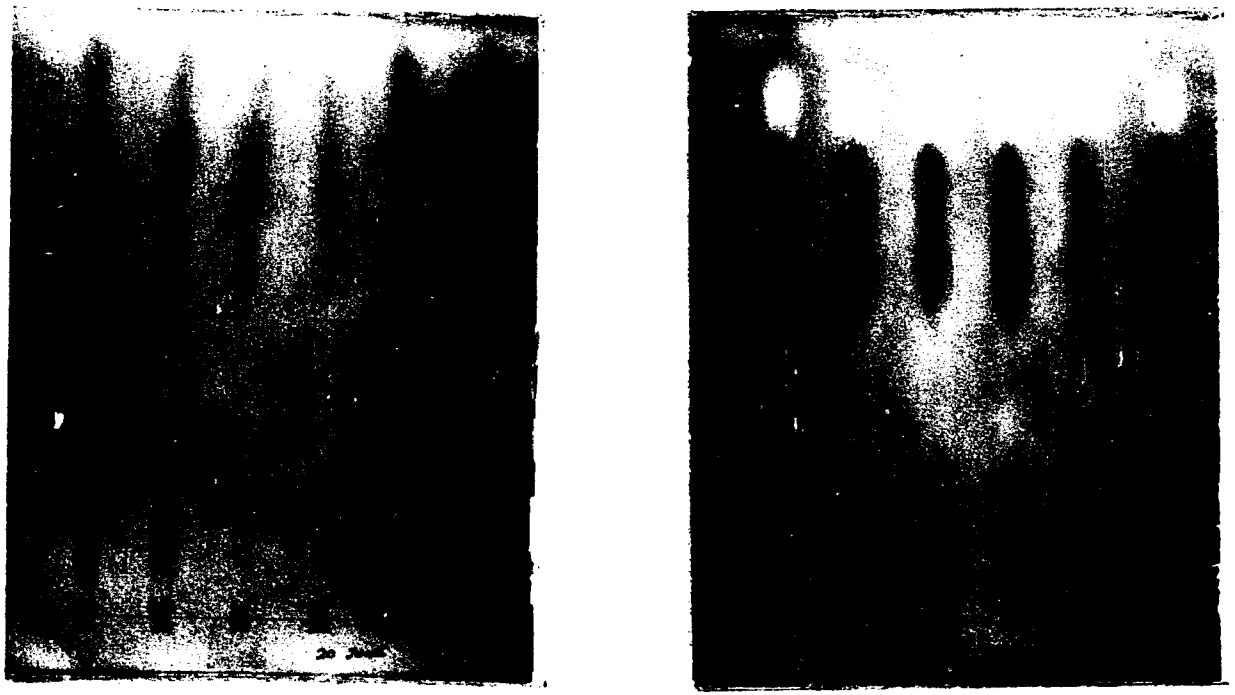


A B

Fig. 3. Comparison of two chromatographic solven systems:

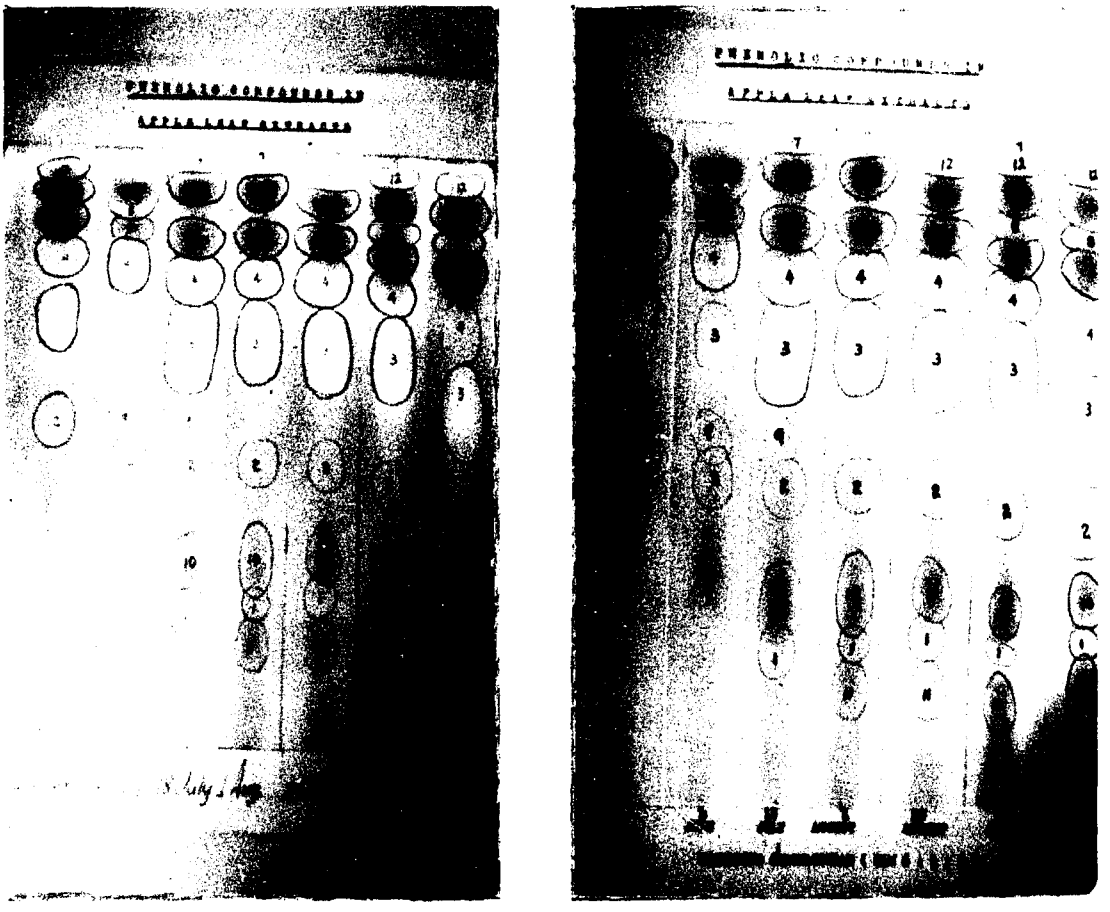
A - Chromatogram of leaf extracts on Whatman No. 3 paper developed in n-butanol:acetic:water (6:1:2 v/v)

B - Chromatogram of similar leaf extracts on Whatman No. 3 paper developed in n-butanol:80% formic (50:50 v/v)



A B

Fig. 4. Appearance of fluorescent materials late in the season. A - Chromatogram of extracts from leaf samples taken on June 20; B - Chromatogram of extracts from leaves taken from the same trees on September 26. Whatman No. 3 paper; n-butanol: 3N formic (50:50 v/v) solvent system. (Photo under short wave U.V)



A B

Fig 5. A and B - chromatograms showing spots of phenolic compounds in extracts from composite leaf samples taken on different dates. Paper: Whatman No. 3; Solvent: n-butanol:acetic acid:water (6:1:2, v/v). (Photo under short wave UV).

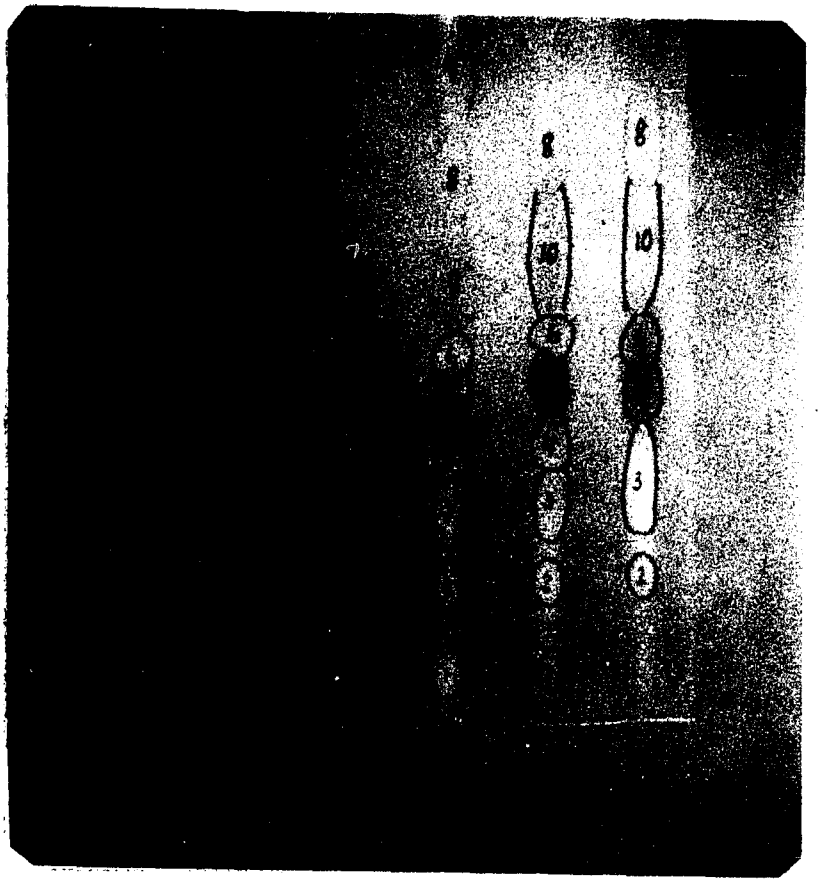


Fig. 6. Chromatogram showing the seasonal variation of phenolic compounds in the acidic fractions of ethyl acetate extracts. Paper: Whatman No. 3; solvent: n-butanol:acetic acid:water (6:1:2, v/v). (Photo under short wave UV).

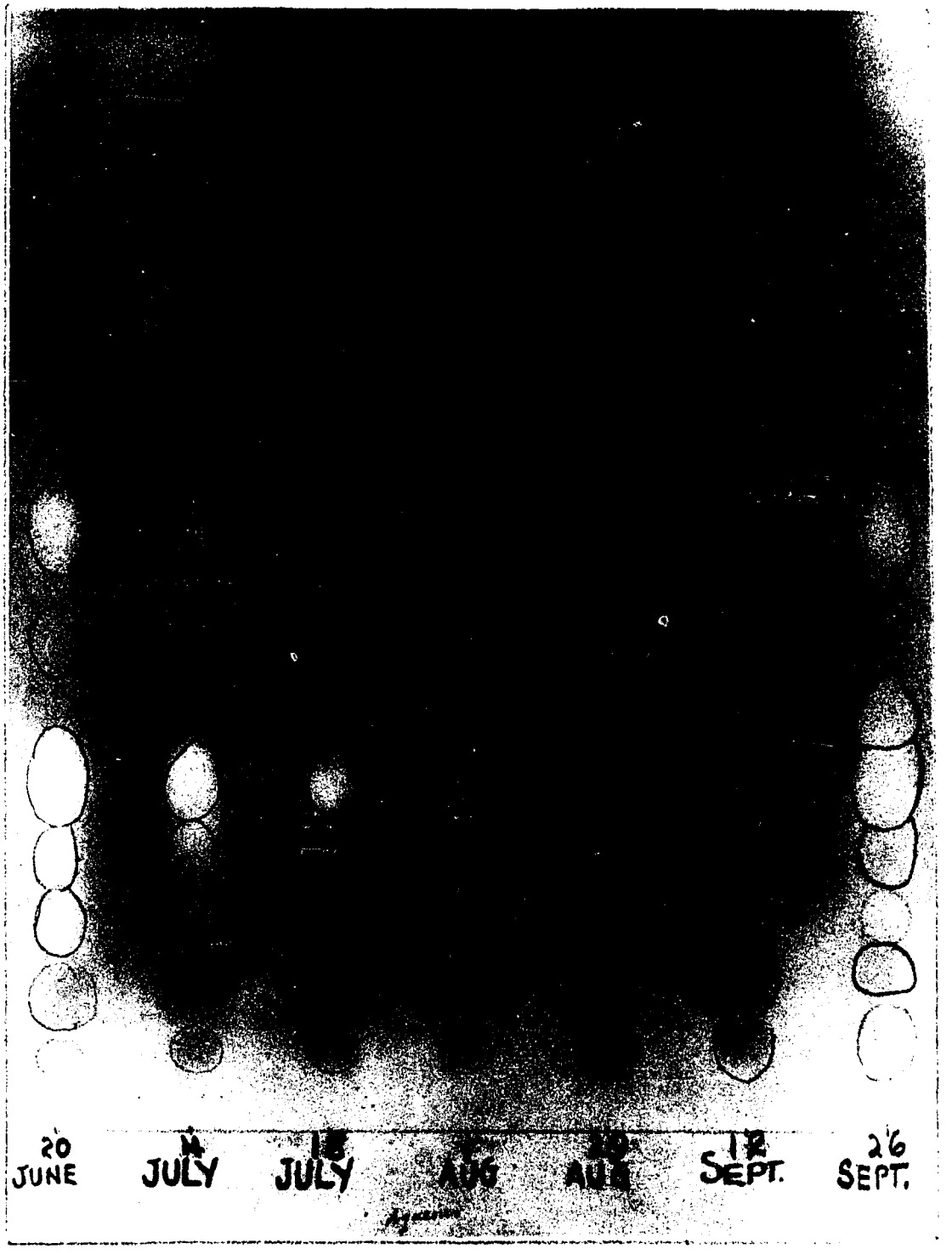


Fig. 7. Seasonal variation of acids that remained in the aqueous fraction after ethyl acetate extraction. C - citric; M - malic (Bromophenol blue treated chromatogram)

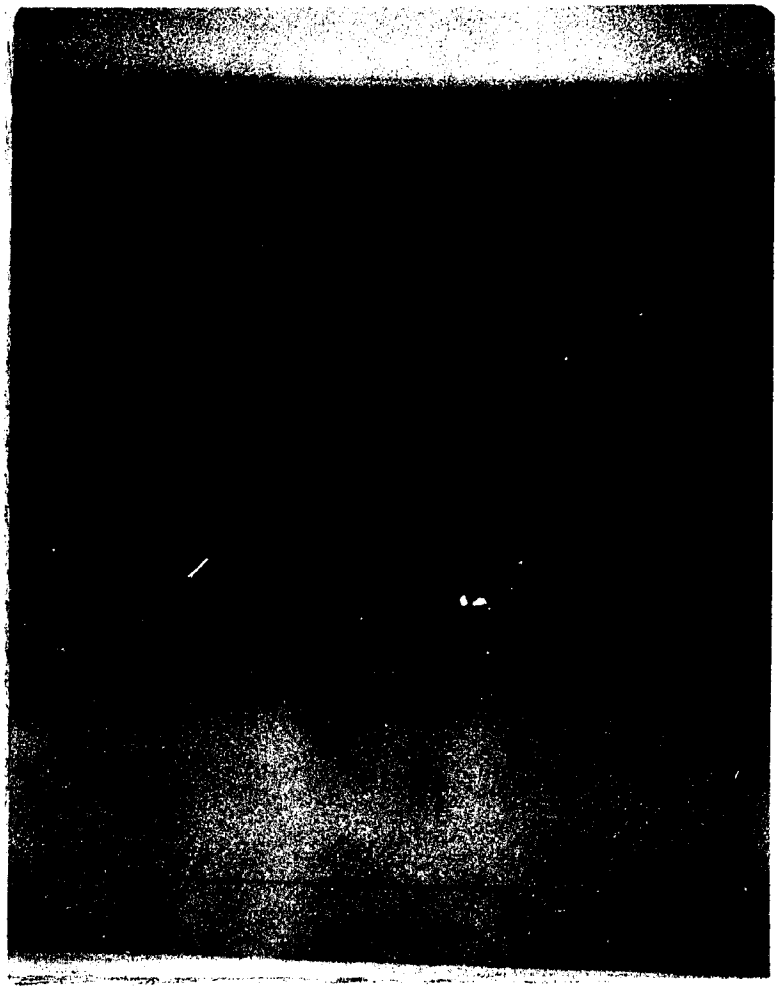


Fig. 8. Comparison of the first fractions of June 20 and September 26 extracts eluted from columns of Dowex 1 x 10 resin in the formate form. C = citric; M = malic; ma = malonic; su = succinic. (Bromophenol blue treated chromatogram)

Table 5. Seasonal variation of titratable acidity per tree in milliequivalents per gram of over dry leaf tissues.

Sampling date		20 June	4 July	18 July	1 Aug.	29 Aug.	12 Sept.	26 Sept.	Mean
Tree No.	207	0.18	0.23	0.19	0.16	0.19	0.24	0.27	0.21
	218	0.19	0.21	0.17	0.13	0.17	0.22	0.26	0.19
	225	0.22	0.20	0.17	0.13	0.17	0.22	0.26	0.20
	236	0.20	0.21	0.18	0.14	0.18	0.23	0.26	0.20
	244	0.18	0.20	0.18	0.13	0.18	0.21	0.24	0.19
	247	0.18	0.21	0.17	0.13	0.18	0.22	0.24	0.19
	253	0.20	0.21	0.16	0.13	0.15	0.22	0.26	0.19
	258	0.19	0.21	0.16	0.13	0.15	0.21	0.27	0.19
	261	0.18	0.21	0.16	0.13	0.16	0.21	0.23	0.18
	272	0.18	0.20	0.15	0.11	0.14	0.22	0.24	0.18
	285	0.17	0.21	0.16	0.13	0.13	0.22	0.23	0.18
	296	0.18	0.21	0.17	0.13	0.13	0.22	0.24	0.18
Mean[y]		0.19[b]	0.20[b]	0.17[a]	0.13	0.16[a]	0.22	0.25	

[y]Means between sampling dates followed by the same superscript are not statistically different at the 5% level (Dundan's new multiple range test).

trees sampled indicate that titratable acidity rose from 0.19 milliequivalent on June 20 to a first peak of 0.20 milliequivalent on July 4. Thereafter, it declined to the lowest value of 0.13 milliequivalent on August 1. From that date there was a steady rise and a value of 0.25 milliequivalent was recorded from samples taken on September 26.

Mineral composition

Tables 6, 7 and 8 for levels of calcium, magnesium and potassium, respectively, may be summarized as follows:

1) The percentage of calcium on September 26, was more than double that of June 20;

2) by September 12, the mean values for magnesium had increased by more than 50 per cent above the level at the beginning of the experiment; and,

3) potassium percentages declined as the season advanced. Fig. 9 summarizes the relationship between titratable acidity and Ca, Mg and K percentages throughout the season.

Table 6. Seasonal variation of calcium in the leaves of McIntosh apple clones on a per cent dry weight basis.

Sampling date		20 June	4 July	18 July	1 Aug.	29 Aug.	12 Sept.	26 Sept.	Mean
Tree No.	207	0.70	0.60	0.95	0.97	1.26	1.23	1.38	1.01
	218	0.60	0.82	0.86	1.05	1.43	1.21	1.39	1.05
	225	0.66	0.80	1.02	1.16	1.30	1.27	1.54	1.11
	236	0.60	0.81	1.02	1.18	1.40	1.40	1.64	1.15
	244	0.71	1.01	1.14	1.28	1.43	1.50	1.68	1.25
	247	0.69	1.21	1.15	1.34	1.53	1.63	1.85	1.34
	253	0.69	1.27	1.27	1.40	1.64	1.71	1.84	1.40
	258	0.71	1.19	1.14	1.19	1.38	1.46	1.60	1.24
	261	0.76	1.07	1.21	1.33	1.46	1.59	1.70	1.30
	272	0.84	1.01	1.18	1.27	1.37	1.56	1.67	1.27
	285	0.60	0.82	1.01	1.10	1.30	1.35	1.38	1.08
	296	0.61	0.92	0.96	1.15	1.18	1.39	1.45	1.09
Mean[y]		0.68	0.96^{a}	1.08^{ab}	1.20^{b}	1.39^{c}	1.44^{c}	1.59	

[y]Means between sampling dates followed by the same superscript are not statistically different at the 5% level (Duncan's new multiple range test).

Table 7. Seasonal variation of magnesium in the leaves of McIntosh apple clones on a per cent dry weight basis.

Sampling date		20 June	4 July	18 July	1 Aug.	29 Aug.	12 Sept.	26 Sept.	Mean
Tree No.	207	0.25	0.24	0.31	0.33	0.34	0.39	0.39	0.32
	218	0.24	0.26	0.31	0.36	0.37	0.41	0.40	0.34
	225	0.26	0.27	0.34	0.38	0.37	0.42	0.42	0.35
	236	0.25	0.28	0.36	0.39	0.41	0.41	0.38	0.35
	244	0.28	0.31	0.35	0.40	0.42	0.43	0.39	0.37
	247	0.26	0.35	0.36	0.39	0.41	0.42	0.41	0.37
	253	0.26	0.37	0.36	0.38	0.43	0.42	0.36	0.37
	258	0.26	0.36	0.34	0.36	0.37	0.39	0.38	0.35
	261	0.27	0.32	0.36	0.38	0.41	0.42	0.35	0.36
	272	0.28	0.30	0.36	0.39	0.39	0.43	0.39	0.36
	285	0.26	0.29	0.34	0.36	0.40	0.39	0.36	0.34
	296	0.25	0.27	0.30	0.34	0.32	0.36	0.32	0.31
Mean[y]		0.26	0.30	0.34	0.37^{a}	0.39^{ab}	0.41^{b}	0.38^{a}	

[y]Means between sampling dates followed by the same superscript are not statistically different at the 5% level (Duncan's new multiple range test).

Table 8. Seasonal variation of potassium in the leaves of McIntosh apple clones on a per cent dry weight basis

Sampling date		20 June	4 July	18 July	1 Aug.	29 Aug.	12 Sept.	26 Sept.	Mean
Tree No.	207	2.14	2.13	1.95	1.83	1.70	1.62	1.48	1.84
	218	1.99	1.80	1.83	1.74	1.56	1.45	1.34	1.67
	225	1.96	1.72	1.66	1.45	1.27	1.10	1.03	1.46
	236	1.97	1.74	1.62	1.51	1.19	1.10	1.01	1.44
	244	1.99	1.68	1.68	1.48	1.26	1.09	1.06	1.46
	247	1.91	1.98	1.51	1.38	1.21	1.07	0.97	1.43
	253	1.86	2.13	1.69	1.52	1.27	1.14	1.07	1.52
	258	2.09	2.26	1.74	1.64	1.43	1.30	1.23	1.67
	261	2.08	1.85	1.75	1.56	1.21	1.20	1.10	1.53
	272	2.24	1.91	1.81	1.53	1.22	1.05	1.02	1.54
	285	1.83	1.71	1.63	1.57	1.46	1.34	1.28	1.55
	296	1.84	1.62	1.68	1.64	1.53	1.45	1.38	1.59
Mean[y]		1.99	1.88	1.71	1.57	1.36[ab]	1.24[a]	1.16[b]	

[y]Means between sampling dates followed by the same letter are not significantly different at the 5% level (Duncan's new multiple range test).

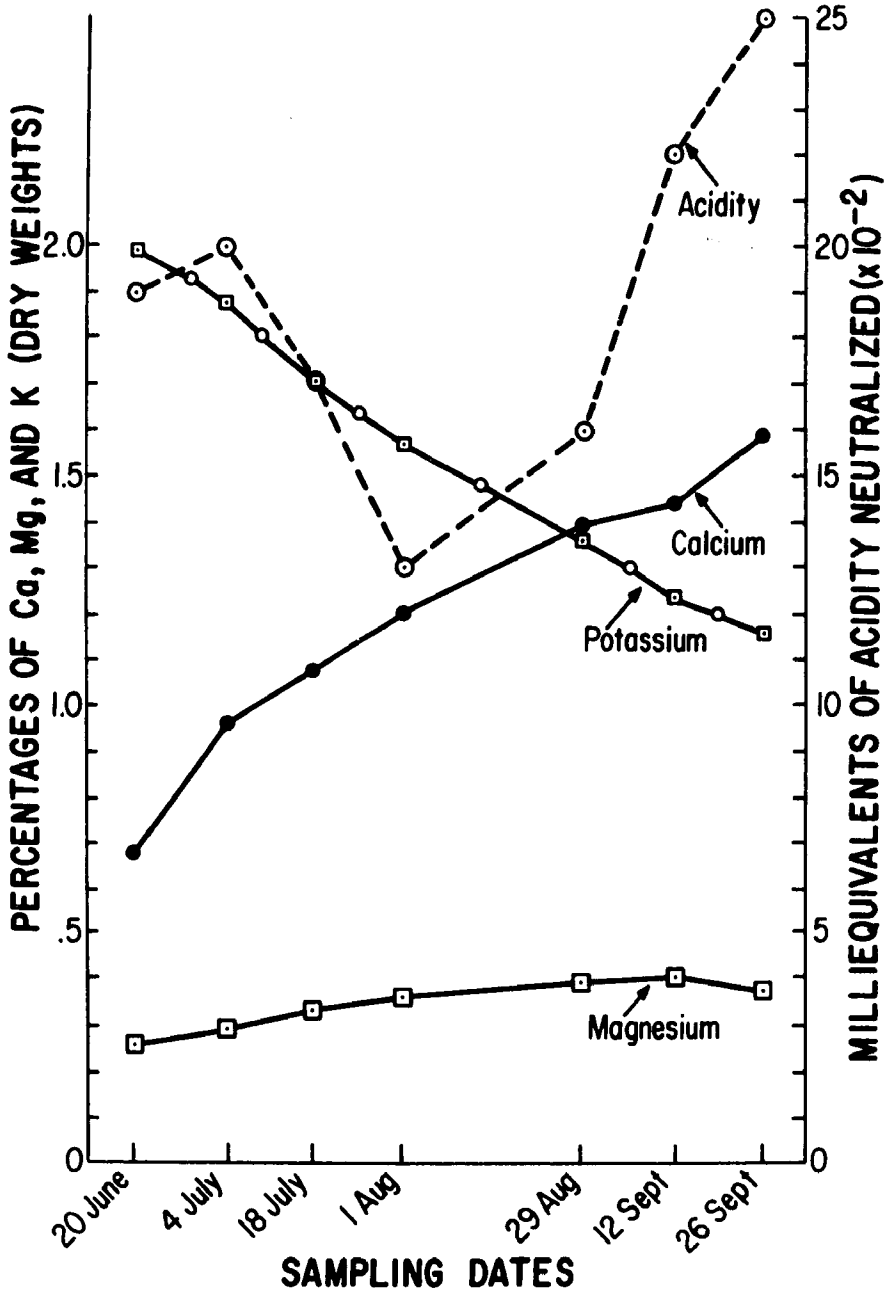


Fig. 9. The relationship between titratable acidity and percentages of Ca, Mg and K in the leaves of selected McIntosh apple clones

DISCUSSION

Data in Table 4 show that the acids detected in the apple leaf extracts included aspartic, citric, fumaric, galacturonic, glutamic, malic, malonic, succinic, caffeic, chlorogenic, phosphoric, and an unidentified phenolic acid.

The formula for solvent system A as used by Markakis, Jarczyk and Krishna (1963) is the upper phase of a mixture of n-butanol plus 3 N formic acid(50:50, v/v). Calculation revealed that 3 N formic acid is a mixture of 13.8% acid and 86.2% water. When it proved impossible to obtain compact and well defined spots with this solvent, it was decided to reduce the amount of water in the system. The basis for this decision was because, for paper chromatography, excepting reversed phase systems, water forms the stationary phase on the cellulose of the paper. If present in excessive amount, water might cause the materials chromatographed to 'trail' and separate poorly. Experimentation showed that 80% formic acid plus n-butanol (50:50, v/v) formed a homogeneous solvent system. This was particularly good for separating distinctly citric from malic acids which merged in other solvent systems. Other materials did separate out as seen in Fig. 3B. The time taken for a chromatogram to develop in a solvent system such as B could be reduced by half when the new solvent system C was used. The unidentified fluorescent spots showing as white in Fig. 4 B are the same as those numbered 8 in Fig. 5 A and B. These materials appeared as faint spots under short wave UV on chromatograms of July 4 extracts but disappeared thereafter. They reappeared on September 12 and 26. These materials accumulated in the leaves as the season advanced. They also have the

same R_f values as those of some growth regulators notably -indole-3-n-butyric and indole propionic acids on paper chromatograms. Therefore, these materials could be of some biological significance in the plant. No authentic abscisin sample was available for comparison.

Fig. 5 A and B shows that phenolic compounds increased in the leaves as the season advanced. Treatment of chromatograms with sulphanilic acid reagent showed that most of the phenolic compounds appearing late in the season were acidic. Some of the phenolic compounds partitioned into the acidic fraction of ethyl acetate extraction and which were detected on chromatograms under UV light are represented in Fig. 6. It is shown that even in that fraction phenolic acids increased as the season advanced. Numbers of spots in Fig. 6 do not necessarily correspond with those in Fig. 5. Chlorogenic and caffeic acids represented by numbers 6 and 10, respectively in Fig. 6, increased as the season progressed.

Chromatographic evidence was obtained to show that citric, malic and succinic were the dominant acids in leaf extracts, (Fig. 7 and 8). Malonic and fumaric acids were present in trace amounts. It was shown that citric and malic acids decreased significantly during the period of July 4 - August 1, as indicated on chromatograms represented in Fig. 7. It was noted that bromophenol blue reactions produced color intensities that were roughly proportional to the acidity curve in Fig. 9.

The ethyl acetate extract of the HCl hydrolysate did not contain phenolic compounds that were detectable under UV light. However, bromophenol blue tests indicated spots on chromatograms in the malic acid region. The spot colors were of about equal intensities throughout the season. This was interpreted as meaning that malic was the major acid involved in glycoside, ester and/or salt formation. To test

whether malic acid was soluble in ethyl acetate, 0.1 g of malic acid powder from the authentic sample on the shelf was mixed with 5 ml of ethyl acetate in a test tube at room temperature. It was found to be readily soluble. It was then questioned whether malic acid had been partitioned in the acidic fraction of the ethyl acetate extraction thereby altering the amount that would be found in the aqueous fraction. Bromophenol blue tests on chromatograms of the acidic fractions on the different sampling dates, showed spots in the malic acid region. Spots on chromatograms of the extracts of leaves sampled on September 12 and 26 were the most intense. This indicated that under similar experimental conditions more malic acid was partitioned in the acidic fraction of extracts from leaves collected later in the season than from those collected at the beginning. This was further proof that malic acid increased toward the end of the season. On the same chromatograms of the acidic fractions, moderately faint spots were detected in the succinic acid region, and they were of equal intensity throughout the season.

Comparison of samples eluted from columns of Dowex 1 x 10 resin in the formate form was made on the first fractions of extracts from June 20 and September 26 leaf samples. In Fig. 8 it is shown that there were more malic and succinic acids in the September 26 extracts than in those of June 20. Fractions 2 through 6 represented in the same figure were from June 20 extracts. It is shown that they consisted of basic materials, and no attempt was made to identify them at this time.

Data for acidity in Table 5 and in Fig. 9, indicate that titratable acidity declined during the period July 4 - August 1 and this correlated exactly with the decline in

citric and malic acids as shown in Fig. 7. To account for the decline in titratable acidity it is proposed that the acids formed in the leaves may have been translocated to developing fruit. Many researchers have shown that malic acid is the principal acid in apple fruit juice. Griffiths, Potter and Hulme (1950) who were studying the effects of storage temperature on Bramley's seedling apple fruit picked at different stages of maturity, obtained data showing that acidity which was 2.47 per cent fresh weight on June 23, had fallen to 1.27 per cent by September 24. This indicated that acidity is high in immature fruit and declines as ripening approaches. Work by Kidd, West and Potter (1951) indicated that the increase of acidity in the fruit on the tree was about 1 per cent at the beginning of June. It rose to a peak value of 2 per cent early in July and fell to 1 per cent toward the end of October. Lee, Salunkhe, Do, and Olson (1966) while investigating factors which affect quality of canned applesauce, recorded in Jonathan apple, a peak value of over 6 milliequivalents of acidity/100 g fresh weight on September 25, after which acidity declined. The results reported for leaves in Table 5 show that by September 26, the increase was 6 milliequivalents of acidity per 100 g dry weight over that on June 20. From the research reported, it is evident that information was being sought on quality of stored or processed fruit. No measurement of titratable acidity was made on foliar samples. In the study reported here, foliar samples were analyzed for titratable acidity without analyzing fruit. To test if fruit acidity is elaborated in leaves, foliar and fruit samples from bearing and non-bearing shoots and spurs, should be taken on the same dates and analyzed to determine the location and direction of acid translocation.

Another possible explanation for the decline in acidity during the period July 4 - August 1, might be that amination and rapid synthesis of proteins occurred from the acids. Using radioactive carbon, Maeatalu (1964) studied the distribution of C^{14} in fractions of products of photosynthesis at 10 minute intervals of CO_2-fixation in the leaves of apple tree shoots during the vegetative period. His experiments showed that during the early stages of growth, sugars are the main products of photosynthesis. In the second stage of growth, the C^{14}-fixed was used to greatest extent in organic acid and amino acid synthesis. The period July 4 - August 1 in this experiment (Fig. 9) might correspond to the latter part of stage 2 in the work of Maeatalu.

Fig. 9 shows that titratable acidity increased as the season advanced beyond August 1. Calcium and magnesium continued to accumulate while potassium declined during the growing season. The value of 0.25 milliequivalent per gram dry weight on September 26 represents an increase of 32 per cent over the acidity present on June 20. The increase of 0.12 milliequivalent is 92 per cent more than the acidity present on August 1. The participation of organic acids in active plant metabolism has been demonstrated (Burris, 1953; Davies, 1959). According to Truog and Meacham (1919) and Robinson (1963), acids which accumulate and do not disappear later may be regarded as waste products. Work by Tiffin and Brown (1961) indicated that some organic acids which accumulate, especially malic and malonic, may function as chelates in the transport of certain cations within plants. Blommaert (1955) found acidic growth-inhibitors in peach buds late in the season. Luckwill (1957) showed that Cortland apple leaves collected between August and November con-

tained three acidic growth-promoting and two acidic growth-inhibiting substances. Robinson, Wareing and Thomas (1963) showed that the growth-inhibitor found in the acid fraction extracted from Acer pseudoplatanus was correlated with short photoperiods. This was in agreement with the results of research by Kawase (1961) which indicated that short day treatments induced production of acidic growth inhibitors in leafy seedlings of Betula pubescens. After August 1, day length is considerably shorter than in June at Durham, New Hampshire. It may be that the total number of phenolic acids which appeared late in the season contributed to the build up of titratable acidity. As indicated in Fig. 7 and 8, there was considerable increase in citric, malic and succinic acids by September 26. The pH value of the extracts, however, remained within the range of 5.5-5.8. This agrees with Small (1955) who reported that the pH in leaf mesophyll of Prunus laurocerasus was between 5.7-5.8.

The percentages in Table 6 indicate that by September 26, calcium increased by more than 100 per cent of the original level. The level on September 26, was 32 per cent greater than the amount of calcium on August 1. This increase corresponds exactly to the overall increase in titratable acidity. Calculation of figures in Table 7 shows that the quantity of magnesium on the last sampling date had increased by 58 per cent. The sum of calcium and magnesium increased by 110 per cent. There was, therefore, a parallelism between titratable acidity and increase in calcium and magnesium as the season advanced beyond August 1. The results reported suggest that calcium and magnesium, which continue accumulating after visible growth has stopped, may be the principal cations associated with the acids which make up the buffer system of the leaf. Since titratable acidity increased

despite a fairly constant pH implies that the total buffer increased. As acidity is part of the total buffer, the increase in acidity requires a corresponding increase in the quantity of bases for neutralization. It may be assumed then, that under conditions of constant pH, titratable acidity reflects the total buffer concentration and, therefore, the concentration of cations associated with the buffer. If there is insufficient magnesium, that which is available may be diverted toward buffering the acidity produced. This occurs at the expense of other functions and magnesium deficiency symptoms thus appear. This suggestion agrees with work by Pierce and Appleman (1943) who found a correlation between ether-soluble organic acids and excess inorganic cations in herbaceous plants. They interpreted it as a means of maintaining cation-anion balance. Work by Ulrich (1941) on excised barley roots under controlled conditions of nutrition showed that when cation absorption was in excess of anion absorption, so long as there was an ample supply of carbohydrates, organic acids were formed to counterbalance the potential physiological alkalinity developed. This was confirmed later by Torii and Laties (1966). On the basis that organic acids are formed in response to excess cation absorption in order to maintain pH relatively constant, it may be assumed that the reverse is also true. If excessive organic acids are produced in response possibly to shorter days, extra cations, when available, would be absorbed to counterbalance the potential physiological acidity produced. Hoagland (1944) reported that organic acids in association with cations of potassium, calcium, magnesium and sodium together with phosphate, amides, amino acids and other compounds, consti-

tute a buffer system which maintains the hydrogen ion concentration fairly constant. He emphasized that the system is not a static one. The cells are capable of responding to causes of change in hydrogen ion concentration through appropriate metabolic reactions that prevent wide fluctuations in pH.

Since measurement of titratable acidity was made on extracts from dry leaf tissues for this experiment, it might be argued that the interpretation of results might not be applicable to living systems. Dunne (1932) investigating buffer systems in wheat plants, divided the experimental material into two lots: the one being dried and ground and the other frozen. Water was added to the dried and ground sample to give the same water content as the fresh extract. He found that the acid buffer curve of the dry extract corresponded exactly to that of the expressed sap from frozen plants.

In his experiments with buckwheat under controlled conditions of nutrition, Dunne (1932) found that a decrease in the Ca content of sap was accompanied by a large increase in K. He explained that the K then served as the main base in equilibrium with organic acids. He suggested that in the buffer system of sap, one base served as well as another provided that enough total base could be absorbed. In terms of growth, the balance among cations is extremely important (Shear, Crane, and Myers, 1948). Research by Cain (1948) confirmed previous work by Lilleland and Brown (1938), showing that a decrease in calcium or magnesium resulted in increased potassium in leaves of fruit trees to satisfy a "cation requirement". Potassium, which declines as the season advances, ordinarily may not be involved in buffering pH of apple leaf tissues late in the season. Under condi-

tions of insufficient calcium and/or magnesium, potassium may partially replace these cations to maintain anion-cation balance. It may, at the same time, upset balance among cations. Insufficient cations, in certain proportions, may result in changes in tissue pH. It is theorized that such changes may be a major factor in the damage characteristic of some mineral deficiency symptoms in apple leaves. Such a theory is supported by the work of Cain (1954) who found that iron-chlorotic blueberry leaves had a higher pH than green leaves of comparable age. He correlated the increase in pH with accumulation of the basic cations of Ca, Mg and K. Further support for this theory comes from work by Iljin (1951) in which three apple species were studied under lime-induced chlorosis. In every instance, it was found that severity of chlorosis was closely associated with leaf content of citric acid.

SUMMARY

Water extracts of dry leaf tissues from selected McIntosh apple clones were analyzed by titration with sodium hydroxide, paper and ion exchange-resin chromatography and by atomic absorption spectrophotometry. A useful solvent system for paper chromatographic separation of organic acids in apple leaf extracts was developed. Citric, malic and succinic were the most predominant of the acids detected. It was shown that titratable acidity decreased during the period July 4 - August 1. This decline corresponded to the disappearance of citric and malic acid spots on chromatograms. Titratable acidity increased as the season advanced beyond August 1. This increase coincided with the re-appearance of citric and malic acids. Succinic, caffeic and chlorogenic acids increased as the season advanced. An unidentified phenolic acid having similar R_f values as those of γ-indole-3-n-butyric and indole propionic acids appeared in significant amount toward the end of the growing season. Mineral element data suggest that calcium and magnesium, which accumulate in leaves as the season advances, may be the principal cations associated with bases involved in the buffering systems which maintain pH relatively constant. The appearance of deficiency symptoms late in the season is attributed to diversion of the available magnesium toward buffering acidity at the expense of other functions. Potassium which accumulates only under conditions of deficient calcium and/or magnesium may not ordinarily be associated with pH buffering systems late in the season. It is theorized that change in pH may be a

major factor in the characteristic damage resulting from certain mineral deficiencies in apple leaves.

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Appendix Table 1. Variance analysis of means in milliequivalents of titratable acidity in McIntosh apple leaves sampled on different dates.

Source	Degrees of Freedom	Mean Square	F
Total	83		
Between dates	6	0.0192	112.9*
Error	77	0.00017	

*Significant at .05

Appendix Table 2. Shortest Range values[a] (5% level) and significance of means in milliequivalents of titratable acidity in McIntosh apple leaves sampled on different dates.

	p	2	3	4	5	6	7
	rp	2.82	2.97	3.07	3.13	3.19	3.21
	Rp	0.011	0.011	0.012	0.012	0.012	0.012
Sampling dates		8/1	8/29	7/8	6/20	7/4	9/12	9/26
Means		.13	.16	.17	.19	.20	.22	.25
Stat. significance at .05								

[a] p = Size ranges; rp = Approximate significant Studentized ranges; Rp = Shortest significant ranges

Means not underscored by the same line are significantly different.

Appendix Table 3. Variance analysis of Calcium percentage means in McIntosh apple leaves sampled on different dates.

Source	Degrees of Freedom	Mean Square	F
Total	83		
Between dates	6	1.18	56.2*
Error	77	0.021	

*Significant at 5% level

Appendix Table 4. Shortest Range values (5% level) and significance of Calcium percentage means in McIntosh apple leaves samples on different dates.

	p	2	3	4	5	6	7
	rp	2.82	2.97	3.07	3.13	3.19	3.21
	Rp	0.118	0.124	0.128	0.131	0.133	0.134
Sampling dates		6/20	7/4	7/18	8/1	8/29	9/12	9/26
Means		0.68	0.96	1.08	1.20	1.39	1.44	1.59
Stat. significance at .05								

Means not underscored by the same line are significantly different.

Appendix Table 5. Variance analysis of Magnesium percentage means in McIntosh apple leaves sampled on different dates.

Source	Degrees of Freedom	Mean Square	F
Total	83		
Between dates	6	0.0327	44.2*
Error	77	0.00074	

*Significant at .05

Appendix Table 6. Shortest Range values (5% level) and significance of Magnesium percentage means in McIntosh apple leaves sampled on different dates.

	p	2	3	4	5	6	7
	rp	2.82	2.97	3.07	3.13	3.19	3.21
	Rp	0.022	0.023	0.024	0.025	0.025	0.025
Sampling dates	6/20	7/4	7/18	8/1	9/26	8/29	9/12
Means	0.26	0.30	0.34	0.37	0.38	0.39	0.41
Stat. significance at .05							

Means not underscored by the same line are significantly different.

Appendix Table 7. Shortest Range Values (5% level) and significance of Potassium percentage means in McIntosh apple leaves sampled on different dates.

Source	Degrees of Freedom	Mean Square	F
Total	83		
Between dates	6	1.22	46.9*
Error	77	0.026	

*Significant at .05

Appendix Table 8. Shortest Range values (5% level) and significance of Potassium percentage means in McIntosh apple leaves sampled on different dates.

	p	2	3	4	5	6	7
	rp	2.82	2.97	3.07	3.13	3.19	3.21
	Rp	0.131	0.138	0.143	0.146	0.149	0.150
Sampling dates	9/26	9/12	8/29	8/1	7/18	7/4	6/20
Means	1.16	1.24	1.36	1.57	1.71	1.88	1.99
Stat. significance at .05							