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ABSTRACT

COMPARATIVE STUDY OF PHENOLIC COMPOUNDS IN CHERRY ROOTSTOCKS

By

Kyung Sang Yu

Sweet cherry trees grafted on Mahaleb rootstocks (*P. mahaleb*) show incompatibility symptoms after several years of growth in the orchard, while those grafted on Mazzard (*P. avium*) do not show such symptoms. The cause of this incompatibility is not known. Several groups of biochemical compounds have been suggested as causal agents: cyanogenic glucosides, alkaloids, proteins, amino acids, and phenolic compounds. In this study, the phenolic compounds occurring in Mazzard and Mahaleb seedlings were evaluated and compared to determine their role in cherry graft incompatibilities.

Total phenolic compounds of leaves, stem, and root bark were measured at two-week intervals during 5 months of active growth. The levels of phenolic compounds in these tissues increased during the first two months, then stabilized. Mahaleb root tissues, however, showed marked variation during this period. The two rootstocks differed

significantly in total phenolic compounds in all tissues tested in that Mazzard tissues contained 1.5 to 3 times more phenolics than Mahaleb. Both Mazzard and Mahaleb had higher levels of total phenolic compounds in the bark than in the leaves. Greenhouse-grown cherry seedlings of both species contained markedly lower levels of total phenolics than those grown in the field.

Methanol extracts and centrifugal sap of fresh tissues were examined for phenolic composition. The following compounds were identified on the basis of (1) color reactions, (2) Rf's in paper chromatography and thin layer chromatography, (3) retention times in gas chromatography, and (4) ultraviolet spectra. Compounds found in only one of the two species are underlined.

Mazzard

Leaf . . . o-coumaric acid, *p-coumaric acid*, *caffeic acid*, *chlorogenic acid*, *p-coumarylquinic acid*, coumarin, kaempferol, *quercetin*, *dihydrowogonin*, d-catechin, epi-catechin.

Stem bark . . . o-coumaric acid, *p-coumaric acid*, coumarin, *chlorogenic acid*, *p-coumarylquinic acid*, *dihydrowogonin*, d-catechin, epi-catechin, leucoanthocyanidin.

Root bark . . . o-coumaric acid, *p-coumaric acid*, coumarin, *chlorogenic acid*, *p-coumarylquinic acid*, *dihydrowogonin*, d-catechin, epi-catechin, leucoanthocyanidin.

Sap . . . coumarin, *dihydrowogonin*.

Mahaleb

Leaf . . . o-coumaric acid, coumarin, herniarin, kaempferol.

Stem bark . . . o-coumaric acid, coumarin, herniarin, d-catechin, epi-catechin, leucoanthocyanidin.

Root bark . . . o-coumaric acid, coumarin, herniarin, d-catechin, epi-catechin, leucoanthocyanidin.

Sap . . . coumarin, herniarin.

The possible roles of these compounds in incompatibility are discussed.

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Dedicated to my
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mother

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INTRODUCTION

Mahaleb (*Prunus mahaleb*) and Mazzard (*P. avium*) are the two rootstocks generally used for sweet cherry (*P. avium*) cultivars. In the nursery Mahaleb seedlings are vigorous, uniform, and easy to bud. Sweet cherry cultivars budded on Mahaleb grow well in the nursery and for the first 4 to 6 years in the orchard. When the trees come into production, however, many show graft-incompatibility. Cultivars budded on Mazzard do not show this abnormality (Argles, 1937).

Several groups of compounds have been suggested as the causal agents of graft-incompatibilities including cyanogenic glucosides (Gur, 1968b), alkaloids (Mothes, 1955), proteins (Crane, 1945), amino acids (Tschiersch, 1963), and phenolic compounds (Thiel, 1954; Gur, 1968b).

Phenolic compounds are ubiquitous and yet specific in higher plants. Many phenolic compounds are phytotoxic (Pridham, 1960), and their inhibitory roles have been shown in germination (Hemberg, 1961; Evenari, 1961), shoot and root growth (Hemberg, 1961; Goodwin and Taves, 1950). The compounds also have been implicated in growth regulation via the IAA-oxidase system (Galston, 1969). Preliminary study showed that Mahaleb and Mazzard rootstocks differed in their phenolic composition. The possible role of phenolics as causal agents of graft-incompatibility prompted us to make a detailed comparison of the compounds in Mazzard and Mahaleb seedlings.

REVIEW OF LITERATURE

I. Graft Incompatibility

A. Definition and types

Graft-incompatibility may be defined as an inherent antagonism or discordant association between certain stocks and scions (Amos, 1936). It can be due to either failure of graft unions or factors other than graft unions (Luckwill, 1962).

Two types are generally recognized, which Mosse (1962) has termed localized vs. translocated incompatibility. The first type requires direct contact between the incompatible tissues. Reciprocal grafts of these are also incompatible. Use of compatible interstems eliminates the incompatibility. The second type does not require direct tissue contact, but occurs despite the presence of an intermediate stem piece. Reciprocal grafts may or may not exhibit incompatibility. Examples of "localized" incompatibility are the pear variety C_8 on quince A (Mosse and Herrero, 1951) and Oullin's Gage plum on Myrobalan B (Herrero, 1951). Examples of "translocated" incompatibility include Hale's Early peach on

Myrobalan B (Mosse, 1955) and lemon on sour orange (Calavan *et al.*, 1951). The incompatibility reaction may be immediate, in which case the graft union either does not form or is short lived as in Cox's Orange Pippin on *Malus theifera* (Luckwill, 1962), and ornamental cherries (*P. serrulata*) on Mahaleb (Amos *et al.*, 1936). Alternatively, it may be delayed until the plant is several years old, as is the case in certain sweet cherry clones budded on Mahaleb (Grubb, 1938). In fruit trees, decline often occurs when the trees begin to fruit (Garavel, 1954; Jimenez, 1957).

B. Incompatibility in cherry

1. Symptoms. The two principal rootstocks for sweet cherry cultivars are wild seedling types of *Prunus avium* (Mazzard) and *P. mahaleb* (Mahaleb). Mahaleb seedlings, which are easily budded and which produce vigorous trees in the nursery, have shown symptoms of incompatibility with sweet cherry cultivars (Grubb, 1938; Simons and Carlson, 1968). The symptoms generally occur only after 8 to 10 years. This fact, together with differences in response in different climates, has raised questions as to the true nature of this incompatibility. However, observations on growth rates and tree losses in many countries confirm that sweet cherry cultivars grafted on Mahaleb are generally shorter lived than those grafted on Mazzard (Argles, 1937; Garner, 1967; Raptopoulus _et al_., 1959; Hilkenbaumer, 1952; Howe, 1927; Quinn, 1935). Symptoms include constriction of the stock immediately below the graft union, premature defoliation, and a decline in vigor. Starch accumulation at the union is a characteristic symptom of incompatibility. However, vascular connections between stock and scion appear to be normal (Herrero, 1951).

2. Effect of scion cultivars. Cultivar effects on graft-incompatibility have not been carefully studied in sweet cherry/Mahaleb combinations. Symptoms do occur in many sweet cherry cultivars budded on Mahaleb. Trees often grow vigorously for a few years, then decline or die in a 5- to 24-year period (Garner, 1967; Howe, 1927; Fowler, 1933; Amos _et al_., 1936). In Michigan, however, Carlson (unpublished data) observed the different responses among sweet cherry cultivars on Mahaleb. Certain cultivars such as "Emperor Francis," "Hedelfingen," and "Stark's Black Hardy Giant" frequently show incompatibility symptoms, whereas cultivars such as "Vista," "Schmidt," and "Windsor" appear to be more compatible with Mahaleb. Philp (1930) reported a case of complete incompatibility among cherry cultivars. In California the sweet cherry 'Chapman' showed lack of affinity with the sour cherry 'Stockton Morello' but this could be overcome by double working. Ornamental cherry trees also showed incompatibility on clonal selections of Mahaleb (Amos et al., 1936). Trees on the Mazzard selections grew vigorously but varieties budded on Mahaleb died by the end of the second season. At East Malling, Herrero (1951) noted that trees of the sweet cherry cultivar 'Frogmore' budded on rootstock F250 (sour cherry selection) were dying in four years. No cause could be found, so death was considered due to incompatibility. Grubb (1938) reported three cultivars 'Early Rivers,' 'Governor Wood,' and 'Waterloo,' incompatible with Mahaleb selections, F6/1/2, F8/1/10, and F8/1/12.

3. Environmental effects. Cherry trees are rather exacting with regard to climatic and soil requirements, growing best on light, moist, well-drained loams. Most of the commercial production in the United States is confined to the Great Lakes area and the Pacific Coast where moderate temperatures and humidity occur (Westwood, 1966).

The behavior of sweet cherries on Mahaleb seems to be closely related to environment. Failure of trees budded on Mahaleb has been reported in England (Garner, 1967), New York (Howe, 1927), Tennessee (McClintock, 1930), Virginia (Anthony et al., 1937), Michigan (Simons and Carlson, 1968), Canada (Anonymous, 1945), Germany (Hilkenbaumer, 1952), Greece (Raptopoulos, 1959), and South Australia (Quinn, 1935). Such trees are short lived and unproductive, whereas trees budded on Mazzard are vigorous, productive, and long lived. However, trees have been successfully grown on Mahaleb in Utah (Coe, 1945) and California (Philp, 1930). In Utah, Coe (1945) found that Mahaleb was definitely superior to Mazzard. In California some growers preferred Mazzard, others Mahaleb (Philp, 1930). Also in California, Brooks (1950) recognized that the best rootstocks for sweet cherries varied according to soil and climatic conditions. There, the percentages of rootstocks used commercially for sweet cherries were Mazzard, 65%; Mahaleb, 30%; and Stockton Morello, 5%.

These striking differences might be related to environmental factors. Soil drainage in particular seemed to be related to tree losses. In Utah, where the orchard soil was a coarse gravelly loam with good drainage and aeration, trees on Mahaleb outgrew and outproduced those on Mazzard (Coe, 1945). Upshall *et al.* (1950) believed that poor soil drainage was the main cause of tree loss. In the Niagara district of Ontario, the authors found that most tree losses (sweet cherry/ Mahaleb) occurred in orchards with poorly drained soils, whereas in well-drained soils, trees on Mahaleb outyielded and grew more than those on Mazzard.

Although the basic causes of graft-incompatibility are genetic, symptom expression may be governed by environmental factors (Chang, 1937). Soil drainage conditions and other environmental factors may thus accelerate or delay expression of incompatibility in sweet cherry trees on Mahaleb. Possibly there is no true incompatibility between these two species; trees may fail simply because Mahaleb cannot tolerate poor soil drainage. In the same area, however, sour cherry (*Prunus cerasus*) trees tolerate poor drainage conditions better than sweet cherries. Therefore an inherent factor in sweet cherry accelerates tree decline.

4. Reciprocal grafts. There are a few reports on reciprocal grafting of sweet cherry on Mahaleb. Hedrick (1914) reported that reciprocal grafts of Mahaleb on any cultivated sweet cherry failed at Geneva, New York. However, Cummings *et al*. (1933) found that Mazzard actually grew better on Mahaleb stock than on its own roots, whereas Mahaleb grew well on both its own roots and on Mazzard during ten years' trial in Vermont.

C. Causes of Incompatibility

1. Anatomical. The underlying causes of graft incompatibility are unknown (Mosse, 1962). Special attention has been paid to anatomical studies on the assumption that the graft union is a mechanical barrier to translocation. Chang (1937), from his studies of compatible and incompatible combinations of pears, plums, peaches, and cherries, noted that incompatible unions reduced the flow of dyes and water. Starch accumulation occurred at the graft union in incompatible combinations (e.g., 'Durondeau' pear/Quince F). Starch depletion was also noted in the roots of the incompatible combinations Hale's Early peach/Myrobalan B and 'Frogmore' cherry/F250 sour cherry (Herrero, 1951). In simulated graft union studies using grafting, banding, and scoring techniques, sweet cherry "Napoleon" on Mahaleb was found to contain higher levels of starch than those on Mazzard (Carlson and Yu, 1969). McClintock (1948), from his study of incompatibility between peaches and Mariana plum found that the roots died first and that the phloem tissues of the plum stock and the peach scion failed to unite. He concluded that this mechanical obstruction at the union was caused by a lack of translocation to the roots leading to their death. Nitrogenous materials were reported to also accumulate at the graft union in incompatible combinations.

This interference with translocation appears to be due to structural defects at the graft union, including one or more of the following:

1. A layer of parenchymatous tissue at the line of union as in 'President' on common plum and some pears on quince rootstocks (Chang, 1937; Proebsting, 1926) rather than normal differentiated tissue.

2. Distortion of vascular tissue at the line of union in pear/quince (Herrero, 1951; Proebsting, 1926).
3. Gummy masses at the union in certain stone fruits (Proebsting, 1928).
4. A cork layer at the union as in 'Reeve's' peach on Myrobalan B (Herrero, 1951).

The initial reaction that leads to vascular discontinuity appears to be related to cambial activity at the point of union. Herrero (1951) noted that primary causes of discontinuity were necrosis of some cells in the cambial region in pear/quince and a slowing down of cambial activity at the line of union in plums. Mosse and Scaramuzzi (1956), working with pear/quince grafts, found that the first necrotic symptoms appeared, not in the cambium, but in 1- to 2-year-old phloem tissues. The necrosis then spread to ray and to cambium cells, breaking vascular continuity in the union. The cause of the degeneration of phloem tissues was not clear. However, the fact is noteworthy that these abnormalities occurred most often at the end of the growing season, when the maximum translocation of metabolites was downward. Herrero (1951) and Mosse and Scaramuzzi (1956), from their anatomical studies, considered that the causes leading to necrosis and breaks in cambial continuity were biochemical rather than anatomical, and were related to seasonal metabolic changes in the tree.

Histological differences between two incompatible tissues were suggested as a causal factor in tree decline, but Herrero (1951) could not correlate such differences, such as relative amounts of vessels, fibers, parenchyma cells and rays, or the size of these elements with the degree of compatibility.

Chang (1937) suggested that different growth characteristics of the stock and scion were a cause of incompatibility. His work suggested that differences in periods of cambial activity, seasonal growth patterns, and growth rates between stock and scion might be responsible. Herrero (1951), however, believed that these characteristics were not directly associated with the primary causes of incompatibility.

2. Role of viruses and mycoplasma. The presence of latent viruses or virus complexes in stone fruits has been demonstrated by Milbrath *et al*. (1945). Several cases have been reported in which viruses were responsible for incompatibility. Toxopeus (1936) noted that sweet orange (*Citrus sinensis*) failed on sour orange (*Citrus aurantium*). Interestingly, failure was limited to Java and South Africa; the same combination was successfully grown elsewhere. Later, the diseases called 'tristeza,' or 'quick decline' was noted in other localities in conjunction with sour orange rootstock. Subsequent study

showed that viruses were responsible for these diseases and for graft failure (Fawcett *et al*., 1946; Bitters *et al*., 1953).

A similar case was reported in apples. The clonal stock Spy 227 proved to be incompatible with certain varieties of apples (Shaw and Southwick, 1944). Gardner *et al*. (1946) pointed out that there was a similarity between this phenomenon and tristeza disease of citrus. Weeks (1948) demonstrated that the toxic principle was viral in nature.

Symptoms of x-disease (buckskin) in sweet cherry on Mahaleb are similar to incompatibility symptoms. Trees infected with this disease, which is caused by a mycoplasm, develop necrotic tissues at the union and distal portions of roots. The trees usually die quickly in late summer after infection. Sweet cherry trees on Mazzard infected with x-disease decline 4 or 5 years before they die. Tissues at the graft union usually remain normal until the tree dies (Granett and Gilmer, 1971; Parker *et al*., 1963).

Mosse (1962) has pointed out the similarities between the symptoms of virus diseases and symptoms of incompatibility. The "translocated" type of graft incompatibility, in particular, has many features in common with virus-induced graft-incompatibility including:

1. Absence of mechanical weakness of the union;
2. Phloem degeneration;

3. Accumulation of starch above, and its absence below, the union;
4. Reduced bud-take;
5. Variability of one-year-old trees;
6. Blistering stem tissues.

However, all "translocated" incompatibilities do not appear to be due to virus. In the case of the incompatible combination, 'Hale's Early' peach on Myrobalan B, where the evidence is strong that translocatable factors are responsible, the virus did not seem to be a causal factor. Herrero (1951) tested the possibility of virus infection by grafting scions from incompatible trees to trees of the compatible combination Hale's Early/Brompton. The test was negative. A similar case was found in incompatible trees of Victoria/President/ Myrobalan B. Normally compatible President/Myrobalan B trees can be made incompatible by topworking with Victoria. However, buds taken from these incompatible trees were fully compatible when budded directly on the Myrobalan B rootstock (Mosse, 1961). These transmission studies do not rule out entirely the possibility that some undetected viruses are responsible. If a virus is involved, the specific requirements for certain grafting combinations are difficult to explain.

3. Biochemical. Anatomical investigation of the graft union of incompatible stock-scion combinations has revealed several facts (Herrero, 1951; Mosse, 1955, 1962; Mosse and Scaramuzzi, 1956). First, cambial breaks occur suddenly at the end of season. The reaction appears to be correlated with the time of maximum translocation of metabolites from the top. Histological characters such as the size and amount of vessels, fibers, parenchyma cells, and the width of ray initials and distance between rays are not critical factors. The abrupt change in starch content was often noted in the adjacent tissues at the union of incompatible combinations, even if there were no apparent structural defects at the union (Mosse, 1962). These observations suggest that the causes of graft-incompatibility are biochemical rather than anatomical. Thiel (1954) agreed that the underlying causes of pear/quince incompatibility were probably biochemical in nature. Robitaille and Carlson (1970) came to the same conclusion in their study of graft union behavior of certain *Malus* and *Prunus* species.

a. Evidence. De Stigter (1956) studied graft-incompatibility using the graft combination, muskmelon (*Cucumis melo*), and malabar gourd (*Cucurbita ficifolia*). The combination of muskmelon as scion and malabar gourd as stock failed completely when the stock was defoliated. However, when stock leaves were left intact, the graft union was strong and phloem degeneration did not occur. In a further study, he double-worked the compatible combination cucumber (Cucumis sativus L.) on gourd (Cucurbita ficifolia) using different lengths of melon stem pieces as interstocks. A long intermediate stem caused incompatibility, but when the intermediate was extremely short, the combination remained healthy. The author concluded that either the muskmelon lacks specific substances for malabar gourd or the composition of metabolites was changed during transport through melon tissue. These changes were assumed to be minimized when the intermediate was very short.

Calavan et al. (1951) showed that the graft combination of lemon/sour orange required the presence of sour orange leaves to overcome incompatibility.

Mosse's work (1960a, 1955) with plum and peach suggests that graft transmissible factors or toxins interfere with normal growth. She induced incompatibility between two normally compatible clones by top working with another variety. For example, the compatible combination 'President'/Myrobalan B became incompatible when top worked with the variety 'Victoria' (Mosse, 1961). The peach variety 'Hale's Early' is compatible with the plum rootstock Brompton but incompatible with Myrobalan B. When a ring of bark of Myrobalan B is grafted on the stem of the stock of 'Hale's Early'/Brompton, symptoms of incompatibility appear on "Hale's Early' scion (Mosse, 1960b). The author suggested that some translocatable factor was responsible for incompatibility.

Relative position affects compatibility of certain graft combinations. 'Conference' pear was compatible with either Quince A or pear C8 when single grafted. When double grafted using all combinations of three clones as scion/interstock/stock, the combination Conference/Quince A/C8 and all other combinations in which any tissue of Quince A occurred above C8 grew very badly, whereas the combination Conference/C8/Quince A grew well (Mosse and Herrero, 1951). This work also suggests that some substances which cause incompatibility move in a polar manner.

Incompatibility may also be triggered by reproductive process. Jimenez (1957) showed that the graft combination *Carica goudotiana* (paw paw)/*Carica cauliflora* was normal until male inflorescences were produced, when both stock and scion declined. However, male trees grew vigorously if the inflorescences were removed. This again suggests that certain biochemical factors are involved in incompatibility.

b. Specific compounds.

Alkaloids: In studies with herbaceous plants, specific substances such as alkaloids are postulated as causal factors in graft failure. Mockaitis (1969) attributed the graft failure between *Ipomoea violacea/ Ipomoea nil* to a specific indole alkaloid compound of the scion. Mothes *et al*. (1955) considered nicotine to be the causal factor in incompatibility in grafts of *Atropa belladona/Nicotiana rustica*. The former is free of nicotine while the latter contains much nicotine. However, if *Atropa belladona* scions were grafted on the low nicotine containing stock, such as *Nicotiana glauca*, the combinations grew vigorously.

Amino acids: Tschiersch (1963) found a relationship between graft failure and the presence of a particular amino acid in some plants of the Leguminosae. *Canavalia ensiformis* contains the amino acid, canavanine. The plant cannot be grafted to *Phaseolus vulgaris L*., which is closely related but free of canavanine. The author suggested that canavanine caused the death of *P. vulgaris*.

Proteins: In explaining the origin of virus in grafting, Darlington (1944) suggested that a stable and useful protein with one plant genotype can act as a destructive agent with another. Crane (1945) proposed from the observation of apple 'Lord Lambourne' grafted on certain stocks that abnormalities in the scion may be due to the invasion of cells of one variety by the proteins of another, which behave as virus in the scion tissues. However, there has been no confirmation of these proposals. Sheldrake and Northcote (1968) noted several enzymes in xylem sap and suggested that phloem sap may contain enzymes. When these enzymes are translocated to foreign tissues (stock or scion), they may influence metabolic activity.

Cyanogenic compounds: The cyanogenic glucoside prunasin was suggested as a causal factor in incompatibility of pear/quince grafts (Gur, 1968b). According to Gur's theory, vascular discontinuity at the graft union is due to the accumulation of hydrogen cyanide, a breakdown product of prunasin, which is a natural component in quince, but absent from pear tissues.

Phenolic compounds: Phenolic compounds have been suggested as causal agents of incompatibility (Gur, 1968b; Thiel, 1954; Williams, 1953; Buchloh, 1958). A separate section is devoted to studies dealing with these compounds.

II. Role of Phenolic Compounds in Graft Incompatibility

A. Physiological functions of phenolic compounds and their seasonal variations

The physiological significance of phenolic compounds is uncertain. Some consider them to be end products of metabolic activity, serving only as cell wall materials (Bate-Smith, 1958). One definite role of phenolics is to furnish plants with diverse color. The complex coloration in flower petals and in fruits is known to aid in fertilization and seed distribution (Harborne, 1967b).

In all vascular plants, the phenolic compounds are essential for structural features such as tracheids, vessels, and fibres. In higher plants, the immediate precursors of phenolic compounds are the aromatic amino acids, phenylalanine, and tyrosine, which are in turn synthesized via the shikimic acid pathway from carbohydrates. The first products of deamination are cinnamic acid and p-coumaric acid, which then serve as precursors of lignin, flavonoids, coumarin, and other types of phenolic compounds (Koukol and Conn, 1961; Neish, 1961). The deposition of lignin in xylem tissues may be one form of cellular excretion of metabolic by-products. Reznik (1960) pointed out that higher plants, unlike microorganisms or animals, do not have an efficient system for excretion of metabolic by-products. Instead, they practice a type of 'local excretion' into the vacuoles and cell walls.

Phenolic compounds might have a protective function in plant tissues. For example, Perrin and Bottomley (1962) identified a phenolic compound 'pisatin' from pea pods, which inhibits the multiplication of an invading fungus. When cactus tissue was mechanically injured, it responded by formation of lignin, which prevents rotting of the tissue (Steelink *et al*., 1967). Feldman and Hanks (1965) reported a quantitative difference in bound phenolic compounds between citrus cultivars, one of which is susceptible to the burrowing nematode (*Radopholus* *similis*) and the other tolerant. After infection, the bound phenolics increased 27-300% in the tolerant cultivars while decreasing 16-34% in the susceptible. Koeppe *et al*. (1969) thought that phenolic compounds may be important in mediating the effects of environmental conditions. Under stress conditions, such as high ultra violet radiation (Lott, 1960; Koeppe *et al*., 1969), 2, 4, dichlorophenoxyacetic acid application (Dieterman *et al*., 1964), lack of boron (Watanabe *et al*., 1964), or low nitrogen (Harborne, 1964), the concentration of certain phenolic compounds increases.

In more recent years, evidence has been presented that the phenolic constituents play a part in dormancy of buds (Hemberg, 1961; Hendershott and Walker, 1959). The phenolic compounds appear to affect plant growth via the IAA-oxidase system (Hare, 1964; Tomaszewski, 1964; Galston, 1969).

The dynamic status of these compounds has been documented. Hillis and Swain (1959) analyzed leaves of *Prunus* *domestica* for total phenols, leucoanthocyanins and flavonols at intervals during the growing season and found that the amounts increased rapidly until the leaves reached maximum size and then decreased. In a study of seasonal levels of phenolic acids in two ferns, Glass and Bohm (1969) found a rapid increase during the early stages of growth. In apple shoots, Harvey (1925) found that phloridzin was most abundant in the apex of the shoot, where metabolic activity is high. However, the maximum concentration lagged about 2 weeks behind the maximum rate of growth.

B. Evidence for the role of phenolic compounds in incompatibility

Gur (1968b) investigated incompatibility of pear cultivars on quince rootstocks and concluded that certain substances moved from the quince rootstocks and were changed into toxic components in the pear bark. Prunasin, a cyanogenic glucoside, occurs in the quince but not in the pear. According to Gur's theory prunasin diffuses upward across the union and is hydrolyzed in the pear bark. The liberated hydrocyanic acid inhibits cambial activity and prevents vascular continuity. The liberation of hydrocyanic acid, however, is dependent on the presence of arbutin, a cofactor for β-glucosidase. The compatibility was quantitative and was inversely correlated with arbutin content of pear cultivars.
Gur (1968b) also postulated that in the bark of pear varieties the ratio of free to bound phenolic compounds was related to the degree of incompatibility with Quince A rootstock.

Buchloh (1960) believed that some factors inhibit lignification at the graft union and that these factors are responsible for vascular discontinuity. In the early stage of graft-union formation, cell walls of stock and scion are joined by a common middle lamella consisting of pectic material. The next step is the formation of the secondary cell wall consisting of cellulose and hemicellulose. In further development, the pectic substances of the middle lamella disappear and are gradually replaced by lignin. However, in the incompatible graft-combination, after the pectic material decomposes, the middle lamella is not replaced with lignin. Buchloh also noted that adjoining cells, and sometimes cell walls, were discolored in both compatible and incompatible unions. The pigment disappears later in compatible unions, but in incompatible unions, the pigment remains, forming a dark brown precipitate. The pigment appeared to be hydrolysed phenolic compounds which subsequently were polymerized to brown precipitates. In the course of development of the graft union, lignification was markedly inhibited when the brown precipitate formed. Buchloh, therefore, concluded that lignification was inhibited either by reactions which give rise to the formation of the brown colored compounds or by the compounds themselves. Thiel (1954) suggested also that the discoloration of tissues and death of cells of incompatible pear/quince graft unions were toxic effects of oxidized phenolic compounds liberated from arbutin.

C. Evidence for translocation

In order for phenolic compounds to be effective in inducing incompatibility, they must reach the tissues of the graft partner. Demonstration of graft transmission is therefore a prerequisite for their implication as causal agents, unless one assumes that cell-to-cell contact is sufficient. The evidence for graft transmission varies, depending upon the species under study.

Friedrich (1958) found no evidence for translocation of polyphenols in reciprocal grafts of apple and pear. Hillis and Swain (1959) could find no direct relationship between phenolics in leaves and those in woody tissues of *Prunus domestica*. The authors suggested that translocation of phenolic compounds between the tissues was unlikely. In *Eucalyptus* wood, polyphenolic compounds were not translocated from one tissue to another but were formed *in situ* from carbohydrates (Hillis and Carle, 1960, 1963). Bate-Smith (1962) believed that polyphenolic constituents were formed from simple precursors in the particular cells in which they occurred. In the examination of sieve tube exudate, he could find no trace of polyphenolic compounds (leucoanthocyanins and flavonols), although these were abundant in leaf tissues.

There are, however, several reports suggesting the possibility of translocation. For example, Hergert and Goldschmid (1958) suggested that quercetine and taxifolin were synthesized and glucosylated in the leaves, then transported down the phloem and laterally to the bark and heartwood. Many of polyphenolics found in oak bark originate in the leaves, according to Hathaway (1959). The author suggested that compounds such as gallocatechin and leucodelphinidin were translocated by the sieve tube system to the cambium and then condensed to tannins which were stored in the bark. In the study of phenolics in the solanaceae, coumarins appeared to be formed in the root from tyrosine and translocated into the shoot (Kala, 1956). Gorz and Haskins (1962) noted movement of coumarins across the graft union in clover, while MacLeod and Pridham (1965) demonstrated the translocation of phenolic compounds which were introduced into the apical leaves of _Vicia_ _faba_. The general translocation rates for phenolics approximated those for C^{14} assimilates. The glycosidic derivatives migrated more rapidly than the phenolic aglycones. The authors also showed the presence of phenolic compounds in aphid stylet exudates from the sieve tubes of _Salix_ and _Vicia_. Favre-Bonvin _et al_. (1966) studied the effect of grafting on the distribution of coumarin in cherry. Chemical analysis of the interspecific graft combination _Prunus_ _mahaleb/P_. _avium_ showed that the normally coumarin-free P. avium roots accumulated coumarin and herniarin. Further study with the phenolic precursors, DL-phenylalanine-C^{14} by these authors (1968) showed that coumarin compounds moved readily between the graft partners.

D. Possible mechanism of action in incompatibility

Phenolic compounds may cause incompatibilities either directly as phytotoxins, or indirectly through their effects upon IAA oxidase.

Phenolic compounds as phytotoxins: Many phenolic compounds are phytotoxic (Pridham, 1960). The inhibitory activity of the compounds has been recognized in germination (Evenari, 1949; Van Sumere, 1960; Varga and Köves, 1959; Börner, 1960; Hemberg, 1961), in bud dormancy (Hendershott and Walker, 1959; Lane and Bailey, 1964), in potato tuber dormancy (Hemberg, 1958; Housley and Taylor, 1958), in shoot growth (Thimann and Bonner, 1959; Hancock et al., 1961; Tomaszewski and Thimann, 1966; Galston, 1969), and in root growth (Goodwin and Taves, 1950). Many components of the β-inhibitor complex have been identified as phenolic compounds and hypotheses have been advanced as to their regulatory roles (Hemberg, 1961; Lane and Bailey, 1964; Moreland et al., 1966). Plants are known to excrete chemical inhibitors which control the growth of other plants (allelopathy). Some examples are: Artemisia *absinthium* (Bode, 1940), *Eucalyptus rostrata* (Evenari, 1961), black walnut (Bode, 1958), and Johnson grass (Abdul-Wahab *et al*., 1967). Some of these inhibitors have been identified as phenolic compounds (Rice, 1965; Abdul-Wahab *et al*., 1967; Köves and Varga, 1958; Börner, 1957; Bautz, 1953; Grümmer and Beyer, 1960).

These compounds, in the free state, have both lipophilic and hydrophilic properties, and Frey-Wyssling (1942) suggested that they could interfere with the function of cell vacuoles and tonoplast membranes. A few of these compounds are reported to interfere with oxidative phosphorylation (Millerd *et al*., 1953; Stenlid, 1963) and with mitosis (Steinegger and Leupi, 1955; Cornman, 1957). Others may inhibit enzyme activity. In the study of the inhibitory action of coumarins on plant growth, Thimann and Bonner (1959) concluded that inhibition of growth involved their reacting with enzyme sulfhydryl groups. Mayer and Poljakoff-Mayber (1961) listed several enzymes whose activities were inhibited by coumarin. In germinating lettuce seeds and seedlings, this compound inhibited the activity of proteinase, lipase, and phytase.

Plant tissues, then, need an efficient system to detoxify the phenolic compounds. Pridham (1960) reported that *Vicia faba* seeds germinated and plants developed to maturity in the presence of relatively large quantities of arbutin. However, the corresponding aglycone, quinol, inhibited germination. Van Sumere (1960) showed that ferulic acid-β-glucoside had no effect on the germination of wheat rust uredospores whereas free ferulic acid was strongly inhibitory. The glycosylation of applied phenolics has been reported in barley and wheat leaves (Nystrom *et al*., 1959), in tobacco leaves (Runeckles et al., 1963), leaves of Solanum species, *Cestrum newellii*, *Raphanue sativus*, *Clematic lawsoniana*, *Lycopersicum esculentum*, and in *Datura knightii* (Harborne and Corner, 1961). Toweres *et al*. (1958) have shown that maleic hydrazide, a compound with phenolic properties, is converted to a glucoside by plant tissues, and they suggest that this is the reason why relatively high concentrations of the reagent are needed for effective growth inhibition. Seedlings of the interspecific hybrid *Lilium aruatum* X *Lilium speciosum* are tumorous and die at an early stage of development. Asen and Emsweller (1962) found that 60% of total ferulic acid in the seed was present as the aglycone. However, in seedlings from intraspecific crosses, which develop normally, only 6% of the ferulic acid was present as the free acid, the remainder being present as the glucose ester. Thus, if a stock or a scion of a certain graft combination does not have an efficient system of detoxification, any condition which allows the accumulation of the phenolic compounds in the tissue may restrict the normal growth and development of the graft.

Phenolic compounds as cofactors or inhibitors of IAA oxidase: Phenolic compounds may affect the growth of stock or scion via the indole-3-acetic acid (IAA) oxidase system. IAA oxidase occurs widely in nature, and is believed to regulate the level of IAA by oxidizing it to inactive products (Hare, 1964). Phenolic compounds either inhibit or synergize with the enzyme depending on their structures. Monohydric phenols act as cofactors for the IAA-oxidase system, while dihydric phenols inhibit oxidation (Stenlid, 1963; Hare, 1964; Tomaszewski and Thimann, 1966). All 4' hydroxyflavonoids were cofactors for the oxidation of IAA, whereas 3', 4' dihydroxyflavonoids inhibited the destruction of IAA (Mumford et al., 1961; Stenlid, 1963). Work by Russell et al. (1969) suggested that phytochrome may control growth of pea seedlings by regulating the synthesis of phenolic compounds which act as cofactors in an IAA oxidase system. In pea seedlings Furuya and Galston (1965) identified kaempferol-3-glucoside and its p-coumaric ester as promoters, and quercetin-3-glucoside and its p-coumaric ester as inhibitors of IAA oxidase. In addition to flavonoids, other types of phenolic compounds have been shown to interact with IAA oxidase. Compounds which promote the enzyme are p-coumaric acid and p-hydroxy benzoic acid (Engelsma, 1964; Tomaszewski and Thimann, 1966; Lee and Skoog, 1965). The inhibitors of IAA oxidase are chlorogenic acid, caffeic acid, ferulic acid, sinapic acid, scopoletin, scopolin, and 3, 4 dihydroxy benzoic acid (Gortner and Kent, 1958; Henderson and Nitsch, 1962; Tomaszewski and Thimann, 1966; Sacher, 1963; Schaeffer, 1967; Varga and Köves, 1962; Gelinas and Postlethwait, 1969; Sequeira, 1964; and Lee and Skoog, 1965).

There is strong evidence that cambial activity and the differentiation of secondary xylem and phloem are controlled by metabolites and growth hormones, especially endogenous auxin, from the developing buds and leaves (Wareing et al., 1964; Larson, 1964; Evert and Kozlowski, 1967; Torrey and Loomis, 1967; Wilson, 1968; Reinders-Gouwentak, 1965). Thus, the radial growth of the stocks of certain graft combinations may be dependent upon the supply of auxins and metabolites from the scions. Phenolic compounds may play a role in incompatibility by regulating the auxin supply to the rootstock via the activation or inhibition of IAA oxidase (Gur and Samish, 1968a).

III. Phenolic Compounds Identified in Cherry Tissues

The fact that plant species contain specific phenolic constituents allows taxonomists and biochemists to detect relationships between plants and groups of plants (Bate-Smith, 1961a). Much research has been done on phenolic compounds in woody tissues of cherry.

Hergert (1962) has reviewed the economic importance of flavonoid compounds occurring in the genus *Prunus*. Flavonoid compounds in cherry wood have been studied in some detail by chemists in France and Japan. In the woody tissues of *P. yedonensis*, Hasagawa *et al*. (1952) identified 4 flavonoid compounds: prunin, genkwanin, naringenin, and d-catechin. They (1954, 1957) also studied tissues of several other *Prunus* species. In the heartwood of *P. avium* 11 flavonoid compounds were identified by elementary analysis, boiling point, and chromatographic technique. These were d-catechin, naringenin, prunin, aromadendrin, eriodictyol, taxifolin, chrysin, aequinocitin, genistein, prunetin, and genistin.

Mentzer *et al*. (1954) found 12 flavonoid compounds while examining ether extracts of wild cherry heartwood. These compounds were identified as chrysin, dihydrochrysin, sakuranetin, tectochrysin, and dihydrotectochrysin. Chopin *et al*. (1957) identified a flavanone from heartwood extract as dihydrowogonin. The glucoside of dihydrowogonin was also detected in pedicels of *P. cerasus* (Wagner *et al*., 1969).

Two water soluble compounds from the heartwood of cherry trees were identified by Pacheco *et al*. (1957) as d-catechin and aromadendrin. These compounds were found to accumulate in the heartwood as the tree increased in age. Chopin and Pacheco (1958) isolated the flavanonol 7-methylaromadendrin from an ether extract of the heartwood of *P. avium*.

In the woody tissue of *P. mahaleb*, Pacheco (1959) identified four flavonoid compounds: naringenin, aromadendrin, genistein, and prunetin. A year later the same author reported that he found a glucoside of aromadendrin in Mahaleb heartwood. Pacheco *et al.* (1960) reported on more flavonoid compounds present in the woody tissue of *P. mahaleb*. These were: a flavanone glucoside prunin, glucosides of aromadendrin, and taxifolin.

Geissman (1956) studied the phenolic composition of normal and virus-infected cherry leaves. Using paper chromatographic methods, he found that the leaf tissue contained glucosides of kaempferol and quercetin, and esters of p-coumaric acid and caffeic acid.

In discussing the taxononomic significance of phenolic compounds in the Rosaceae, Bate-Smith (1961b) noted certain common features in the distribution of phenolic compounds among genera and subgenera. For example, in the genus *Prunus* leucoanthocyanidins, flavonols, and hydroxycinnamic acids were found. The sub-genus '*cerasus*' contained a 'cherry factor' which was identified as o-coumaric acid. Mahaleb leaves were shown to have the specific 'mahaleb factor,' herniarin (7 methoxy coumarin).

Favre-Bonvin et al. (1966, 1968) studied the site of synthesis of coumarins by interspecific grafting between Mazzard and Mahaleb. Using the radioactive precursor phenylalanin-3-C^{14}, they demonstrated that leaves, fruits, and root-wood synthesized both coumarin and herniarin. The stem wood could also synthesize herniarin. For convenience certain of these findings are summarized in Table 1.

Table 1. Phenolic compounds occurring in cherry trees.

Common Name	Chemical Structure	P. avium			P. mahaleb			References
		Leaf	Bark	Wood	Leaf	Bark	Wood	
Flavonoids								
Catechin	d-catechin			+				Hasagawa (1957), Pacheco (1957)
Pinocembrin (Dihydrochrysin)	5,7 dihydroxy flavanone			+				Mentzer (1954)
Pinostrobin (Dihydrotectochrysin)	5 hydroxy 7 methoxy flavanone			+				Mentzer (1954)
Naringenin	4',5,7 trihydroxy flavanone			$+^{1}$			$+^{2}$	1. Hasagawa (1957) 2. Pacheco (1959)
Prunin	Naringenin 7 glucoside			$+^{1}$			$+^{2}$	1. Hasagawa (1957) 2. Pacheco (1959)
Sakuranetin	4',5 dihydroxy 7 methoxy flavanone			+				Mentzer (1954)
Dihydrowogonin	5,7 dihydroxy 8 methoxy flavanone			+				Chopin (1957) Wagner (1969)
Eriodictyol	3',4',5,7 tetrahydroxy flavanone			+				Hasagawa (1957)
Aromadendrin	4',5,7 trihydroxy flavanonol		$+^{1}$	$+^{2}$			$+^{3}$	1. 2. Pacheco (1957) Hasagawa (1957) 3. Pacheco (1959)
Pinobanskin	5,7 dihydroxy flavanonol		+					
Taxifolin (Dihydroquercetin)	3',4',5,7 tetrahydroxy flavanonol		$+^{1}$	$+^{2}$			$+^{3}$	1. 2. Hasagawa (1957) 3. Pacheco (1960)
Chrysin	5,7 dihydroxy flavone			+				Hasagawa (1957) Mentzer (1954)
Tectochrysin	5 hydroxy 7 methoxy flavone			+				Mentzer (1954)
Genkwanin	4',5 dihydroxy 7 methoxy flavone							
Genistein	4',5,7 trihydroxy isoflavone			+			+	1. Hasagawa (1957) 2. Pacheco (1960)
Genistin	Genistein 7 glucoside			+				Hasagawa (1957)
Prunetin	4',5 dihydroxy 7 methoxy isoflavone			+			+	1. Hasagawa (1957) 2. Pacheco (1960)
Kaempferol	4',5,7 trihydroxy flavonol	$+^{1,2}$			$+^{1}$			1. Bate-Smith (1961) 2. Geissman (1956)
Quercetin	3',4',5,7 tetrahydroxy flavonol	$+^{1,2}$			$+^{1}$			1. Bate-Smith (1961) 2. Geissman (1956)
Cyanidin	3,5,7,3',4' pentahydroxy flavylium	$+^{1}$						1. Bate-Smith (1961)
Coumarins								
Coumarin					$+^{1,2}$	$+^{1}$		1. Favre Bonvin (1966) 2. Bate-Smith (1961)
Herniarin	7 methoxy coumarin				$+^{1,2}$	$+^{1}$		1. Favre Bonvin (1966) 2. Bate-Smith (1961)
Cinnamic acids								
o-Coumaric acid	2 hydroxy cinnamic acid	+			+			Bate-Smith (1961)
p-Coumaric acid	4 hydroxy cinnamic acid	$+^{1,2}$			?			1. Bate-Smith (1961) 2. Geissman (1956)
caffeic acid	3,4 dihydroxy cinnamic acid	$+^{1,2}$						1. Bate-Smith (1961) 2. Geissman (1956)

MATERIALS AND METHODS

I. Seasonal Variations of Total Phenolic Compounds

A. Plant material

One-year-old Mahaleb and Mazzard seedlings were planted in the field in May, 1969. Uniform nursery-grown seedlings were obtained from Hilltop Nursery, Hartford, Michigan. At two-week intervals three trees of each species were harvested and fresh weights of leaves, roots, and stems recorded. Leaves, stem bark, and root bark were put immediately in the oven and dried overnight at 85°C. The dried tissue was ground to pass through a number 4 mesh screen and stored in closed bottles until extraction.

B. Extraction

One gram of ground tissue was shaken for 3 hours with 50 ml 80% aqueous methanol. The solvent was then decanted and the same procedure repeated twice for a total of 150 ml solvent. The extract was filtered through Whatman No. 1 filter paper and made up to final volume of 200 ml.

C. Quantitative determination

The method used for measurement of total phenolic compounds (Swain and Hillis, 1959) involves the reduction of compounds containing sexivalent tungsten and molybdenum to colored products. A 0.1 ml aliquot (= 500 ug tissue) of the sample solution was diluted with 6.9 ml distilled water. After mixing, 0.5 ml of 0.25 N Folin-Ciocalteau reagent (Bray and Thorpe, 1954) was added. The samples were shaken for 3 minutes and then 1.0 ml of 1N Na_2CO_3 solution was added. The solution was again thoroughly mixed, made up to 10 ml with distilled water, and stored one hour in the dark before measurement of absorbance in a Klett-Summerson photoelectric colorimeter, using a No. 66 red filter (725 mu). For a standard curve, ferulic acid was used.

II. Identification of Phenolic Compounds in Methanol Extracts, and Determination of Relative Amounts

A. Plant material

Uniform Mazzard and Mahaleb seedlings were planted in the greenhouse May 30, 1969, and grown until October 30, when they were placed at 35°F to satisfy the chilling requirement. At the end of January, 1970, the seedlings were again planted in the greenhouse and grown until May. They were then removed from the pots, washed thoroughly with tap water, and separated into leaves, stems, and roots. Leaves were harvested with petioles attached. Bark tissues were obtained by cutting the stems and roots into small segments and peeling the bark from the wood. The wood was discarded. Both leaves and bark were weighed and frozen in liquid nitrogen. Special care was taken to discard any dead tissues such as the epidermal layer. The samples were kept frozen until used.

B. Extraction

The weighed samples (Table 2) were macerated in 400 ml absolute methanol, and the macerate was filtered through Whatman No. 1 paper. Each sample residue was extracted by shaking for 16 hours successively with five 200 ml portions of methanol on an oscillating shaker. The combined methanol extracts (1400 ml) were filtered through celite and stored in brown bottles (34°F). The detailed extraction procedure is shown in Figure 1.

Table 2. Weights of tissues used for extraction of phenolic compounds.

Species	Tissue	Fresh weight (g)
Mazzard	leaf	100
	stem bark	77
	root bark	35
Mahaleb	leaf	100
	stem bark	100
	root bark	77

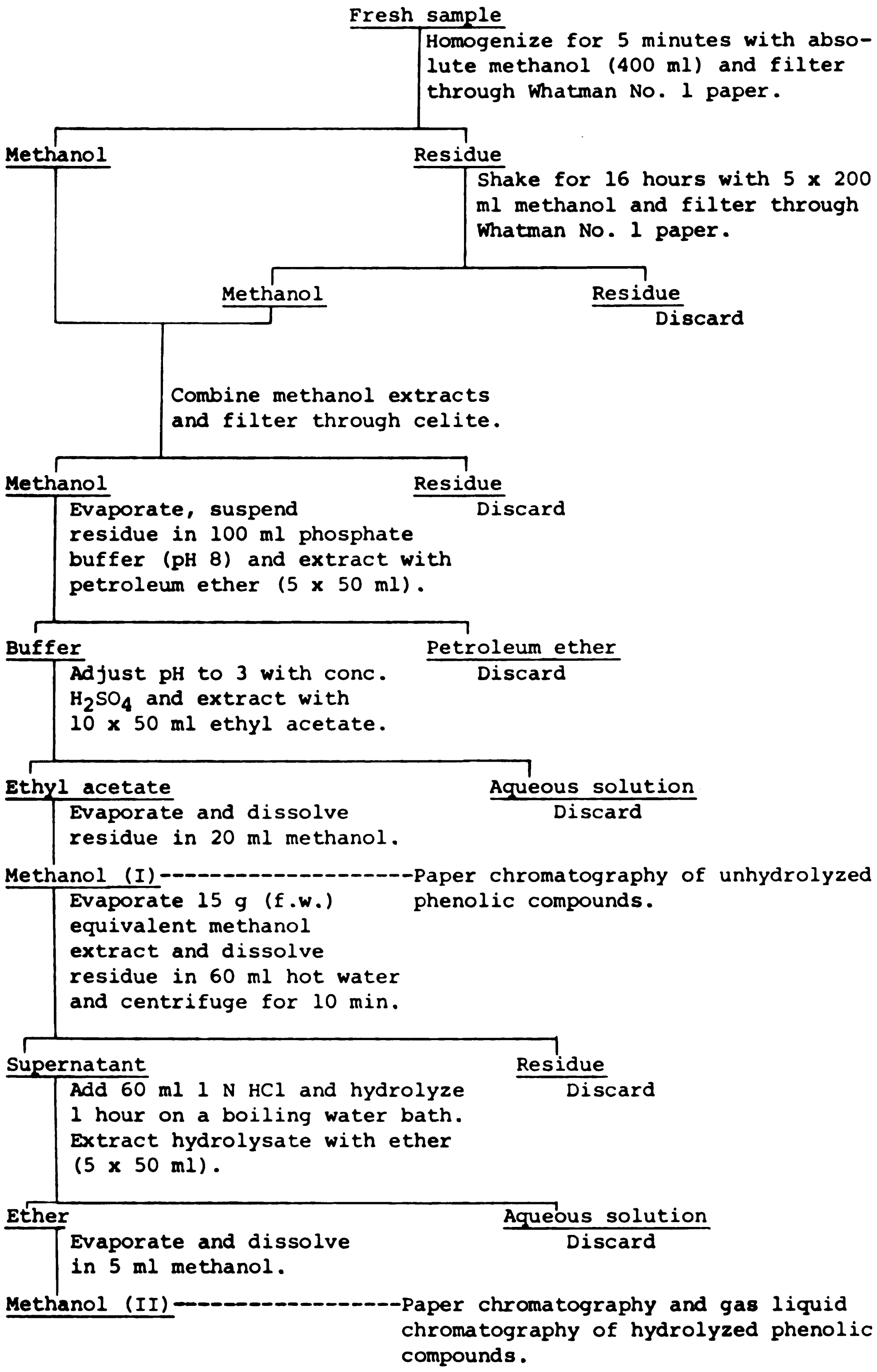


Figure 1. Procedure for extraction and purification of phenolic compounds.

C. Hydrolysis

Aliquots of the crude methanol extract representing 15 g (f.w.) of tissue were evaporated under vacuum, and the residues were dissolved in 60 ml hot water. The water was centrifuged to remove the chlorophyll precipitate, and the supernatant was extracted with chloroform (5 x 20 ml) to eliminate carotenoids, chlorophyll, and lipids. The water phase was transferred to a 300 ml volumetric flask, and 60 ml of 1 N HCl was added. The solution was then heated for one hour on a boiling water bath. After cooling to room temperature the aqueous solution was extracted with ether (5 x 50 ml). The ether extract was evaporated and the residue dissolved in 5 ml methanol (Fig. 1). This methanolic solution was used directly for either paper chromatography or for gas liquid chromatography.

D. Purification and identification

1. Paper chromatography. Twenty to fifty µl (60 to 150 mg fresh weight of tissue) of extract was spotted on the upper left-hand corner of Whatman No. 1 papers (46 x 57 cm) for chromatography. The diameter of the spots (less than 1 cm) was minimized by using a 5 µl pipette and a stream of air. The first solvent was n-butanol: acetic acid: water, 6:1:2 (BAW) which irrigated the long direction of the paper for 17 to 18 hours at room

temperature. The air-dried paper was then run for 3.5 to 4 hours in 2% acetic acid (HOAc) in the short direction.

The air-dried chromatograms were examined under ultraviolet light before and after exposure to ammonia vapor. Replicate chromatograms were then treated with 4 different reagents (Table 3). One chromatogram was dipped in a freshly prepared solution of equal volumes of 0.5% $FeCl_3$ and 0.5% $K_3Fe(CN)_6$, then rinsed with 2% HCl solution followed by distilled water. The phenolic compounds appeared as blue spots on the chromatogram. A second chromatogram was sprayed with diazotized p-nitroaniline in 2 N HCl, 5% $NaNO_2$, and 20% sodium acetate (w/v) in a ratio of 1:10:30. A third chromatogram was sprayed with freshly prepared Hoepfner's reagent (5% $NaNO_2$ plus 5% acetic acid). With this reagent phenolic acids give characteristic colors. A fourth chromatogram was sprayed with 2 N NaOH, and after 3 to 5 minutes it was viewed under ultraviolet light. Coumarins gave a characteristic greenish yellow fluorescence. The chromatogram was then sprayed with DPNA, which reacts with coumarins to form distinctively colored products.

In order to prepare enough material for further characterization, the crude methanol extract was applied as a line on Whatman No. 3 paper. Chromatograms were developed with 2% acetic acid for 3.5 hours, then air-dried and examined under ultraviolet radiation. The

Table 3. Reagents used for phenolic compounds.

Reagents	Composition	Specificity	Reference
$FeCl_3$-$K_3Fe(CN)_6$	0.5% Fe Cl_3 and 0.5% $K_3Fe(CN)_6$(1:1,v/v)	All phenolic compounds	Keppler (1957)
Diazotized p-nitroaniline (DPNA)	0.5% p-nitroaniline 5% $NaNO_2$ 20% sodium acetate (1:10:30, v/v)	Most phenolic compounds; characteristic colors with phenolic acids	Swain (1953)
Vanillin-HCl	1% vanillin in ethanol, and conc. HCl (1:1, v/v)	Leucoanthocyanidins Catechins	Swain <u>et al.</u> (1959)
Tetrazotized benzidine	0.5% benzidine in dil. HCl, and 10% $NaNO_2$ (1:1, v/v)	Phloroglucinol-resorcinol type compounds	Smith (1960)
$NaBH_4$-HCl	2% $NaBH_4$ in methanol Fume chromatogram with HCl vapor	Flavanones	Horowitz (1957)
Hoepfner	5% $NaNO_2$ and 5% acetic acid (1:1, v/v)	Phenolic acids	Walker (1962)
Folin-Ciocalteau	10 g sodium tungstate and 2.5 g sodium molybdate in 70 ml. Add 5 ml phosphoric acid (85%) and 10 ml conc. HCl. Boil and add 15 g lithium sulphate and 1 drop of bromine. Made up to 100 ml with water (stock solution). 0.25 N Folin-Ciocalteau, then 1 N Na_2CO_3	All phenolic compounds	Swain <u>et al.</u> (1959) Waldi (1965)
2 N NaOH-DPNA	2 N NaOH, then DPNA	Coumarins	Swain (1953)

distinctive color bands were marked with a pencil. A few more bands were detected when the paper was sprayed with $FeCl_3$-$K_3Fe(CN)_6$ and DPNA reagents.

Zones from identical unsprayed chromatograms were cut out and eluted by shaking with 3 successive 100 ml portions of 95% ethanol. The eluates were filtered and evaporated in a rotary film evaporator under vacuum. Each fraction was rechromatographed on Whatman No. 1 paper in BAW (first direction) followed by 2% acetic acid (second direction). Bands which fluoresced under ultraviolet light, or which reacted with DPNA and $FeCl_3$-$K_3Fe(CN)_6$ on parallel chromatograms, were cut from the chromatograms, and eluted with ethanol (3 x 10 ml). The eluates were filtered through Whatman No. 1 paper and evaporated under vacuum. The residues were dissolved in 0.5 ml ethanol, spotted on Whatman No. 1 paper, and chromatographed one dimensionally in the following solvent systems:

butanol: acetic acid: water, 6:1:2 (BAW)
2% acetic acid (HOAc)
butanol: pyridine: water, 10:3:3 (BPW).

2. Thin layer chromatography. Aliquots (5 µl) of the partially purified compounds were spotted on Eastman thin layer plates (silica gel G) and run in one direction in the following solvent systems:

benzene: methanol: acetic acid, 45:8:4 (BMA),

11% methanol in $CHCl_3$,

toluene: ethyl acetate: formic acid, 5:4:1 (TEF).

The chromatographic chamber was lined with filter paper. When the solvent reached 10 cm above the origin, the plates were removed and air-dried. They were then viewed under ultraviolet and visible light before and after the application of phenolic reagents.

3. Gas liquid chromatography. Samples were gas chromatographed on a 6 foot by 2 mm column of 2% QF-1 on Chromosorb W using a Packard gas chromatograph Series 7300 equipped with a hydrogen flame ionization detector. Column temperature was 160°C and those of detector and injection block were 250°C. Nitrogen was used as a carrier at a flow rate of 40 ml per minute.

Methylation was based on the method of Schlenk and Gellerman (1960). The methylation apparatus consisted of three test tubes with side arms. A stream of nitrogen gas was saturated with ether in the first tube and this carried the CH_2N_2 generated in the second tube into the sample tube where esterification took place. The components in the three tubes were as follows:

Tube 1: ether (20 ml),

Tube 2: 0.7 ml carbitol (diethylene glycol), 0.7 ml ether, 1.0 ml 60% KOH,

Tube 3: sample (equivalent of 3 g fresh weight) in 1 ml ethanol, 3.0 ml ether, 0.3 ml methanol.

The reaction was begun by adding a small quantity of N methyl-N-nitroso p-toluene sulfonamide (Diazald) in 0.5 ml ether to tube number 2. The tubes were stoppered and the reaction was allowed to proceed for 3 to 10 minutes until a yellow tinge appeared in tube number 3. After evaporating the solvent, the methylated sample was dissolved in 0.5 ml acetone and 0.6 μl aliquots were injected into the gas chromatograph.

Flavonoid compounds were silylated by adding 50 μl Tri-Sil (Pierce Chemical Co., a premixed trimethylsilylation reagent containing hexamethyldisilazane and trimethylchlorosilane in pyridine) to a 15 ml conical centrifuge tube which contained 1 mg of the standard compound. After 15 minutes at room temperature, this solution was injected directly into the column.

4. Ultraviolet absorption spectra. Absorption spectra of the purified compounds in 95% ethanol were obtained with a Beckman DB spectrophotometer.

5. Sources of reference compounds. The sources of the reference compounds used are given in Table 4.

E. Quantitative determination

In most cases, the compounds were so close to each other on paper chromatograms that it was difficult to elute and quantify them by absorption spectroscopy. The coumarin derivatives did not give responses similar to

Table 4. Sources of reference compounds.

Source	Compounds
S. A. Brown Department of Chemistry Trent University Peterborough, Ontario	Umbelliferone Aesculetin Herniarin
A. Grouiller Institut National des Sciences Appliques de Lyon	Taxifolin Aromadendrin 7 Methyl aromadendrin
J. Favre-Bonvin Museum National d'Histoire Naturelle, Paris	Dihydrowogonin Prunetin Pinocembrin Genistein
H. Erdtman Kungl. Tekniska Högskolan Stockholm, Sweden	Chrysin Tectochrysin
Sigma Chemical Company St. Louis, Missouri	Naringenin Naringin Salicin Chlorogenic acid Rutin Scopoletin Kaempferol Hesperetin
Eastman Organic Chemicals Rochester, N.Y.	o-Coumaric acid Ferulic acid Quercetin
Aldrich Chemical Company	Apigenin Caffeic acid p-Coumaric acid
R. F. Carlson Department of Horticulture	Coumarin

other phenolic compounds when the eluates were treated with common Folin-Ciocalteau reagent or with DPNA, which also complicated the measurement.

Therefore, visual estimation of the relative amounts of phenolic compounds was made. The scale was based on the area and intensity of the individual spots on a two-dimensional paper chromatogram after it was sprayed with $FeCl_3$-$K_3Fe(CN)_6$ or with 2 N NaOH plus DPNA. A spot of o-coumaric acid of Mazzard leaf extract was arbitrarily assigned a value of 5, and other spots were rated 1 to 12 in relation to the size of the o-coumaric acid spots.

III. Identification and Relative Amounts of Phenolic Compounds in Centrifugal Sap

A. Plant material

Uniform one-year-old seedlings grown in the greenhouse for 5 months were used. In the case of Mazzard, 128 gram of fresh weight was used and for Mahaleb, 110 grams.

B. Extraction

The sap was extracted as follows (Goldschmidt and Monselise, 1968). Stems were cut into 5 cm segments and placed in centrifuge tubes with bases down. Twenty per cent methanol was added to cover the basal ends of the stems, and the tubes were centrifuged at 1,500 x g for

20 minutes. The methanol was evaporated and the remaining aqueous solution diluted to 20 ml with water (Fig. 2).

C. Fractionation of extract

The aqueous solution from (B) above was extracted first with ether (3 x 10 ml) and then with ethyl acetate (4 x 10 ml). The remaining aqueous solution was acidified to pH 2 and extracted again with ethyl acetate (4 x 10 ml). Each fraction was evaporated and the residue dissolved in 2 ml methanol. These methanol fractions were used for paper and for gas liquid chromatography as described in Section II above.

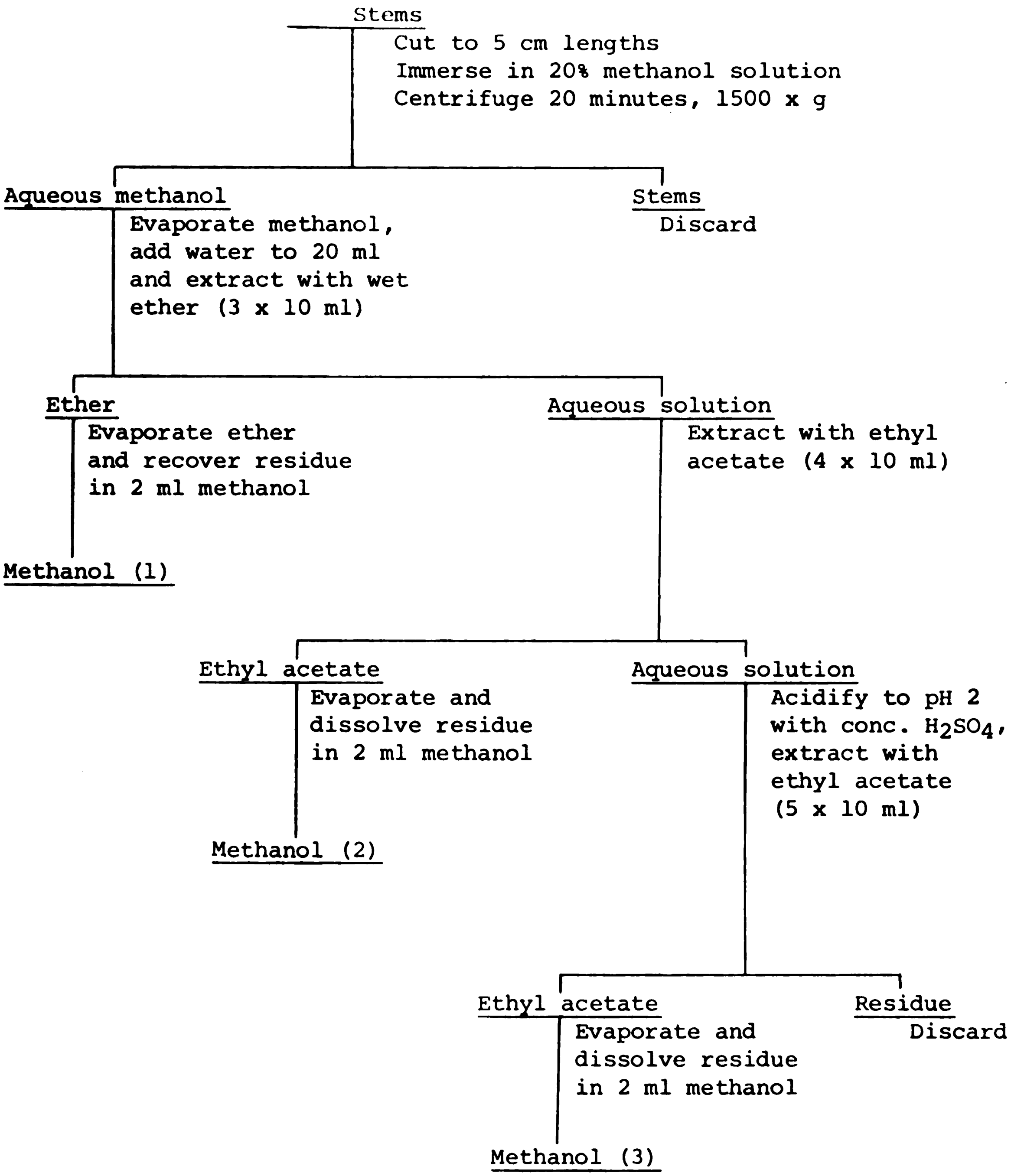


Figure 2. Procedure for extraction and purification of centrifugal sap.

RESULTS AND DISCUSSION

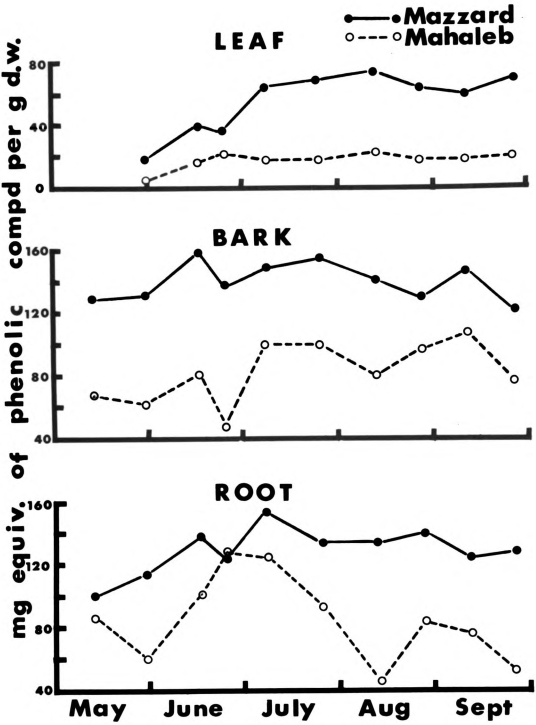
I. Seasonal Variations of Total Phenolic Compounds in Field-Grown Seedlings

Mazzard tissues were found to contain higher levels of phenolic compounds than Mahaleb tissues (Fig. 3). Average amounts for all sampling dates for leaf, stem bark, and root bark of Mazzard were 55, 141, 129 mg equivalents of ferulic acid per gram dry weight respectively, compared with 17, 82, and 86 mg for Mahaleb.

Leaves. Among the tree tissues sampled, the leaf tissue contained the least amount of methanol extractable phenolic compounds. One week after bud break, the small leaves contained low amounts of phenolic compounds. As the leaves grew, the phenolic compounds increased. In Mahaleb the phenolic level increased slowly until the latter part of June, then remained constant to the end of September. In Mazzard the level of phenolic compounds increased at a more rapid rate until early July and then stabilized (Fig. 3).

Stem bark. Mazzard contained 1.5 to 2 times more phenolics in the bark than Mahaleb. As in the leaf tissue, Phenolics increased slightly to the first part of June, then remained relatively stable after July 1.

Figure 3. Seasonal variations of total phenolic compounds in field-grown cherry seedlings. Average values of phenolics in leaf, bark, and root were significantly different at 1% level between two rootstocks.


Mazzard
Mahaleb
LEAF
BARK
ROOT
mg equiv. of phenolic compd per g d.w.
80
40
0
160
120
80
40
160
120
80
40
May
June
July
Aug
Sept

Root bark. Phenolic compounds increased in bark of Mazzard root until late June and then leveled off. Bark of Mahaleb root showed the greatest variation in phenolics of the tissues tested. Two peaks in concentration were found, one in the latter part of June and the other at the end of August. During the first two weeks after planting, the level of phenolics dropped from 86 to 60 mg, then increased rapidly until June 24. The concentration declined in mid-July, reaching a low of 46 mg per gram of tissue in mid-August. A smaller rise was noted in late August through early September.

Seasonal variations of phenolic compounds have been studied in plum (Hillis and Swain, 1959), oak (Feeney, 1968), and fern (Gloss and Bohm, 1969). Phenolics increased rapidly during the early part of the growing season, then declined after the leaf attained its maximum size. The bark and woody tissues in plum showed less variation than the leaf during the growing season.

Transport of some phenolic compounds has been reported (Gorz and Haskins, 1962; Favre-Bonvin, 1968; Macleod and Pridham, 1965). However, it is unlikely that all the phenolic compounds in root or bark were translocated from the leaf tissues, because these tissues contained a high level of phenolic compounds before bud break. In higher plants, the immediate precursors of phenolic compounds are the aromatic amino acids phenylalanine and tyrosine, which are in turn synthesized via the shikimic acid pathway from carbohydrates (Brown, 1964). In Mahaleb the supply of precursors in the top may affect the concentrations of phenolic compounds in the root tissues. However, in Mazzard, the root tissue may be able to mobilize local storage metabolites for synthesis of phenolics. The sudden drop of phenolics in Mahaleb root in August is difficult to explain. The phenolic compounds may have been transported to other tissues or changed into methanol insoluble forms.

The patterns of lignification could differ in the two species. In Mahaleb active lignification may lag behind growth but be coincident with it in Mazzard.

II. Identification of Phenolic Compounds in Methanol Extracts of Greenhouse-Grown Cherry Seedlings, and Determination of Relative Amounts

A. Total phenolic compounds

The total methanol extractable phenolic compounds in fresh leaf, stem bark, and root bark tissues of Mazzard and Mahaleb varied from 200 μg equivalents of ferulic acid per gram fresh weight in Mahaleb leaf to 5667 μg in Mazzard root bark (Table 5). This was based on one sampling in May following 4 months of growth in the greenhouse. Mazzard tissues consistently contained more phenolics than Mahaleb tissues, in agreement with the previous experiment.

Table 5. Total phenolic compounds in Mazzard and Mahaleb tissues from greenhouse-grown seedlings.[a]

Species	Tissues	Total phenolic compounds (μg ferulic acid equivalents/g fresh weight)
Mazzard	Leaf	583
	Stem bark	5175
	Root bark	5667
Mahaleb	Leaf	200
	Stem bark	2517
	Root bark	2833

[a] Average of 3 determinations based on ferulic acid standard curve.

However, the absolute values were approximately 10% of those obtained in the field study on a dry weight basis.

The difference may be due to differences in sample preparation or in growing conditions. The drying of samples in the first study may have released more 'bound' phenolic compounds. Phenolic compounds in the living tissues usually occur as glucosides, esters, or other bound forms (Harborne, 1964; El-Basyouni et al., 1966). The free phenolic compounds can be released by hydrolysis. Beck (1964), studying isoflavones in clover leaves, found extremely low levels of isoflavones when the tissue was extracted immediately with boiling ethanol. If the crushed leaves were allowed to stand for less than one minute before extraction, high levels of isoflavones were obtained.

Environmental factors might also account for the discrepancy. Light intensity, light quality, and nutritional status are known to affect the synthesis of phenolic compounds in intact plants and tissue slices (Siegelman, 1964). Lott (1960) reported that field-grown tobacco plants contained more phenolic compounds than greenhouse-grown plants. He attributed the differences to the high intensity of ultraviolet light in the field. Ultraviolet light increases the levels of phenolic compounds in tabacco and sunflower (Koeppe et al., 1969). Thus, the high levels of phenolic compounds in field-grown cherry seedlings may be due to higher ultraviolet irradiation in the field.

B. Paper chromatography of hydrolysed leaf extracts

Paper chromatograms of hydrolysed extracts of Mazzard and Mahaleb leaves are illustrated in Figure 4. Most of the compounds moved rapidly in BAW (6:1:2), suggesting that they were aglycones. Fourteen spots were distinguishable on chromatograms of Mazzard leaf extracts after spraying with $FeCl_3$-$K_3Fe(CN)_6$ reagent. In Mahaleb extracts 7 spots were noted. All compounds were colorless under visible light; however, some fluoresced under ultraviolet light. The color reactions of phenolic compounds from both species are shown in Table 6. Rf values are given in Tables 7 and 8, and relative quantities in

Figure 4. Paper chromatograms of hydrolysed phenolic compounds extracted from Mazzard and Mahaleb leaves. The chromatograms were developed first with BAW (6:1:2) and secondly with 2% HOAc. The spots marked with solid lines were more intense than spots marked with broken lines. Shaded spots were common to both species. Spots restricted to one species are marked with Z (Mazzard) or H (Mahaleb). Thirty µl of extracts (equivalent to 90 mg fresh weight) was spotted at the origin.

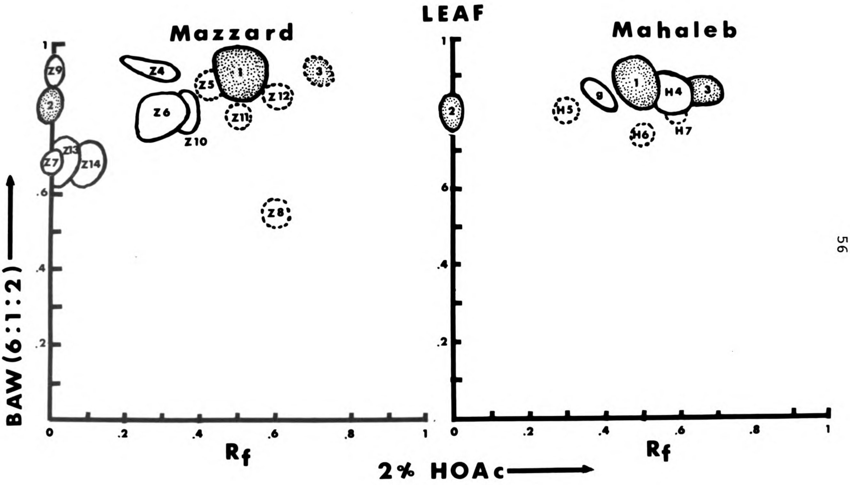

LEAF
Mazzard
Mahaleb
BAW (6:1:2)
2% HOAc
Rf
Rf
0
.2
.4
.6
.8
1
Z4
Z5
1
3
Z6
Z10
Z11
Z12
Z8
Z9
2
Z7
Z13
Z14
9
H4
H5
H6
H7

Table 6. Color reactions of phenolic compounds extracted from Mazzard and Mahaleb leaves, hydrolyzed and purified by paper chromatography. Color characteristics were compared with those of authentic compounds.[a,b,c]

Spot no.	Tentative identification	UV light[d]			Visible light			
		-	$+NH_3$	2N NaOH	DPNA	$FeCl_3$ $K_3Fe(CN)_6$	2N NaOH DPNA	Hoepfner
Mazzard leaf								
1	o-coumaric acid	stW	stYfl	stYGfl	Y→Pu	B	Pu	stY
2	kaempferol	Y	O	Yfl	Y	B	Y	lBr
3	coumarin	(D)	(D)	YGfl	lPu	B	Pu	pY
Z4	dihydrowogonin	pH	pD	lY	stY	B	lY	C
Z5	p-coumaric acid	C	stV	V	lY→Gray	lB	C	C
Z6	caffeic acid	B	B	W	lT→lBr	B	lBr	stT
Z7	quercetin	Y	O	Yfl	O	B	lY	lY
Z8	chlorogenic acid	B	G	G	fBr	B	lBr	Y
Z9	unknown	pBr	pBr	pBr	Y	B	pBr	lBr
Z10	unknown	B	C	C	lBr→lY	pB	C	C
Z11	unknown	V	C	C	C	B	C	Y
Z12	unknown	B	B	C	C	B	C	C
Z13	unknown	pB	G	lW	C	B	C	lY
Z14	unknown	pB	G	lW	C	B	pY	lY
Mahaleb leaf								
1	o-coumaric acid	stW	stYfl	stYGfl	Y→Pu	B	C	Y
2	kaempferol	Y	O	stY	Y	B	Y	C
3	coumarin	(D)	(D)	stYGfl	lPu	B	lR V	C
H4	herniarin	V	V	stBfl	Y→Gray	C	stV	C
H5	unknown	B	B	C	lPu	B	C	C
H6	unknown	C	C	C	C	B	C	pY
H7	unknown	C	C	C	C	B	C	C
Reference compounds								
	o-coumaric acid	W	GY	stYGfl	Pu	B	Pu	Y
	kaempferol	Y	stY	Yfl	Y	B	Y	G
	coumarin	(D)	(D)	stYGfl	lPu	C	Pu	C
	dihydrowogonin	C	C	lY	stY	B	lY	C
	p-coumaric acid	C	stV	stV	Y→lGray	B	lV	stY
	caffeic acid	B	B	fWB	lT→lBr	B	lBr	T→Br
	quercetin	Y	Y	Yfl	lO	B	fY	lY
	chlorogenic acid	B	G	fG	lT→Br	B	lBr	stO
	herniarin	V	V	stBfl	C	C	stV	C
	ferulic acid	B	B	stB	P	B	T→lB	Y

[a] None of the compounds gave a color reaction with $NaBH_4$-HCl reagent.

[b] Only spot H5 gave a color reaction with Vanillin-HCl.

[c] B, Blue; Br, Brown; C, Colorless; D, Dark; G, Green; O, Orange; Pu, Purple; R, Red; T, Tan; Y, Yellow; W, White; p, Pale; l, light; br, bright; st, strong; fl, fluorescence.

[d] Colors under long UV except '( )' showing colors under short UV.

Table 7. Rf values of phenolic compounds extracted from Mazzard and Mahaleb leaves, hydrolyzed and separated by two-dimensional paper chromatography. The compounds were rechromatographed together with reference compounds in six different solvent systems.[a,b,c]

Compound	Tentative identification	Thin layer			Paper		
		BMA	M-C	TEF	BAW	2%HOAc	BPW
Mazzard leaf							
1	o-coumaric acid	.39	.50	s	.84	.59	.88
2	kaempferol	.29	.36	.21	.80	.00	.88
3	coumarin	.62	.53	.56	.88	.75	.87
Z4	dihydrowogonin	.60	.57	.56	.86	.0-.42	.90
Z5	p-coumaric acid	.41	.44	s	.84	.51	.86
Z6	caffeic acid	.27	.27	s	.72	.40	.78
Z7	quercetin	.12	--	--	.65	.00	--
Z8	chlorogenic acid	.02	.00	.01	.48	.65	.25
Mahaleb leaf							
1	o-coumaric acid	.43	.47	s	.84	.57	.88
2	kaempferol	.36	--	.19	.80	.00	.88
3	coumarin	.62	.48	.60	.88	.73	.87
H4	herniarin	.62	.48	.60	.89	.61	.86
Reference compounds							
	o-coumaric acid	.43	.45	s	.85	.61	.88
	kaempferol	.30	.37	.21	.81	.00	.87
	coumarin	.63	.54	.61	.89	.77	.87
	dihydrowogonin	.61	.57	.57	.87	.0-.42	.90
	p-coumaric acid	.42	.40	s	.85	.54	.86
	caffeic acid	.26	.29	s	.73	.41	.79
	quercetin	.16	.20	.08	.65	.00	.83
	chlorogenic acid	.02	.00	.01	.48	.71	.25
	herniarin	.63	.50	.60	.89	.64	.86
	ferulic acid	.45	.50	s	.82	.59	.78

[a] Chambers were saturated with developing solvents and chromatograms were developed at room temperature (22 ± 1°C).

[b] Each value is the average of 3 determinations.

[c] BMA (benzene:methanol:acetic acid, 45:8:4), M-C (11% methanol in $CHCl_3$), TEF (toluene:ethyl acetate:formic acid, 5:4:1), BAW (buthanol:acetic acid:water, 6:1:2), 2% HOAc (2% acetic acid), BPW (buthanol:pyridine:water, 10:3:3), s, streak.

Table 8. Rf values of phenolic compounds extracted from Mazzard and Mahaleb leaves, hydrolysed and separated by two dimensional paper chromatograms.

Compounds	Solvent system	
	BAW (6:1:2)	2% HOAc
Mazzard		
1	.86	.45
2	.82	.00
3	.86	.74
Z4[a]	.90	.25
Z5	.81	.35
Z6	.76	.25
Z7	.65	.00
Z8	.58	.59
Z9	.89	.00
Z10	.90	.31
Z11	.80	.49
Z12	.81	.54
Z13	.66	.05
Z14	.69	.12
Mahaleb		
1	.90	.49
2	.82	.00
3	.91	.75
H4[a]	.88	.62
H5	.82	.26
H6	.78	.50
H7	.82	.60

[a] Prefix Z or H means that the compound was present only in Mazzard or Mahaleb respectively.

[b] See Table 7 for notations of chromatographic conditions.

Table 9. Spectral characteristics of partially purified compounds from Mazzard and Mahaleb leaves, compared with those of reference compounds.

Compound	Tentative identification	max (mμ)	min (mμ)
Mazzard leaf			
1	o-coumaric acid	320,271	300,244
2	kaempferol	368,268	280,240
3	coumarin	276	252
Z4	dihydrowogonin	332,290	320,256
Z5	p-coumaric acid	270	248
Z6	caffeic acid	320,243	260,230
Z7	quercetin	370,256	286,240
Z8	chlorogenic acid	327	265
Mahaleb leaf			
1	o-coumaric acid	325,274	300,246
2	kaempferol	368,260	314,
3	coumarin	310,275	300,244
H4	herniarin	320	260
Reference Compounds			
	o-coumaric acid	325,274	300,243
	kaempferol	369,268	290,240
	coumarin	312,276	298,242
	dihydrowogonin	336,290	320,254
	p-coumaric acid	310,226	246
	caffeic acid	326,243	264,230
	quercetin	372,256	286,238
	chlorogenic acid	332,244	268
	herniarin	320,	260

Table 10. Ultraviolet absorption maxima and minima are shown in Table 9. From these data, some of the compounds in the leaf extracts were identified as follows:

Spot 1: This compound, occurring in both species, appeared bright white under ultraviolet light. In the presence of ammonia, it fluoresced bright yellow. The compound reacted with diazotized p-nitro aniline to form a purple color, and turned a bright yellow on treatment with Hoepfner reagent. The Rf values in both thin layer chromatography and paper chromatography were comparable to those of standard o-coumaric acid. The compound had an absorption spectrum similar to that of authentic o-coumaric acid, with maxima at 320 and 271 mμ and minima at 300 and 243 mμ. Thus, compound 1 was tentatively identified as o-coumaric acid.

Spot 2: This compound occurred in both tissues in similar concentrations. It appeared yellow under ultraviolet light, changing to orange on fuming with ammonia. Further tests with other reagents indicated that the compound might be a flavonoid. It moved rapidly in BAW (Rf .80) but not in 2% acetic acid (Rf .00). This is characteristic of planar flavonoid aglycones (flavone and flavonol) (Seikel, 1964). Its Rf values and ultraviolet absorption spectrum were similar to those of kaempferol. Therefore, spot 2 was identified as kaempferol.

Table 10. Relative amounts of phenolic compounds in Mazzard and Mahaleb leaves, based on spot size and color intensity on two dimensional paper chromatography after application of $FeCl_3$-$K_3Fe(CN)_6$ reagent.

Compound	Tentative identification	Relative amount	
		Mazzard	Mahaleb
1	o-coumaric acid	+++++	++++++
2	kaempferol	++++	+++
3	coumarin	+	+++++
Z4	dihydrowogonin	++++	-
Z5	p-coumaric acid	++	-
Z6	caffeic acid	+++++	-
Z7	quercetin	++	-
Z8	chlorogenic acid	++	-
Z9	unknown	+++	-
Z10	unknown	+	-
Z11	unknown	++	-
Z12	unknown	++	-
Z13	unknown	++	-
Z14	unknown	++	-
H4	herniarin	-	++++++++++++
H5	unknown	-	+
H6	unknown	-	+
H7	unknown	-	+

Spot 3: Compound 3 was also common to both leaf tissues. However, the concentration of the compound was higher in Mahaleb than in Mazzard. It was colorless under long and absorbed under short ultraviolet light, with or without ammonia. When the spot was sprayed with 2 N NaOH, it fluoresced a bright yellow-green a typical color reaction of coumarin. Subsequent treatment with DPNA produced the characteristic purple color obtained with coumarin (Swain, 1953). Rf values and spectral characteristics were similar to those of coumarin. The compound was therefore identified as coumarin.

Spot Z4: Compound Z4 occurred only in Mazzard leaf extracts and was colorless under ultraviolet light with or without ammonia. However, a distinctive yellow color was obtained when the compound was sprayed with diazotized p-nitro aniline reagent (DPNA). The Rf values of the compound in both thin layer and paper chromatography were comparable to those of dihydrowogonin (Table 7). Therefore, Z4 was identified as dihydrowogonin.

Spot Z5: Z5, also detected only in Mazzard extracts, showed no color under ultraviolet in the absence of ammonia, but fluoresced bright violet in the presence of ammonia. Although Rf values were comparable with those of standard p-coumaric acid, the ultraviolet absorption spectrum differed. The compound may have been either contaminated with other compounds or too low in concentration to be detected spectrophotometrically.

Spot Z6: This compound, unique to Mazzard tissues, fluoresced bright blue under ultraviolet with or without ammonia. With Hoepfner reagent it gave a bright tan color, and with DPNA a light tan, later turning to light brown. Rf values of the compound in six different solvent systems were similar to those of standard caffeic acid, as were spectral characteristics, with maxima at 320 and 243 and minima at 260 and 230 mμ. It was thus identified as caffeic acid.

Spot Z7: Z7 occurred in Mazzard and showed flavonoid characteristics in its color reactions. The compound fluoresced yellow under ultraviolet light with or without ammonia. Rf values were 0.65 in BAW and 0.00 in 2% acetic acid. Maximum absorption occurred at 370 and 256, minimum at 280 and 240 mμ. These characteristics agreed with those of the standard compound quercetin and so it was identified as quercetin.

Spot Z8: Z8, occurring only in Mazzard, fluoresced blue under ultraviolet light. When the chromatogram was fumed with ammonia, the color changed to green, which is typical of chlorogenic acid. The Rf values of the compound corresponded to those of standard chlorogenic acid.

Its spectral characteristics, however, differed from those of chlorogenic acid. The low concentration present in the hydrolyzed extract resulted in poor recovery during purification and the absorption spectrum was poor. However, color reactions and Rf values suggest that Z8 is chlorogenic acid.

Spot H4: H4 was one of the most distinctive compounds in Mahaleb tissue. It fluoresced bright violet under ultraviolet light, changing bright blue after spraying with 2N NaOH. Subsequent treatment with DPNA gave a bright violet color under visible light. These are typical color reactions of herniarin (7 methoxy coumarin) (Swain, 1953). The Rf values of the compound in six different solvent systems were comparable with those of standard herniarin, as was the absorption spectrum (maximum at 320 mμ and minimum at 260 mμ). Therefore, the compound was identified as herniarin.

The results obtained by extracting phenolic compounds from Mazzard and Mahaleb leaves are summarized in Table 10. Relative amounts of each compound are also given.

Three compounds occurred in both Mazzard and Mahaleb tissues: o-coumaric acid, kaempferol, and coumarin. The first two compounds were present in similar concentrations in both Mazzard and Mahaleb leaves. However, the coumarin level was much higher in Mahaleb than in Mazzard. The Mazzard leaves contained several specific phenolic compounds, including caffeic acid, dihydrowogonin, quercetin, chlorogenic acid, and p-coumaric acid. The first two were most prominent. The Mahaleb leaves contained four major phenolic compounds, three of these being o-coumaric acid, kaempferol, and coumarin. Herniarin, specific to Mahaleb, was the dominant compound that appeared on two-dimensional paper chromatograms.

C. Paper chromatography of hydrolyzed stem bark extracts

Phenolic compounds extracted from living bark tissues were acid hydrolyzed and chromatographed on paper. Among the 13 phenolic compounds that appeared on chromatograms of Mazzard extracts, only three corresponded with those found in Mahaleb bark (Fig. 5). The paper chromatogram of Mahaleb bark showed 11 phenolic spots.

The color reactions of phenolic compounds from Mazzard and Mahaleb bark are shown in Table 11; Rf values and relative concentrations are reported in Table 12. In the previous section some of the spots were identified in leaf extracts on the basis of color reactions and Rf values. On the chromatograms of Mazzard bark extracts, five spots exhibited color reactions and Rf values identical with those of Mazzard leaf phenolic compounds. Those spots were o-coumaric acid, coumarin, dihydrowogonin, p-coumaric acid, and Z9. Mahaleb bark also contained some

Figure 5. Paper chromatograms of hydrolyzed phenolic compounds extracted from Mazzard and Mahaleb stem bark. The chromatograms were developed first with BAW and secondly with 2% HOAc. The spots marked with solid lines were present in larger amounts than those marked with broken lines. Shaded spots were common to both tissues. Species specific spots are marked with Z (Mazzard) or H (Mahaleb). 30 µl of extract (equivalent to 90 mg fresh weight) was spotted at the origin.

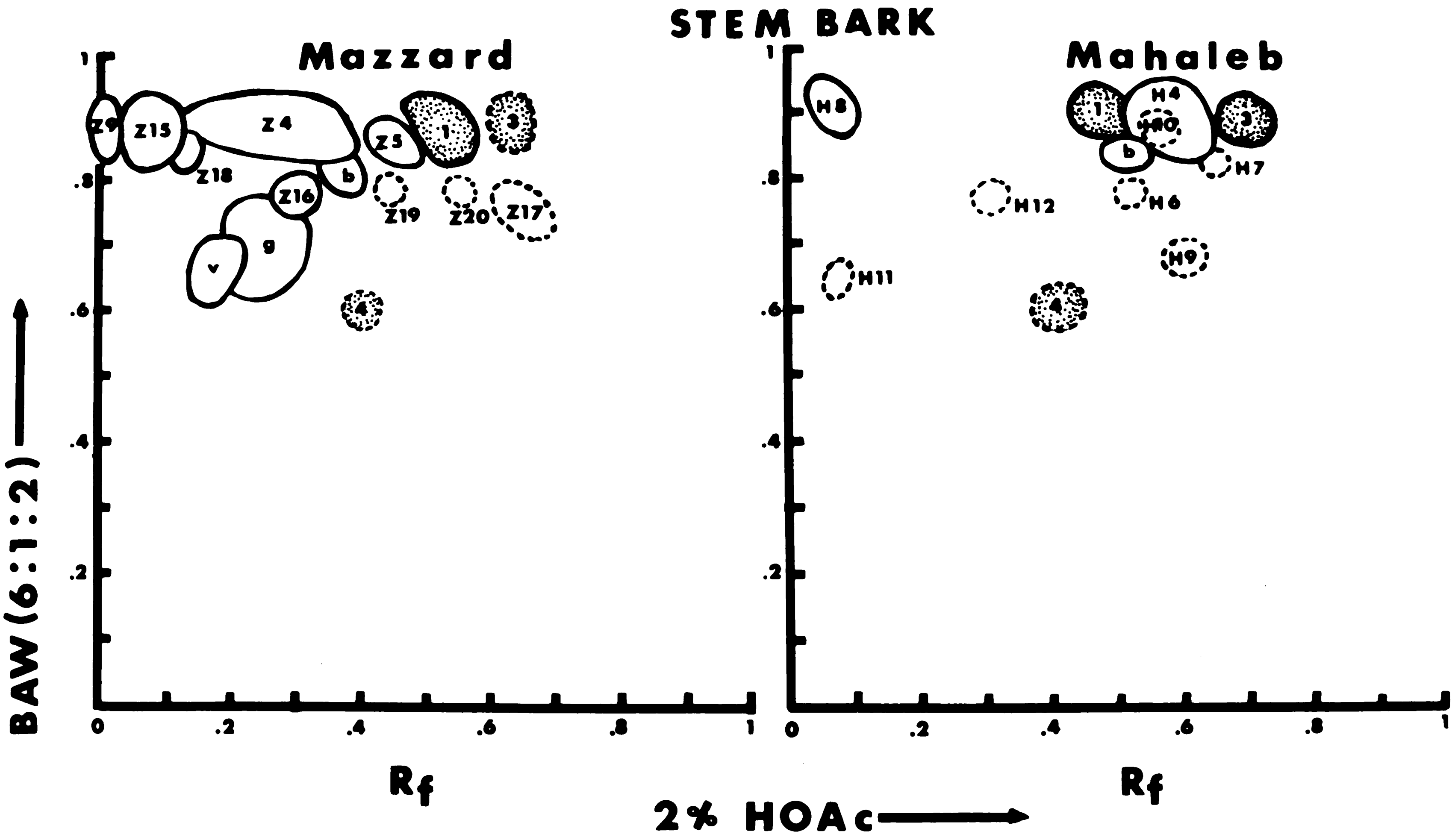

STEM BARK
Mazzard
Mahaleb
Z9
Z15
Z4
Z18
Z5
1
3
b
Z16
Z19
Z20
Z17
g
v
4
H8
H4
H10
H7
H6
H12
H9
H11
BAW (6:1:2)
2% HOAc
Rf
Rf
0
.2
.4
.6
.8
1

Table 11. Color reactions of phenolic compounds extracted from Mazzard and Mahaleb stem bark, hydrolyzed and chromatographed on a two-dimensional paper chromatogram.[a,b]

Spot no.	Tentative identification	UV light			Visible light				
		-	+NH_3	+2N NaOH	$FeCl_3$-$K_3Fe(CN)_6$	DPNA	2N NaOH + DPNA	Hoepfner	Vanillin HCl
<u>Mazzard</u>									
1	o-coumaric acid	W	Y	stYGfl	B	Pu	C	C	C
3	coumarin	(fD)	C	stYGfl	C	Y-Pu	C	lY	C
4	catechin	C	C	C	B	O	fY	C	P
Z4	dihydrowogonin	lD	lD	lY	B	stY	Y	C	C
Z5	p-coumaric acid	C	stBV	stV	B	Y→Gray	C	Y	C
Z9	unknown	Br	Br	C	B	Y	lY	lY	C
Z15	unknown	VB	VB	C	B	O	lY	fY	C
Z16	unknown	C	C	Br	B	lBr	lY	Y	C
Z17	unknown	C	C	Y	B	lY	C	C	C
Z18	unknown	(D)	(D)	C	B	lY	lO	C	C
Z19	unknown	C	C	C	B	C	C	Y	C
Z20	unknown	C	C	C	B	C	C	C	C
<u>Mahaleb</u>									
1	o-coumaric acid	W	Y	stYGfl	B	Pu	C	C	C
3	coumarin	C	C	YG	C	lPu	lRV	C	fP
H4	herniarin	V	V	stBfl	C	Y→Gray	V	C	C
4	catechin	C	C	C	B	Y	fY	lY	lP
H6	unknown	C	C	C	B	C	C	Y	C
H7	unknown	C	C	lY	B	C	C	lY	C
H8	unknown	(D)	(D)	C	B	Y	C	C	C
H9	unknown	C	C	C	B	T	C	C	lO
H10	unknown	C	C	C	B	P	C	fY	C
H11	unknown	C	C	C	B	C	C	lY	C
H12	unknown	C	C	C	B	Y	C	lY	C

[a] None of the compounds gave a color reaction with $NaBH_4$-HCl reagent. [b] See Table 6 for notation.

Table 12. Rf values and relative concentrations of phenolic compounds extracted from Mazzard and Mahaleb stem bark, hydrolyzed and chromatographed on a two-dimensional paper chromatogram.[a]

Spot No.	Tentative identification	Rf value		Relative concentration
		BAW (6:1:2)	2% HOAc	
Mazzard				
1	o-coumaric acid	.85	.42	+++
3	coumarin	.89	.65	+
4	catechin	.62	.40	++
Z4	dihydrowogonin	.89	.00-.39	++++++++++++
Z5	p-coumaric acid	.85	.37	+++
Z9	unknown	.86	.00	++
Z15	unknown	.88	.05	++++
Z16	unknown	.76	.31	+++
Z17	unknown	.75	.65	+
Z18	unknown	.87	.13	+++
Z19	unknown	.78	.42	+
Z20	unknown	.77	.55	+
Mahaleb				
1	o-coumaric acid	.91	.41	+
3	coumarin	.91	.75	+++
H4	herniarin	.90	.62	+++++++++
4	catechin	.62	.40	++
H6	unknown	.79	.48	+
H7	unknown	.81	.55	+
H8	unknown	.89	.86	+
H9	unknown	.70	.60	+
H10	unknown	.87	.62	++
H11	unknown	.64	.08	+
H12	unknown	.77	.28	+

[a] See Table 7 for notations and chromatographic conditions.

of the phenolic compounds present in the Mahaleb leaf tissues, namely o-coumaric acid, coumarin, herniarin, and two unknown spots H6 and H7.

Compound 4 was found in both Mazzard and Mahaleb bark. It was colorless under ultraviolet light with or without ammonia, but gave a pink color with vanillin-HCl reagent, which is specific for flavonoids with a phloroglucinol nucleus such as catechin and leucoanthocyanidins (Swain and Hillis, 1959). The absorption spectrum of the compound had the characteristics of d-catechin with maximum absorption at 280 mμ and minimum at 250 mμ (Jurd, 1962). Rf values were comparable with those of catechin reported by Luh et al. (1967). Therefore compound 4 was identified as d-catechin.

The living bark tissues of the rootstocks differed as to type of phenolic compounds. Mazzard bark appeared to be high in cinnamic acids and flavanones (p-coumaric acid, o-coumaric acid, and dihyrowogonin) while Mahaleb bark was exceptionally high in coumarins.

The spots marked v, g, and b in Figure 5 did not appear to be phenolic compounds, for they were negative to $FeCl_3$-$K_3Fe(CN)_6$ and DPNA reagents. However, v and g were useful markers for Mazzard bark extracts, since they gave distinctive colors (v, violet; g, green) under ultraviolet light. The two spots were absent from Mazzard leaf extracts.

D. Paper chromatography of hydrolyzed cherry root bark extracts

Phenolic compounds found in root bark were similar to those found in bark of stem tissues (Fig. 6). Color reactions, Rf values and relative concentrations of phenolic compounds from both seedlings are shown in Tables 13 and 14.

Eleven phenolic spots appeared on the paper chromatogram of Mazzard extracts, and ten spots on that from Mahaleb. Root bark tissues of both rootstocks contained the three common phenolic compounds which were found in stem bark of both Mazzard and Mahaleb, namely o-coumaric acid, coumarin, and catechin.

In Mazzard, dihydrowogonin was the dominant compound followed by Z15, o-coumaric acid, and p-coumaric acid. In Mahaleb, herniarin was the dominant compound but coumarin and catechin were also prominent.

E. Gas liquid chromatography of hydrolyzed phenolic compounds

Gas liquid chromatography was used to confirm and extend the results obtained with paper chromatography.

The standard phenolic compounds and the unknown compounds were methylated with CH_2N_2 (Schlenk *et al.*, 1960). Among the different standard phenolic compounds studied, only the cinnamic acid series was successfully methylated. Since the flavonoid compounds did not respond to

Figure 6. Paper chromatograms of hydrolyzed phenolic compounds extracted from Mazzard and Mahaleb root bark. The chromatograms were developed first with BAW (6:1:2) and secondly with 2% HOAc. The spots marked with solid lines were present in larger amounts than those marked with broken lines. Shaded spots were common to both species. Species specific spots are marked with Z (Mazzard) or H (Mahaleb). 30 µl extract (equivalent to 90 mg fresh weight) was spotted at the origin.

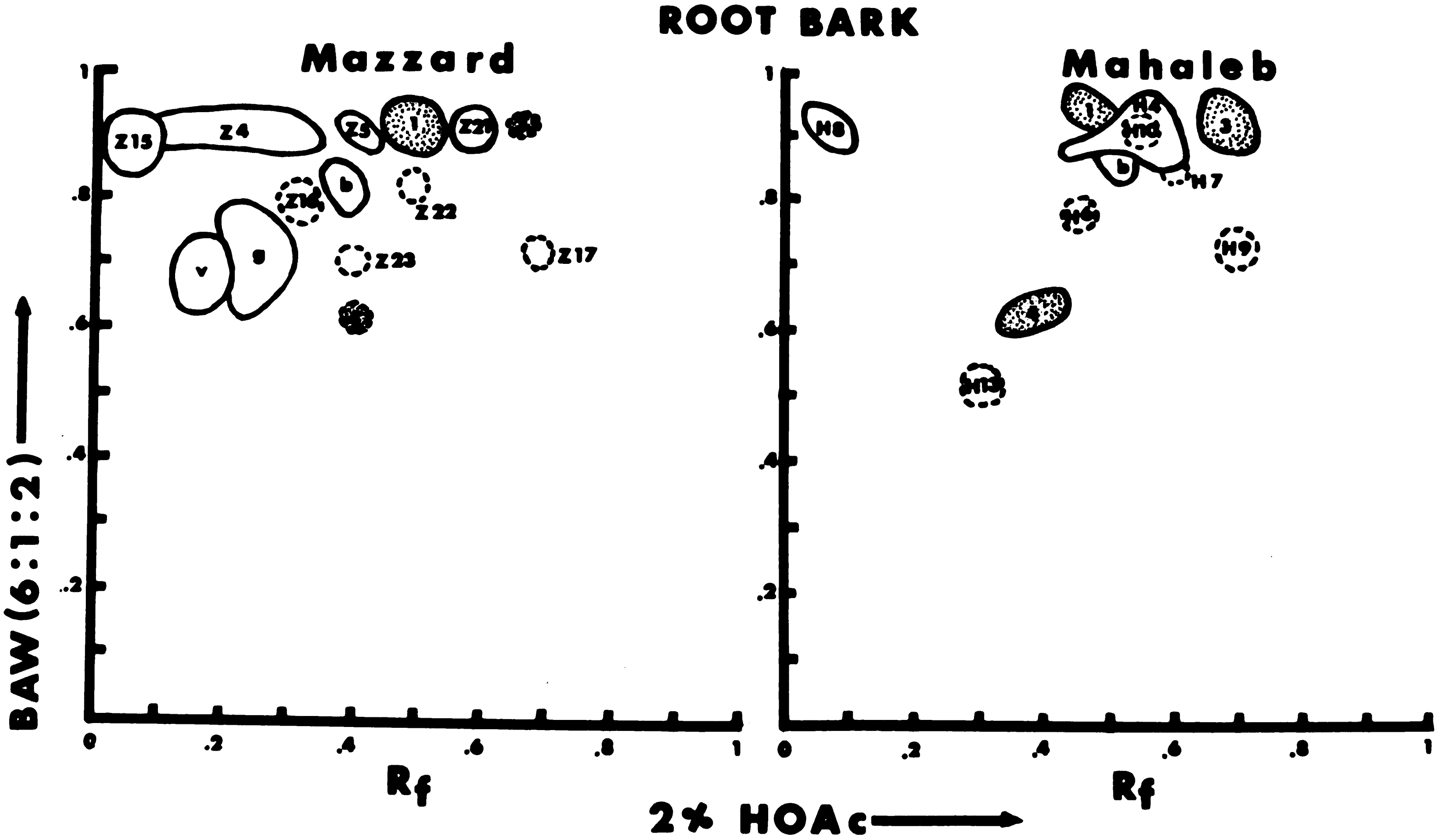

ROOT BARK
Mazzard
Mahaleb
BAW (6:1:2)
2% HOAc
Rf
Rf
0
.2
.4
.6
.8
1
Z15
Z4
Z5
1
Z20
b
Z16
Z22
s
v
Z23
Z17
H8
1
H4
b
H7
3
H9
4

Table 13. Color reactions of phenolic compounds extracted from root bark of Mazzard and Mahaleb, hydrolyzed and chromatographed on a two-dimensional paper chromatogram.[a,b]

Spot no.	Tentative identification	UV light			Visible light				
		-	$+NH_3$	2N NaOH	DPNA	$FeCl_3$ $K_3Fe(CN)_6$	2N NaOH DPNA	Hoepf-ner	Vanil-lin HCl
Mazzard									
1	o-coumaric acid	W	GY	YGfl	Pu	B	C	C	C
3	coumarin	C	C	lYGfl	Pu	fB	RV	C	C
4	catechin	C	C	C	Y	B	C	C	P
Z5	p-coumaric acid	C	stBV	stBV	Y→Gray	B	C	C	C
Z15	unknown	VB	VB	C	stO	B	lO	fB	C
Z16	unknown	C	C	C	lBr	B	C	lY	C
Z17	unknown	C	Y	lY	C	B	C	lY	C
Z21	unknown	V	V	B	C	B	C	C	C
Z22	unknown	C	C	C	C	B	C	lY	C
Z23	unknown	C	C	C	C	B	C	C	C
Z4	dihydrowogonin	C	C	C	stY	B	lY	C	C
Mahaleb									
1	o-coumaric acid	W	Y	YGfl	Pu	B	C	C	C
3	coumarin	(D)	(D)	YGfl	lPu	C	lPu	C	C
4	catechin	C	C	C	Y	B	Y	lY	lP
H4	herniarin	V	V	stB	lBr	C	stV	C	C
H6	unknown	C	C	C	Y	B	C	lY	C
H7	unknown	C	C	C	C	B	C	lY	C
H8	unknown	(D)	(D)	Y	Y	B	Y	C	C
H9	unknown	C	C	C	T→O	B	C	lY	lO
H10	unknown	C	C	C	P	B	C	lY	C
H13	unknown	C	C	C	Pu	B	C	lY	C

[a] None of the compounds gave a color reaction with $NaBH_4$-HCl.

[b] See Table 6 for notation.

Table 14. Rf values and relative concentrations of phenolic compounds extracted from Mazzard and Mahaleb root bark, hydrolyzed and chromatographed on two-dimensional paper chromatograms.[a]

Spot No.	Tentative identification	Rf value		Relative concentration
		BAW (6:1:2)	2% HOAc	
Mazzard				
1	o-coumaric acid	.90	.49	+++
3	coumarin	.90	.66	+
4	catechin	.64	.42	+
Z4	dihydrowogonin	.91	.16-.38	+++++++++++
Z5	p-coumaric acid	.89	.40	++
Z15	unknown	.89	.05	++++
Z16	unknown	.77	.32	+
Z17	unknown	.70	.67	+
Z21	unknown	.89	.57	+
Z22	unknown	.75	.51	+
Z23	unknown	.71	.40	+
Mahaleb				
1	o-coumaric acid	.93	.42	+
3	coumarin	.92	.72	+++
4	catechin	.61	.40	+++
H4	herniarin	.91	.52	++++++++++++
H6	unknown	.80	.50	+
H7	unknown	.82	.62	+
H8	unknown	.89	.06	+
H9	unknown	.70	.60	+
H10	unknown	.89	.60	+
H13	unknown	.49	.30	+

[a] See Table 7 for notation and chromatographic conditions.

methylation, silylation was employed. Different columns and a wide range of temperatures were used, but no response could be demonstrated by this method.

Figure 7 shows a trace obtained with the methyl esters of the standard phenolic compounds. Five compounds were chromatographed successfully. Coumarin was eluted first, followed by o-coumaric acid, p-coumaric acid, caffeic acid, and herniarin. Two peaks of different concentration were observed with o-coumaric acid, with p-coumaric acid, and with caffeic acid. This probably was due to impurities or to incomplete methylation.

Typical gas chromatograms for leaf, stem bark, and root bark extracts are shown in Figures 8, 9, and 10. Retention times and concentrations of phenolic compounds are given in Table 15.

o-Coumaric acid occurred in all tissues of both rootstocks, and similar levels were present in both. Leaves contained higher levels of the compound than did the bark tissues. p-Coumaric acid occurred in all Mazzard tissues tested, but the level of the compound was much higher in the bark than in the leaves. Coumarin was present in all the tissues tested. In stem and root bark, Mahaleb contained 8 to 10 times more coumarin than did Mazzard. Caffeic acid was detectable only in Mazzard leaves. The level was 0.11 mg per gram fresh weight of leaves. Herniarin occurred only in Mahaleb tissues. As in the case

Figure 7. Gas liquid chromatogram of a mixture of standard phenolic compounds. Compounds were methylated before chromatography on 2% QF-1 on Chromasorb W. Column temperature was 160°C. Coum = coumarin; O-C = o-coumaric acid; p-C = p-coumaric acid; Caf = caffeic acid; Her = herniarin.

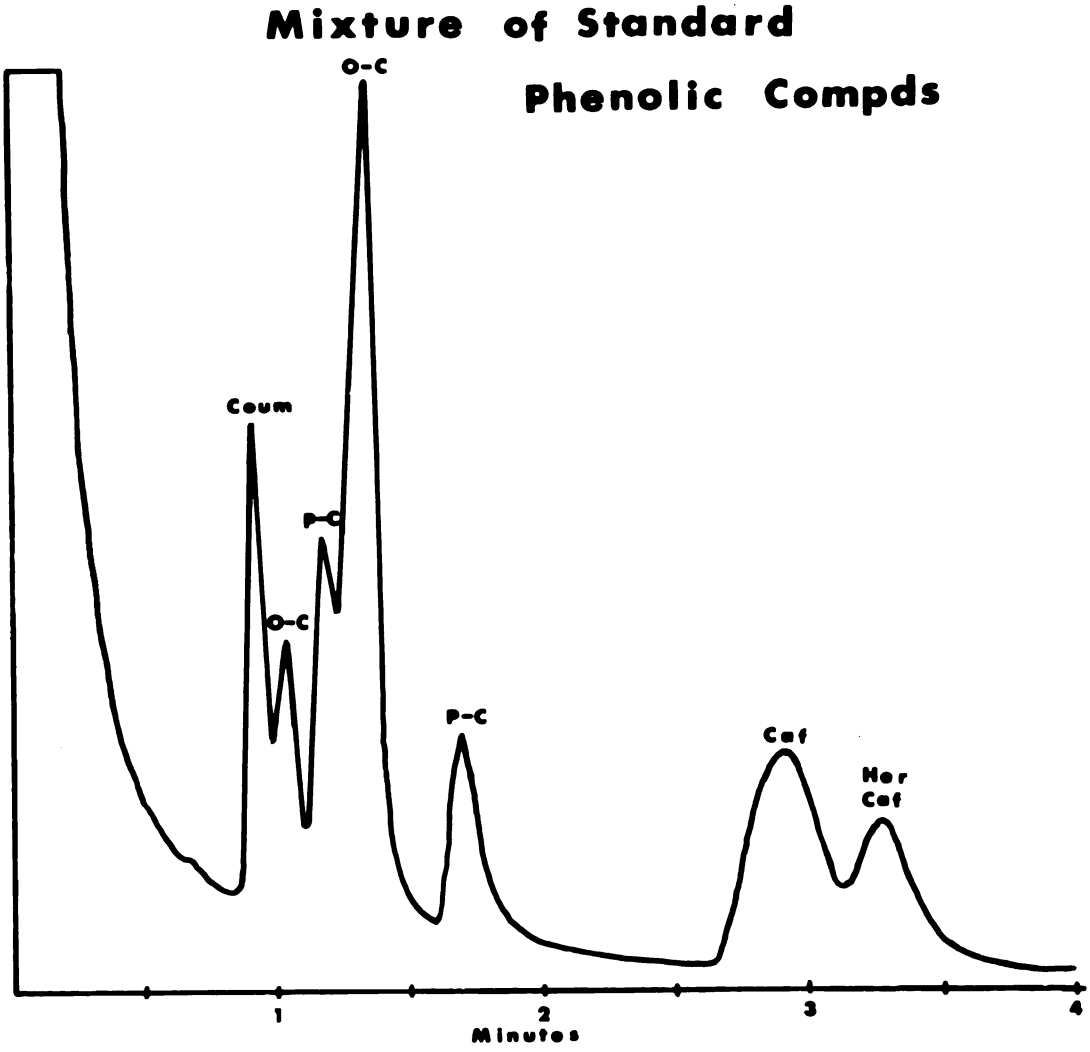

Mixture of Standard
Phenolic Compds
O-C
Coum
P-C
O-C
P-C
Caf
Her
Caf
1
2
3
4
Minutes

Figure 8. Gas liquid chromatograms of hydrolyzed phenolic compounds extracted from Mazzard and Mahaleb leaves. Compounds were methylated before chromatography on 2% QF-1 on Chromasorb W. Column temperature was 160°C.
1 = coumarin; 2 = o-coumaric acid; 3 = p-coumaric acid; 4 = caffeic acid; 5 = herniarin.


Mazzard Leaf
1
2
3
4
1X
3X
0
1
2
3
4
5
Minutes

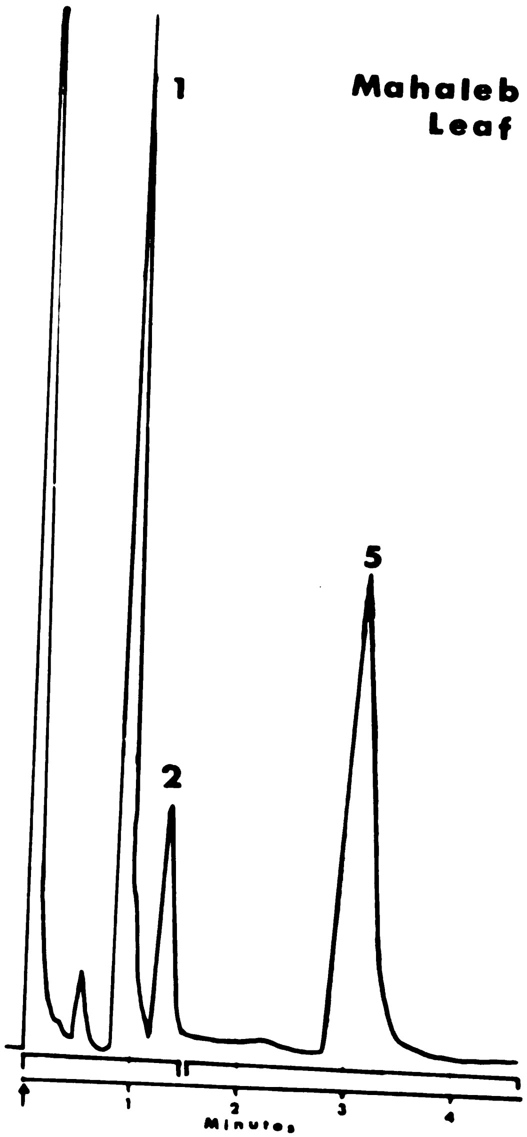
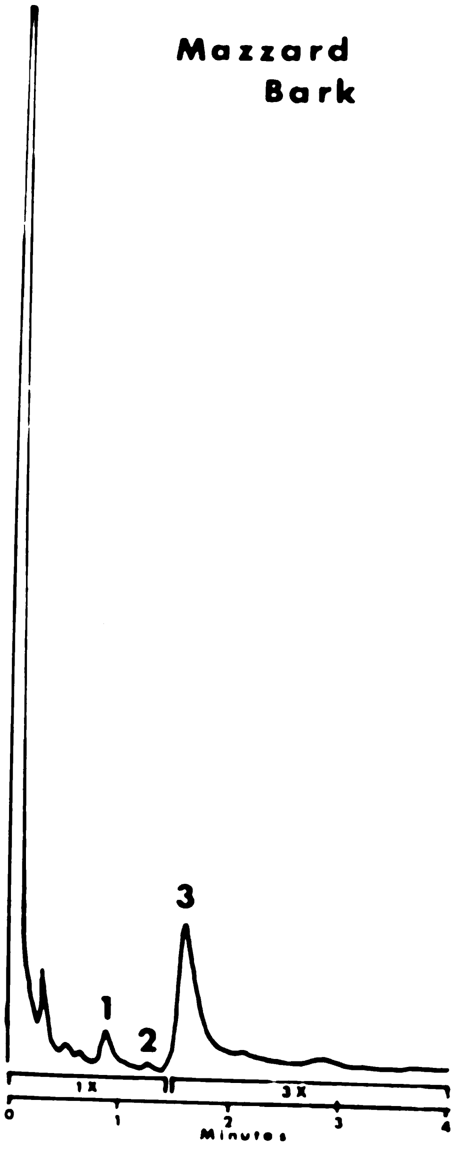

Mahaleb Leaf
1
2
5
1
2
3
4
Minutes

Figure 9. Gas liquid chromatograms of hydrolyzed phenolic compounds extracted from Mazzard and Mahaleb stem bark. Compounds were methylated before chromatography on 2% QF-1 on Chromasorb W. Column temperature was 160°C.
1 = coumarin; 2 = o-coumaric acid; 3 = p-coumaric acid; 4 = caffeic acid; 5 = herniarin.


Mazzard
Bark
1
2
3
1X
3X
0
1
2
3
4
Minutes

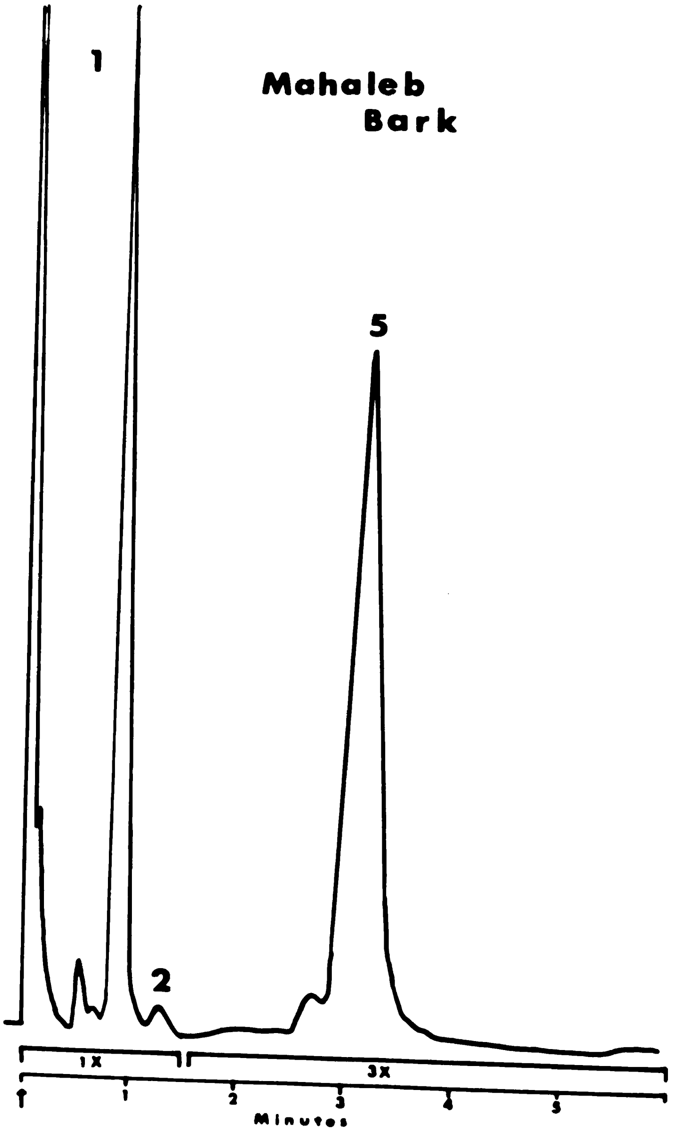
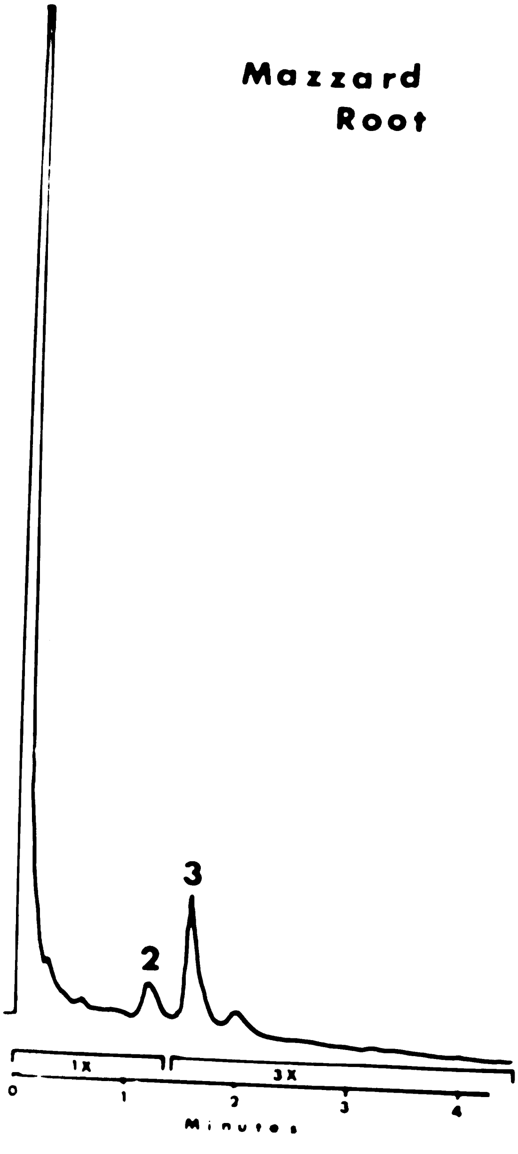

1
Mahaleb
Bark
5
2
1X
3X
1
2
3
4
5
Minutes

Figure 10. Gas liquid chromatograms of hydrolyzed phenolic compounds extracted from Mazzard and Mahaleb root bark. Compounds were methylated before chromatography on 2% QF-1 on Chromasorb W. Column temperature was 160°C. 1 = coumarin; 2 = o-coumaric acid; 3 = p-coumaric acid; 4 = caffeic acid; 5 = herniarin.


Mazzard
Root
3
2
1X
3X
0
1
2
3
4
Minutes

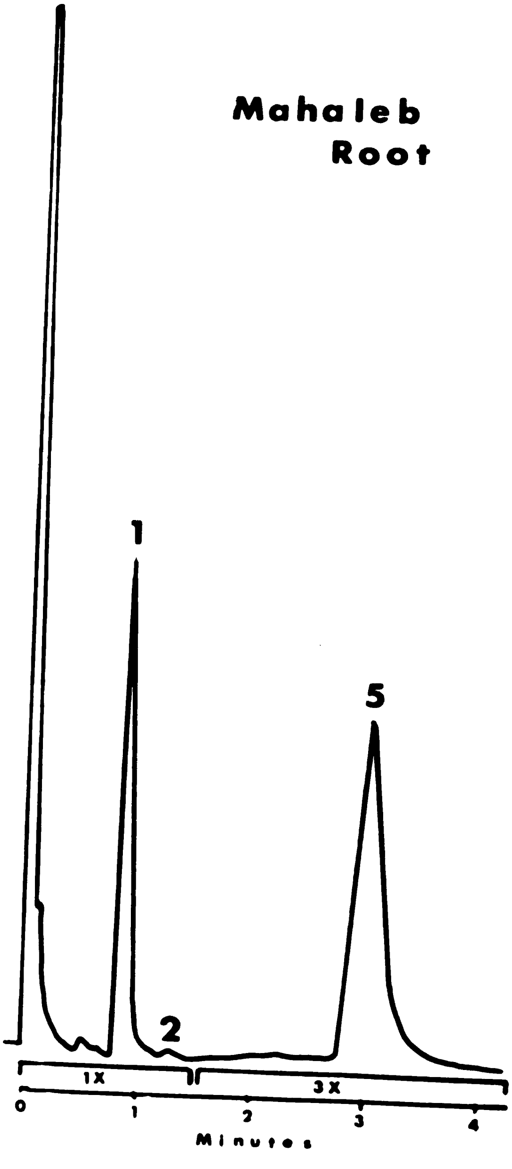

Mahaleb
Root
1
5
2
1X
3X
0
1
2
3
4
Minutes

Table 15. Retention times and concentrations (mg/g fresh weight) of phenolic compounds extracted from leaves, bark, and root bark of Mazzard and Mahaleb seedlings as determined by gas liquid chromatography.[a,b]

Compound[c]	Retention time (min)	Leaf		Stem bark		Root bark	
		Maz	Mah	Maz	Mah	Maz	Mah
coumarin	.86	.76	.76	.08	.64	.06	.52
o-coumaric acid	1.24	.21	.22	.06	.08	.08	.06
p-coumaric acid	1.61	.13	-	.28	-	.26	-
caffeic acid	2.80	.11	-	-	-	-	-
herniarin	3.10	-	.44	-	.50	-	.38

[a] Average of two determinations.

[b] 2% QF-1 on Chromasorb W, column temperature 160°C and flow rate 40 ml/min.

[c] Compounds were methylated before chromatography.

of coumarin, all the tissues tested contained a high level of the compound. Mahaleb stem bark had the highest level with 0.5 mg per gram fresh weight followed by leaves with 0.44 mg and root bark with 0.38 mg. Table 16 summarizes the hydrolyzed phenolic compounds occurring in leaf, bark, and root bark tissues of Mazzard and Mahaleb seedlings.

III. Identification and Relative Amounts of Phenolic Compounds in Centrifugal Sap

A. Ether fraction

The ether fraction of the sap showed several phenolic substances (Fig. 11). In Mazzard, ten spots appeared on two-dimensional paper chromatograms. Four of these (3, 5, 6, 7) had the same color characteristics and Rf values as compounds occurring in Mahaleb extracts. Compound 3 was readily identified as coumarin on the basis of color reactions and Rf values. Compounds 5, 6, and 7 gave strong color reactions with $FeCl_3$-$K_3Fe(CN)_6$; however, they were not identified.

Spot Z4, which reacted strongly with both DPNA and $FeCl_3$-$K_3Fe(CN)_6$ reagents, had color characteristics and Rf values identical with those of authentic dihydrowogonin.

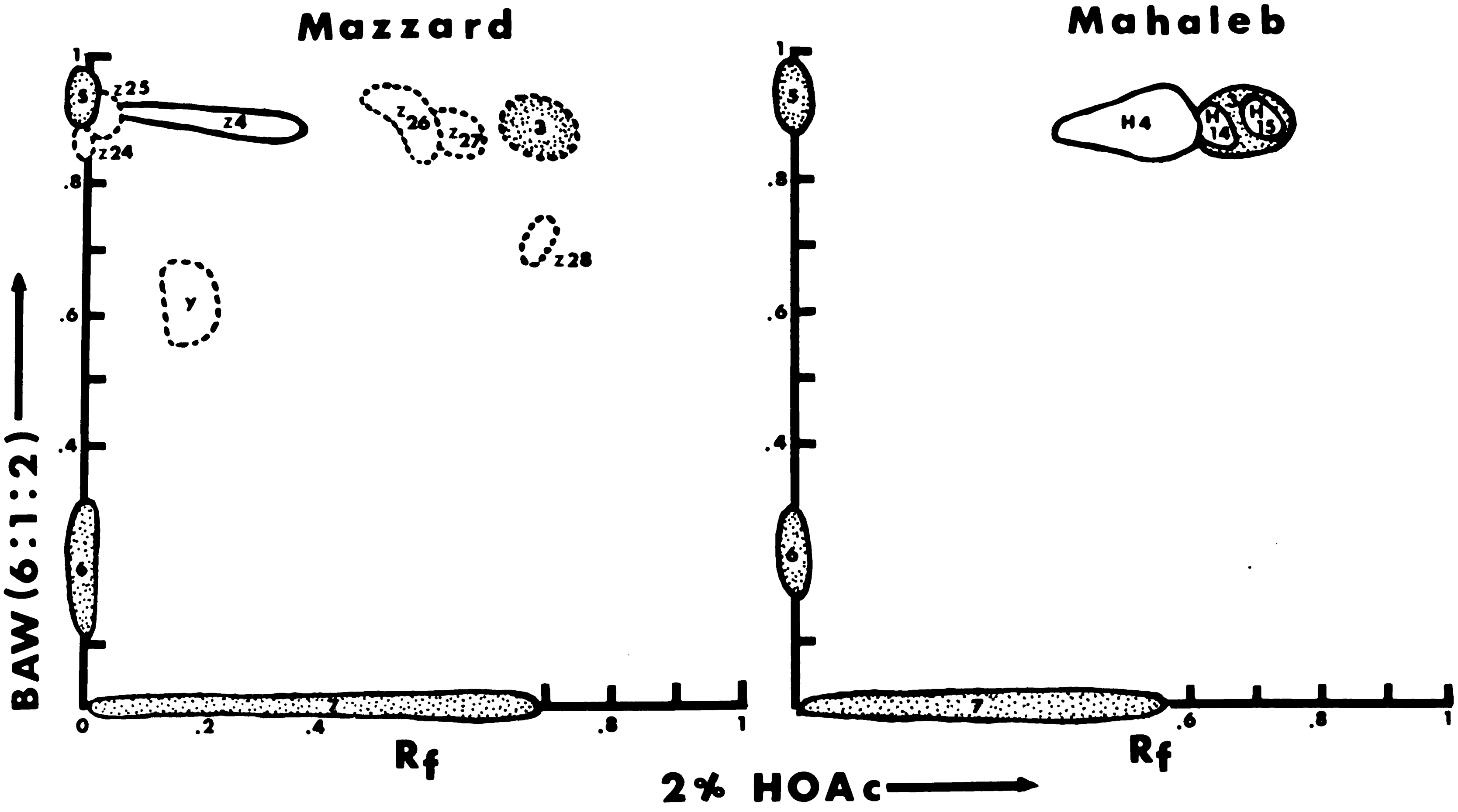
In Mahaleb, among 7 spots on paper chromatograms, only three spots appeared to be specific to Mahaleb extracts (H4, H14, and H15). Compound H4, one of the prominent spots, had the same color reactions and Rf

Table 16. Comparison of hydrolyzed phenolic compounds in extracts of leaf, stem bark, and root bark of Mazzard and Mahaleb seedlings.

Spot No.	Compound	Leaf		Stem bark		Root bark	
		Maz	Mah	Maz	Mah	Maz	Mah
1	o-coumaric acid	+	+	+	+	+	+
2	kaempferol	+	+	-	-	-	-
3	coumarin	+	+	+	+	+	+
4	catechin	+	-	+	+	+	+
Z4	dihydrowogonin	+	-	+	-	+	-
Z5	p-coumaric acid	+	-	+	-	+	-
Z6	caffeic acid	+	-	-	-	-	-
Z7	quercetin	+	-	-	-	-	-
Z8	chlorogenic acid	+	-	-	-	-	-
Z9	unknown	+	-	+	-	-	-
Z10	unknown	+	-	-	-	-	-
Z11	unknown	+	-	-	-	-	-
Z12	unknown	+	-	-	-	-	-
Z13	unknown	+	-	-	-	-	-
Z14	unknown	+	-	-	-	-	-
Z15	unknown	-	-	+	-	+	-
Z16	unknown	-	-	+	-	+	-
Z17	unknown	-	-	+	-	+	-
Z18	unknown	-	-	+	-	-	-
Z19	unknown	-	-	+	-	-	-
Z20	unknown	-	-	+	-	-	-
Z21	unknown	-	-	-	-	+	-
Z22	unknown	-	-	-	-	+	-
Z23	unknown	-	-	-	-	+	-
H4	unknown	-	+	-	+	-	+
H5	unknown	-	+	-	-	-	
H6	unknown	-	+	-	+	-	+
H7	unknown	-	+	-	+	-	+
H8	unknown	-	-	-	+	-	+
H9	unknown	-	-	-	+	-	+
H10	unknown	-	-	-	+	-	+
H11	unknown	-	-	-	+	-	-
H12	unknown	-	-	-	+	-	-
H13	unknown	-	-	-	-	-	+

Figure 11. Paper chromatograms of phenolic compounds in the ether fraction of Mazzard and Mahaleb sap extracts. The chromatograms were developed first with BAW (6:1:2) and secondly with 2% HOAc. The spots marked with solid lines were present in larger amounts than spots marked with broken lines. Shaded spots were common to both rootstocks. 100 µl extract (equivalent to 6.4 g. fresh weight in Mazzard and 5.5 g in Mahaleb) was spotted at the origin.

SAP (ETHER)


Mazzard
Mahaleb
BAW (6:1:2)
2% HOAc
Rf
Rf
1
.8
.6
.4
0
.2
.4
.8
1
.6
5
6
7
z25
z24
z4
y
z26
z27
3
z28
H4
H14
H15

values as herniarin. Compounds H14 and H15 reacted strongly with $FeCl_3$-$K_3Fe(CN)_6$, DPNA, and benzidine, but were not identified. The separation of these compounds from coumarin was not possible with two-dimensional paper chromatography. The compounds were differentiated only with the use of diazotized benzidine reagent (Table 17).

The identification of coumarin derivatives in Mahaleb sap was further confirmed by gas liquid chromatography. The sap contained compounds which had retention times identical to those of coumarin and herniarin (Table 19). The concentration of coumarin was 11 μg and of herniarin 24 μg per gram fresh weight. Neither compound was present in Mazzard sap.

Several compounds occurring in the chromatograms of ether extracts appeared to be free aglycones; these included herniarin, coumarin, and spots 5, H14, and H15 in Mahaleb sap, and dihydrowogonin and spot 5 in Mazzard sap. In the living tissues, aglycones rarely occur in the free state but rather as glycosides or esters. If they occur in higher plants, they are present only in storage tissues or dead tissues (Harborne, 1964), for their solubility is usually too low to occur in the cell sap. Therefore the compounds were probably hydrolyzed during the extraction procedure. However, the possibility that they occur naturally cannot be ruled out completely. Free aglycones can originate from autolysis of xylem and

Table 17. Color reactions of phenolic compounds in the ether fraction of Mazzard and Mahaleb sap extract. The compounds were chromatographed on two-dimentional paper chromatograms and sprayed with phenolic reagents.[a]

Spot no.	Tentative identification	UV light			Visible light			
		-	+NH_3	2N NaOH	$FeCl_3$+ $K_3Fe(CN)_6$	DPNA	2N NaOH +DPNA	Benzidine
Mazzard								
3	coumarin	C	C	fYG	C	C	fPu	-
5	unknown	BW	BW	C	stB	fY	Y	Br
6	unknown	lV	Y	C	B	C	C	C
7	unknown	C	C	C	B	C	C	C
Z4	dihydrowogonin	C	C	Y	B	Y	fY	-
Z24	unknown	D	C	C	B	C	C	-
Z25	unknown	G	C	C	fB	lY	C	-
Z26	unknown	C	stB	C	fB	C	C	-
Z27	unknown	V	V	lB	C	C	C	-
Z28	unknown	C	C	Y	C	C	C	C
Mahaleb								
3	coumarin	C	C	stYG	C	lPu	lPu	C
5	unknown	BW	BW	B	stB	fY	Y	Br
6	unknown	lV	Y	fBr	B	C	C	C
7	unknown	C	C	C	B	C	C	C
H4	herniarin	stV	stV	stB	C	fT	V	C
H14	unknown	C	O	stYG(?)	B	V	lV	R
H15	unknown	C	C	stYG(?)	B	lPu	lPu	YO

[a] See Table 6 for notations.

Table 18. Rf values of phenolic compounds in the ether fraction of Mazzard and Mahaleb sap extracts on a two-dimensional paper chromatogram.[a]

Spot No.	Tentative identification	Solvent	
		BAW (6:1:2)	2% HOAc
Mazzard			
3	coumarin	0.86	0.70
5	unknown	0.93	0.00
6	unknown	0.10-0.30	0.00
7	unknown	0.00	0.00-0.70
Z4	dihydrowogonin	0.90	0.00-0.30
Z24	unknown	0.85	0.00
Z25	unknown	0.89	0.04
Z26	unknown	0.88	0.51
Z27	unknown	0.58	0.87
Z28	unknown	0.70	0.70
Mahaleb			
3	coumarin	0.87	0.68
5	unknown	0.92	0.00
6	unknown	0.22	0.00
7	unknown	0.00	0.00-0.57
H4	herniarin	0.87	0.53
H14	unknown	0.86	0.64
H15	unknown	0.87	0.72

[a] See Table 7 for notations and chromatographic conditions.

Table 19. Retention times and concentrations of coumarins extracted from centrifugal sap of Mazzard and Mahaleb as determined by gas liquid chromatography.[a]

Compound	Retention time (min)		Relative concentration (μg/g f.w.)
	2% OV-1	1% SE-30	
Mazzard sap			
Peak 3	3.7	-	-
Mahaleb sap			
Peak 1	0.2	1.3	11
Peak 2	0.5	3.4	24
Standard compound			
Coumarin	0.2	1.3	
Herniarin	0.5	3.4	
Column temp. (°C)	220	180	
Detector and injector temp. (°C)	250	250	
N_2 flow rate (ml/min)	40	80	

[a] Average of 2 determinations.

phloem tissues. The reported presence of hydrolytic enzymes in xylem sap could result in accumulation of free aglycones (Sheldrake and Northcote, 1968).

B. Neutral and acid ethyl acetate fractions

After extraction with ether, the sap was further extracted with ethyl acetate without changing the pH. The aqueous layer was then acidified and again extracted with ethyl acetate. The content of phenolic compounds was essentially the same in all 3 fractions. In Mazzard, one additional spot (Z29) appeared in the neutral fraction (Fig. 12, Table 20) and 4 more in the acid fraction (Fig. 13, Table 20). In Mahaleb, spot H16 occurred in the acid fraction (Fig. 13, Table 20). Most of the dihydrowogonin, coumarin, and herniarin appeared in the ether fraction. However, spots 5, 6, 7, H14, H15 occurred in all three fractions.

The occurrence of phenolic compounds in Mazzard and Mahaleb sap supports the theory that they can be translocated. Phenolic compounds are thought to be metabolically inert, non-translocatable secondary metabolites, which are synthesized _in situ_ from non-aromatic precursors such as carbohydrates (Hillis and Cale, 1962; Bate-Smith, 1962). This may be true for certain compounds which are limited to specific tissues. For example, anthocyanin appears in petals and fruit tissues but may

Figure 12. Paper chromatograms of phenolic compounds in the neutral fraction of Mazzard and Mahaleb sap extracts. The chromatograms were developed first with BAW (6:1:2) and secondly with 2% HOAc. The spots marked with solid lines were present in larger amounts than spots marked with broken lines. Shaded spots were common to both rootstocks. 100 µl extract (equivalent to 6.4 g fresh weight in Mazzard and 5.5 g in Mahaleb) was spotted at the origin.

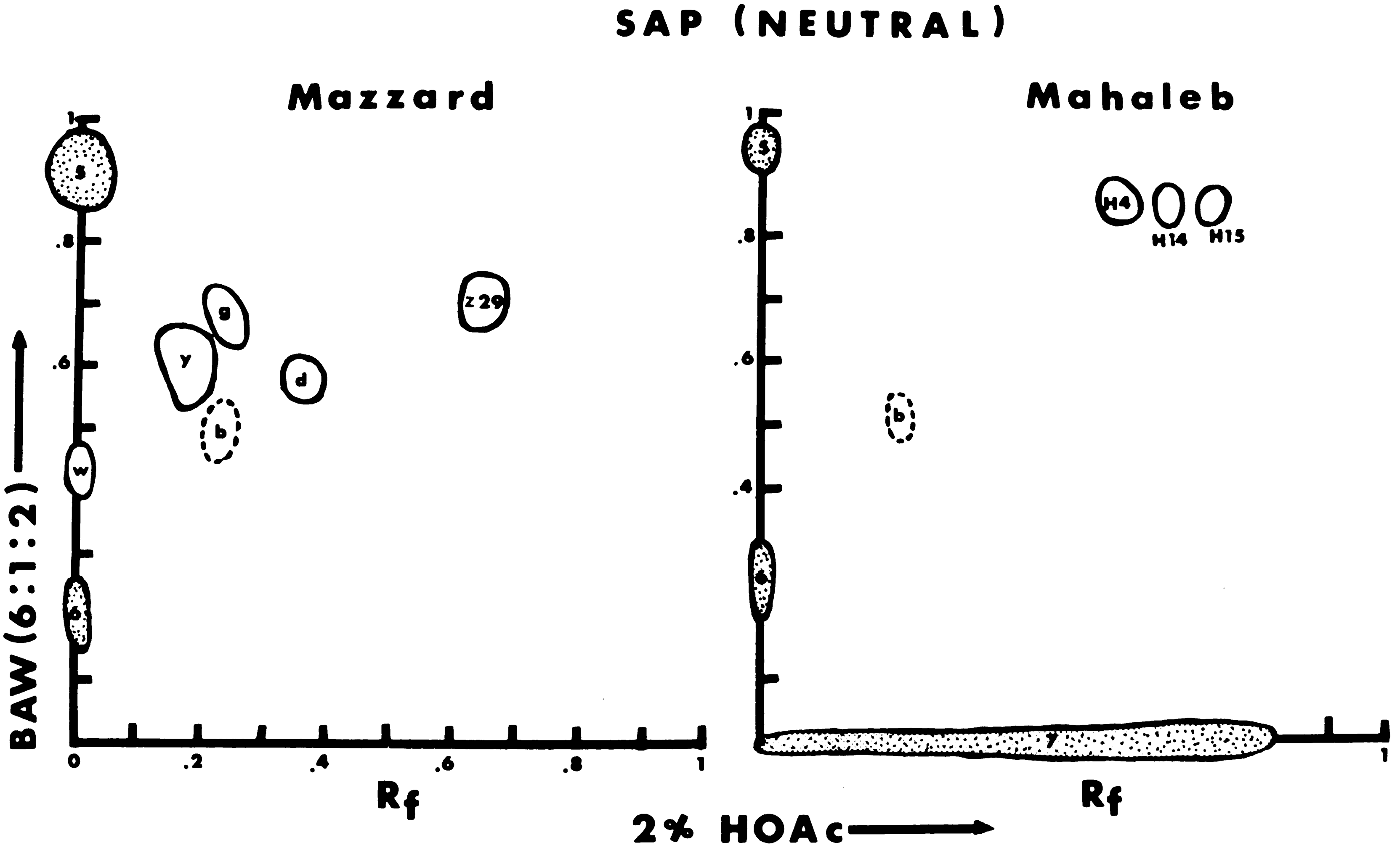

SAP (NEUTRAL)
Mazzard
Mahaleb
BAW(6:1:2)
2% HOAc
Rf
Rf
0
.2
.4
.6
.8
1
.8
.6
.4
1
S
6
w
y
g
d
b
z29
H4
H14
H15
7

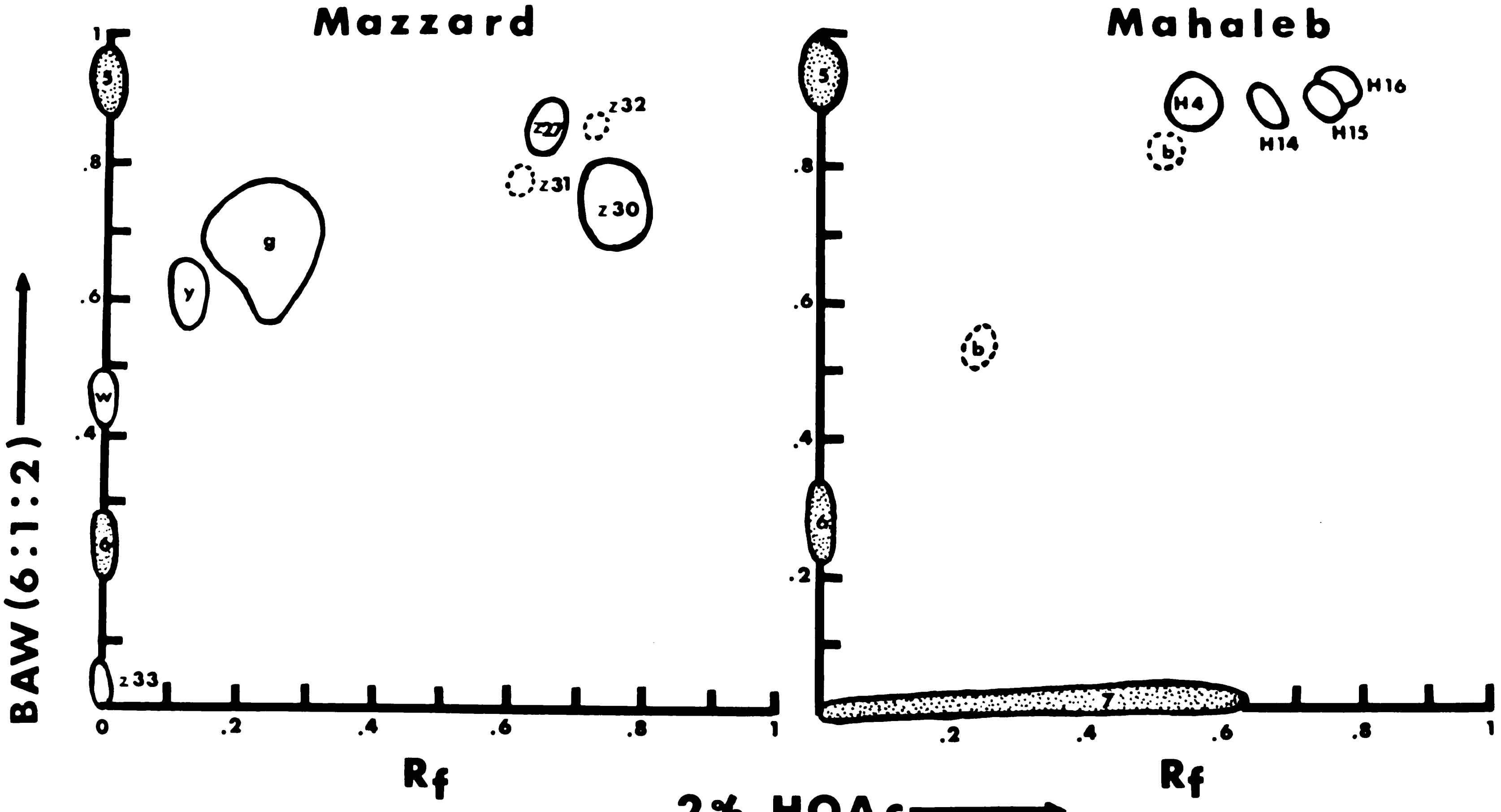
Table 20. Color reactions of phenolic compounds occurring in neutral and acidic ethyl acetate fractions of Mazzard and Mahaleb sap. Compounds that occurred in the ether fraction were omitted.[a]

Compound	UV light			Visible light			
	-	+NH_3	2N NaOH	$FeCl_3$ $K_3Fe(CN)_6$	DPNA	2N NaOH	Benzidine
Mazzard (neutral)							
Z29	D	Br	C	B	P→Br	C	R
Mazzard (acid)							
Z30	G	C	C	C	1T	C	1R
Z31	C	C	C	fB	C	C	C
Z32	C	C	C	fB	C	C	C
Z33	C	C	C	fB	C	C	C
Mahaleb (acid)							
H16	C	C	C	B	1T	C	YO

[a] See Table 6 for notation.

Figure 13. Paper chromatograms of phenolic compounds in the acid fraction of Mazzard and Mahaleb sap extracts. The chromatograms were developed first with BAW (6:1:2) and secondly with 2% HOAc. The spots marked with solid lines were present in larger amounts than spots marked with broken lines. Shaded spots were common to both rootstocks. 100 µl extract (equivalent to 6.4 g fresh weight in Mazzard 5.5 g in Mahaleb) was spotted at the origin.

SAP (ACID)


Mazzard
Mahaleb
BAW (6:1:2)
2% HOAc
Rf
Rf
1
.8
.6
.4
.2
0
.2
.4
.6
.8
1
5
6
w
z33
y
g
z27
z31
z32
z30
7
b
b
H4
H14
H15
H16

be absent from the adjacent leaves. Polyphenolic compounds in heartwood are different from those found in bark or cambium tissues. The translocation of these compounds appears to be unlikely. However, other kinds of phenolic compounds are translocatable. Aromatic amino acids such as phenylalanine and tyrosine are found in xylem and phloem sap along with sugars (Zimmerman, 1958; Hardy and Possingham, 1969). Cinnamic acids were also reported in sieve tube exudates (Macleod and Pridham, 1965). The accumulation of coumarin in normally coumarin-free tissues when they are grafted to coumarin-rich tissues also strongly suggests translocation (Gorz and Haskins, 1962; Favre-Bonvin *et al.*, 1966). Table 21 summarizes the findings of phenolic compounds in the sap of Mazzard and Mahaleb seedlings.

IV. Unhydrolyzed Phenolic Compounds in the Leaf, Stem Bark, and Root Bark of Mazzard and Mahaleb Seedlings

A. Stem bark extracts

Methanol extractable phenolic compounds were separated by two-dimensional paper chromatography. Figure 14 shows the distribution of phenolic compounds from Mazzard and Mahaleb stem bark. Both bark tissues contained 10 spots which were similar in color characteristics and Rf values (Fig. 14, Tables 22 and 23). Among these components two compounds (spots 6 and 7) were most prominent. Spot 7

Table 21. Comparison of phenolic compounds in Mazzard and Mahaleb sap extract.

Spot No.	Compound	Mazzard	Mahaleb
3	coumarin	+	+
5	unknown	+	+
6	unknown	+	+
7	unknown	+	+
Z24	unknown	+	-
Z25	unknown	+	-
Z26	unknown	+	-
Z27	unknown	+	-
Z28	unknown	+	-
Z29	unknown	+	-
Z30	unknown	+	-
Z31	unknown	+	-
Z32	unknown	+	-
Z33	unknown	+	-
Z4	dihydrowogonin	+	-
H4	herniarin	-	+
H14	unknown	-	+
H15	unknown	-	+
H16	unknown	-	+

Figure 14. Paper chromatograms of unhydrolyzed phenolic compounds in Mazzard and Mahaleb stem bark. The chromatograms were developed first with BAW (6:1:2) and secondly with 2% HOAc. The spots marked with solid lines were more intense than spots marked with broken lines. Shaded spots were common to both species. Spots restricted to one species are marked with Z (Mazzard) or H (Mahaleb). 10 µl extract of Mazzard stem bark (equivalent to 40 mg fresh weight) was spotted at the origin. In Mahaleb, 15 µl extract (65 mg fresh weight) was applied.

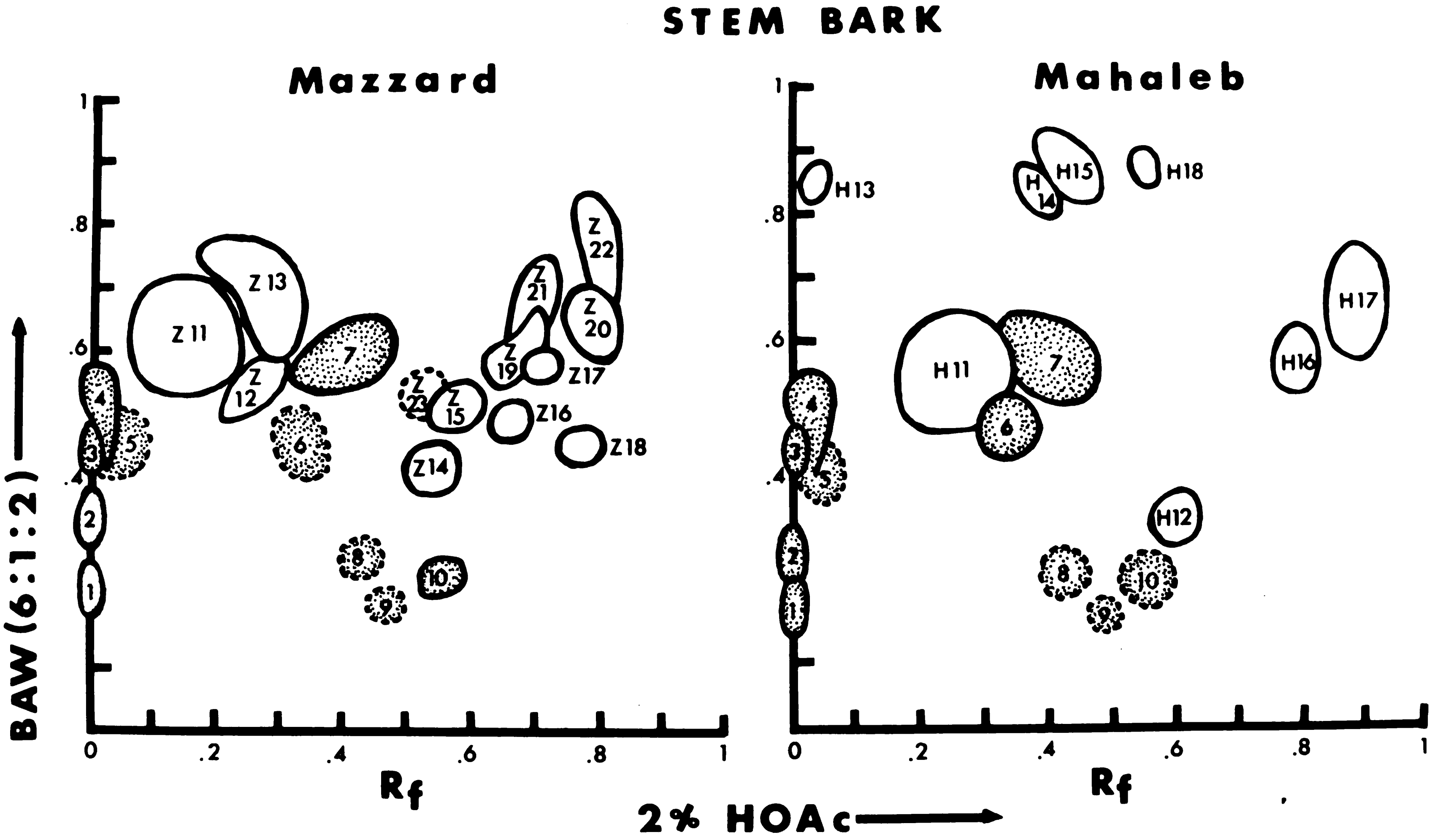

STEM BARK
Mazzard
Mahaleb
BAW (6:1:2)
2% HOAc
Rf
Rf
0
.2
.4
.6
.8
1
Z 11
Z 12
Z 13
Z 14
Z 15
Z16
Z17
Z18
Z 19
Z 20
Z 21
Z 22
Z 23
H11
H12
H13
H 14
H15
H16
H17
H18
1
2
3
4
5
6
7
8
9
10

Table 22. Color reactions of unhydrolyzed phenolic compounds occurring in Mazzard and Mahaleb root and stem bark extracts.[a]

Spot no.	Tentative identification	UV light			Visible light				
		-	+NH_3	+NaOH	$FeCl_3$ $K_3Fe(CN)_6$	DPNA	Vanillin HCl	2N NaOH	2N NaOH DPNA
Common compounds									
1	unknown	Y	O	O	B	pBr	C	C	C
2	unknown	B	Y	Y	B	pBr	C	C	C
3	unknown	Y	Y	C	B	pBr	C	C	C
4	unknown	B	B	B	C	C	Y	C	C
5	unknown	D	D	Br	C	Y	C	C	C
6	epi-catechin	C	C	Br	B	Br	pRP	Br	pBr
7	d-cathechin	(D)	(D)	C	stB	Br	P	Br	pBr
8	leucoanthocyanidin	C	C	C	B	Br	pP	Y	C
9	leucoanthocyanidin	C	C	C	B	Br	pP	Y	C
10	leucoanthocyanidin	C	C	C	B	Br	pP	Y	C
Mazzard compounds									
Z11	unknown	Br	stY	stY	pB	Br	Y	C	C
Z12	unknown	C	B	C	C	Br	C	C	C
Z13	unknown	G	B	W	C	pT	C	C	C
Z14	chlorogenic acid	pB	G	C	pB	C	C	C	C
Z15	chlorogenic acid	B	G	C	pB	C	C	C	C
Z16	chlorogenic acid	pB	G	C	pB	C	C	C	C
Z17	chlorogenic acid	B	G	C	pB	C	C	C	C
Z18	unknown	BV	B	Y	C	C	C	C	C
Z19	p-coumarylquinic acid	B	stB	stB	pB	C	C	C	C
Z20	p-coumarylquinic acid	V	stB	stB	pB	pT	C	C	C
Z21	unknown	RBr	YBr	Y	C	T	C	C	C
Z22	unknown	pB	B	C	C	C	C	C	C
Z23	unknown	C	C	C	B	O	C	Br	Br
Z24	unknown	C	C	C	B	Y	C	C	C
Z25	unknown	C	C	C	B	Y	C	C	C
Mahaleb compounds									
H11	unknown	pG	stB	C	stB	stT	C	C	C
H12	leucoanthocyanidin	C	C	C	B	Br	pP	Y	C
H13	unknown	pD	pD	C	B	pBr	C	C	C
H14	unknown	pW	B	B	C	C	C	C	C
H15	herniarin	V	V	B	C	C	C	C	V
H16	unknown	pV	C	C	C	C	C	C	C
H17	unknown	(D)	V	C	C	C	C	C	C
H18	coumarin	C	C	stYGfl	C	C	C	C	pPu
H19	unknown	C	C	B	B	Y	C	C	C

[a]See Table 6 for notations.

Table 23. Rf values of unhydrolyzed phenolic compounds occurring in extracts of Mazzard and Mahaleb root and stem bark. Compounds were chromatographed on a two-dimensional paper chromatogram.[a]

Spot No.	Tentative identification	BAW (6:1:2)	2% HOAc
Common spots			
1	unknown	.20	.00
2	unknown	.29	.00
3	unknown	.43	.00
4	unknown	.52	.02
5	unknown	.42	.06
6	epi-catechin	.45	.33
7	d-catechin	.58	.42
8	leucoanthocyanidin	.26	.43
9	leucoanthocyanidin	.19	.48
10	leucoanthocyanidin	.23	.55
Mazzard spots			
Z11	unknown	.60	.16
Z12	unknown	.53	.25
Z13	unknown	.52	.51
Z14	chlorogenic acid	.41	.52
Z15	chlorogenic acid	.50	.55
Z16	chlorogenic acid	.48	.64
Z17	chlorogenic acid	.56	.70
Z18	unknown	.44	.76
Z19	p-coumarylquinic acid	.57	.64
Z20	p-coumarylquinic acid	.63	.77
Z21	unknown	.67	.68
Z22	unknown	.74	.80
Z23	unknown	.53	.52
Z24	unknown	.43	.33
Z25	unknown	.38	.62
Mahaleb spots			
H11	unknown	.56	.57
H12	leucoanthocyanidin	.34	.59
H13	unknown	.85	.04
H14	unknown	.85	.41
H15	herniarin	.84	.46
H16	unknown	.57	.80
H17	unknown	.69	.89
H18	coumarin	.88	.55
H19	unknown	.34	.62

[a] See Table 7 for notations and chromatographic conditions.

appeared to be d-catechin. The compound gave a pinkish red color with vanillin-HCl reagent, which is characteristic of a flavan-3-ol or a flavan 2, 3 diol (Swain and Hillis, 1959). The Rf values of the compound were comparable to the values reported for catechin (Luh <u>et al</u>., 1967). Spectral characteristics of the compound also were similar to those of catechin with the maximum peak at 280 and minimum at 250 mμ (Jurd, 1962). Spot 6 gave color reactions similar to those of spot 7. According to the Rf values of the compound it is most likely epicatechin (Luh <u>et al</u>., 1967).

Spots 8, 9, and 10 did not fluoresce under ultraviolet light and did not change color in the presence of ammonia vapor. When sprayed with the vanillin-HCl reagent, they appeared a pinkish-red. The low mobility of the compounds in BAW (Rf 0.19-0.27) and the moderate mobility in 2% acetic acid (Rf 0.42-0.54) indicated that the compounds were not catechins but rather leucoanthocyanidins (Luh <u>et al</u>., 1967). The other common spots (1, 2, 3, 4, and 5) were not identified.

There were 13 species-specific spots in the Mazzard extract and 8 in the Mahaleb extract. Mazzard tissues appeared to contain several cinnamic acid derivatives, which were absent from Mahaleb. Color reactions and Rf values of spots Z14, Z15, Z16, and Z17 were comparable to those of the chlorogenic acids (Luh <u>et al</u>., 1967;

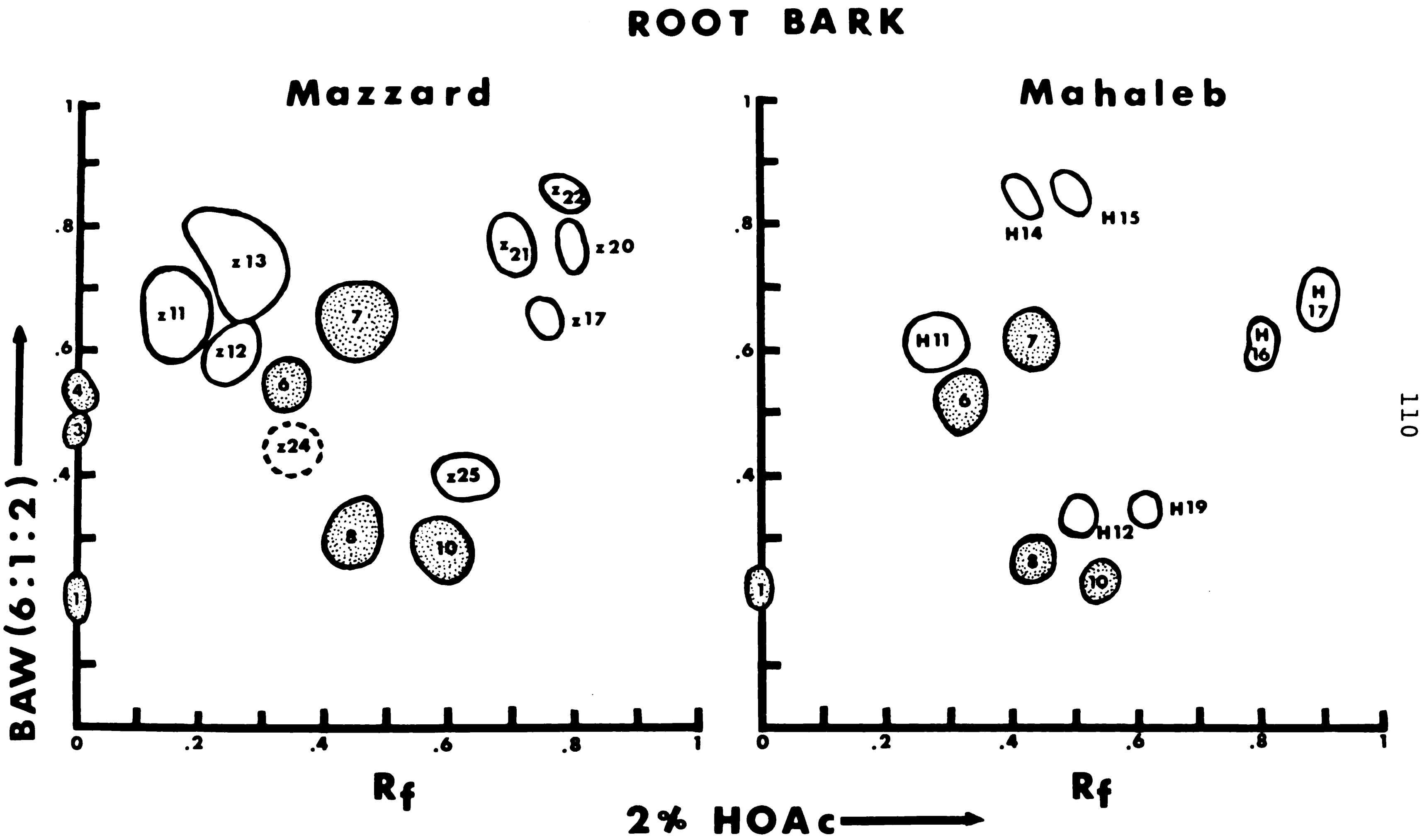
Roberts, 1962; Smith, 1960). Compounds Z19 and Z20 gave color reactions and Rf values similar to those of p-coumaryl quinic acid (Luh et al., 1967; Roberts, 1962; Smith, 1960). Other compounds in Mazzard bark extracts (Z11, Z13, Z18, Z21, and Z23) were not identified.

Among the eight Mahaleb-specific spots, only two spots were identified. These were spots H15 and H18, which had the same color characteristics and Rf values as herniarin and coumarin. Spot H12 appeared to be a leucoanthocyanidin on the basis of color reactions and Rf values. Other spots (H11, H13, H14, H16, and H17) were not identified.

B. Root bark extracts

Extracts from root bark resembled extracts from stem bark. Both Mazzard and Mahaleb root bark extracts contained epi-catechin (spot 6), d-catechin (spot 7), leucoanthocyanidins (spots 8, 9, 10) and an unknown (spot 1) (Fig. 15 and Tables 22 and 23). The Mazzard root also contained chlorogenic acid (Z17) and p-coumarylquinic acid (Z20). Other spots common to Mazzard stem and root bark were Z11, Z12, Z13, Z21, and Z22. Mazzard root bark extracts contained two additional spots, Z24 and Z25 Mahaleb root bark extracts contained 11 of the phenolic compounds found in stem bark (Fig. 14), and one additional compound (H19).

Figure 15. Paper chromatograms of unhydrolyzed phenolic compounds in Mazzard and Mahaleb root bark. The chromatograms were developed first with BAW (6:1:2) and secondly with 2% HOAc. Shaded spots were common to both species. Spots restricted to one species are marked with Z (Mazzard) or H (Mahaleb). 15 µl extract (equivalent to 26 mg in Mazzard and to 58 mg in Mahaleb) was spotted at the origin.


ROOT BARK
Mazzard
Mahaleb
BAW (6:1:2)
2% HOAc
Rf
Rf
0
.2
.4
.6
.8
1
1
3
4
6
7
8
10
z11
z12
z13
z17
z20
z21
z22
z24
z25
H11
H12
H14
H15
H16
H17
H19

C. Leaf extracts

Leaf phenolics differed from those of found in stem bark and root bark (Fig. 16, Table 24). First, there were markedly fewer compounds in the leaves. Secondly, no compounds were common to both species. Thirdly, leucoanthocyanidins and catechins were low in concentration or completely lacking (Fig. 16). The Mazzard leaf contained epi-catechin (spot 6), catechin (spot 7), chlorogenic acid isomers (Z15, Z16), and p-coumaryl quinic acid (Z19). The Rf values and color reactions of spot Z28 were similar to those of standard caffeic acid. Several other unidentified spots (Z26, Z27, Z30, and Z31) appeared in the leaf extracts (Fig. 16). Only 7 spots were distinguishable in Mahaleb leaf extracts. Four of these spots (H14, H15, H16, and H17) also occurred in stem and root bark. Three additional spots (H19, H20, and H21) were not identified.

All the phenolic compounds which were observed in unhydrolyzed extracts of leaf, bark, and root bark are listed in Table 25. Within species, bark tissues, whether from root or stem, were similar in their phenolic composition. However, the phenolic composition of leaf tissue differed markedly from that of the bark tissue. Extracts from Mazzard and Mahaleb bark contained several phenolics in common whereas the leaf extracts contained none in common. Certain compounds were

Figure 16. Paper chromatograms of unhydrolyzed phenolic compounds in Mazzard and Mahaleb leaf tissues. The chromatograms were developed first with BAW (6:1:2) and secondly with 2% HOAc. 30 µl extract (equivalent to 3.75 g fresh weight) was spotted at the origin.

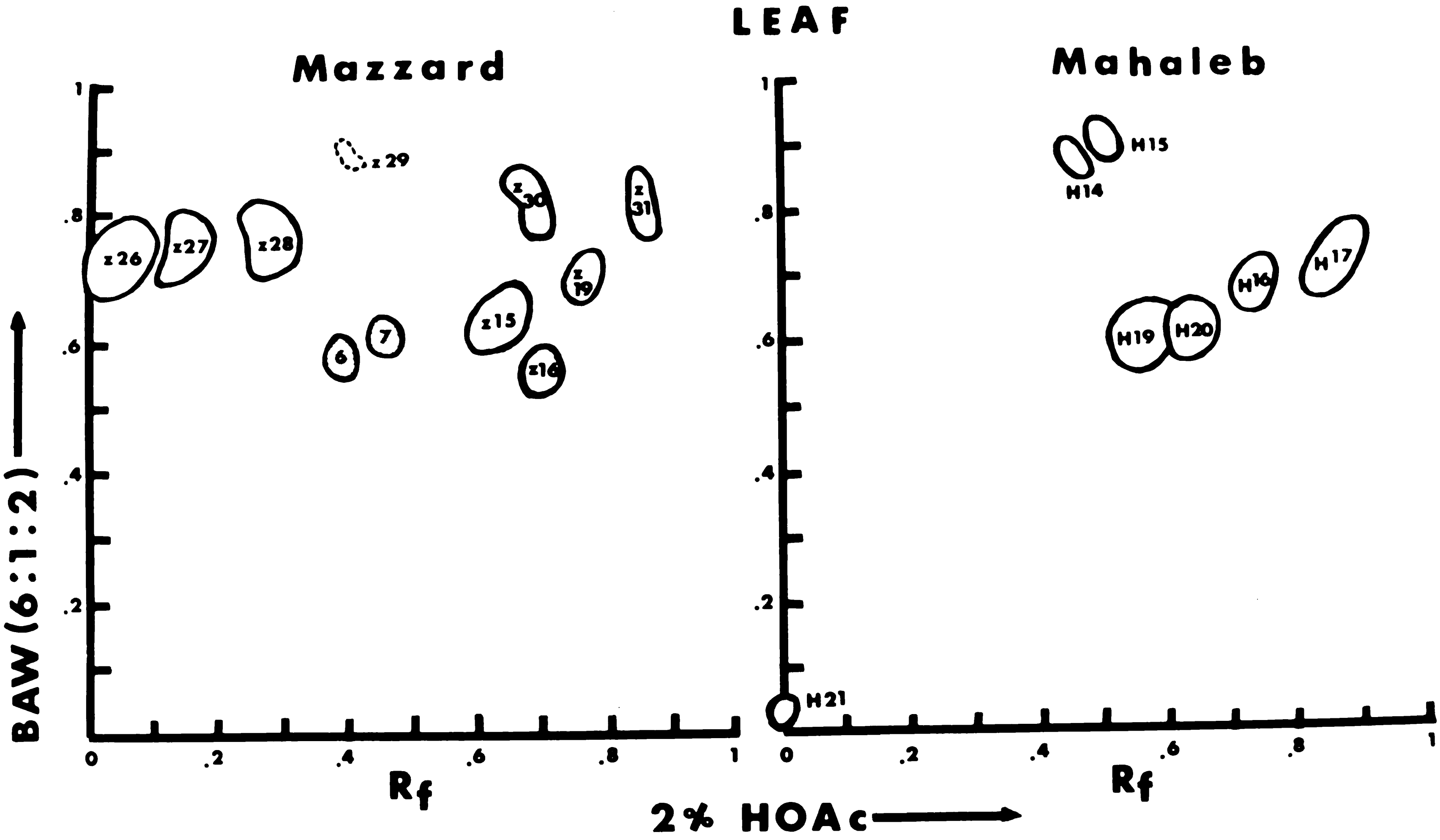

LEAF
Mazzard
Mahaleb
z26
z27
z28
z29
6
7
z15
z16
z
30
z
19
z
31
H14
H15
H16
H17
H19
H20
H21
BAW (6:1:2)
Rf
Rf
2% HOAc
0
.2
.4
.6
.8
1

Table 24. Color reactions and Rf values of unhydrolyzed phenolic compounds occurring in Mazzard and Mahaleb leaf extracts. Spots occurring in stem bark were omitted.[a]

Spot No.	Tentative identification	Color reaction under				Rf values	
		UV light		Visible light			
		-	+NH_3	$FeCl_3$ $K_3Fe(CN)_6$	DPNA	BAW	2% HOAc
Mazzard leaf							
Z26	unknown	B	B	B	C	.75	.05
Z27	unknown	B	stG	B	C	.76	.16
Z28	caffeic acid	B	B	B	T	.77	.28
Z29	unknown	pB	stB	C	C	.90	.40
Z30	unknown	pB	stB	C	C	.82	.69
Z31	unknown	D	D	C	C	.80	.85
Mahaleb leaf							
H20	unknown	(D)	(D)	C	C	.61	.57
H21	unknown	lB	lB	C	C	.62	.63
H22	unknown	C	B	C	C	.02	.00

[a] See Tables 6 and 7 for notations.

Table 25. Comparison of unhydrolyzed phenolic compounds in extracts of leaf, stem bark, and root bark of Mazzard and Mahaleb.

Spot No.	Compound	Mazzard			Mahaleb		
		Leaf	Stem bark	Root bark	Leaf	Stem bark	Root bark
1	unknown	-	+	+	-	+	+
2	unknown	-	+	-	-	+	-
3	unknown	-	+	+	-	+	-
4	unknown	-	+	+	-	+	-
5	unknown	-	+	-	-	+	-
6	epi-catechin	+	+	+	-	+	+
7	d-catechin	+	+	+	-	+	+
8	leucoanthocyanidin	-	+	+	-	+	+
9	leucoanthocyanidin	-	+	+	-	+	+
10	leucoanthocyanidin	-	+	+	-	+	+
Z11	unknown	-	+	+	-	-	-
Z12	unknown	-	+	+	-	-	-
Z13	unknown	-	+	+	-	-	-
Z14	chlorogenic acid	-	+	-	-	-	-
Z15	chlorogenic acid	+	+	-	-	-	-
Z16	chlorogenic acid	+	+	-	-	-	-
Z17	chlorogenic acid	-	+	+	-	-	-
Z18	unknown	-	+	-	-	-	-
Z19	p-coumarylquinic acid	+	+	-	-	-	-
Z20	p-coumarylquinic acid	-	+	+	-	-	-
Z21	unknown	-	+	+	-	-	-
Z22	unknown	-	+	+	-	-	-
Z23	unknown	-	+	-	-	-	-
Z24	unknown	-	-	+	-	-	-
Z25	unknown	-	-	+	-	-	-
Z26	unknown	+	-	-	-	-	-
Z27	unknown	+	-	-	-	-	-
Z28	caffeic acid	+	-	-	-	-	-
Z29	unknown	+	-	-	-	-	-
Z30	unknown	+	-	-	-	-	-
Z31	unknown	+	-	-	-	-	-
H11	unknown	-	-	-	-	+	+
H12	leucoanthocyanidin	-	-	-	-	+	+
H13	unknown	-	-	-	-	+	+
H14	unknown	-	-	-	+	+	+
H15	herniarin	-	-	-	+	+	+
H16	unknown	-	-	-	+	+	+
H17	unknown	-	-	-	+	+	+
H18	coumarin	-	-	-	?	+	?
H19	unknown	-	-	-	-	-	+
H20	unknown	-	-	-	+	-	-
H21	unknown	-	-	-	+	-	-
H22	unknown	-	-	-	+	-	-

widespread in their occurrence. For example, all Mazzard tissues sampled contained chlorogenic acid, p-coumaryl quinic acid, catechin, and epi-catechin. The Mahaleb tissues shared herniarin and possibly coumarin, and 3 other compounds (H14, H16, H17). The differences noted between leaf and bark tissues indicate that all the phenolic compounds found in bark tissues are not translocated from the leaves. However, some of the phenolic compounds may be translocated, especially coumarin and herniarin, and possibly p-coumarylquinic acid and chlorogenic acid.

Table 26 summarizes the phenolic compounds identified from the studies of sections II, III, and IV.

Table 26. Comparison of known phenolic compounds in extracts of leaf, stem bark, and root bark of Mazzard and Mahaleb seedlings.

Compounds	Leaf		Stem bark		Root bark		Sap	
	Maz[a]	Mah[a]	Maz	Mah	Maz	Mah	Maz	Mah
Phenolic acids								
o-coumaric acid	+	+	+	+	+	+	-	-
p-coumaric acid	+	-	+	-	+	-	-	-
caffeic acid	+	-	-	-	-	-	-	-
p-coumarylquinic acid	+	-	+	-	+	-	-	-
chlorogenic acid	+	-	+	-	+	-	-	-
Coumarins								
coumarin	+	+	+	+	+	+	-	+
herniarin	-	+	-	+	-	+	-	+
Flavonoids								
dihydrowogonin	+	-	+	-	+	-	+	-
kaempferol	+	+	-	-	-	-	-	-
quercetin	+	-	-	-	-	-	-	-
d-catechin	+	-	+	+	+	+	-	-
epi-catechin	+	-	+	+	+	+	-	-
leucoanthocyanidin	-	-	+	+	+	+	-	-

[a] Maz = Mazzard; Mah = Mahaleb.

DISCUSSION

Mahaleb rootstocks, which show graft-incompatibility symptoms with certain sweet cherry cultivars, differ in phenolic composition from Mazzard rootstocks, which are fully compatible with sweet cherries. The former contained lower levels of phenolic compounds in their leaves, root bark, and stem bark (Fig. 3). These differences have implications when sweet cherries are grafted on Mahaleb rootstocks. Mahaleb tissues may not have an efficient system for handling large quantities of phenolics, which could interfere with physiological processes (Mayer et al., 1961; Goodwin and Taves, 1950; Stenlid, 1963; Frey-Wyssling, 1942). Mahaleb roots may be more sensitive to sweet cherry phenolics, which might accumulate in the soil as a result of leaching or decomposition of leaf tissues.

There are also qualitative differences in phenolic compounds in the two rootstocks (Tables 16, 21, 24, 25, and 26). Mazzard tissues contain a greater variety of phenolics. For example, in unhydrolyzed tissues 31 compounds were present in Mazzard as determined by paper chromatography, but only 22 in Mahaleb. Qualitative differences were particularly noted in phenolic acids, coumarins, and flavonoids (Table 26).

Phenolic acids. Mazzard tissues contained high levels of hydroxy cinnamic acid derivatives, including p-coumaric, o-coumaric, caffeic, p-coumaryl quinic, and chlorogenic acids, whereas Mahaleb tissues contained only o-coumaric acid. In his survey of leaf phenolics in Prunus, Bate-Smith (1961b) found p-coumaric, o-coumaric, and caffeic acids in Mazzard. In Mahaleb, o-coumaric acid was the only phenolic acids identified, although the presence of p-coumaric acid was suspected.

The two rootstocks may have different biosynthetic scheme of phenolic acids by differing in their hydroxylation pattern of cinnamic acid, which is known to be an important precursor of phenolic acids in higher plants (Steck, 1968; Neish, 1964). Mahaleb tissues, which contain large amounts of coumarin and herniarin, apparently hydroxylate only the ortho position of cinnamic acid, hence p-coumaric and caffeic acids are lacking. Mazzard tissues, however, appear to be able to hydroxylate both the ortho and the para position of the benzene ring of cinnamic acid.

The esters of p-coumaric and caffeic acids may regulate levels of IAA in plant tissues by influencing its decarboxylation. Tomaszewski and Thimann (1966) showed that polyphenols such as chlorogenic acid and caffeic acid reduced the inactivation of IAA, whereas monophenols such as p-hydroxy benzoic acid and p-coumaric acid increased the inactivation. In Mazzard, caffeic acid was mainly located in leaf tissues, whereas p-coumaric acid was high in stem and root bark and low in leaf tissue. Thus, translocation of p-coumaric acid from a sweet cherry scion to a Mahaleb stock could conceivably favor decarboxylation of IAA and thereby reduce the vigor of the rootstock.

Coumarins. The two rootstocks differed markedly in coumarins. All Mahaleb tissues tested contained high levels of both coumarin and herniarin, whereas Mazzard contained no herniarin and showed high coumarin activity only in leaf tissues. Favre-Bonvin _et al_. (1966) considered that _P_. _avium_ was incapable of synthesizing coumarin. However, o-coumaric acid can be easily transformed to coumarin in acid solution or on exposure to light (Bate-Smith, 1961b; Edwards and Stoker, 1968). Therefore, the coumarin observed in Mazzard hydrozates may be an artifact.

The inhibitory activity of coumarins in root growth, in germination and in mitosis (Goodwin and Taves, 1950; Thimann and Bonner, 1949; Mayer and Poljakoff-Mayber, 1961) suggest a role for these compounds in the graft-incompatibility of *P. avium*/*P. mahaleb*. This is unlikely, however, for the following reasons. The major sites of synthesis of coumarins are the aerial parts, especially the young leaves (Gorz and Haskins, 1962; Favre-Bonvin *et al*., 1966, 1968). After the fourth year of grafting of *P. avium* on *P. mahaleb*, Favre-Bonvin *et al*. (1966) noted the complete disappearance of coumarins from the stocks. During this period the trees grow vigorously with no symptoms of incompatibility (Garner, 1967).

At low concentrations, coumarins stimulate plant growth (Mayer and Poljakoff-Mayber, 1961). Instead of being inhibitory, they might be essential for normal growth. Their disappearance from root tissues after several years may be related to the loss of vigor of the tree. Coumarin derivatives such as scopolin and scopoletin are known to inhibit IAA-oxidase (Andreae, 1952; Gortner and Kent, 1958; Schaeffer *et al*., 1967). Thus, the absence of coumarins in Mahaleb rootstocks may allow the destruction of IAA. Alternatively, their absence may limit lignin synthesis, leading to incompatibility. Kosuge and Conn (1961) reported that labelled coumarin was rapidly transformed into β-glucosides of o-hydroxy cinnamic acids. The latter are known to be incorporated into lignin (Neish, 1964; El-Basyouni *et al*., 1966).

Flavonoids. Mazzard tissues contain three flavonoids, dihydrowogonin, kaempferol, and quercetin. The latter two are found only in leaf tissues. Kaempferol also occurs in Mahaleb leaf tissues, but quercetin was not found. Bate-Smith (1961b) found both quercetin and kaempferol in hydrolysates of Mahaleb leaves. The concentration of quercetin in leaves of the Mahaleb seedlings used in the present study may have been too low to be detectable, or occurrence may depend upon tree age. Dihydrowogonin occurred in all Mazzard tissues tested, as well as in sap. Mature cherry trees are known to contain various other flavonoids in the heartwood (see Table 1). None of these compounds was noted in the extracts of either bark or leaf tissues of Mazzard and Mahaleb seedlings.

Flavonoids act as synergists or antagonists to IAA *in vitro*. Kaempferol conjugates promote IAA oxidation while quercetin conjugates inhibit its oxidation (Furuya and Galston, 1965). The relatively high amount of kaempferol in Mahaleb suggests that it may have a higher rate of decarboxylation of IAA than Mazzard. The activity of flavonoids in IAA decarboxylation appears to depend on the hydroxylation pattern in the B ring. The effect of dihydrowogonin on IAA oxidase is difficult to estimate because of the lack of a hydroxyl group in the B ring.

However, the wide occurrence of the compound in Mazzard tissue and its presence in the sap suggest that it may be important in graft-incompatibility.

SUMMARY AND CONCLUSIONS

Phenolic compounds in leaf, bark, and root tissues from Mazzard and Mahaleb seedlings were examined. In field-grown seedlings, Mazzard tissues contained much higher levels of total phenolic compounds than did the Mahaleb seedlings during a five-month growing period. The phenolic level increased during the first two months, then stabilized for the remaining period. However, the Mahaleb root tissue varied during the season, in that the phenolic content rose rapidly at beginning of growth, then dropped sharply during the latter part of the season. Furthermore, the bark tissues contained higher levels of phenolic compounds than did the leaf tissues.

These facts apparently relate to the imperfect graft of some sweet cherry cultivars on Mahaleb rootstocks. Since sweet cherry cultivars are closely related to Mazzard, and since Mazzard contained 3 times as much total phenolic compounds than Mahaleb, it can be proposed that (1) the abnormal amounts of phenolics in sweet cherry species are toxic to the Mahaleb, (2) that the phenolics translocate across the graft union from sweet cherry to the Mahaleb or (3) the phenolic balance between these 2 species interferes with normal growth.

In summary it also should be noted that the levels of phenolic compounds were consistently higher in Mazzard than in Mahaleb tissues when grown under greenhouse conditions. Furthermore, the greenhouse-grown seedlings contained only 10% as much total phenolics as the field-grown seedlings.

Thus, this data strongly indicates that, the climatic conditions under which the trees are grown play an important role in the normal synthesis of phenolic compounds both in the rootstock and in the scion. The so-called incompatibility conditions have been observed to be more prevalent in some fruit-growing areas than in others.

Variable amounts of phenolic compounds were found in the different tissues of Mazzard and Mahaleb. For example, dihydrowogonin was found in all the tissues tested of Mazzard, but not in Mahaleb; herniarin was in all tissues tested of Mahaleb, but none in Mazzard; and caffeic acid was found only in the Mazzard leaf tissue.

As in the application of balanced nutrient requirements of plants, so must there apparently also be an internal balance of phenolic components in the 2 plants which are combined by grafting.

SUGGESTIONS FOR FURTHER STUDY OF GRAFT RELATIONSHIPS IN CHERRY CLONES

1. Seasonal levels of phenolic compounds in the phloem sap of sweet cherry/Mahaleb;
2. The effect of dihydrowogonin and p-coumaric acid on cambial activity of Mahaleb stem;
3. Differences in phenolic compounds of Mahaleb clones, compatible and incompatible;
4. The effects of soil accumulated phenolic compounds on the growth of Mahaleb root;
5. Nature of metabolites in phloem above and below graft union;
6. The effect of reproductive activity on the distribution of metabolites between two compatible and incompatible rootstocks;
7. Extensive field survey of the behavior of sweet cherry/Mahaleb in relation to factors such as soil drainage and climactic conditions.

8. The relationship between the loss of synthetic ability of coumarins and the vigor of Mahaleb roots;
9. The biological effects of dihydrowogonin on IAA oxidase, root growth, germination, and stem growth;
10. Graft-incompatibility of sweet cherry/Mahaleb may be due to either lack of metabolites or toxic effects of one of the graft partners:
    a. Toxic action of scion on stock,
    b. Toxic action of stock on scion,
    c. Lack of metabolites in scion for stock,
    c. Lack of metabolites in stock for scion.

This could be studied with various graft combinations between stock and scion.

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LITERATURE CITED

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