A STUDY OF CRUDE AND FRACTIONATED WILLOW EXTRACTS FOR ROOTING

by

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Short Title

WILLOW EXTRACTS AS ROOTING AID

ABSTRACT

M.Sc. LUCE DAIGNEAULT Plant Science

A STUDY OF CRUDE AND FRACTIONATED WILLOW EXTRACTS FOR ROOTING

The influence of crude water extract of willow as a rooting aid and its interaction with auxins was studied with cuttings of 13 woody ornamental species. Three of four Group 1 species (shrubs) and one of five Group 3 species (evergreens) showed distinct positive response to willow extracts while none of four Group 2 (trees) species showed any response. All Group 3 species showed positive rooting response to auxins while three out of four Group 1 species were adversely affected by auxin.

Using mung bean rooting tests in a controlled environment, fractionation techniques, and paper chromatography, attempts were made to identify and characterize the nature of the root promoting activity in willow extracts. Rooting activity was greater in extracts from plant materials collected in winter months than in those of the summer months. Positive correlations were obtained between root number of mung bean cuttings and total, dihydroxy, and alkali-labile phenol contents in seasonal willow extracts.

Water extracts or their fractions were superior in rooting activity to those of methanol or ethyl acetate counterparts. Indoleacetic acid was detected in the ethyl acetate sub-fractions and an indole compound in both the ethyl acetate and water sub-fractions.

RESUME

M.Sc. LUCE DAIGNEAULT Plant Science

ETUDE DES EXTRAITS DE SAULE NON-RAFFINES ET FRACTIONES POUR L'ENRACINEMENT

L'influence d'extraits aqueux non-raffinés de saule comme agent d'enracinement et leur interaction avec des auxines ont été étudiées sur des boutures de 13 espèces arbustives ornementales. Trois des quatre espèces du Groupe 1 (arbustes) et une des cinq espèces du Groupe 3 (conifères) démontrent une réponse positive distincte aux extraits de saule tandis qu'aucune des espèces du Groupe 2 (arbres) y réagissent. Toutes les espèces du Groupe 3 répondent positivement aux auxines tandis que trois des quatre espèces du Groupe 1 y réagissent négativement.

A l'aide de tests d'enracinement avec des fèves mung en environnement contrôlé, de techniques fractionnelles et de chromatographie sur papier, des essais ont été menés afin d'identifier et de caractériser la nature de l'activité promotrice d'enracinement des extraits de saule. L'activité d'enracinement des extraits est plus forte dans les extraits provenant de matériels végétaux prélevés pendant les mois d'hiver que durant les mois d'été. Des corrélations positives ont été obtenues entre le nombre de racines par bouture de fèves mung et les contenus en phénol total, dihydroxique, et alcali-labile des extraits saisonniers.

Des extraits aqueux ou leurs fractions ont une activité d'enracinement supérieure à leur contrepartie méthanolique ou d'acétate d'éthyle. L'acide indoleacétique a été détecté dans les sous-fractions d'acétate d'éthyle et un composé indolé, et dans les sous-fractions d'acétate d'éthyle et d'eau.

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LIST OF ABBREVIATIONS

ABA	abscisic acid
chloramine-t	N-chloro-p-toluene sulfonamide sodium
DiOH	dihydroxy
EToAc	ethyl acetate
F_W	water sub-fractions
F_E	ethyl acetate sub-fractions
GA_3	gibberellic acid
IAA	indoleacetic acid
IAN	indoleacetonitrile
IBA	indolebutyric acid
NAA	napthtaleneacetic acid
ortho	o
para	p
PG	phloroglucinol
RN	mean root number
RL	mean root length
RP	rooting percentage
TLC	thin layer chromatography
WE	willow extract

INTRODUCTION

Propagation by stem cuttings is one of the most important and basic areas of nursery culture (Girouard and Hess 1964; Hartmann and Kester 1975; Hess 1963). As a vegetative means of propagation, this technique preserves the phenotypic and genotypic characteristics of ornamental plants (Hartmann and Kester 1975). However, numerous factors influence rooting of cuttings.

The time of the year at which cuttings are taken can have a dramatic influence on rooting (Hartmann and Kester 1975), but little is known about the controlling mechanism of this response (Lanphear 1963). The seasonal change in rootability of cuttings appears to be related to the level of endogenous hormones (Alvim et al. 1976; Nanda and Jain 1972), rooting cofactors (Lanphear and Meahl 1966), and inhibitors in the buds (Fadl and Hartmann 1967).

Van Overbeek (1941) showed that only 5% of the plant auxin exists in a free form while most of the indoleacetic acid (IAA) is bound in the plant as an IAA-conjugate or IAA rooting cofactor complex (Cohen and Bandurski 1982). The ability of exogenously applied natural and synthetic auxins to promote adventitious root formation on stem cuttings of several species has been recognized for many years (Cooper 1935; Thimann and Koepli 1935; Went and Thimann 1937). Studies have also demonstrated the lack of successful rooting of certain species when treated with auxins.

The complexity and variability in rooting response indicated that

there is an interaction of several substances (Thimann 1977) which include components such as auxin, rooting cofactors, and inhibitors (Hartmann and Kester 1975). Some of these rooting cofactors have been shown to be phenols (Hess 1963; Mosella Chancel and Macheix 1979), and others have been proposed to be carbohydrates (Atman and Wareing 1975; Hansen 1976), lipids (Heuser and Hess 1972) or other factors (Hemberg 1953; Van Overbeek and Gregory 1945).

In comparison with easy-to-root species, hard-to-root species are either lacking endogenous auxin, some rooting cofactors or contain some inhibitors not found in the easy-to-root form (Ashiru and Carlson 1938; Biran and Halevy 1973; Fadl and Hartmann 1967a; Hess 1961; Van Overbeek and Gregory 1945).

Chemlar (1974) tested the rooting capacity of 107 willow taxa (Salix sp.) and found that most of them rooted readily. Willows have been shown to have preformed lateral root primordia at their nodes (Carlson 1938). Kawase (1964, 1970, 1971, 1981), Kikuchi et al. (1983), and Richer-Leclerc and Chénier (1983) have demonstrated the root-promoting activity of the water extracts from Salix alba, S. kariyanagi, S. bakko, and S. alba tristis on rooting of mung beans and many woody plant species. Kawase suggested that the willow extracts contain large amounts of endogenous cofactors, as yet unidentified, and the right balance of hormone and rooting substances capable of improving rooting.

The purpose of this study was to investigate the influence of crude willow extracts as rooting promoters and the influence of these plant extracts with auxins on rooting of woody ornamental cuttings. In an attempt to identify the rooting substances and the nature of their rooting

activity in willow extracts (a) a systematic series of experiments using mung bean rooting tests under controlled environment were conducted with willow extracts that were subjected to progressive steps of greater purification, and (b) selected extracts were analyzed for the presence of phenolic and auxinic compounds.

LITERATURE REVIEW

1. Factors influencing rooting of cuttings

Various physiological, anatomical and environmental factors influence rooting of cuttings. Many of these factors can be controlled or modified: the time of the year in which cuttings are taken; the source and the type of wood selected for cuttings, such as length and width of cuttings, lateral versus terminal shoots, different parts of the shoot; the environmental conditions and nutritional status during growth of stock plants, such as light intensity and photoperiod; the environmental conditions during rooting such as humidity control by misting; the composition and temperature of the rooting medium; light intensity and duration during rooting (Hackett 1969; Hartmann and Kester 1975; Stromquist and Eliasson 1979).

Other factors may be less amenable to control: genetic origin, growth habit and age of stock plants; physiological condition; endogenous composition of hormones, rooting cofactors, and(or) inhibitors (Hartmann and Kester 1975).

Woody shrub and tree species are generally more difficult to propagate than herbaceous species (Hartmann and Kester 1975). It is within this context of difficult-to-root species that a review of factors influencing rooting of trees and shrubs is directed.

1.1 Genetic differences

Differences in ease of rooting and seasonal rooting pattern were found among three cultivars of holly: 'Arden', 'Old Hale and Hearty', and 'Cumberland' (Childers and Snyder 1957). Profound differences in rooting were also reported between cherry cultivars: 'Malaheb', 100%; 'Montmorency', 90%; 'Stockton Morello', 77%; 'Black Tartarian', 40%; 'Bing', 20%; and 'Napoleon', 0% (Hartmann and Brooks 1958). Varietal variations were also observed in Olea europaea (Loreti and Hartmann 1964), Pyrus communis (Fadl and Hartmann 1967b), Vaccinium angustifolium (O'Rourke 1944), Acer, Azalea, Cotoneaster, Cotinus, Eleagnus, Forysthia, Juniperus, Magnolia, Rhododendron and Viburnum (Lamb and Kelly 1982). Patton and Riker (1958) observed variation in rooting ability of 10 clones of 12-year-old Pinus strobus. Howard and Shepherd (1978) reported large differences in rooting among individual trees of Tilia cordata, Tilia europaea, and Acer campestris and even among cuttings from the same plant. Miller et al. (1982) observed genotypic variation in rooting percentage, and in number and length of roots of Fraser fir cuttings.

Howard and Shepherd (1978) observed variation in rooting response of hardwood cuttings of 21 woody tree species treated with 5000 ppm indolebutyric acid (IBA). Treatments with IBA between 0 and 5000 ppm greatly enhanced rooting of 'Myrobalan B' and 'St-Julien' plums with optimum rooting at 5000 ppm (Howard and Nahlawi 1969). With 'Bramptom' and 'EA.16' only a slight enhancement was noted, with no improvement beyond treatment with 1250 ppm IBA (Howard and Nahlawi 1969).

1.2 Growth phases

Davies et al. (1982) found that juvenile cuttings of Ficus pumila rooted easily whereas the mature cuttings did not root at all. This phenomenon was found to be a common occurrence in 30 woody tree species tested by Gardner (1929). One-year-old seedlings of all species rooted, and rooting potential tended to decrease rapidly with increasing age of plants (Gardners 1929). Vieitez (1968) indicated that, for some species like chestnut, successful rooting of cuttings was restricted to the seedling phase. In experiments with cuttings of 6- to 12-years-old Norway spruce, Roulund (1975) showed that there was a decrease in rooting with increasing age: 4% per year in trees 7- to 9-years-old, 6.3% per year in trees 9- to 13-years-old, and 1.3% per year in trees 13- to 21-years-old. Thus the age of the stock plant is an important consideration especially for plants in the more difficult-to-root category.

Beakbane (1969) proposed that juvenility in relation to rooting may be explained by the presence of anatomical barriers. Blair et al. (1956) showed that cuttings of juvenile phase Malus robusta, which rooted much more readily than the adult phase, had less phloem fibers. Davies and Joiner (1980) observed that juvenile cuttings of Ficus pumila required 1000-1500 ppm of IBA for best rooting, whereas mature cuttings required 2000-3000 ppm. They proposed that juvenile cuttings contained more endogenous auxin than the adult ones. Thimann and Delisle (1939) showed that juvenile plants contained more rooting cofactors, and that some mature plants were devoid of such factors. Hess (1962) demonstrated the

presence of four rooting cofactors in the easily-rooted, juvenile form of Hedera helix whereas the difficult-to-root mature form contained less root-promoting cofactors.

In adult Eucalyptus deglupta, Paton et al. (1970) reported the presence of three inhibitors determined to be naturally occurring derivatives of the 2,3-dioxabicycle(4,4,0)decane system; similar compounds were not found in the juvenile phase. According to Paton et al. (1970), phase change in relation to rooting may be explained by the increasing production of inhibitors as plants increase in age.

Several authors reported a juvenility gradient within a tree (Passecker 1949; Schaffalitzky de Muckadell 1954). While Passecker (1949) assigned three main zones in a tree (juvenile, intermediate, and mature zones), Schaffalitzky de Muckadell (1954) indicated that the physiological adult characteristics arose first at the periphery of the tree while the interior portion around the stem base remained juvenile for a long time. Roulund (1975) showed that the rooting ability of Norway spruce cuttings increased within a tree from the top to the lower part of the crown.

Sussex (1976) indicated that juvenile to adult phase changes did not have their basis at the cellular level in the meristems per se and thus were not only ontogenetic; these changes were imposed upon the meristem by the remainder of the organism in response to environmental and nutritional factors.

1.3 Selection of cuttings

Differences in rooting ability due to location of shoots used for cut-

tings has been recognized for many years (Hartmann and Kester 1975). O'Rourke (1944) showed that in three blueberry cultivars, rooting of cuttings increased progressively from the terminal to the basal position. Similarly, Loreti and Hartmann (1964) reported that sub-terminal and basal sections of two olive cultivars rooted more readily than soft terminal sections. On the contrary, Hartmann and Brooks (1958) reported that softwood terminal cuttings from three cherry cultivars rooted better than basal cuttings. Gardner and Hatcher (1955) also reported that terminal cuttings of apple and plum rootstocks planted directly into the nursery soil in autumn rooted easier than more distal (sub-terminal) cuttings. Miller et al. (1982) found that the lateral cuttings from Fraser fir rooted better than terminal ones. These results were associated with earlier breaking of dormancy of lateral buds in the spring (Miller et al. 1982).

Howard and Nahlawi (1969) showed that position of cuttings within shoots of three plum cultivars did not influence rooting, although 'Myrobalan B' plum rootstocks showed increased rooting from terminal to sub-terminal positions. These researchers also showed that thin shoots of all cultivars rooted more readily than thick shoots.

It appears that in the more difficult-to-root species, the choice of cuttings from shoots that are in either a flowering or vegetative condition is an important factor (Hartmann and Kester 1975). In blueberry (Vaccinium atrococcum), O'Rourke (1940, 1944) observed that hardwood cuttings from shoots bearing flower buds rooted less readily than those bearing only leaf buds. Antagonism between regenerating capacity and flow-

ering also has been reported for Armoracia rusticana (Doré 1953) and Rubus idaeus (Hudson 1953); the regenerating capacity was low during the months of flowering.

1.4 Seasonal effects

The time of the year in which cuttings are taken exert a profound influence on rooting (Hartmann and Kester 1975), but little is known about the controlling mechanism of this response (Lanphear 1963).

While certain species, such as Ligustrum, rooted readily when softwood, semi-hardwood or hardwood cuttings were taken any time during the year (Hartmann and Loreti 1965), softwood cuttings of deciduous woody species taken during spring or summer tend to root more readily than hardwood cuttings taken in mid-winter (Hartmann and Kester 1975). Hard-to-root species such as Rhododendron, Syringa, Prunus, and Tilia rooted better than softwood cuttings taken in early spring (Olieman et al. 1971; Hartmann and Brooks 1958; Still 1981). On the other hand, in the northern hemisphere, coniferous cuttings root best when collected between late fall and budbreak the following season (Hansen and Ernsten 1982). This increased capacity of coniferous cuttings to initiate roots during the period from October to April has been reported for several coniferous species: Pinus (Hansen and Ernsten 1982), Picea (Deuber and Farrar 1940), Abies (Bhella and Roberts 1974; Thimann and Delisle 1942), Taxus, and Juniperus (Lanphear and Meahl 1966).

According to Alvim et al. (1976) and Nanda and Jain (1972), seasonal

changes in rootability of cuttings appeared to be related to the level of endogenous hormones. A variation in auxinic content of Populus x robusta cuttings during different seasons was associated with a decline or an increase in rootability (Smith and Wareing 1972). Lanphear and Meahl (1966) reported noticeable relationships between the rooting cofactors and auxin activity present in Juniperus horizontalis 'Plumosa' and their seasonal rooting pattern. Fadl and Hartmann (1967b) reported that the fluctuation in rooting of cuttings is most likely correlated with changes in inhibitor activity of the buds. Inhibitor levels in buds increased progressively in late summer (pre-resting stage) reaching a maximum in late fall (resting stage) and then decreased to a minimum during the winter (post-resting stage). According to Smith and Wareing (1972), removal of bud dormancy after a period of chilling increased the rooting ability of some plants.

Guerriero and Loreti (1975), on the other hand, showed that in peach hardwood cuttings, there was no clear relationship between dormancy and rootability. Howard (1968) showed that the removal of buds to study their effect on rooting leads to the release of a wound-induced stimulus which promoted rooting. Howard (1968) argued that this effect can be mistakenly attributed to the removal of supposedly inhibitory buds.

Seasonal rooting pattern has been related to the level of endogenous hormone such as auxin (Alvim et al. 1976; Nanda and Jain 1972), level of rooting cofactors (Lanphear and Meahl 1966), and level of inhibitors (Fadl and Hartmann 1967b). According to Nanda and Anand (1970), the seasonal rooting pattern in Populus nigra was determined by a proper balance of growth inhibitors, auxins, and rooting cofactors. Bhella and Roberts

(1974) reported that one factor that particularly affected seasonal response of stem cuttings of Abies balsamea was photoperiod.

2. Hormones and other rooting substances

The complexity and variability in rooting response indicated that there is an interaction of several substances (Thimann 1977). Hartmann and Kester (1975) summarized the hypothetical relationship of various components such as auxin, rooting cofactors, and inhibitors with regards to the rooting process (Fig. 1).

2.1 Plant hormones

2.1.1 Auxin

In the 1930's, IAA was the first natural auxinic compound discovered and identified (Thimann 1935; Thimann and Went 1934; Went 1934; Went 1935). The ability of this hormone to promote adventitious root formation on stem cuttings of several species was soon documented (Cooper 1935; Thimann and Koepfli 1935; Went and Thimann 1937). Soon thereafter, synthetic auxins were tested for root promoting activity on stem segments and their root stimulating ability on cuttings was demonstrated (Cooper 1935; Thimann 1977). IBA and naphtaleneacetic acid (NAA) were more effective in promoting root initiation than the natural IAA (Hartmann and Kester 1975; Wareing 1973; Zimmermann and Wilcoxon 1935), and were more stable, less degradable by oxidase enzymes, and more mobile than naturally occurring

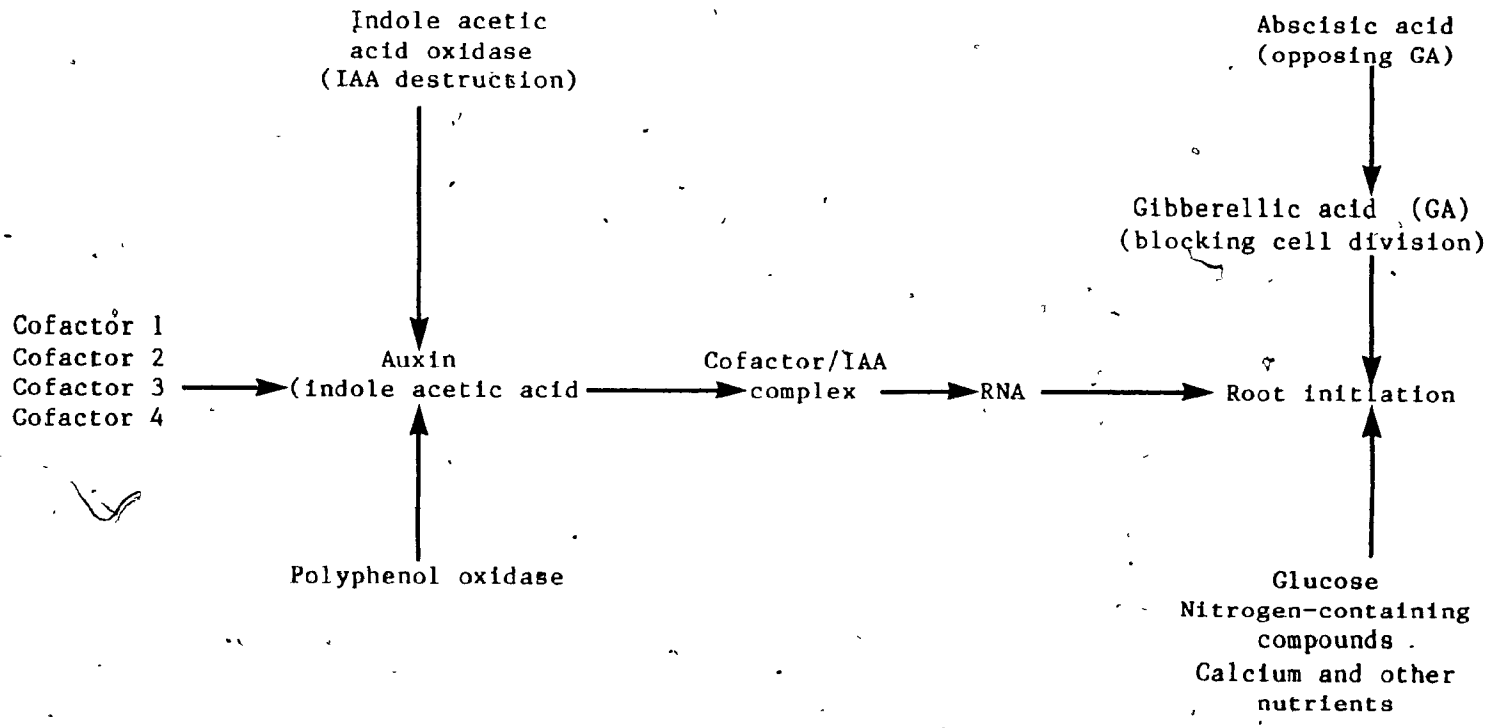


Figure 1. Hypothetical relationships of various components leading to adventitious root initiation (Hartmann and Kester 1975).

auxins (Tukey 1979; Wareing 1973). Several studies were conducted to compare the effect of auxin formulation on uptake in cuttings (Dirr 1982; Heung and McGuire 1973; Hitchcock and Zimmermann 1939; Kelly 1978). Today, IBA and NAA are commonly used in cutting propagation (Doran 1957).

Auxin is synthesized mainly in young leaves, developing buds, root tips, pollen and fruit (Tukey 1979) and is usually transported basipetally (Goldsmith 1977; Went 1929). According to the classical concept of auxin transport, auxin moves in the cytoplasm and is secreted at the lower end of each cell by a carrier-mediated energy-dependent process (Goldsmith 1977). Auxin enhances cell division, elongation, and differentiation (Haissig 1970), and shows an effect on nucleic acid metabolism (Key 1969). For instance, it appears that endogenous accumulation of natural and exogenously applied auxins and auxin synergists at the base of cuttings are required to initiate ribonucleic acid synthesis during the initiation of root primordium cells (Haissig 1971; Haissig 1972; Wareing 1973).

Van Overbeek (1941) and Bandurski and Shulze (1974) showed that most of the IAA in a plant exists bound in the form of a precursor. Hangarter and Good (1980) proved that the biological activity of IAA conjugates was related to the rate at which these were hydrolyzed by the tissue. Apart from being a form of storage and regulation of the concentration of IAA in the plant, an IAA-conjugate protects IAA from enzymatic degradation and aids transport of IAA (Cohen and Bandurski 1982). Although the free form appeared to exert auxinic activity, several studies reported that successful application of synthetic IAA-conjugates such as aryl esters of indole

acids (Haissig 1979), phenyl-IBA (Davies 1982), N-phenyl indolyl-3-butyramide, and phenyl indole-3-thio-butyrate (Haissig 1983) promoted adventitious root primordium initiation and development.

2.1.2 Rhizocaline

Thimann and Went (1934) noticed that substances other than auxin, but structurally very similar to it, are active in both root formation and promotion of cell elongation. Based on these observations, Went (1938) postulated the presence of a new specific hormone-like factor 'rhizocaline' from the cotyledon. Apparently, rhizocaline caused root formation in the presence of endogenous auxin. Other authors also reported the presence of 'rhizocaline' (Bouillenne and Bouillenne-Walrand 1955; Cooper 1938; Galston 1948; Fadl and Hartmann 1967a; Girouard 1969; Hess 1962; Kawase 1964; Libbert 1956; Thimann and Delisle 1939; Van Overbeek and Gregory 1945). Van Overbeek and Gregory (1945) found that hard-to-root, white-flowered Hibiscus rosa-sinensis hybrid 'Ruth Wilcox' failed to root in comparison with the red species because it was lacking two essential factors in the cuttings: auxin and a factor, or combination of factors, found to be present in the leafy shoots of the red species. Cooper (1938) also reported that amounts of some naturally occurring root-forming substances other than auxin, as yet unidentified but essential for root initiation, accounted for the ease of rooting of lemon. Both apple and lemon species had similar levels of auxin, but apple cuttings which failed to root were lacking an unidentified natural substance(s) necessary for root formation (Cooper 1938). Later studies supported the 'rhizocaline' theory although this sub-

stance has never been isolated and identified (Girouard and Hess 1964; Hartmann and Kester 1975; Kawase 1964).

2.1.3 Cytokinin

Cytokinins are involved in cell growth, division and differentiation (Hartmann and Kester 1975). According to Torrey (1976) and Wittwer and Dedolph (1963), exogenously applied cytokinin often inhibited root growth and formation. Chin et al. (1969) showed that kinetin at 0.1, 1.0 and 5.0 µg/mL greatly reduced rooting of Phaseolus aureus stem cuttings. Heide (1965) reported an inhibitory effect of kinetin on root formation of Begonia leaves, and showed that the effect of auxin and cytokinin together was dependent on the levels of each other. Humphries (1963) showed that the root-inhibitory effect of kinetin in Phaseolus vulgaris was reflected on the phosphorus metabolism. This suggested that kinetin played a role in cellular differentiation. Stenlid (1982), on the other hand, showed that the inhibitory and the regulatory action of cytokinin on root growth was related to the synthesis and action of ethylene.

On the contrary, Fridborg (1971) reported that cytokinin had little or no effect on root formation, but a synergistic effect when used in combination with NAA or IAA. When kinetin and 2,4-dichlorophenoxyacetic acid were omitted from the culture medium of in vitro cultured Asparagus cells, large numbers of shoots developed with few or no roots (Wilmar and Hellendorn 1968). The effect of cytokinin has been shown to vary according to the timing of application during the root initiation phase (Andersen and

Hansen 1975; Eriksen 1974).

2.1.4 Gibberellin

Gibberellins are known primarily for their effects on stem elongation (Jones 1973). Most studies showed that gibberellins inhibit root formation (Brian et al. 1955; Brian 1960; Mosella et al. 1980; Schraudolf and Reinert 1959). Gaspar et al. (1977) showed that at concentrations greater than 10^{-8}M, gibberellic acid (GA_3) inhibited the rooting of Pinus radiata when applied at the induction phase. Apparently, the inhibition by GA_3 was a direct local effect (Brian 1955, 1960).

On the contrary, Meyer et al. (1973) observed that at low concentrations, some gibberellins favoured root initiation and elongation in certain species. Hansen (1976) found that in cuttings taken from stock plants grown at low irradiance, low concentrations of GA_3 (10^{-8} and 10^{-7}M) promoted rooting. This suggested that the effect of GA_3 on root formation was dependent on the irradiance previously given to the stock plant (Andersen et al. 1975; Hansen 1976; Veierskov et al. 1976). Tukey (1979) showed that gibberellins favoured root development because of growth competition for metabolites, but had no effect on initiation. According to Key (1969) and Jones (1973), gibberellins interferred with the regulation of deoxyribonucleic acid, ribonucleic acid, and protein metabolism.

It has been proposed that the inhibitive effect of gibberellin is counteracted by abscisic acid (ABA) (Chin et al. 1969).

2.1.5 Abscisic acid

The effects of ABA tend to oppose those of auxins and gibberellins (Cornforth et al. 1966; Sankhla and Sankhla 1968), but the mechanism of action of ABA or its interaction with the auxins with regards to root formation is not known. Heide (1968) showed that ABA had little effect on rooting. Chin et al. (1969) reported promotive effect of ABA on root formation in stem cuttings of Phaseolus aureus and Hedera helix. Although there was no apparent synergism or additive effects between ABA and IAA, ABA suppressed the effect of gibberellin. On the contrary, Basu et al. (1970) reported that ABA promoted the rooting of Phaseolus aureus and Lycopersicon esculentum, but not of Phaseolus vulgaris; ABA showed an additive effect with IAA or IBA depending on the species tested.

2.2 Rooting cofactors and other substances

As shown in Fig. 1, the rooting phenomenon is a result of complex interactions and a balance between several hormones, rooting cofactors, and inhibitors (Nitsch 1957; Thimann 1977; Waxman 1957). Besides the hormones described above, several endogenous substances seem to act as promoters or inhibitors of rooting (Basu et al. 1969; Girouard 1967; Hess 1962; Went 1938).

2.2.1 Phenolic compounds

Phenolic compounds have been proposed to be involved in the rooting process (Hartmann and Kester 1975; Hess 1962; Mosella 1980; Shiboaka 1971;

Snyder 1974). Many workers have extracted from woody plants root promoting cofactors characterized as phenolic (Bassuk et al. 1981; Bassuk 1981a; Fadl and Hartmann 1967b; Girouard 1969; Hess 1962; Hess 1965; Howard et al. 1981). Several phenolic compounds have been demonstrated to act synergistically with auxins (Hitchcock and Zimmerman 1942; Mosella 1979; Bojarczuk 1978; Hartmann and Kester 1975; Lee and Tukey 1971). For instance, Hackett (1970) observed a strong synergism between catechol and IAA, but not NAA, in promoting root initiation in juvenile shoot tips of Hedera helix. Anthocyanins and flavonoids were reported to enhance root formation in cuttings (Bachelard and Stowe 1962). A high correlation between the total amount of anthocyanin present in the leaves of Acer rubrum cuttings and the number of roots formed in cuttings treated with IBA was also shown (Bachelard and Stowe 1962).

The stimulatory effect of phloroglucinol(PG) and phloridzin breakdown products on in vitro rooting has been reported in apples (Hegedus and Phan 1983; James and Thurbon 1981; Jones 1976; Jones and Hatfield 1976; Zimmermann and Broomes 1981), plums, cherries (Jones and Hopgood 1979), and raspberries (James 1979; James et al. 1980). James (1979) observed that PG alone promoted rooting (over 80%) in Fragaria and Rubus cultures. Basu et al. (1969) and Jones and Hatfield (1976) found that various phenolic compounds in the absence of auxin were ineffective as root promoters. Other researchers have also indicated that inclusion of phenolic compounds in the tissue culture medium provided little or no benefit, or was even detrimental (Abbott and Whitely 1976; Zimmermann 1978).

Hess (1962) showed that the structural qualification for a phenolic

compound to stimulate root initiation was associated with the presence of at least two hydroxyl groups in an ortho (o) relationship and that the para (p) position must be free. Gorter (1962) showed that monophenols inhibited root formation while o-diphenols promoted it. Thimann (1977) confirmed that monophenols inhibited both stem and root growth while o-diphenols promoted both. Hitchcock and Zimmerman (1942) and Wells and Marth (1953) showed that the introduction of one or more substitutions in the ring of monophenols, particularly the halogens, Cl and Br, increased the root-inducing activities, with descending order of activity for mono-substituted phenoxyacetic acids being para, ortho, and meta.

Basu et al. (1969) indicated that concentration markedly determined the synergistic effect of a phenolic. McRae and Bonner (1952, 1953) proposed that anti-auxinic phenolic compounds resulted when an active auxin molecule capable of consumating two-point attachment, was so modified that it was only able to undergo attachment at a point to the auxin receptive entity within the plant, and they described such changes.

Phenols acted as auxin conjugate (Cohen and Bandhurski 1982). Mosella (1980) demonstrated that the addition of specific phenolic compounds during the root initiation phase led to an increase in auxin within the plant. Thimann (1977) and Thimann et al. (1962) proposed that the synergistic effect of phenols with auxin is due to the inhibition of IAA-oxidase by the IAA-oxidase system.

Researchers demonstrated that phenolic synergists and phenolic inhibitors acted through inhibition of oxidation of the IAA-oxidizing system (Zenk and Muller 1963; Lee and Skoog 1965; Tomaszewski and Thimann 1966;

Lee 1980; Lee et al. 1981; Waygood 1956; Doumenjou 1978). For instance, Fox (1965) showed that in the absence of H_2O_2, the complex peroxidase-H_2O_2 was formed when Mn^{2+}, a monophenol, and IAA were present. Once the complex was built, it oxidized first the o-diphenol and subsequently the IAA. However, Vieitez et al. (1966) observed an inhibition and a promotion of IAA-oxidizing system by different concentrations of p-hydroxybenzoic acid presented evidence against the theory of IAA decarboxylation or oxidation as a general explanation for the growth and root formation stimulating or inhibiting activity of all phenols.

Leopold and Plummer (1961) observed the formation of an IAA-quinone complex upon the addition of polyphenol peroxidase enzyme on several phenols and proposed that the quinone might add to the N of the indole ring or to one of the carbonations of the furane ring.

Gorter (1969) summarized the influence of phenolics as follows: a) its inhibition of IAA oxidase system; b) a cofactor of tryptophane to IAA conversion.

Such involvement on polyphenols in the formation of auxin from tryptophane has been shown by Gordon and Paleg (1961). They observed a primary oxidation of phenols to the o-quinoid form and the reaction of this quinone with tryptophane to form indolepyruvate and subsequently to IAA.

Stenlid (1963) proposed a correlation with the activity of some phenols and the uncoupling of oxidative phosphorylation. Stenlid (1976) and Marigo and Boudet (1977) showed that phenolic compounds could act on rooting and growth processes through the regulation of polar transport of auxin.

2.2.2 Carbohydrates

The presence of a high initial carbohydrate content, translocated from leaves to stem, is associated in some instances with increased rooting (Breen and Muraoka 1973; Hartmann and Kester 1975; Haissig 1984; Snyder 1974). Reid (1926) and Basu and Ghosh (1974) reported that a high C/N ratio favoured rooting of Lycopersicon esculentum and Justicia gendarussa cuttings, respectively. Several researchers demonstrated that exogenous carbohydrates, such as sucrose and glucose, supplied to cuttings, increased the carbohydrate pool in cuttings and improved rooting (Andersen et al. 1975; Evans 1971; Howard and Sykes 1966; Loach and Whalley 1978; Went and Thimann 1937). According to Atman and Wareing (1975) and Hansen (1976), the root stimulating effect of hormone such as IAA, and in some instances GA_3, could be a consequence of a mobilization of low molecular weight carbohydrates for root formation. However, Moore et al. (1972) found that external supply of sucrose to excised radish cotyledons suppressed the formation of roots.

2.2.3 Mineral nutrients

Gorter (1958) considered B as the most important inorganic compound in the rooting process. Hemberg (1951) showed that rooting of Phaseolus vulgaris cuttings was inhibited in the absence of B. Apparently, B stimulated root elongation, but had no influence on root initiation (Hemberg 1951; Albert 1975). Similar enhancement of root growth by boron was observed on softwood cuttings of Pelargonium zonale and Ribes americanum

(Murray et al. 1957). However, Weiser and Blaney (1960) found a synergistic B-IBA interaction resulting in increased rooting percentage, root number and to a lesser extent root length in Ilex aquifolium cuttings. The use of B in combination with IBA, also accelerated the rooting process of Clematis and Ilex aquifolium cuttings, suggesting an effect of B on root initiation and root growth (Weiser 1959). Weiser and Blaney (1967) reported that B enhanced rooting through an influence on oxidative processes, possibly through increased mobilization of oxygen rich citric and iso-citric acids into the rooting tissues.

Van Overbeek and Gregory (1945) demonstrated that nitrogenous substances from the leaves were involved in the root initiation process of Hibiscus. Thimann and Poutasse (1941) showed that two organic forms of nitrogen, asparagine and adenine, were effective in stimulating rooting of Phaseolus vulgaris leaf cuttings. Good and Tukey (1967) found that N was required during root elongation of chrysanthemum cuttings, but not during root initiation. On the contrary, greater root length and healthier root appearance were found in cuttings under N-deficient regime (Swanson and Davis 1977).

Swanson and Davis (1977) showed that P-deficient cuttings of Plectranthus australis showed shorter roots and lower rooting percentage. Kramer (1969) showed that calcium deficiencies characteristically suppressed root growth. The favourable effect of Fe added to the rooting medium on rooting of peach almond hybrids was reported by Bindra (1976).

2.2.4 Other compounds

Hemberg (1953) showed that vitamins K and H, when used in conjunction with IAA, promoted auxinic action by increasing the rooting of bean cuttings. A similar enhancement of rhizogenesis by vitamins D_2, D_3, and some of their analogs such as dihydrotachysterol on softwood cuttings of Populus tremula and Cynara, woody cuttings of Populus nigra and tremula, and cuttings of Phaseolus vulgaris was reported by Buchala and Schmid (1979) and Moncousin and Gaspar (1982). The B vitamins also were found to play a role in root initiation (Bachelard and Stowe 1961; Bonner 1937). According to Keevers et al. (1982), vitamins such as D_2 and D_3 exerted their root-promoting effect with auxins by interacting with the calcium-controlled secretory process of peroxidase. Stowe and Obreiter (1962) suggested that isoprenoid vitamins act via cytochrome oxidation.

Krishnamoorthy (1970) found that application of ethephon ((2-chloroethyl) phosphonic acid), an ethylene-generating substance, to mung bean cuttings stimulated root formation. On the contrary, Schier and Campbell (1978) reported that ethephon did not stimulate rooting of dormant stem cuttings of Populus tremula although it did increase callus formation and inhibited decay in the stem cuttings.

Shiboaka et al. (1967) reported that heliangine, a sesquiterpenic lactone, isolated from the leaves of Helianthus tuberosus, promoted root formation in Phaseolus and Azukia cuttings. Portulal, a bicyclic diterpene, isolated from leaves of Portulaca grandiflora, was also showed to exhibit root promoting activity in Azukia, Vigna, and Phaseolus cuttings (Mitsuhashi et al. 1969).

2.2.5 Plant extracts

Researchers have observed biological activity in extracts from plants or plant parts. Went (1929) observed root promoting activity of Carica leaf extract on Acalypha plants. Bouillenne and Went (1933) found substances (presumed to be rhizocaline) in cotyledons, leaves and buds which stimulated rooting of cuttings. Rhizocaline, obtained from various products and extracts, was found to be quite similar in effect to auxin but not necessarily identical (Thimann and Went 1934; Went 1934; Went 1938). Van Overbeek and Gregory (1945) found that a hard-to-root white-flowered Hibiscus failed to root in comparison with an easy-to-root red-flowered species; the white-flowered species was lacking auxin and a factor, or combination of factors, found to be present in the leafy shoots of the red-flowered species. Nelson (1959) showed that liquid extract of alfalfa contained an unknown active substance, 'chloromone'; which stimulated rooting of junipers.

Using mung bean bioassay, Hess (1961a) obtained from extracts of easy-to-root, juvenile form of Hedera helix and red flowering Hibiscus rosa-sinensis, four root-promoting substances which he referred to as rooting cofactors 1,2,3 and 4. Hess (1961b) also showed that chromatograms from hard-to-root, mature Hedera and white-flowering form of Hibiscus either lacked these cofactors or contained smaller quantities. Rooting cofactors also have been found in Chrysanthemum, Camellia, Castanea, Euonymus, Pyrus, and Rhododendron (Fadl and Hartman 1967a; Hess 1963; Lee et al. 1969; Lee and Tukey 1971; Luckwill 1956; Vieitez 1976).

Hackett (1970) found three peaks of root promoting activity from methanolic extracts of juvenile and adult ivy stem tissue, although cofactor 4 was missing; there was no difference between juvenile and adult extracts in terms of amounts and composition of cofactors. Fadl and Hartmann (1976a) isolated an endogenous root promoting factor from basal sections of hardwood cuttings of an easily rooted pear cultivar 'Old Home'. High levels of this cofactor were found during the period of optimum rooting while high levels of inhibitors were observed during their rest period. Extracts from basal segments of hard-to-root 'Bartlett' pear did not show this rooting cofactor, but showed high levels of inhibitors throughout most of the year. Differences in amounts of rooting inhibitors accounted for difference in rooting of hard-to-root 'Orpheo' and easy-to-root 'Choot Ashani' dahlia (Biran and Halevy 1973), and between two Eucalyptus species, grandis and deglupta (Paton et al. 1970).

Ouellet (1962) extracted seeds of barley and wheat, and dried barley plants and pieces of elm stem steeped in water, and obtained increased rooting of stem cuttings of Ulmus americana treated with these extracts; however, root promotion was less than that due to IBA treatment.

Kawase (1964) obtained a strong root promoting activity on mung bean cuttings by applying centrifugal diffusate of Salix alba cuttings. The diffusate was strongly synergistic with IAA in inducing mung bean rooting. Kawase (1970) also extracted with water, some rooting substances from freeze-dried Salix alba similar to those found in the centrifugal diffusate. Water-soluble substances from diverse plants such as Cotoneaster racemiflora soogorica, Euonymus fortunei carrieri, Symplocos

paniculata, Lonicera maackie, Ilex opaca, Physocarpus amurensis, Taxus cuspidata, and Viburnum bukwoodii also were found to promote rooting when tested in mung bean bioassays (Kawase 1971). Kawase (1981) reported that the crude extract from only 9.45 grams of willow twig stimulated production of 12 times as many roots per mung bean cuttings. Richer-Leclerc et al. (1984) treated Juniperus sabina and Thuya occidentalis with willow and popular extracts with or without IBA. Although certain treatments solely with extracts of poplar and(or) willow were as effective as IBA, the highest rooting response occurred of extract treatments in combinations with 5000 ppm IBA.

MATERIALS AND METHODS

1. Influence of crude willow extracts on rooting of woody ornamental cuttings

1.1 General Details

1.1.1 Propagating environment

Experiments were conducted at Macdonald College under intermittent mist controlled by electronic leaf (Mac Penney, Plastic, Engineers, Ltd., Worthing, W. Sussex), either outdoors in shaded frames during the growing season or in greenhouses during the winter with 21°C day temperature and 18°C night temperature. Indoor benches (each 2.5 m long x 1.1 m wide) and outdoors benches (each 7.3 m long x 1.1 m wide) were provided with bottom heat thermostatically set at 24°C (Hartmann and Kester 1975).

The standard rooting medium consisted of horticultural grade perlite and vermiculite mixed in equal volumes contained in fiber flats (18 cm long x 13 cm wide x 7 cm deep). The defoliated basal ends (3 cm) of cuttings were treated with extracts and (or) auxins, as described below in each experiment, and placed into the rooting medium under intermittent mist. Benlate 50% WP (methyl-1 (butylcarbamoyl)-2-benzimidazole carbamate was applied at a rate of 1.5 L/m^2 (2g/L) of bench space to prevent against rotting of cuttings. Thereafter, Captan 50% WP (cis-N-((trich-

loromethyl)thio)-4-cyclohexene-1,2-dicarboximide) or Benlate, mixed and applied as described above, was applied alternatively every week.

1.1.2 Rooting evaluation

In each experiment, cuttings were evaluated according to the following parameters: (a) rooting percentage; (b) mean root length (cm) per cutting; (c) mean root number per cutting.

1.1.3 Preparation of willow extract

The preparation and extraction of willow extracts were, according to the method of Kawase (1964), modified as described by Richer-Leclerc and Chong (1982, 1983).

On the dates, 28 February, 1981, 25 November 1982, 28 February 1983, 7 July 1983, and 3 August 1983, twigs (20 to 45 cm in length) were harested from a 22-year-old weeping willow (Salix alba tristis) tree growing on the Macdonald College campus. Twigs were taken from the same tree on all collection dates to prevent inter-tree variation.

Defoliated twigs were cut into 1.0-1.5 cm pieces, immediately stored in a freezer for 48 hours at -15°C in tightly-covered plastic containers, then freeze-dried for 48 hours (125 μ vacuum at -35°C, Labconco freeze-dryer Model 5) and kept frozen at -15°C. Immediately before each experiment, freeze-dried twigs were ground in a Wiley mill (Arthur H. Thomas Co., Philadelphia, Pa.) to pass through a 40-mesh wire screen. Crude extracts were prepared by adding 100 mL distilled water to various amounts

of powder as described in each experiment. The mixture was then shaken (Eberback Co., Ann Arbor, Michigan) at 270 to 280 strokes per min for one hour at 4°C to reduce possible enzymatic reactions.

1.2 Experiments

In the summer of 1982 and the winter of 1983, leafy cuttings of four Group 1 (shrubs), four Group 2 (trees), and five Group 3 (evergreens) species were taken from current season's terminal growth on the dates shown in Table 1.

In Group 1 species, 8 to 10 cm long softwood cuttings collected randomly from five plants of each species were used. These species were located on the Macdonald College campus and were approximately 11-years-old.

In Group 2 species, 10 to 12 cm long semi-hardwood cuttings were used. Cuttings of Salix alba tristis and Tilia cordata were collected from four plants of each species, one tree per replication, located on the Macdonald College campus; cuttings of Betula pendula 'Gracilis' and Malus rinkii 'Royalty' were collected randomly from 40 trees, located at Cramer's Nursery, Les Cèdres. Malus rinkii 'Royalty' trees were five years old, Betula pendula 'Gracilis' 6 years old, and Tilia cordata 20 years old. Trees of Salix alba tristis were 15, 18, 22, and 30 years old.

In Group 3 species, 10 to 12 cm long hardwood cuttings were used. Cuttings of 8-years-old Taxus media and Juniperus chinensis 'Mountbatten' (8 December sample, Table 1) were collected from plants, one plant per replication, at Macdonald College campus and Cramer's Nursery, res-

Table 1. Propagation starting dates and rooting periods of Group 1, 2, and 3 species.

Species	Starting Date	Rooting Period (weeks)
GROUP 1 (SHRUBS)		
Cotoneaster acutifolia	10 June 1982	6
	25 July 1982	6
Cornus alba 'Elagantissima'	11 June 1982	4
	2 August 1982	4
Ribes alpinum	14 June 1982	4
	29 July 1982	4
Philadelphus coronarius 'Avins'	16 June 1982	4
	5 August 1982	5
GROUP 2 (TREES)		
Salix alba tristis	22 July 1982	2
Tilia cordata	17 August 1982	6
Betuala pendula 'Gracilis'	22 July 1983	5
Malus rinkii 'Royalty'	22 July 1983	10
GROUP 3 (EVERGREENS)		
Taxus media	9 December 1982	8
Juniperus chinensis 'Mountbatten'	8 December 1982	12
	10 March 1983	14
Juniperus virginiana 'Skyrocket'	10 March 1983	14
Pinus mugho 'Mughus'	10 March 1983	12
Pinus sylvestris	10 March 1983	14

pectively; cuttings of 10-year-old Juniperus chinensis 'Mountbatten' (10 March sample, Table 1), 8-year-old Juniperus virginiana 'Skyrocket', 7-year-old Pinus mugho 'Mughus' and 9-year-old Pinus sylvestris were taken randomly from hedge plantings at Cramer's Nursery, Les Cèdres. During rooting, cuttings were exposed to 16-hour photoperiod (0600 to 2200) using high pressure sodium lamps (Phillips HDK 602 Lu 400) suspended one meter above the rooting medium. The average light intensity at the level of the cuttings was 8000 lux as measured by a light meter (LI-COR, Quantum radiometer/Photomer, Model LI-185).

1.2.1. Rooting treatments

Group 1 cuttings were tested with willow extract treatments of 0 (35% ethanol control), 1.6, 4.0 and 10.0 g/100 mL alone or in combination with 5000 ppm IBA. The willow extract of twigs collected from 28 February 1981 was used in this experiment. The experimental design was a split-plot in time (main factor), with two subplot factors (willow extract and IBA) arranged in a randomized complete block design with five replications and 15 cuttings per experimental unit.

Group 2 cuttings were tested with willow extract treatments of 0 (35% ethanol control), 1.6, and 4.0 g/100 mL alone or in combination with 5000 ppm IBA solution, or with 0.4% IBA powder (Stim-Root, No. 2). In this experiment, the willow extract of twigs collected from 19 July 1982 was tested on Salix alba tristis, Malus rinkii 'Royalty', and Betula pendula 'Gracilis', and from 3 August 1982 for Tilia cordata. The experimental

design was a two factor factorial with four replications arranged in randomized complete blocks. Factor A with three levels was willow extract and factor B with two levels was IBA. For each species, there were 10 cuttings in each experimental unit.

Two Group 3 species, Juniperus chinensis 'Mountbatten' and Taxus media were subjected to the following seven treatments: control (35% ethanol), 5000 ppm IAA, 5000 ppm IBA, 5000 ppm NAA, and each of these auxin treatments in combination with willow extract (25 November collection date) at 4.0 g/100 mL. The experiment was arranged in randomized complete blocks with four replications per species and 10 cuttings in each experimental unit.

In a related experiment, all Group 3 species (except Taxus media) were subjected to the following eight treatments: control (35% ethanol), 5000 ppm IAA, 5000 ppm IBA, 5000 ppm NAA, willow extract (28 February 1983) at 4.0 g/mL, and each of the above auxin treatments in combination with willow extract at 4.0 g/100 mL. The experiment was a two factor factorial arranged in randomized complete blocks. Factor A with two levels was the willow extract and factor B with five levels was the type of auxins. There were four replications per species and 10 cuttings in each experimental unit.

Rooting percentage and root number were transformed to obtain normality and homogeneity of variance as described below:

(i) rooting percentage (RP): arcsine $\sqrt{RP}$

(ii) root number (RN): $\sqrt{RN + 0.5}$

Transformed data were subjected to analysis of variance. This statistical

manipulation was not required for data of root length. In all experiments described above, differences among means were compared by the LSD test (Steel and Torrie 1980).

2. Rooting activity of willow extracts on mung bean cuttings

2.1 General details

The nature of the root promoting activity of willow extracts was studied using the mung bean rooting test described by Hess (1961) and Kawase (1964) but modified slightly to present experimental conditions.

2.1.1 Germination and rooting test

Mung beans (Phaseolus aureus Roxb.) were germinated in vermiculite. Shoots were allowed to elongate during a 7-day period in controlled environment cabinets (Conviron Model E-15, Controlled Environment Ltd., Winnipeg) at constant temperature of 24°C and 18-hour photoperiod (0600 to 2400). Seedlings were provided with 400 lux of incandescent light.

In rooting tests, 7-9 cm long mung bean cuttings, each with a 5 cm long hypocotyl, a 2-3 cm long epicotyl, and a pair of true primary leaves, were obtained from mung beans germinated as described above. The cotyledons were removed from each cutting (if they had not abscised at the time the cuttings were made) to reduce amounts of endogenous rooting substances (Hess 1961; Kawase 1964). During the rooting tests, which lasted for 7 days, mung bean cuttings were placed in 15 mL of rooting test solutions or

extracts, contained in glass vials (7 cm x 2.5 cm) prepared as described below in section 2.2.1. In all experiments, the original 15 mL volume was maintained by daily addition of distilled water. All experiments were conducted in growth cabinets, described earlier, under a light intensity of 14000 lux obtained from a combination of incandescent and cool white fluorescent bulbs (ratio 25% incandescent and 75% fluorscent in wattage) throughout the rooting test period.

Rooting activity was evaluated by counting the number of roots longer than 1 mm on each cutting (Kawase 1964).

Unless specified, all experiments were arranged in a randomized complete block design with one to three main factors and with four replications per experimental treatment unit (glass vial). Each treatment unit consisted of six cuttings.

Unlike experiments in section 1.2, transformation of data was not required.

2.2 Analytical

2.2.1 Total phenols

A. Reagents

Folin-Ciocalteu. --(Anachemia)

100% methanol

50% methanol. --25mL of 100% methanol with 25 mL of distilled water.

17% sodium carbonate. --To 1.7 g of Na_2CO_3, 10 mL of distilled water was added.

Phenols standard solution. --To 5 mg of L(-)Tyrosine in a 50 mL volumetric flask, 50 mL of 50% methanol was added; aliquots with concentrations of 0,1,2,3,4, and 5 mg were used for the standard of tyrosine.

B. Procedure

Total phenol content was determined colorimetrically by a modification of the method of Swain and Hillis (1959). Selected willow extracts or sub-fractions (described in sections 2.3.1 and 2.3.2) prepared at a concentration of 7.5 mg per mL of distilled water were diluted to 1/5 of its volume with distilled water. To a 0.5 mL aliquot of this mixture in a 10 mL graduated centrifuged tube was added 6.5 mL of distilled water. The contents were mixed well, 0.5 mL of the Folin-Ciocalteu reagent added, and the tubes thoroughly reshaken (Vortex mixer). After 3 minutes, 1.5 mL of Na_2CO_3 was added and the mixture again reshaken. After an additional one hour, the absorbance at 700 nm was determined by a spectrophotometer (Bausch and Lamb Spectrophotometer, Model Spectronic 20) using 1 cm cells. A calibration curve was prepared with standard solutions containing from 0 to 5 mg tyrosine per g dry weight. The content of phenols was expressed in terms of mg tyrosine per mL of extracts.

2.2.2 Dihydroxy and alkali-labile phenols

A. Reagents

Folin-Ciocalteu. --(Anachemia)

100% methanol

50% methanol. --Id. to section 2.2.3.1

0.1 N sodium hydroxide

2% sodium carbonate. --To 2.0 g of Na_2CO_3, 100 mL of distilled water was added.

Phenols standard solution. --Id. to section 2.2.3.1

B. Procedure

Dihydroxy and alkali-labile phenol contents of selected willow extracts and sub-fractions (described in sections 2.3.1 and 2.3.2) were determined colorimetrically by the method of Jennings (1981). This method is based on the Folin-Ciocalteu reagent. Contents of both phenols and alkali-labile phenols were determined. Thereafter, the difference in the values produced by the two procedures yielded a measure of the amount of orthodihydroxy and other alkali-labile phenols in the sample solution at room temperature.

Determination of total phenols

The samples to be analyzed prepared at a concentration of 7.5 mg per mL of distilled water were diluted to 1/5 of its volume with distilled water. To a 0.5 mL aliquot of this mixture in a 10 mL graduated centrifuge tube was added 0.5 mL of distilled water and thereafter 0.5 mL of the Folin-Ciocalteu reagent. The contents were thoroughly mixed (Vortex mixer). After 10 minutes, 5 mL of the Na_2CO_3 reagent was added and the solution reshaken. After an additional 30 minute, the absorbance at

760 nm was determined. A calibration curve was prepared with standard solutions containing from 0 to 5 mg tyrosine per g dry weight. The content of phenols was expressed in terms of mg tyrosine per mL of extracts.

Determination of alkali-labile phenols

An aliquot of 0.5 mL (prepared as for total phenol determination above) was treated with 5 mL of the Na_2CO_3 solution and mixed. After 15 minutes, 0.5 mL of Folin-Ciocalteu reagent was added and the solution reshaken. After an additional 30 minutes, the absorbance at 760 nm was measured as for total phenols.

2.2.3 Thin layer chromatography for qualitative identification of phenols

Phenols were separated for qualitative identification on ascending thin layer chromatography (TLC), and detected using reagents, according to the method described by Hamel (1972) and Zweig (1972).

Separation by TLC

A) solvents for one-dimensional chromatography

Benzene-dioxane-acetic acid: (90:25:4, v/v)

Benzene-methanol-acetic acid: (45:8:4, v/v)

Benzene-methanol: (95:5, v/v)

Ethyl acetate-isopropanol-water: (9:1:1, v/v)

Ethyl acetate-ammonium-water: (9:1:1, v/v)

B) solvents for two-dimensional chromatography

All possible combinations of each pair of solvents in A above.

Detection

Detection agents for one- and two-dimensional chromatography

Folin-ciocalteu (Anachemia) --(Stahl 1969)

Benzidine, diazotized. --(Stahl 1969)

N-chloro-p-toluene sulfonamide sodium salt (chloramine-T). --(Bajaj 1976)

Iodine crystals. --(Stahl 1969)

Ultra-violet lamp. --254 nm (Stahl 1969)

Procedure

Selected ethyl acetate (EToAc) and water sub-fractions (described in section 2.3.2) were subjected to ascending TLC in one- or two-dimensions. Each sub-fraction was spotted 10 times on a polygram 0.25 plastic plate (12 cm x 12 cm) covered with silica gel layer (SLF/UV_{254} Kodak Eastman Company, Rochester, N.Y., U.S.A.). Each chromatogram plate was introduced into a closed development chamber (10 cm x 22 cm x 22 cm) sealed with silicone lubricant; the solvent was put into the chamber one hour before to obtain a saturated atmosphere. The separation process was conducted at room temperature over a distance of 10 cm, after which the chromatogram was removed and dried at room temperature.

Rf values were calculated for each spot and compared with Rf values of known phenols according to Zweig (1972). The procedure was repeated four times for each chromatogram.

2.2.4 Thin layer chromatography for qualitative identification of IAA and indole groups

IAA and indole groups were separated for qualitative identification on ascending TLC, and detected using reagents, according to the method described by Hamel (1972).

Separation by TLC

Solvents

Ethyl acetate-isopropanol-water: (65:24:1, v/v)

Detection

Spray reagent

Perchloric acid-ferric chloride.

Procedures

Selected EToAc and water sub-fractions (described in section 2.2.2) were subjected to one-dimensional TLC as described above. The chromatograms were dried at room temperature, placed in an oven for 5 minutes at 65°C, and then sprayed with a solution of perchloric acid- ferric chloride (Stahl 1969).

The Rf values were calculated for each spot and compared with an IAA control. The procedure was repeated four times for each chromatogram. The IAA control had an Rf value of 0.64 and developed a pinkish-purple color in the chromatograms (Fig. 2 a,b). Spots with similar Rf values and color reaction to the IAA control were detected only on the chromatogram for EToAc sub-fractions (Fig. 2 a). Spots with an Rf value of 0.50 and similar color reaction, which suggested the presence of an indole compound, were detected in both EToAc and water sub-fractions (Fig. 2 a,b).

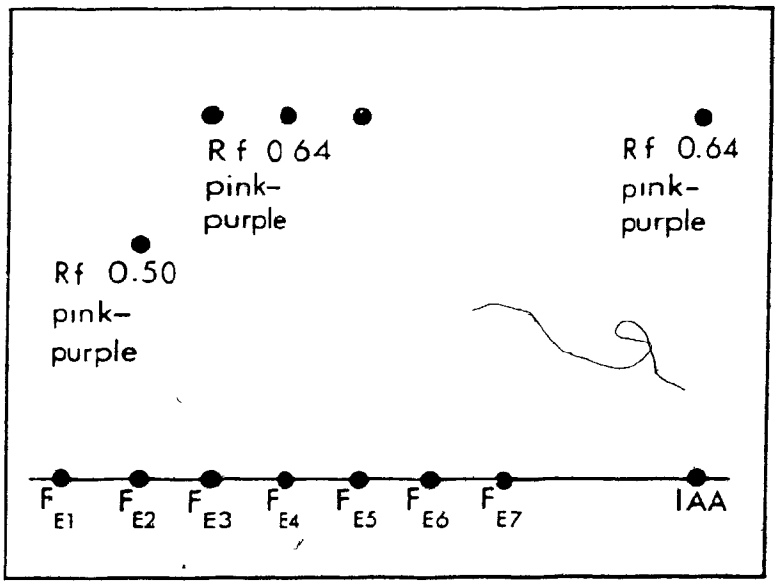
2.3 Experiments

In an attempt to identify the rooting substances and the nature of their activity in willow extracts, a systematic series of experiments were conducted using extracts that were subjected at each progressive step to greater purification according to a modification of Kawase's method (1970).

2.3.1 Crude versus clarified

As shown in Fig. 3, crude willow extracts were obtained by adding 100 mL of distilled water to willow powder (concentrations varied between experiments) and the mixture shaken (Eberback Co., Ann Arbor, Michigan) at 270 and 280 strokes per minute or one hour at 4°C. The crude willow extract was then centrifuged for 15 min at 10,000 rpm in an automatic refrigerated centrifuge (Sorvall, rc2-P, 4°C) to produce a supernatant

a. EToAc SUB-FRACTIONS



b. WATER SUB-FRACTIONS

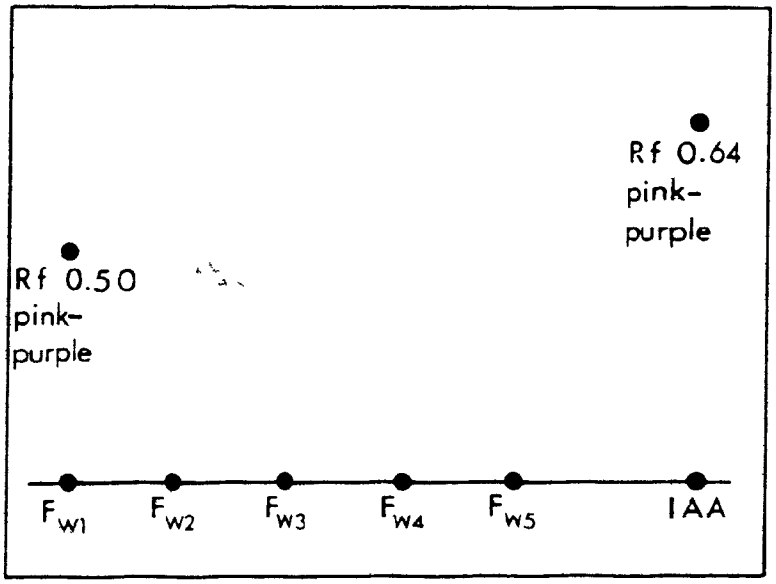


Figure 2. TLC for IAA and indole group using the solvent system, ethyl acetate-isopropanol-water (65:24:1), and the spray reagent, ferric chloride.

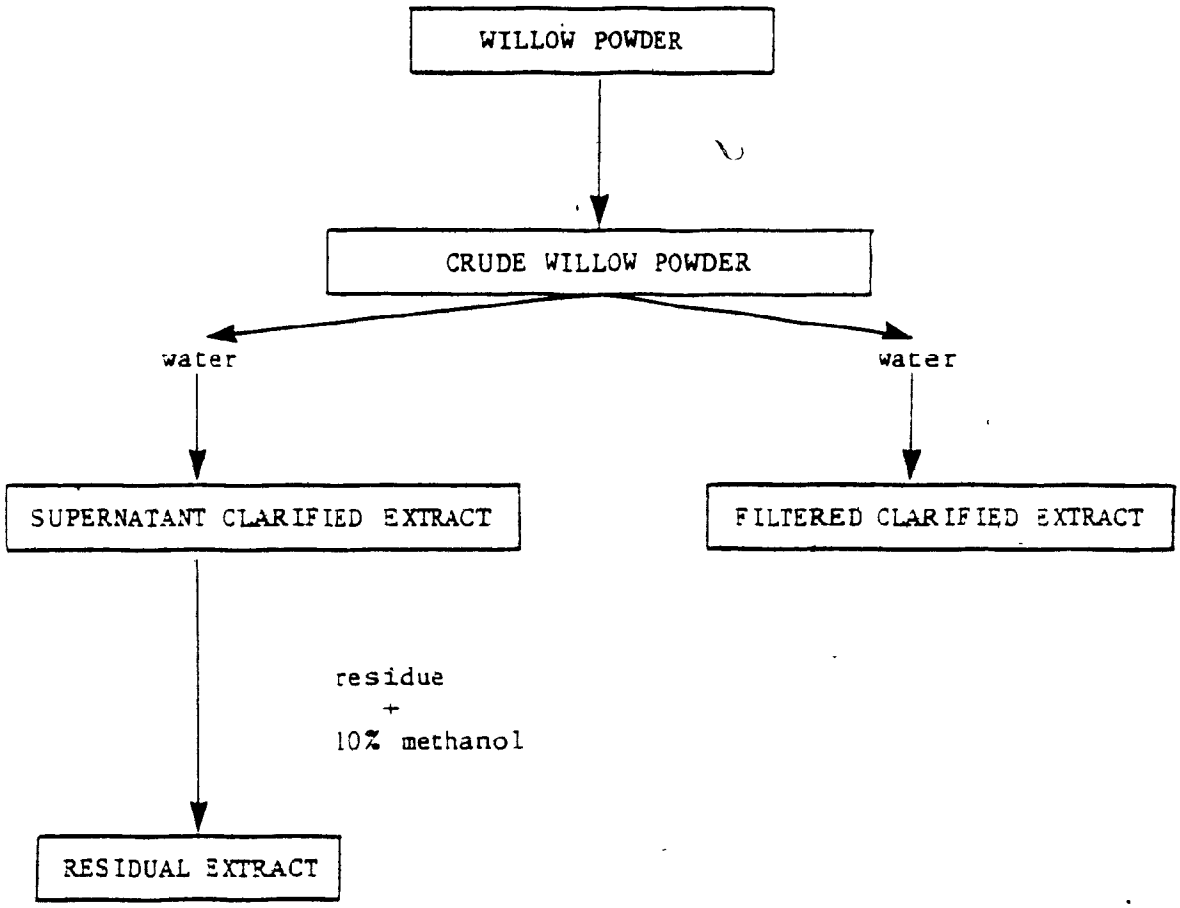


Figure 3. Derivation of clarified supernatant and filtered extracts and residual extract from crude willow extract.

clarified extract, or filtered through Whatmann No. 1 filter paper *in vacuo* to produce a filtered clarified extract.

The residue from the supernatant extract was extracted with 10% methanol (residual extract) to recover as much as possible the water insoluble substances (Hess 1961). Preliminary investigations using methanol concentrations between 1 and 100% indicated toxicity of extracts in which methanol was used above 10%.

Total phenol contents were determined in crude willow extracts obtained from 12 collection dates over a one-year period: (a) 25 October 1982, (b) 25 November 1982, (c) 21 December 1982, (d) 31 January 1983, (e) 28 February 1983, (f) 13 March 1983, (g) 11 April 1983, (h) 31 May 1983, (i) 28 June 1983, (j) 3 August 1983, (k) 30 August 1983, and (l) 24 October 1983.

Dihydroxy and alkali-labile phenol contents also were determined in crude willow extract obtained over a one-year period but only in samples collected at 2-month intervals. The collection dates were: (a) 25 October 1982, (b) 21 December 1982, (c) 28 February 1983, (d) 11 April 1983, (e) 28 June 1983, and (f) 3 August 1983.

Mung beans for this series of investigations were supplied by Dr. George Kuo of the Asian Vegetable Research and Development Center, Taiwan. Mung bean rooting tests were conducted to determine the optimum concentrations and seasonal activity of the extracts and to compare the activity of crude versus clarified extracts. Details with regards to the rooting treatments are provided under results (section 2.1).

2.3.2 Fractionated extracts

The willow powder was extracted and partitioned with water, methanol, and ethyl acetate solvents according to a modification of Jalal's method (1982) (a) to elucidate the type of rooting substances and their rooting activity in these extracts and fractions, and (b) to extract the greatest amount of phenolic rooting substances. These steps are outlined in Fig. 4.

Five grams of willow powder (25 November 1982) were extracted four times sequentially by shaking at 270 to 280 strokes per min at 4°C with water or methanol-water (4:1, v/v) using 50 mL for 30 minutes the first time, and 25 mL for 15 minutes thereafter. Water extract A or methanol-water extract B was obtained after centrifugation.

Methanol-water extract B was evaporated *in vacuo* to the aqueous phase (25 mL) which was transferred to a test tube and partitioned three times using 10 mL of ethyl acetate each time. The combined decantations resulted in ethyl acetate (EToAc) fraction D. The aqueous (bottom layer) remaining after decantations became water fraction C.

EToAc fraction D was evaporated to dryness *in vacuo* and taken up in 5 mL of methanol resulting in methanol-soluble EToAc fration G. Similarly, water fraction C was evaporated to dryness *in vacuo* and taken up in 5 mL of methanol. A precipitate appeared and was separated from the methanol-soluble fraction by centrifugation at 2400 rpm for 5 minutes resulting in methanol-insoluable water fraction F and methanol-soluble fraction E.

Methanol-soluble EToAc fraction G and water fraction F were each passed separately through a chromatographic column of Sephadex LH-20 (50.0

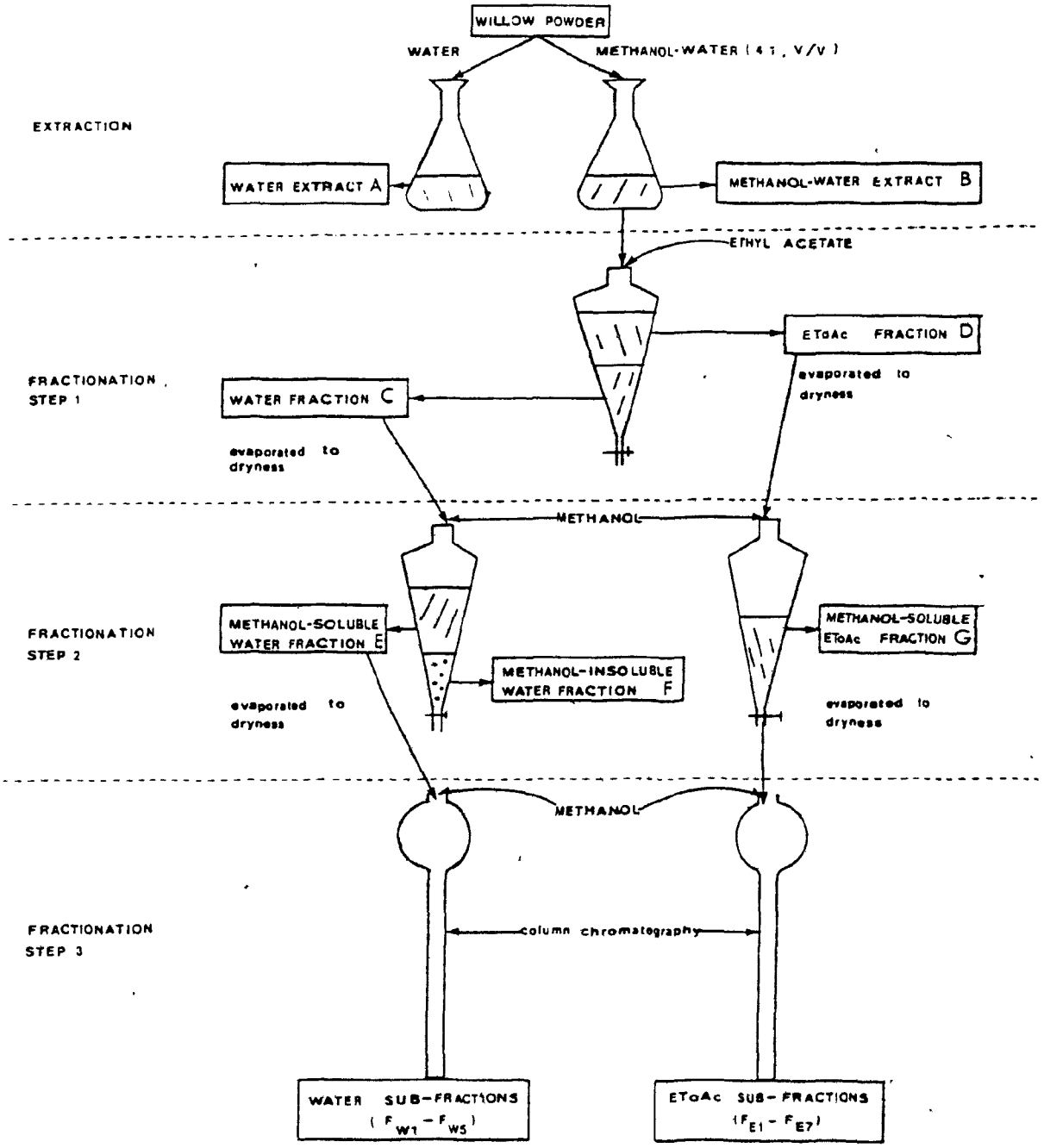


Figure 4. Fractionation of willow extracts.

cm x 2.8 cm) at a flow rate of 5 mL/8 min using methanol as eluent. The partitioning was done at room temperature. Seven hundred millilitres of eluate were collected automatically in successive 6 mL volumes, and the absorbance of each aliquot determined spectrophotometrically at 280 nm.

All extracts and fractions subjected to mung bean rooting tests were evaporated to dryness in vacuo and were taken up with distilled water to a concentration equivalent to 7.5 mg of willow powder per mL of distilled water, unless otherwise stated.

Mung beans for this series of investigations were obtained from W.H. Perron, Chomedy, listed as No. 100 in the catalog.

The EToAc and water sub-fractions (Fig. 3) were subjected to TLC and subsequent qualitative tests for IAA, indole groups and phenolic compounds. Details of these techniques were outlined in sections 2.2.3 and 2.2.4.

EToAc sub-fractions (Fig. 3) were analyzed for total, dihydroxy, and alkali-labile phenols as described in sections 2.2.1 and 2.2.2.

RESULTS AND DISCUSSION

1. Rooting of woody ornamental species with crude willow extract

1.1 Results

1.1.1 Group 1 species (shrubs)

Data for the rooting performance (rooting percentage, root number and root length) of Group 1 species (Cotoneaster acutifolia, Ribes alpinum, Cornus alba 'Elegantissima' and Philadelphus coronarius 'Aureus'), (Tables 2-5), varied widely in response to treatments of crude willow extract and their concentrations between 0 and 10 g/100 mL with or without 5000 ppm IBA.

IBA had a more profound influence on rooting of these species than willow extract, and rooting tended to be better on the earlier date of propagation (Tables 2-5). IBA increased rooting performance of Cotoneaster with regard to all three rooting parameters (Table 2) but tended to have the opposite influence on the other three species, Ribes (Table 3), Cornus (Table 4), and Philadelphus (Table 5). Rooting occurred above the treated basal area which showed browning.

While willow extract concentrations had no influence on the rooting of Cotoneaster (Table 2), root numbers of the other species were significantly influenced. In Ribes (Table 3), and Philadelphus (Table 5), root num-

Table 2. Rooting percentage (RP), mean root number (RN), and mean root length (RL) of Cotoneaster acutifolia cuttings in response to willow extract (WE) and IBA treatments.

WE	RP			RN			RL (cm)		
(g/ 100 mL)	IBA (ppm) 0	5000	Mean	IBA (ppm) 0	5000	Mean	IBA (ppm) 0	5000	Mean
				10 June 1982					
0	1 (0.05)+	47 (0.70)	24 (0.37)	0 (0.72)z	1.6 (1.34)	0.8 (1.03)	0	1.8	0.4
1.6	3 (0.11)	46 (0.70)	25 (0.41)	0 (0.73)	2.4 (1.57)	1.2 (1.15)	0	2.4	1.2
4.0	19 (0.29)	39 (0.63)	29 (0.46)	0.7 (0.97)	1.6 (1.36)	1.1 (1.17)	0.8	1.8	1.3
10.0	0 (0.00)	61 (0.95)	31 (0.47)	0 (0.71)	2.3 (1.64)	1.1 (1.17)	0	2.7	1.3
Mean	6 (0.11)	48 (0.75)	27 (0.43)	0.2 (0.78)	2.0 (1.48)	1.1 (1.13)	0.2	2.1	1.1
				25 July 1982					
0	28 (0.50)	57 (0.85)	43 (0.67)	0.4 (0.92)	1.8 (1.49)	1.1 (1.21)	0.3	1.2	0.7
1.6	13 (0.37)	77 (1.09)	45 (0.73)	0.2 (0.81)	2.5 (1.73)	1.3 (1.27)	0.1	2.2	1.1
4.0	13 (0.37)	81 (1.14)	47 (0.75)	0.1 (0.80)	2.8 (1.81)	1.5 (1.31)	0.1	2.5	1.3
10.0	25 (0.52)	68 (0.97)	47 (0.75)	0.4 (0.94)	2.2 (1.63)	1.3 (1.29)	0.3	1.6	0.9
Mean	20 (0.44)	71 (1.01)	45 (0.73)	0.3 (0.87)	2.3 (1.67)	1.3 (1.27)	0.2	1.9	1.1
LSD (P=0.05)									
WE		NS@			NS			NS	
IBA		0.12			0.39			0.14	
Date		0.35			NS			NS	
Date x WE		NS			NS			NS	
Date X IBA		NS			NS			NS	
WE x IBA		NS			NS			NS	
Date x WE x IBA		NS			NS			NS	

+ Data transformed to arcsine$\sqrt{RP}$ for analysis of variance.
z Data transformed to$\sqrt{RN + 0.3}$ for analysis of variance.
@ Not significantly different.

Table 3. Rooting percentage (RP), mean root number (RN), and mean root length (RL) of Ribes alpinum cuttings in response to willow extract (WE) and IBA treatments.

WE	RP			RN			RL (cm)		
(g/ 100 mL)	IBA (ppm) 0	5000	Mean	IBA (ppm) 0	5000	Mean	IBA (ppm) 0	5000	Mean
				14 June 1982					
0	99 (1.52)+	31 (0.57)	65 (1.05)	13.1 (3.66)z	1.1 (1.24)	7.1 (2.45)	1.2	0.1	0.7
1.6	100 (1.57)	52 (0.81)	76 (1.19)	11.9 (3.51)	5.7 (2.36)	8.8 (2.93)	1.2	0.2	0.7
4.0	99 (1.52)	28 (0.49)	63 (1.01)	11.2 (3.40)	1.7 (1.40)	6.5 (2.40)	0.9	0.1	0.5
10.0	99 (1.52)	48 (0.76)	73 (1.14)	13.4 (3.72)	5.7 (2.30)	9.5 (3.01)	1.0	0.2	0.6
Mean	99 (1.53)	40 (0.66)	69 (1.10)	12.4 (3.57)	3.5 (1.83)	7.9 (2.70)	1.1	0.1	0.6
				29 July 1982					
0	100 (1.57)	9 (0.24)	55 (0.91)	23.8 (4.92)	0 (0.73)	11.9 (2.83)	1.6	0	0.8
1.6	100 (1.57)	17 (0.41)	59 (0.99)	20.7 (4.60)	1.4 (1.27)	11.1 (2.93)	1.6	0.1	0.9
4.0	99 (1.52)	19 (0.44)	59 (0.98)	19.5 (4.45)	0.7 (1.08)	10.5 (2.77)	1.8	0.1	0.9
10.0	99 (1.52)	16 (0.36)	57 (0.94)	19.6 (4.47)	0.5 (0.95)	10.1 (2.71)	1.6	0.1	0.9
Mean	99 (1.55)	15 (0.36)	57 (0.95)	20.9 (4.61)	0.7 (1.01)	10.8 (2.81)	1.7	0.1	0.9
LSD (P=0.05)									
WE		NS@			0.09			NS	
IBA		0.07			0.08			0.08	
Date		0.07			NS			0.08	
Date x WE		NS			0.13			NS	
Date X IBA		0.09			0.09			0.11	
WE x IBA		NS			0.13			NS	
Date x WE x IBA		NS			NS			NS	

+ Data transformed to arcsine$\sqrt{RP}$ for analysis of variance.
z Data transformed to$\sqrt{RN + 0.5}$ for analysis of variance.
@ Not significantly different.

Table 4. Rooting percentage (RP), mean root number (RN), and mean root length (RL) of *Cornus alba* 'Elegantissima' cuttings in response to willow extract (WE) and IBA treatments.

WE	RP			RN			RL (cm)		
(g/ 100 mL)	IBA (ppm) 0	5000	Mean	IBA (ppm) 0	5000	Mean	IBA (ppm) 0	5000	Mean
				14 June 1982					
0	95 (1.39)+	65 (0.96)	80 (1.17)	8.0 (2.89)z	9.4 (3.05)	8.7 (2.97)	1.4	0.3	0.9
1.6	95 (1.39)	74 (1.04)	85 (1.21)	7.0 (2.73)	9.3 (3.07)	8.1 (2.90)	1.2	0.4	0.8
4.0	97 (1.46)	63 (0.93)	80 (1.19)	8.9 (3.03)	8.8 (2.93)	8.9 (2.98)	1.4	0.3	0.9
10.0	97 (1.47)	67 (0.97)	82 (1.21)	9.3 (3.06)	10.3 (3.37)	9.8 (3.21)	1.4	0.4	0.9
Mean	96 (1.43)	67 (0.98)	82 (1.21)	8.3 (2.93)	9.5 (3.08)	8.9 (3.01)	1.4	0.3	0.8
				29 July 1982					
0	93 (1.37)	7 (0.17)	50 (0.77)	7.0 (2.73)	0.4 (0.89)	6.0 (2.17)	1.5	0.1	0.8
1.6	71 (1.06)	32 (0.58)	51 (0.82)	7.6 (2.74)	5.4 (2.29)	6.5 (2.51)	1.8	0.9	1.3
4.0	92 (1.35)	31 (0.58)	61 (0.97)	10.9 (3.35)	5.3 (2.32)	8.1 (2.83)	2.5	0.4	1.5
10.0	81 (1.17)	59 (0.88)	70 (1.03)	10.9 (3.37)	9.0 (2.99)	10.0 (3.18)	2.0	9.0	5.5
Mean	84 (1.24)	32 (0.55)	58 (0.89)	10.3 (3.23)	5.0 (2.12)	7.7 (2.67)	2.0	2.6	2.3
LSD ($P=0.05$)									
WE		NS@			0.41			NS	
IBA		0.29			0.29			5.77	
Date		NS			NS			5.77	
Date x WE		NS			NS			NS	
Date x IBA		0.13			0.41			NS	
WE x IBA		0.18			0.58			NS	
Date x WE x IBA		0.25			0.82			NS	

+ Data transformed to arcsine$\sqrt{RP}$ for analysis of variance.
z Data transformed to $\sqrt{RN + 0.5}$ for analysis of variance.
@ Not significantly different.

Table 5. Rooting percentage (RP), mean root number (RN), and mean root length (RL) of *Philadelphus coronarius* 'Aureus' cuttings in response to willow extract (WE) and IBA treatments.

WE	RP			RN			RL (cm)		
(g/ 100 mL)	IBA (ppm) 0	5000	Mean	IBA (ppm) 0	5000	Mean	IBA (ppm) 0	5000	Mean
				16 June 1982					
0	72 (1.08)+	28 (0.51)	50 (0.79)	7.8 (2.76)z	14.5 (3.49)	11.1 (3.13)	0.9	0.2	0.5
1.6	72 (1.08)	25 (0.52)	49 (0.80)	8.6 (2.91)	10.7 (3.21)	9.7 (3.06)	1.0	0.2	0.6
4.0	68 (1.03)	20 (0.45)	44 (0.74)	7.0 (2.60)	10.0 (3.08)	8.5 (2.84)	0.9	0.1	0.5
10.0	69 (1.00)	63 (0.94)	66 (0.97)	6.9 (2.73)	40.0 (6.28)	23.5 (4.51)	10.0	0.4	0.7
Mean	70 (1.05)	34 (0.61)	52 (0.83)	7.6 (2.75)	18.8 (4.01)	13.2 (3.38)	1.0	0.2	0.6
				5 August 1982					
0	81 (1.18)	1 (0.05)	41 (0.61)	4.1 (2.12)	0.5 (0.91)	2.3 (1.51)	1.6	0.0	0.8
1.6	71 (1.06)	33 (0.56)	52 (0.81)	3.0 (1.82)	2.5 (1.60)	2.7 (1.71)	1.3	0.5	0.9
4.0	73 (1.06)	24 (0.49)	49 (0.77)	3.6 (2.00)	2.1 (1.49)	2.9 (1.75)	1.7	0.5	1.1
10.0	78 (1.10)	32 (0.59)	55 (0.85)	4.2 (2.15)	6.4 (2.46)	5.3 (2.31)	2.1	0.5	1.3
Mean	76 (1.10)	23 (0.42)	49 (0.76)	3.7 (2.02)	2.9 (1.61)	3.3 (1.82)	1.7	0.4	1.1
LSD (P=0.05)									
WE		NS@			0.54			NS	
IBA		0.12			0.38			8.56	
Date		NS			0.38			8.56	
Date x WE		NS			NS			NS	
Date X IBA		0.16			0.54			NS	
WE x IBA		0.23			0.77			NS	
Date x WE x IBA		NS			NS			NS	

+ Data transformed to arcsine$\sqrt{RP}$ for analysis of variance.
z Data transformed to $\sqrt{RN + 0.5}$ for analysis of variance.
@ Not significantly different.

ber was slightly increased or decreased depending on date of propagation, whereas in Cornus (Table 4), root number was increased slightly.

1.1.2. Group 2 species (trees)

Data for rooting of two Group 2 species, Salix alba tristis and Betula pendula 'Gracilis' are shown in Tables 6 and 7, respectively. No rooting occurred in the other species (Malus rinkii 'Royalty' and Tilia cordata) in this group.

Willow extract concentrations between 0 and 4.0 g/100 mL had no influence on rooting percentage, root number or root length of Salix alba tristis (Table 6). However, 5000 ppm IBA increased root number and decreased root length, but showed no influence on rooting percentage of this species.

Similar to Salix, willow extract concentrations had no influence on rooting of Betula (Table 7). IBA solution (5000 ppm) increased slightly rooting percentage, root number and root length, and was more consistent in its effect than powdered (0.4%) IBA.

1.1.3 Group 3 species (evergreens)

Data for rooting of two Group 3 species, Juniperus chinensis 'Mountbatten' and Taxus media, in response to 5000 ppm each of IAA, IBA, and NAA and to willow extract at 4.0 g/100 mL are shown in Table 8.

All rooting parameters of Taxus were increased by all three auxins. Corresponding data for Juniperus were increased slightly and moderately by

Table 6. Rooting percentage (RP), mean root number (RN), and mean root length (RL) of *Salix alba tristis* cuttings in response to six treatments with willow extract (WE) and IBA.

WE	RP			RN			RL (cm)		
(g/ 100 mL)	IBA (ppm) 0	5000	Mean	IBA (ppm) 0	5000	Mean	IBA (ppm) 0	5000	Mean
0	60 (0.90)+	38 (0.59)	49 (0.75)	2.7 (1.71)z	3.8 (1.93)	3.3 (1.82)	0.5	0.1	0.3
1.6	63 (0.93)	53 (0.77)	58 (0.85)	2.4 (1.55)	9.2 (2.38)	5.8 (1.97)	0.5	0.4	0.5
4.0	63 (0.92)	48 (0.81)	55 (0.87)	2.2 (1.67)	6.4 (2.80)	4.3 (2.23)	0.5	0.3	0.4
Mean	62 (0.92)	46 (0.72)	54 (0.82)	2.4 (1.64)	6.5 (2.37)	4.5 (2.01)	0.5	0.3	0.4
LSD (P=0.05)									
WE		NS@			NS			NS	
IBA		NS			3.71			0.18	
WE x IBA		NS			NS			NS	

+ Data transformed to arcsine$\sqrt{RP}$ for analysis of variance.
z Data transformed to$\sqrt{RN + 0.5}$ for analysis of variance.
@ Not significantly different.

Table 7. Rooting percentage (RP), mean root number (RN), and mean root length (RL) of Betula pendula 'Gracilis' cuttings in response to six treatments with willow extract (WE) and IBA.

WE	RP				RN				RL (cm)			
(g/	IBA (ppm)				IBA (ppm)				IBA (ppm)			
100 mL)	0	5000[a]	0.4%[b]	Mean	0	5000[a]	0.4%[b]	Mean	0	5000[a]	0.4%[b]	Mean
0	3 (0.08)+	20 (0.68)	0 (0.00)	8	0 (0.72)z	0.7 (1.03)	0 (0.72)	0.2 (0.84)	0	1.0	0	0.3
1.6	0 (0.00)	20 (0.68)	18	13	0 (0.71)	0.8 (1.33)	0.4 (0.95)	0.4 (0.95)	0	0.6	1.1	0.6
4.0	0 (0.00)	40 (0.39)	5	15	0 (0.71)	1.3 (1.06)	0.1 (0.77)	0.5 (1.00)	0	1.1	0.1	0.4
Mean	1 (0.03)	27 (0.49)	8	2	0 (0.71)	0.9 (1.14)	0.2 (0.84)	0.4 (0.94)	0	0.9	0.4	0.4
LSD (P=0.05)												
WE		NS@				NS				NS		
IBA		NS				NS				NS		
WE x IBA		NS				NS				NS		

+ Data transformed to arcsine $\sqrt{RP}$ for analysis of variance.
z Data transformed to $\sqrt{RN + 0.5}$ for analysis of variance.
@ Not significantly different.
a Solution
b Powder

Table 8. Rooting percentage (RP), mean root number (RN), and mean root length (RL) of *Juniperus chinensis* 'Mountbatten' and *Taxus media* in response to seven rooting treatments, including willow extract (WE) and auxins.

Treatments	Rooting performance		
	RP	RN	RL (cm)
	***Juniperus chinensis* 'Mountbatten'**		
Control	3 (0.08)+	0.2 (0.83)z	0.2
IAA[a]	13 (0.45)	0.5 (0.93)	0.8
IBA[b]	33 (0.59)	2.5 (1.63)	2.7
NAA[a]	3 (0.08)	0.2 (0.79)	0.2
IAA + WE[b]	18 (0.42)	0.2 (0.83)	1.1
IBA + WE[b]	35 (0.63)	1.3 (1.28)	3.7
NAA + WE[b]	57 (0.84)	8.2 (2.90)	3.4
LSD (P=0.05)	0.34	0.64	2.4
	Taxus media		
Control	10 (0.28)+	0.1 (0.79)z	0
IAA[a]	88 (1.32)	11.1 (3.23)	1.1
IBA[a]	90 (1.34)	20.7 (4.23)	1.1
NAA[a]	85 (1.22)	30.5 (5.50)	0.8
IAA + WE[b]	70 (1.00)	3.2 (1.85)	0.6
IBA + WE[b]	53 (0.81)	3.1 (1.81)	0.3
NAA + WE[b]	78 (1.21)	19.9 (4.09)	0.7
LSD (P=0.05)	0.42	1.99	15.8

\+ Data transformed to arcsine $\sqrt{RP}$ for analysis of variance
z Data transformed to $\sqrt{RN + 0.5}$ for analysis of variance
a 5000 ppm solution
b 4.0 g/mL

IAA and IBA, respectively.

Willow extract added to the auxins showed small or variable response in terms of each rooting parameter. While rooting percentage, root number and root length of Taxus were suppressed moderately by these treatments, all rooting parameters of Juniperus were increased markedly by NAA + WE (Table 8). Treatments with IBA + WE also increased root number and root length of Juniperus.

In related investigations of Juniperus chinensis 'Mountbatten' and of three other Group 3 species (Juniperus virginiana 'Skyrocket', Pinus sylvestris and Pinus mugho 'mughus') rooting data are shown in Tables 9, 10, 11, and 12, respectively.

Willow extract at 4.0 g/100 mL increased rooting percentage and root length of Juniperus chinensis (Table 9) and decreased root number and root length of Juniperus virginiana (Table 10), while it had no influence on each rooting parameter of Pinus sylvestris (Table 11) and Pinus mugho (Table 12). Similar to Group 2 species in Table 8, the influence of auxin on rooting on Pinus and Juniperus species were small or moderate, but variable with regards to species, rooting parameters or auxin types.

1.2 Discussion

The present study surveyed 13 woody species for their rooting response to willow extract, auxins, or both. According to the summary in Table 13, three Group 1, and one Group 3 species showed distinct positive response to auxins. While all Group 3 species showed positive response to auxins, three out of four Group 1 species were adversely affected by these treatments.

Table 9. Rooting percentage (RP), mean root number (RN), and mean root length (RL) of *Juniperus chinensis* cuttings in response to willow extract (WE) and auxins.

Auxin	RP			RN			RL (cm)		
(5000 ppm)	WE (g/100 mL) 0	4.0	Mean	WE (g/100 mL) 0	4.0	Mean	WE (g/100 mL) 0	4.0	Mean
0	3 (0.08)+	13 (0.34)	8 (0.21)	0 (0.72)z	0.2 (0.79)	0.1 (0.75)	0.1	1.3	0.7
IAA	8 (0.20)	40 (0.68)	24 (0.44)	0.4 (0.91)	2.4 (1.78)	1.3 (1.35)	1.0	5.2	3.1
IBA	55 (0.85)	55 (0.84)	55 (0.85)	6.2 (2.23)	1.8 (1.49)	4.0 (1.86)	5.2	8.7	6.9
NAA	27 (0.59)	50 (0.86)	39 (0.73)	9.3 (3.37)	17.5 (4.42)	13.4 (3.89)	1.9	3.0	2.5
Mean	23 (0.43)	39 (0.68)	31 (0.55)	4.0 (1.81)	5.5 (2.14)	4.7 (3.65)	2.1	4.5	3.3
LSD (P=0.05)									
WE		0.19			NS@			3.3	
Auxin		0.26			0.26			NS	
WE x Auxin		NS			0.37			NS	

\+ Data transformed to arcsine$\sqrt{RP}$ for analysis of variance.
z Data transformed to$\sqrt{RN + 0.5}$ for analysis of variance.
@ Not significantly different.

Table 10. Rooting percentage (RP), mean root number (RN), and mean root length (RL) of *Juniperus virginiana* cuttings in response to willow extract (WE) and auxins.

Auxin	RP			RN			RL (cm)		
(5000 ppm)	WE (g/100 mL) 0	4.0	Mean	WE (g/100 mL) 0	4.0	Mean	WE (g/100 mL) 0	4.0	Mean
0	48 (0.72)+	48 (0.72)	48 (0.72)	1.4 (1.33)z	1.1 (1.23)	1.3 (1.28)	4.1	4.4	4.3
IAA	60 (0.91)	62 (0.88)	61 (0.89)	1.8 (1.47)	1.6 (1.44)	1.7 (1.45)	4.8	3.7	4.2
IBA	78 (1.09)	55 (0.79)	67 (0.94)	5.8 (2.50)	1.9 (1.52)	3.9 (2.01)	5.9	3.9	4.9
NAA	30 (0.48)	25 (0.72)	27 (0.60)	3.3 (1.82)	1.7 (1.41)	2.5 (1.61)	1.1	2.0	1.5
Mean	54 (0.80)	47 (0.78)	51 (0.79)	3.1 (1.78)	1.6 (1.41)	2.3 (1.59)	4.0	3.5	3.7
LSD (P=0.05)									
WE		NS@			0.33			1.9	
Auxin		0.28			0.47			NS	
WE x Auxin		NS			NS			NS	

+ Data transformed to arcsine$\sqrt{RP}$ for analysis of variance.
z Data transformed to$\sqrt{RN + 0.5}$ for analysis of variance.
@ Not significantly different.

Table 11. Rooting percentage (RP), mean root number (RN), and mean root length (RL) of Pinus sylvestris cuttings in response to willow extract (WE) and auxins.

Auxin	RP			RN			RL (cm)		
(5000 ppm)	WE (g/100 mL) 0	4.0	Mean	WE (g/100 mL) 0	4.0	Mean	WE (g/100 mL) 0	4.0	Mean
0	28 (0.46)+	28 (0.46)	28 (0.46)	0.5 (0.99)z	0.7 (1.06)	0.6 (1.03)	4.0	4.0	4.0
IAA	10 (0.23)	33 (0.51)	21 (0.37)	0.2 (0.83)	0.6 (1.04)	0.4 (0.93)	1.3	7.5	4.4
IBA	40 (0.68)	38 (0.65)	39 (0.67)	1.1 (1.25)	1.0 (1.22)	1.1 (1.23)	9.0	4.0	6.5
NAA	0 (0.00)	13 (0.31)	7 (0.15)	0 (0.71)	0.5 (0.98)	0.3 (0.85)	0	1.4	0.7
Mean	19 (0.34)	28 (0.50)	23 (0.42)	0.5 (0.95)	0.7 (1.08)	0.6 (1.01)	3.6	4.2	3.9
LSD (P=0.05)									
WE		NS@			NS			NS	
Auxin		0.24			0.22			3.41	
WE x Auxin		NS			NS			NS	

+ Data transformed to arcsine$\sqrt{RP}$ for analysis of variance.
z Data transformed to$\sqrt{RN + 0.5}$ for analysis of variance.
@ Not significantly different.

Table 12. Rooting percentage (RP), mean root number (RN), and mean root length (RL) of Pinus mugho 'Mughus' cuttings in response to willow extract (WE) and auxins.

Auxin	RP			RN			RL (cm)		
(5000 ppm)	WE (g/100 mL) 0	4.0	Mean	WE (g/100 mL) 0	4.0	Mean	WE (g/100 mL) 0	4.0	Mean
0	20 (0.38)+	30 (0.56)	25 (0.23)	9.7 (3.19)z	1.1 (1.22)	5.4 (2.22)	0.7	4.6	2.7
IAA	43 (0.70)	65 (0.95)	54 (0.83)	3.3 (1.90)	0.5 (0.98)	1.9 (1.44)	2.4	4.3	3.3
IBA	38 (0.68)	25 (0.52)	31 (0.60)	3.7 (1.89)	2.4 (1.64)	3.1 (1.77)	0.6	1.5	1.1
NAA	13 (0.31)	12 (0.36)	13 (0.33)	0.5 (0.98)	0.8 (1.09)	0.7 (1.03)	0.2	0.4	0.3
Mean	29 (0.52)	33 (0.60)	31 (0.56)	4.3 (1.74)	1.2 (1.58)	2.8 (1.66)	1.0	2.7	1.9
LSD (P=0.05)									
WE		NS@			NS			NS	
Auxin		0.22			NS			1.90	
WE x Auxin		NS			NS			NS	

+ Data transformed to arcsine$\sqrt{RP}$ for analysis of variance.
z Data transformed to$\sqrt{RN + 0.5}$ for analysis of variance.
@ Not significantly different.

Table 13. Summary rooting response of 13 woody nursery species to willow extract (WE) and auxins.

SPECIES	WE	AUXIN	WE X AUXIN INTERACTION
GROUP 1 (SHRUBS)			
Cotoneaster	NS	+	NS
Cornus	+	-	*
Ribes	+	-	*
Philadelphus	+	-	*
GROUP 2 (TREES)			
Salix	NS	+	NS
Tilia	NS	NS	NS
Betula	0	0	0
Malus	0	0	0
GROUP 3 (EVERGREENS)			
Taxus	-	+	a
Juniperus 'Mountbatten'	+	+	*
Juniperus 'Skyrocket'	-	+	NS
Pinus mugho	NS	+	NS
Pinus sylvestris	NS	+	NS

* Interaction was significant
NS Not significantly different
+ or - Influence was classified as positive (+) or negative (-) when one or more of the three parameters (rooting percentage, mean root number and root length) was significantly increased or decreased, respectively.
0 No rooting
a The interaction was not measured.

In a previous study, Richer-Leclerc and Chong (1983) studied the effect of willow extracts on rooting of Philadelphus coronarius 'Aureus', Ribes alpinum, and Cornus alba 'Elegantissima'. They related enhanced root formation of Philadelphus and Ribes to the favorable presence of rooting cofactors or related substances in the willow extract. Although the same three species were tested in the present study, there were variations in the results for Ribes and Cornus. In the study of Richer-Leclerc (1983), cuttings of the three species were collected on August 20, in the present study Ribes cuttings were collected on June 14 and July 29, and Cornus on June 11 and August 2 (Table 2). This difference may explain the discrepancy in these results.

The decrease in rooting response and apparent (basal browning) injury with auxin treatments indicated auxin toxicity to the cuttings as previously described by Richer-Leclerc and Chong (1983). According to Chong (1982), softwood cuttings taken too early in the spring or cuttings obtained from easier-to-root species are more likely to be injured by IBA treatment.

In the more difficult-to-root Group 2 and Group 3 species, response to willow extract was minimal, whereas response to auxins was more pronounced. Similarly, in the present study (Table 13) and that of Richer-Leclerc and Chong (1983), rooting of Cotoneaster was not affected by willow extract treatment but was increased by IBA. However, Juniperus chinensis 'Mountbatten' and Juniperus virginiana 'Skyrocket' failed to root, even with high IBA concentrations (Chong 1982). Gil-Albert and Boix (1978) found J. virginiana to be a very difficult-to-root species. It is noteworthy that Malus and Tilia failed to root regardless of the rooting

treatment applied (Table 13). Other researchers have demonstrated the beneficial effect of high IBA concentrations on the rooting of Malus and Tilia species: Malus 'Hopa' with 20000-40000 ppm IBA (Chong 1982), Malus floribunda with 10000-30000 ppm IBA (Brown and Dirr 1976), Malus 'Selkirk' and Malus sieboldii zumi var. calocarpa with 10000-40000 ppm IBA (Still 1981).

The rooting of Salix alba tristis, an easy-to-root species (Hartmann and Kester 1975), was improved by auxin but not by willow extract treatments. Kawase (1964) did not obtain any root promoting effect when he treated Salix alba cuttings with willow extracts. He suggested that Salix alba cuttings were deficient in auxin, but not in rooting cofactors.

This study confirms the favorable use of plant extracts and its interaction with auxins for stimulating rooting of certain woody species.

In view of the variation in response of willow extracts due to species, further studies to determine the rooting or identification of the substances requires that studies be conducted on a more controlled system.

2. Rooting of mung beans with crude or purified willow extract

All results reported in this section were preceded by preliminary work.

2.1 Crude or clarified extract

2.1.1 Results

2.1.1.1 Optimum concentration

This experiment determined the concentration of crude willow extract (28 February 1983) (Fig. 3) yielding optimal rooting activity. A series of 16 different concentrations were tested: 0 (distilled water control), 2.5×10^{-2}, 5.0×10^{-2}, 7.5×10^{-2}, 1.0×10^{-1}, 2.5×10^{-1}, 5.0×10^{-1}, 7.5×10^{-1}, 1.0×10^{0}, 2.5×10^{0}, 5.0×10^{0}, 7.5×10^{0}, 1.0×10^{1}, 2.5×10^{1}, 5.0×10^{1}, 7.5×10^{1} mg powder per mL of distilled water.

In comparison with the distilled water control, concentrations of crude willow extract between 1 and 75 mg/mL increased the rooting response of mung bean cuttings; the optimum concentration was 7.5 mg/mL (Figure 5). Concentrations of extract greater than 75 mg/mL were not tested because of the pasty, almost solid, consistency of the extracts.

A related investigation with filtered extracts (concentrations between 0 and 10.0 mg/mL) collected at 2-month intervals over a one-year period confirmed that the optimum rooting of this extract (7.5 mg/mL) (Fig. 6) was similar to that of the crude extract (Fig. 5). Unlike results for the supernatant extract (Fig. 8), there was no consistent seasonal rooting pattern with the filtered extract within each of the different concentrations tested (Fig. 6).

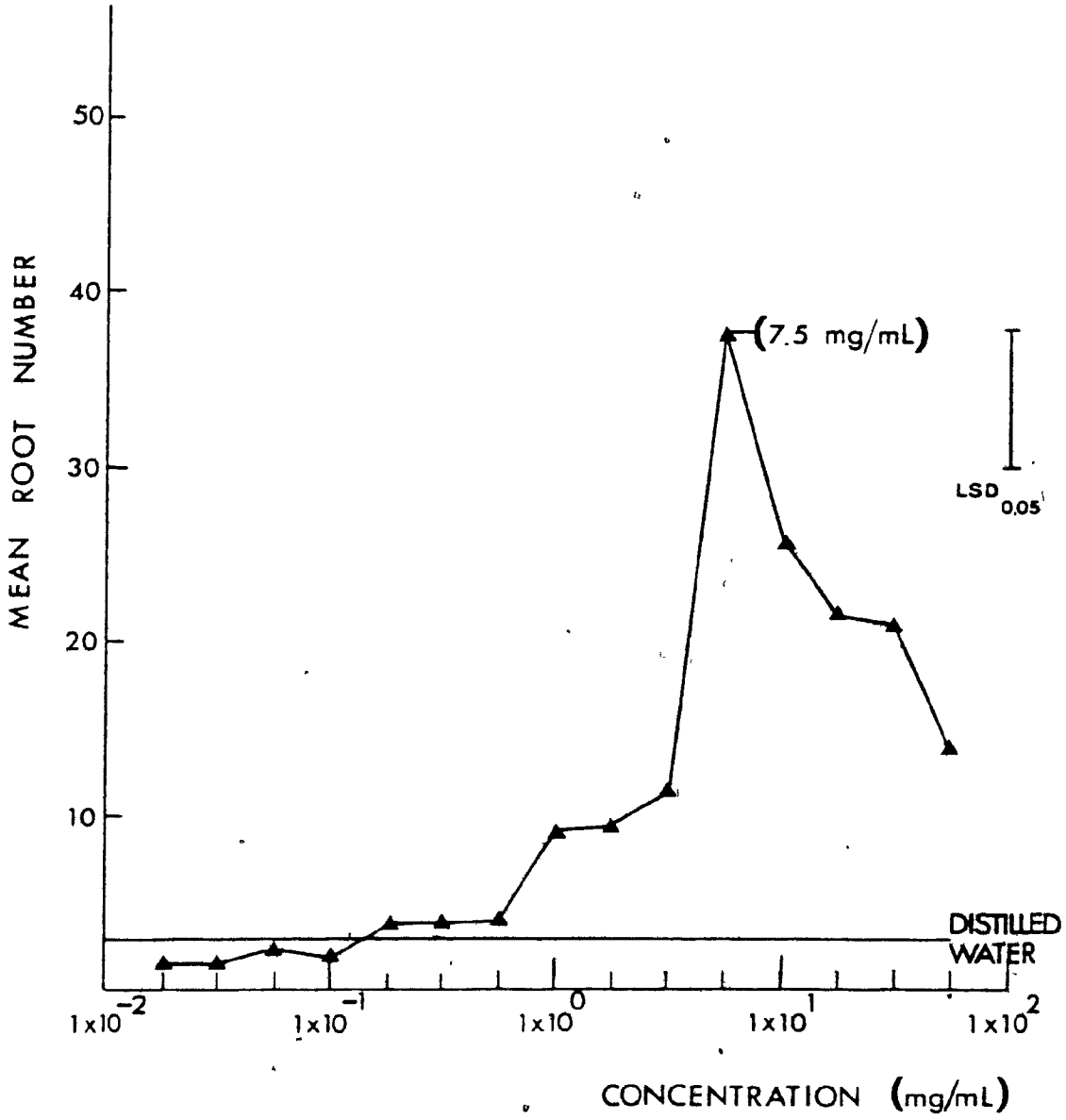


Figure 5. Mean root number per mung bean cutting in response to a series of 16 concentrations of crude willow extract.

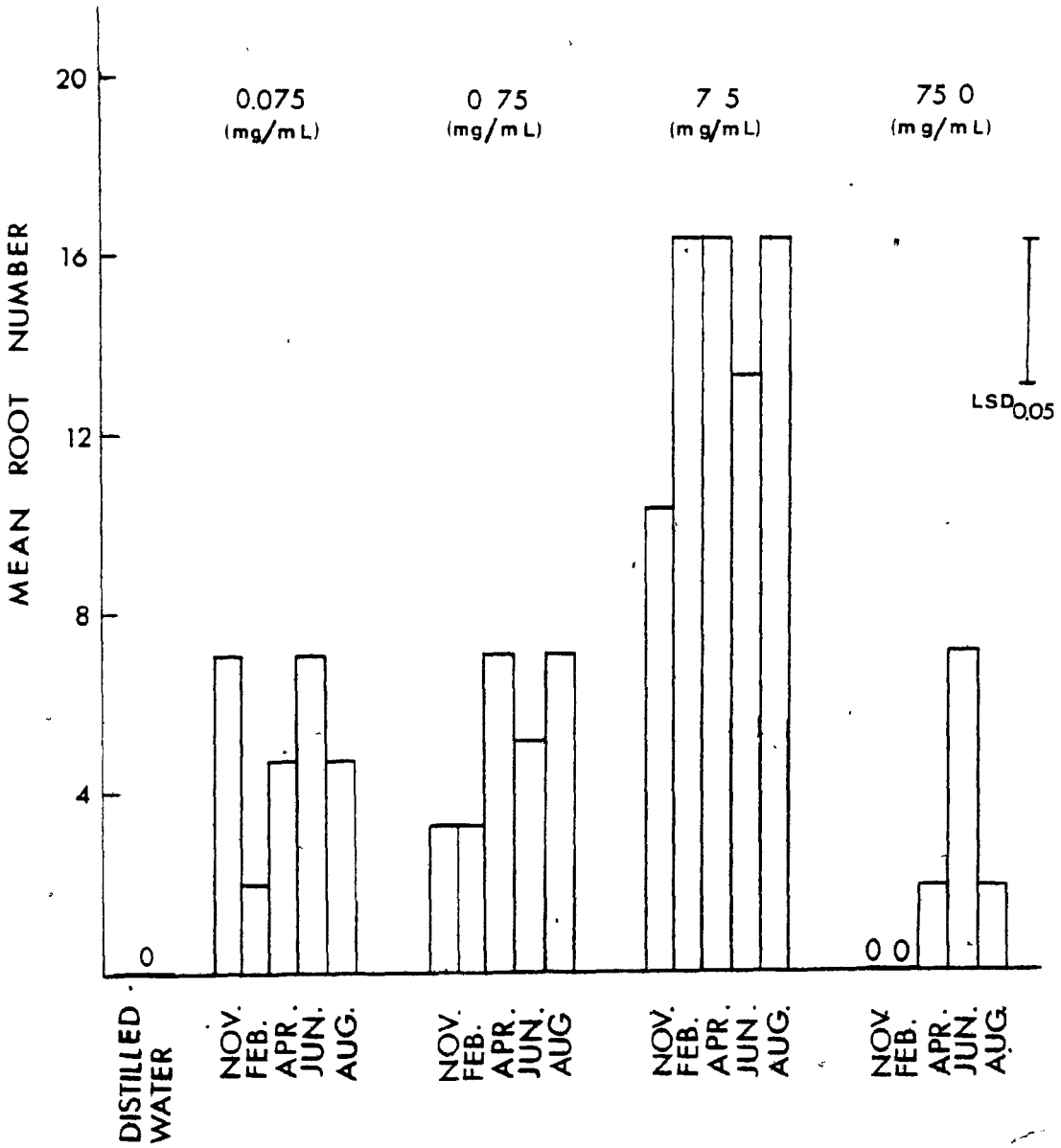


Figure 6. Mean root number per mung bean cutting in response to clarified filtered willow extract at different concentrations over a one-year period. Vertical bar represents LSD for concentration. There was no statistical difference for date of collection and no interaction between concentration x date of collection.

2.1.1.2 Crude versus clarified

This experiment compared the rooting activity of crude extract and clarified extracts, derived as shown in Fig. 2 (page 42). There were six different rooting test solutions (28 February 1982), each at a concentration of 7.5 mg/mL of distilled water; (a) crude willow extract; (b) supernatant clarified extract; (c) filtered clarified extract; (d) filtered, supernatant clarified extract; (e) residual extract redissolved in 100 mL of 10% methanol. A treatment consisting of distilled water was also included.

In comparison with the crude extract, yielding 25.1 root per mung bean cutting, the clarified water extracts (supernatant, filtered, or both) yielded 36-38 roots per mung bean cutting or a mean increase of 46% (Fig. 7). The 10% methanolic residual extract decreased rooting in comparison with the crude extract by 64%, although the residual extract was slightly promotive in rooting activity compared with distilled water.

2.1.1.3 Seasonal activity

To determine the seasonal activity of willow extracts collected on different dates, mung bean rooting tests were conducted on supernatant extracts (Fig. 3) obtained over a one-year period from the following dates of collection: (a) 25 October 1982; (b) 25 November 1982; (c) 21 December 1982; (d) 31 January 1983; (e) 28 February 1983; (f) 13 March 1983; (g) 11 April 1983; (h) 31 May 1983; (i) 28 June 1983; (j) 3 August 1983; (k) 30 August 1983; (l) 24 October 1983. A treatment consisting of

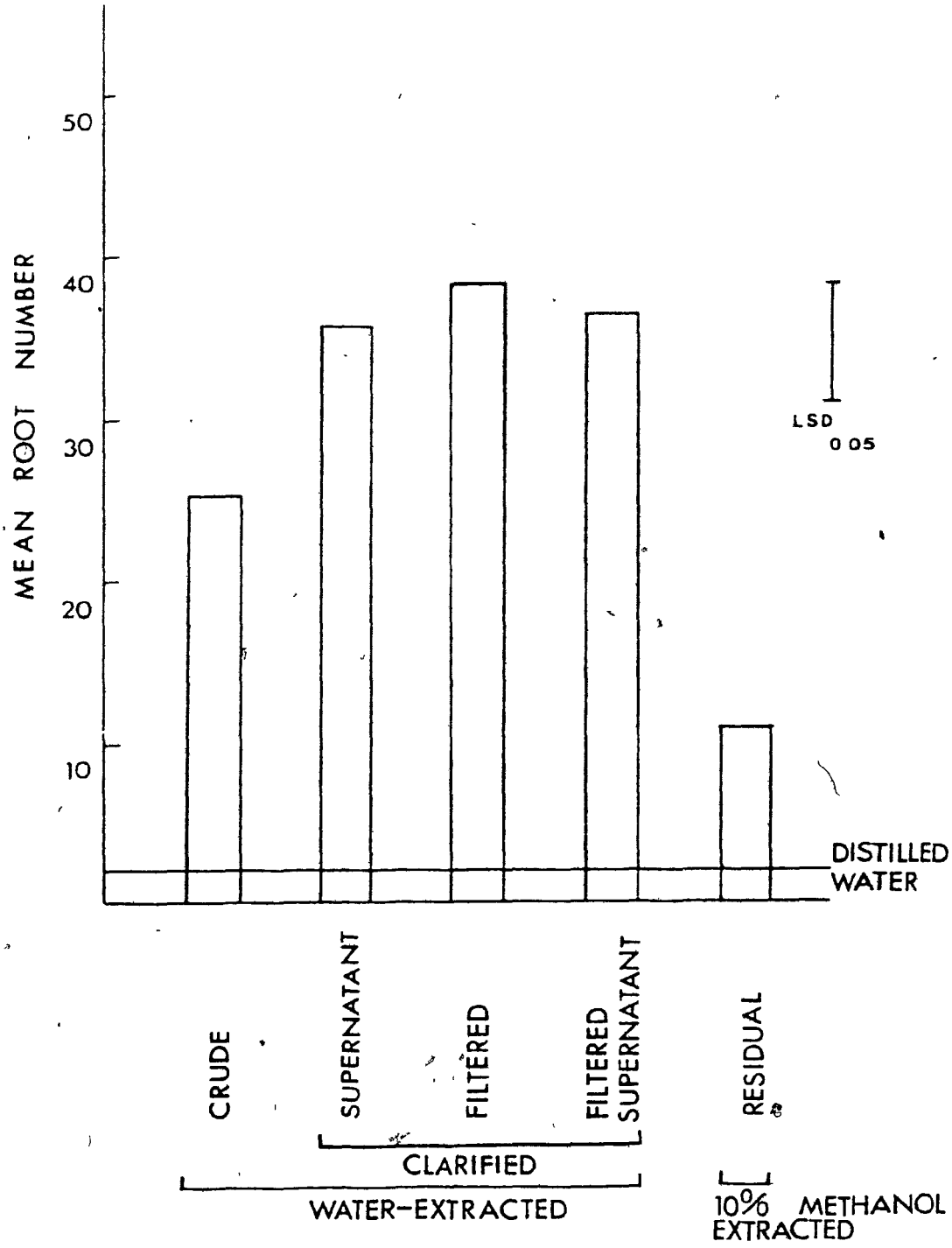


Figure 7. Mean root number per mung bean cutting in response to crude and clarified willow extract.

distilled water was also included. The basic concentration of the extracts used in this study was 7.5 mg mL of distilled water.

The rooting response of seasonal extracts fluctuated greatly during the one-year period Fig. 8 increasing between October and November, decreasing thereafter until January, and increasing to a peak in April. There was a rapid decrease in rooting activity between May and June, followed thereafter by a progressive but sharp rise in activity. The mean response between October and April 35.6 roots cutting) was considerably higher than that between May and October 22.5 roots cutting).

There were similar trends in seasonal rooting activity and content of total phenols analyzed in the supernatant extract (Fig. 8). In fact, correlation coefficient of seasonal data between root number per mung bean cutting and the total phenol content was $r = 0.658$ ($P \leq 0.05$). A similar significant response was observed between seasonal contents of alkali-labile phenols and root number per mung bean cutting ($r = 0.778$, $P \leq 0.05$) (Table 14). There was also indication of a similar relationship between the seasonal content of dihydroxy phenols and the mung bean rooting response, but because of the limited data ($df = 4$), a significant correlation was not found (Table 14).

2.1.2 Discussion

Although the residues of willow powder were not expected to be absorbed or involved per se in root-inducing activity, it is possible that decreased rooting activity of extract from this fraction might be related

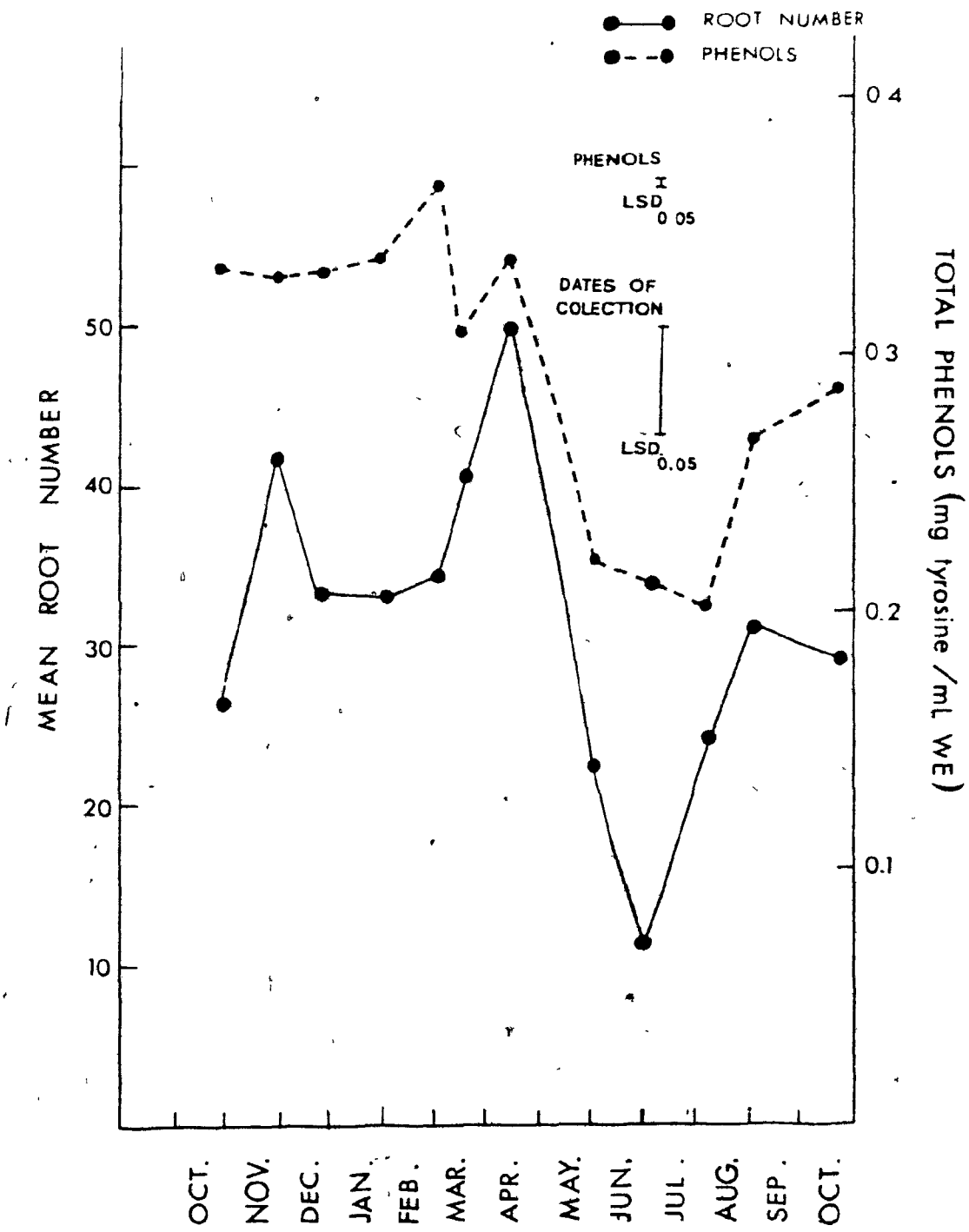


Figure 8. Seasonal rooting activity and total phenol content of extracts from plant materials collected over a one-year period.

Table 14. Seasonal contents of dihydroxy (DiOH) and alkali-labile phenols in willow extracts and correlation with mean root number per mung bean cutting.

Treatments date	Alkali-labile mg tyrosine/mL WE	DiOH phenol mg tyrosine/mL WE
25 Dc 82	0.223	0.202
2 De 82	0.238	0.214
28 Fe 83	0.251	0.231
11 Ap 83	0.256	0.200
28 Jn 83	0.166	0.146
3 Au 83	0.158	0.138
Correlation coefficient[a] of root numbers	0.778*	0.570[NS]
df	4	4

WE Willow extract
NS Not significant
* Significant at $P \leq 0.05$

to solubility and(or) absorption of biological substances in the milieu of this substance. Jackson (1938) attributed poor rooting of Bougainvillea cuttings treated with various synthetic auxin to decreased absorption or non-absorption of these substances by the cuttings. According to Hitchcock and Zimmermann (1939), the absorption process of powdered growth promoting substances is dependent upon the mechanical fineness of the powder. In studies of Kawase (1970, 1971), willow twigs were ground twice in a Wiley Mill to 20 mesh size, whereas in the present study, willow twigs were ground once in a Wiley Mill to 40 mesh size (Materials and Methods section 1.1.3). Because of the larger size of the powder used in the present study, the absorption and the effectiveness of the crude extracts may be lessened. This would help to explain the rooting improvement obtained with the removal of the crude particles.

The present studies also indicated greater root-promoting activity in water-soluble than in the water-insoluble (methanolic) fractions. Similar to results of this study (Fig. 7), enhancement in the rooting response of mung bean cuttings was obtained with supernatant water extract from lyophilized tissues (Kawase 1970) and with centrifugal diffusate (Kawase 1964) of Salix alba. In Kawase's study, the water extract and centrifugal diffusate yielded 57.6 and 43.2 roots per mung bean cutting at optimum concentrations of 2g/vial and 30 cuttings/vial, respectively. The concentration of Kawase's water extract was equivalent to 133 mg/mL; an equivalent concentration of the centrifugal diffusate in mg/mL basis can not be determined. These differences in optimal concentrations of rooting extracts may be attributed to factors such as different conditions of

extraction, different times of the year in which plant material was collected, and different genotypes of Salix used. The lack of seasonal trend at the optimal concentration of 7.5 mg/mL with filtered willow extract (Fig. 6) may be related to the release of an unknown root promoting substance(s) present on the Whatman filter paper no. 1 (Kawase 1970, 1971) used to filter the extracts.

Despite differences in climatic conditions (Italy) and species (Picea glauca), seasonal rooting activity of Picea glauca (Tognoni et al. 1977) was similar to that of willow extract used in this study (Fig. 8). Richer-Leclerc (1982) also observed a tendency for water-soluble willow extracts to have greater rooting activity in the winter months.

While Van der Lek (1925) demonstrated that the rooting capacity of Salix species was high in spring when buds are sprouting and low in winter when buds are dormant, Gumpelmayer (1949) found that the seasonal variations in rooting responses of Salix fragilis cuttings were more complex. The maximum number of roots were produced in the months of August, December and April, and the minimum in the months of October, November, February, and May (Gumpelmayer 1949).

Kikuchi et al. (1983) and Lanphear and Meahl (1963) did not find any correlation between the seasonal change in rooting ability of cuttings of Salix kariyanagi, Salix bakko, Taxus cuspidata 'Nana', and Juniperus horizontalis 'Plumosa' and seasonal rooting activity of the water-soluble substance(s) present in extracts of these species.

As in Fig. 8 and Table 14, Bassuk and Howard (1981a, 1981c) also found a strong correlation between an abundant phenolic (phloridzin) and

rooting cofactors with the seasonal rooting patterns of the cuttings. Cortizo (1981) and Cortizo and Mantilla (1981) related the high level of phenols in stems in winter to low meristematic and hydrolytic enzyme activity of the plant. Forrest (1975) who observed similar seasonal trends in total and o-dihydroxy phenol content as in the present study (Fig. 8, Table 14) related the phenolic content to the state of lignification of the tissue. In summer and spring, young tissues of Sitka spruce were low in phenols and contained mainly monomerics types while in the fall and winter period, the lignified tissues were richer in phenols, especially in polymeric types (Forrest 1975).

Interestingly, it has been shown in several instances that monophenols inhibited root formation while o-diphenol and polyphenols promoted it (Bojarczuk 1978; Borjarczuk 1979; Forter 1962; Hess 1962; Hitchcock and Thimann 1977; Wells and Marth 1953). This suggested that the low rooting potential of the willow extracts from May to September can be attributed to their low phenolic contents, mainly monophenolic in contrast to the winter tissues which contained more phenolic compounds, especially of the o-dihydroxy types.

Foong and Barnes (1981), however, did not find any correlation between the total and o-dihydroxy phenol contents of the tissues and the rooting ability of the cuttings. Similarly, the seasonal change in rooting ability of Salix kariyanagi and Salix bakko was not correlated to the activity of the total phenol content. However, one should emphasize that they extracted the phenolic compounds with 80% alcohol and thus obtained the alcohol- and water-soluble phenols whereas only the water-soluble phenols

of the willow was determined in this study (Materials and Methods section 2.2.3.1). Nevertheless, this indicates that the role played by phenolic rooting cofactors remains controversial (Basu et al. 1969; Thimann 1977).

Several authors who also obtained higher root promoting activity in winter months attributed this high activity to the accumulation of rooting cofactors in the stem after leaf drop (Smith and Chiu 1980) or to the accumulation of inhibitors such as ABA which interacted with endogenous auxin to promote rooting (Alvim et al. 1976; Chin 1969). Gesto et al. (1981) demonstrated that there was alteration of some of the rooting inhibitors into promoters upon cold storage of extracts. In the present study, the willow powder was cold stored. It would be interesting to investigate the stability of the cold stored extract and to see if there are any chemical changes which alter the rooting activity of the extracts. Alternatively, Vieitez and Pena (1969) who found that, on the average, rooting activity of acidic Salix atrocinerea extracts was lowest in the summer months (June to August) related seasonal rooting pattern of the extracts to the amount of endogenous IAA. Supra-optimal level of endogenous auxin in June was also associated with the low rooting of cuttings (Nanda and Anand 1970).

2.2 Fractionated extracts

2.2.1 Results

In the extraction step (Fig. 4, page 45), water-extract A and methanol-water extract B of willow were examined for their root promoting

activity. In comparison with distilled water (control), the rooting response of mung bean cuttings was increased by both water and methanol-water extracts (Fig. 9). There was no statistical difference in the rooting activity of these two extracts.

In the first fractionation step (1) (Fig. 4), EToAc fraction D and water fraction C were examined for their root promoting activity. In comparison with distilled water control, the water fraction signficantly promoted the rooting of mung bean cuttings while the EToAc fraction had no root promoting activity (Fig. 10).

In the second fractionation step (2) (Fig. 3), methanol-soluble EToAc fraction G, methanol-soluble water fraction E, and methanol-insoluble water fraction F, each with or without the presence of IAA (5×10^{-6}M, Kawase 1964), were tested to determine the root promoting activity and also their interaction with this auxin. As shown in Fig. 11a, b, the methanol-soluble EToAc fraction had no root promoting activity. Both methanol-soluble and methanol-insoluble water fractions significantly promoted rooting (Fig. 11a). In the presence of IAA, the promotive effect of the methanol-insoluble water fraction was enhanced even further (Fig. 11b).

In the third fractionation step (3) (Fig. 4), spectrophotometric determination of eluates from column chromotography indicated seven distinct sub-fractions (F_{E1} - F_{E7}) (Fig. 12) in the methanol-soluble EToAc fraction and five sub-fractions (F_{W1} - F_{W5}) (Fig. 13) in the methanol-soluble water fraction.

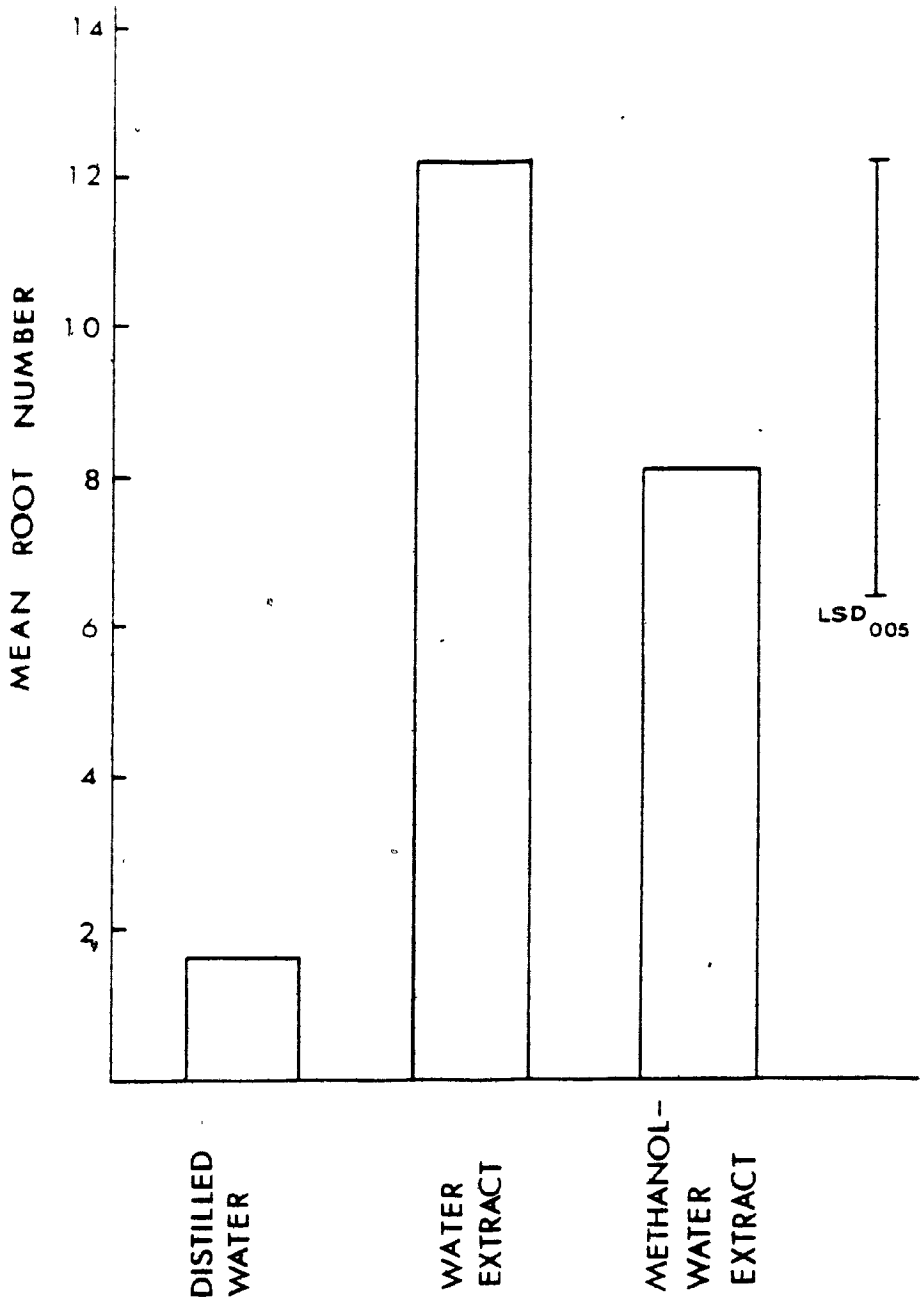


Figure 9. Mean root number per mung bean cutting in response to water and methanol-water extracts.

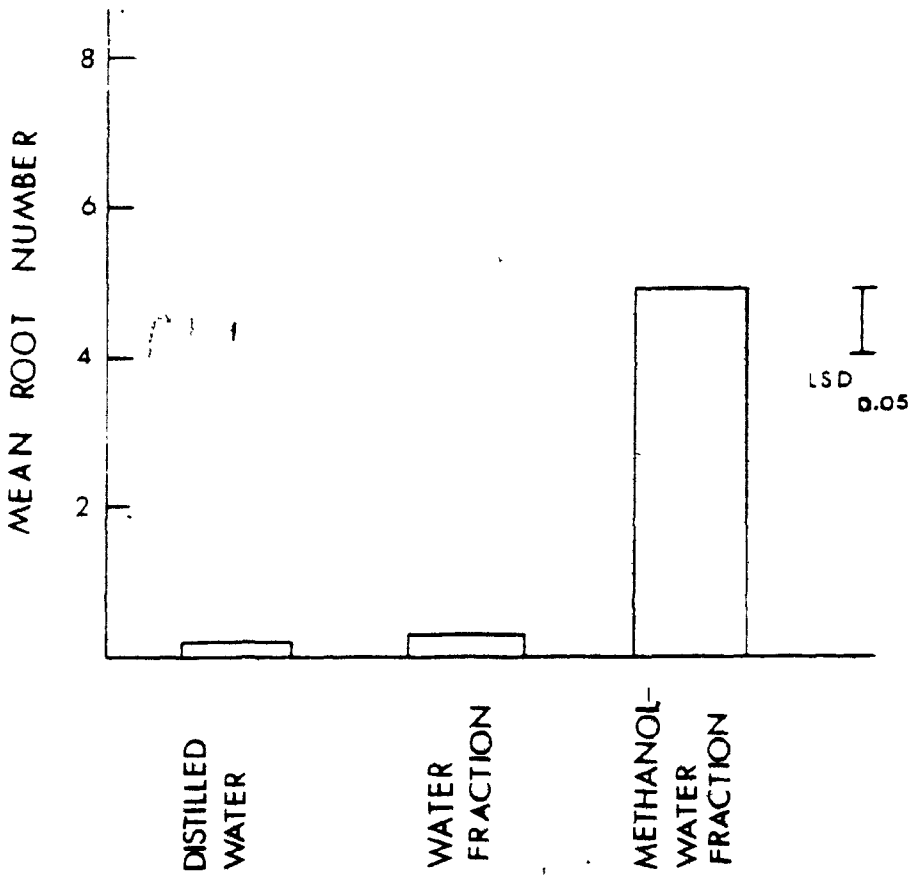


Figure 10. Mean root number per mung bean cutting in response to EToAc and water fractions step 1.

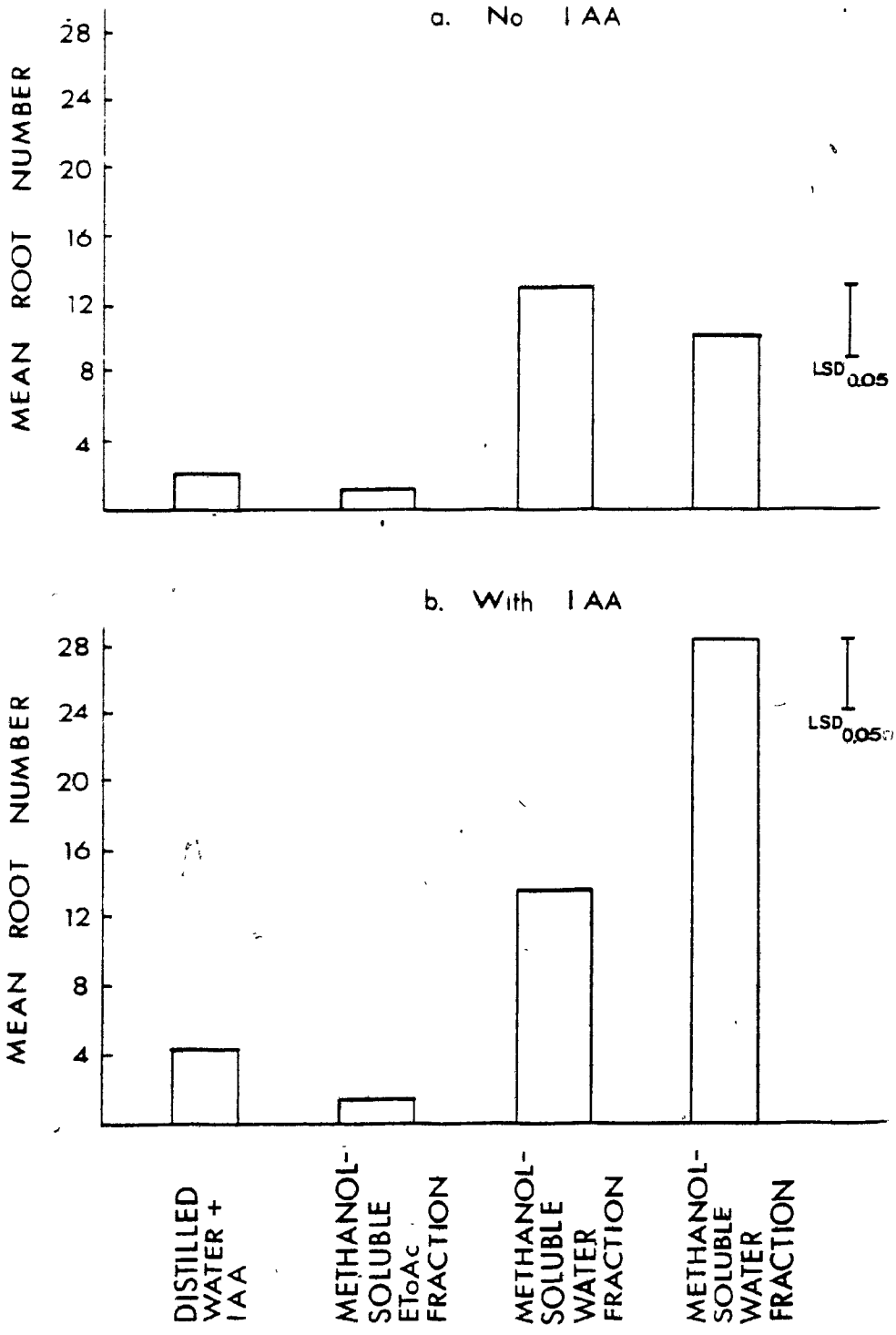


Figure 11. Mean root number per mung bean cutting in response to methanol-soluble EToAc, methanol soluble and insoluble water fractions step 2. The LSD was 4.1 for extract, 2.6 for IAA. There was no interaction of extract x IAA.

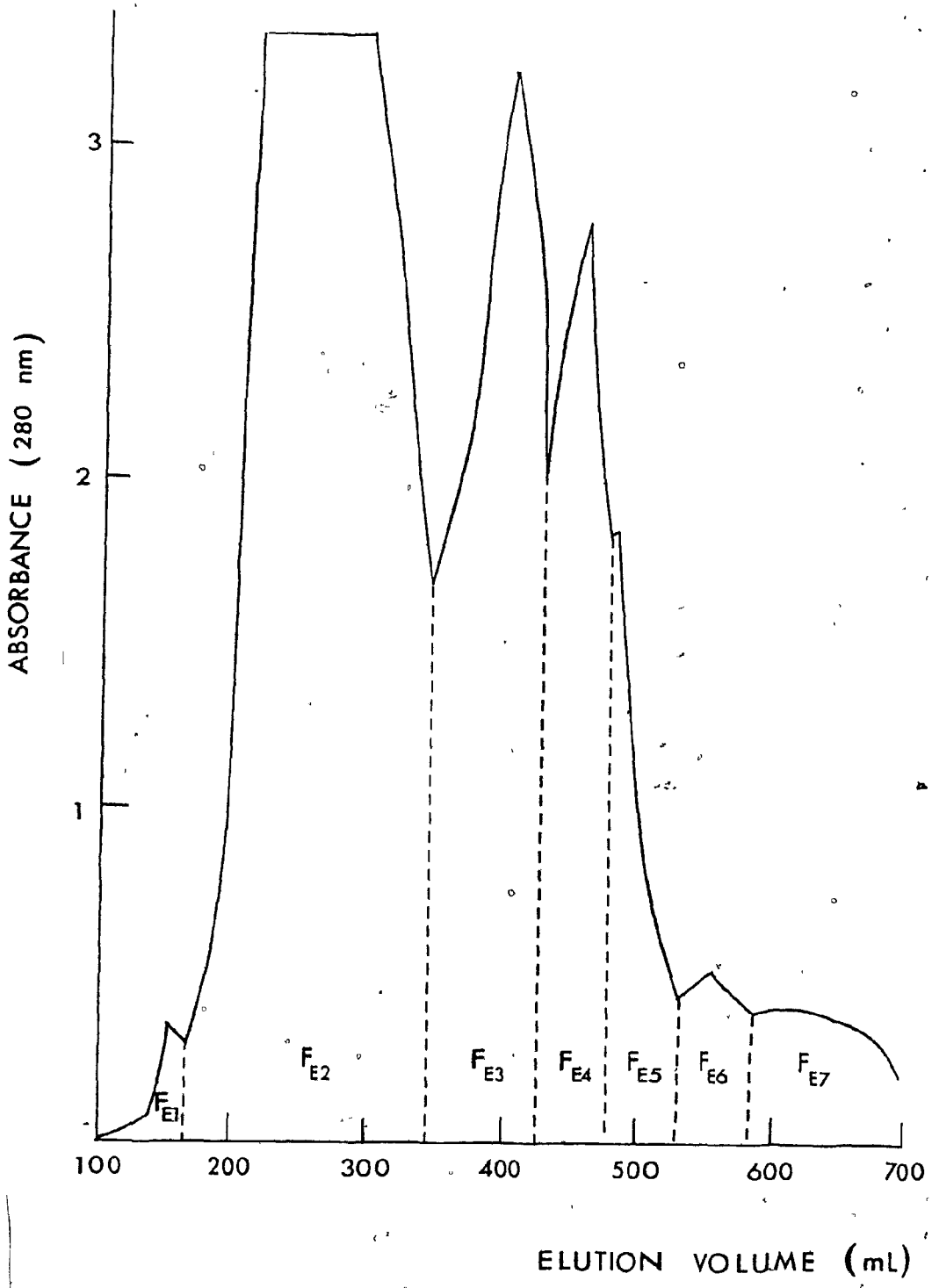


Figure 12. EToAc sub-fractions (F_{E1}-F_{E7}) chromatograms.

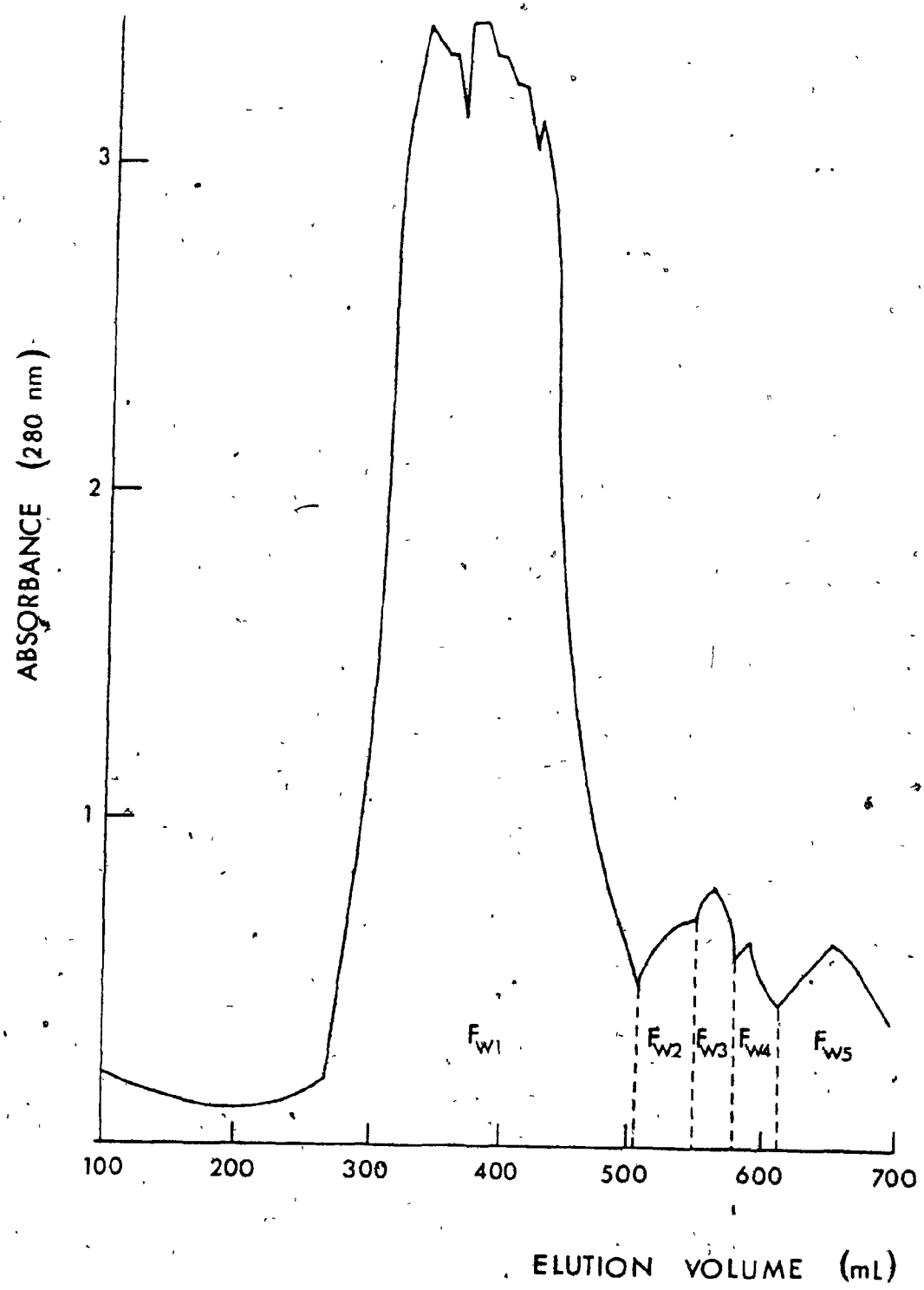


Figure 13. Water sub-fractions (F_{W1}-F_{W5}) chromatograms.

To determine their rooting activity, the F_{E1} - F_{E7} sub-fractions were tested at two concentrations: 0.75 and 7.5 mg/mL of distilled water with or without IAA (5×10^{-6} M). A control treatment containing distilled water fractionated through the Sephadex LH-20 column was also included. The F_{W1} - F_{W5} sub-fractions were tested only at a concentration of 7.5 mg/mL of distilled water.

As shown in Fig. 14, all five F_W sub-fractions showed increased rooting activity over the distilled water control. Interestingly, chromatographic analysis of the sub-fractions indicated the presence of indole group in the F_{W1} sub-fraction, the one with the greatest root promoting activity.

Fig. 15 and 16 show the rooting activity of the F_E sub-fractions tested at the higher concentration (7.5 mg/mL) with or without IAA, respectively. Fig. 17 and 18 show the rooting activity of the same sub-fractions tested at a lower concentration (0.75 mg/mL) with or without IAA, respectively.

The rooting response of each sub-fraction was variable and dependent upon the concentration and the presence of exogenously applied IAA. The LSD ($P \leq 0.05$) for data in Fig. 15 - 18 was 1.8 for the extract for sub-fraction, 0.9 for IAA, and 0.9 for concentration; there was no interaction of extract IAA, extract x concentration or extract x IAA x concentration.

At the higher concentration without IAA (Fig. 15), the F_{E1}, F_{E2}, F_{E5}, and F_{E6} sub-fractions showed moderate to high promotive effect with the highest activity in the F_{E2} sub-fractions; the F_{E3}, F_{E4}, F_{E7} sub-fractions were slightly inhibitive. With IAA (Fig. 16), the

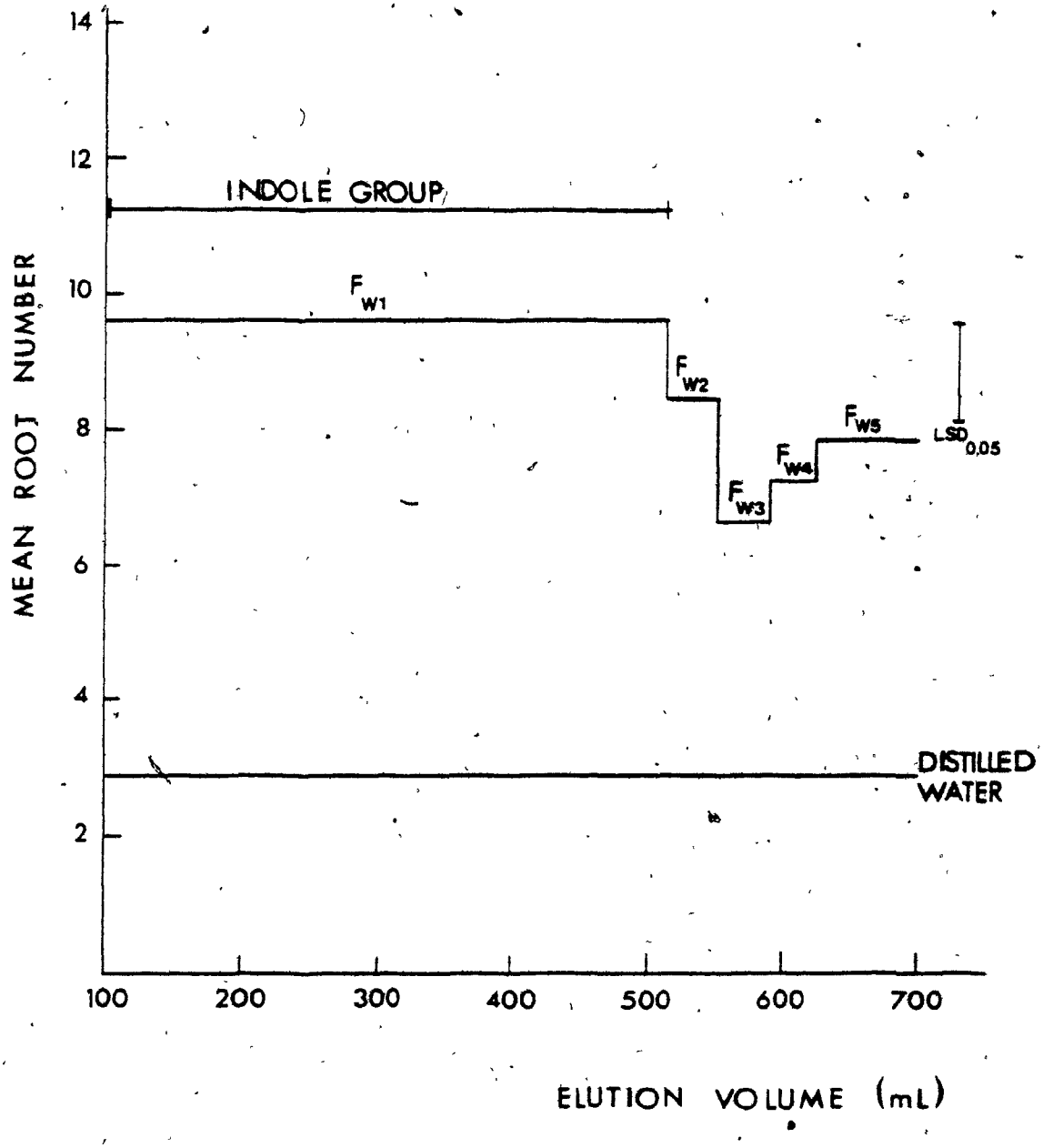


Figure 14. Mean root number per mung bean cutting in response to water sub-fractions (7.5 mg/mL).

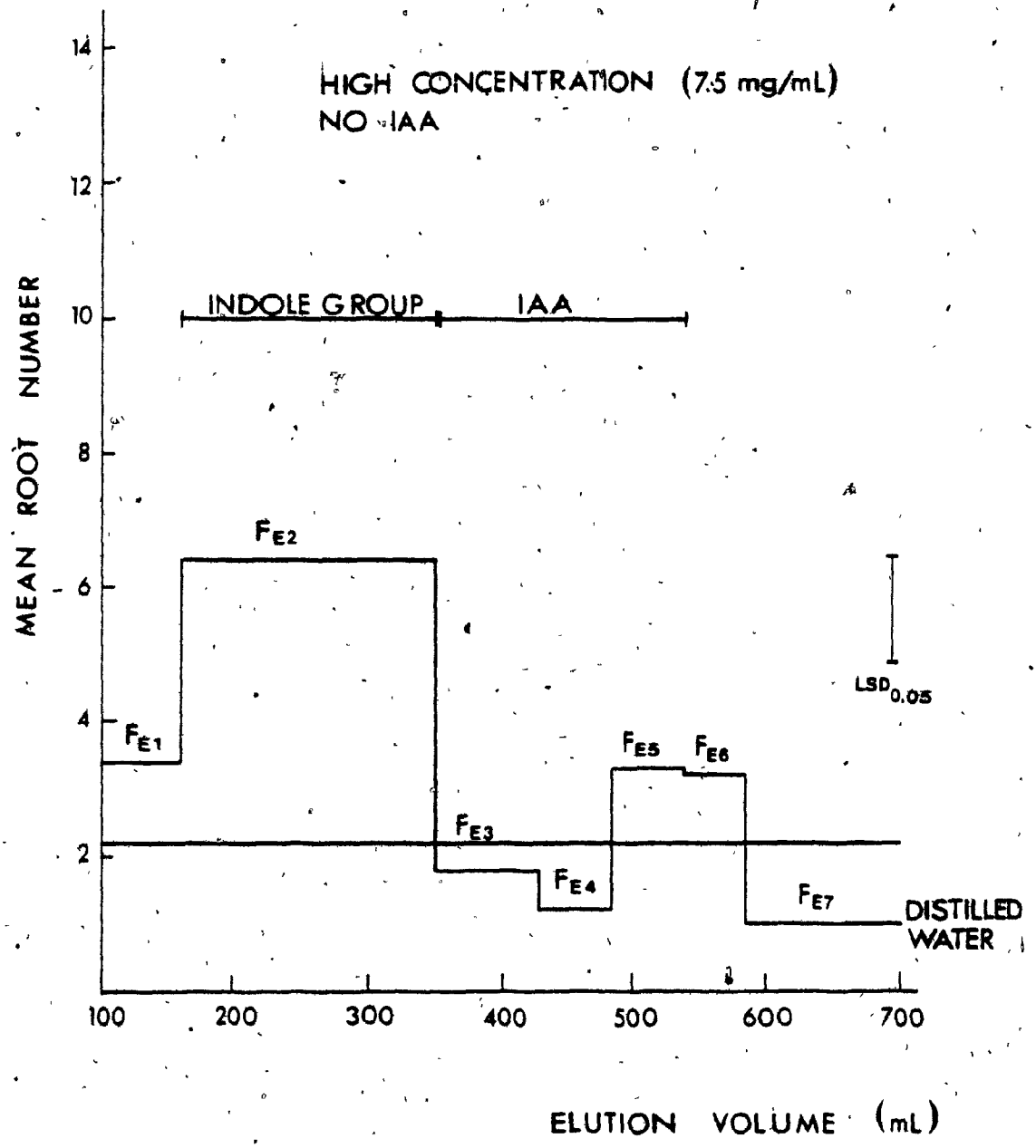


Figure 15. Mean root number per mung bean cutting in response to EToAc sub-fractions (7.5 mg/mL, without IAA).

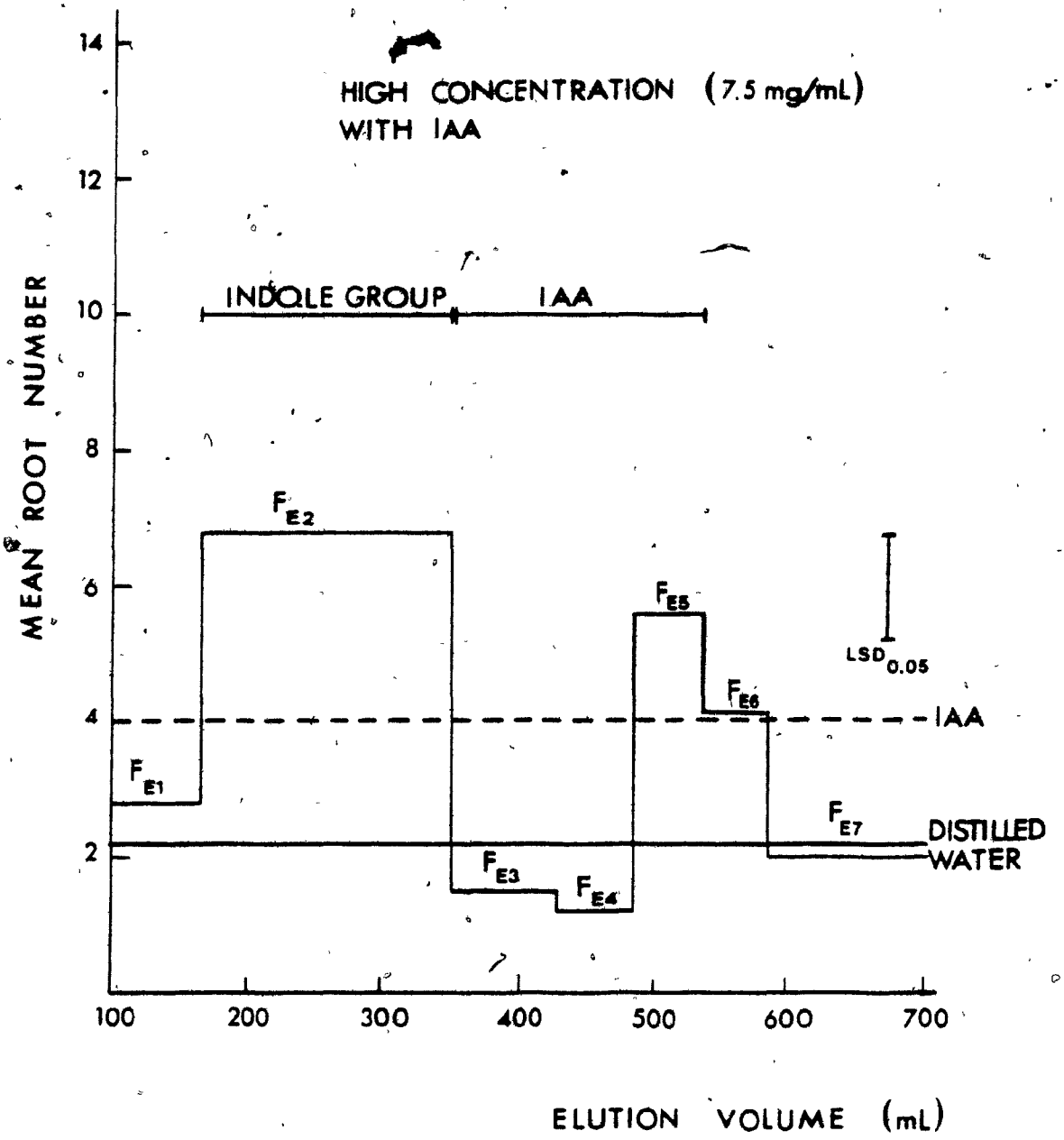


Figure 16. Mean root number per mung bean cutting in response to EToAc sub-fractions (7.5 gm/mL, with IAA).

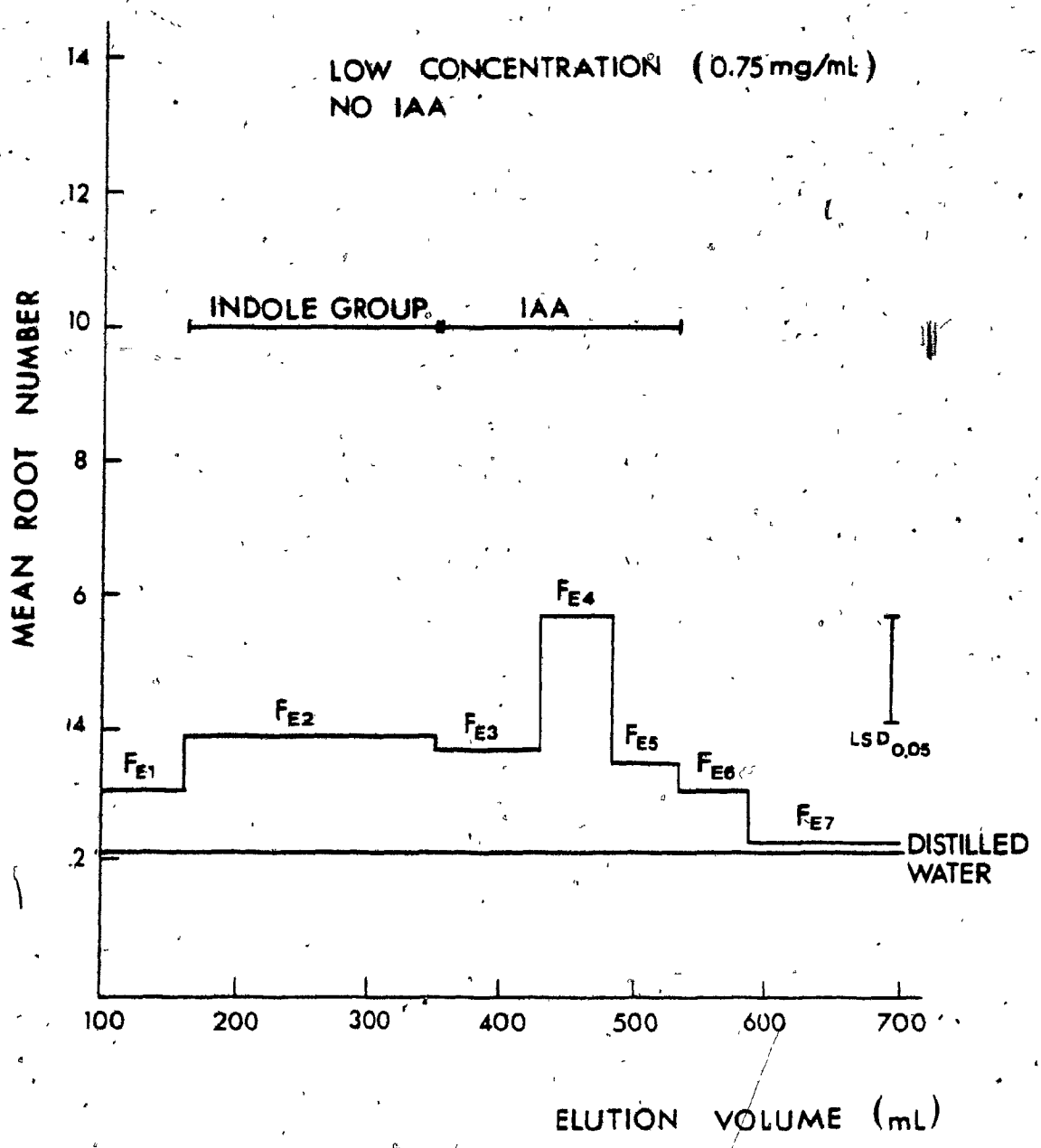


Figure 17. Mean root number per mung bean cutting in response to EToAc sub-fractions (0.75 mg/mL, without IAA).

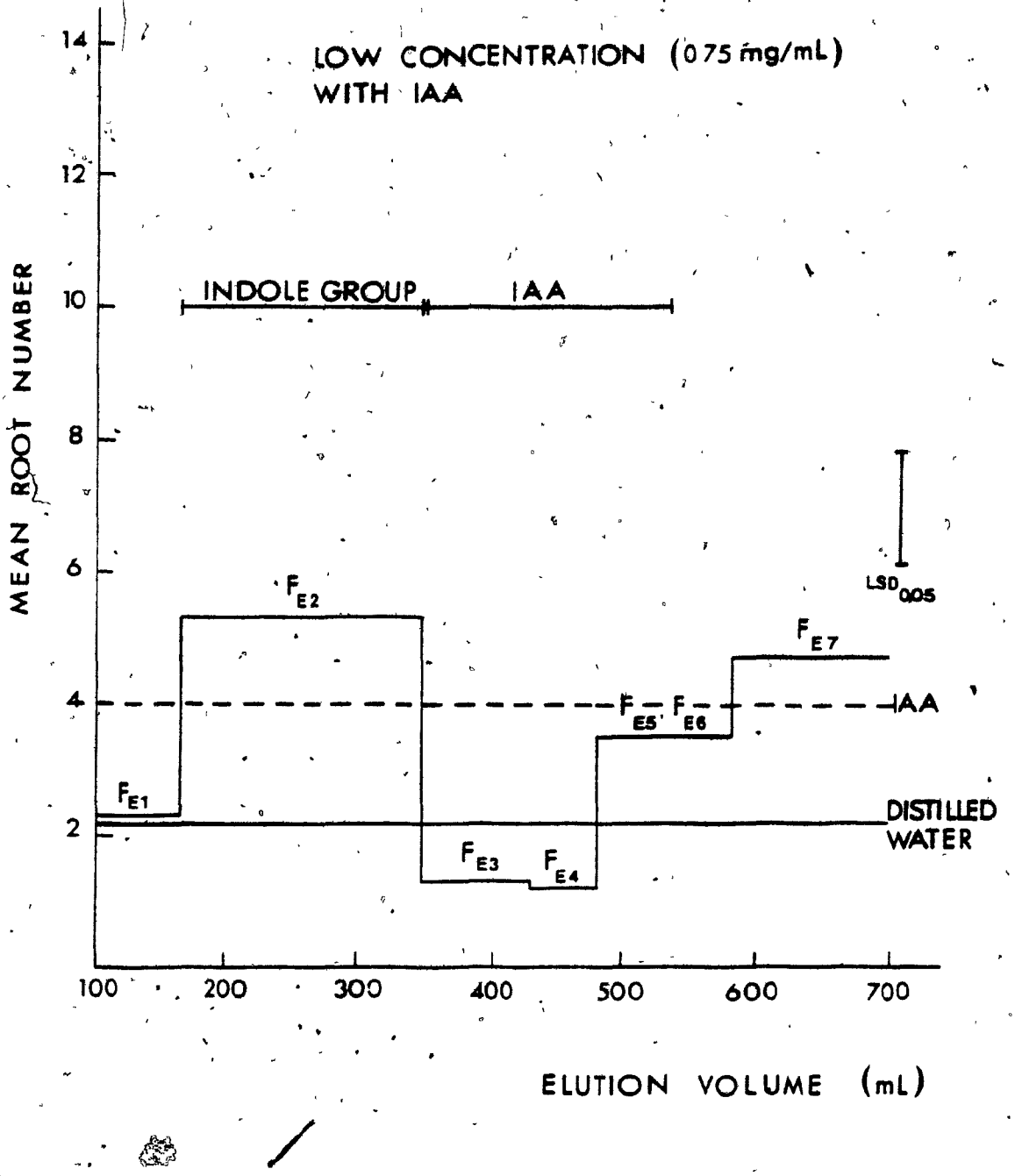


Figure 18. Mean root number per mung bean cutting in response to EToAc sub-fractions (0.75 mg/mL, with IAA).

relative strengths of each sub-fractions were similar. However, the F_{E5} and F_{E6} sub-fractions in the presence of IAA (Fig. 16) showed a marked increase in activity over the same sub-fractions in the absence of IAA (Fig. 15). Similar to the F_{W1} sub-fractions (Fig. 14), indole groups were also detected in the F_{E2} sub-fractions (Fig. 15, 16). However, the presence of IAA was also detected in the F_{E3}, F_{E4}, and F_{E5} sub-fractions (Fig. 15, 16).

At the lower concentration without IAA (Fig. 17) all seven F_E sub-fractions showed small to high promotive effect with the highest activity found in the F_{E4} sub-fraction. However, in the presence of IAA (Fig. 18), the promotive effect of the sub-fractions differed greatly. In fact, the F_{E3} and F_{E4} sub-fractions were inhibitory relative to distilled water, with the F_{E4} sub-fraction having the greatest inhibitory effect. The F_{E7} sub-fraction which had the smallest promotive effect without IAA (Fig. 17) showed a significant increase in promotive activity in the presence of IAA (Fig. 18). Similar as at the higher concentrations (Fig. 15, 16), indole groups were detected in the F_{E2} sub-fraction and IAA in the F_{E3}, F_{E4}, and F_{E5} sub-fractions (Fig. 17, 18).

The presence of phenols was detected in all of the EToAc and water sub-fractions with the Folin-Ciocalteu and with the chloramine-T spray reagents. However, the phenols were not clearly separated by TLC to permit precise identification.

No correlation was found between root number per mung bean cuttings and total or dihydroxy phenol contents analyzed in the EToAc sub-fractions.

2.2.2 Discussion

In previous studies, many attempts were made to characterize the nature of rooting substances(s) in diverse plant species. As shown in Table 15, water (hydrophilic) extracts or fractions were found to contain between two to four fractions or zones of rooting or growth promoting activity. Kawase (1964, 1970, 1971) found four promotive fractions in willow extracts, all insoluble in lipophilic solvents such as chloroform and ethyl ether; although the nature of the rooting substance(s) in his fractions was unknown, the most active one was located near the solvent front at Rf 0-0.1. Of the four rooting cofactors found in methanolic extracts by Hess (1961, 1963), three were shown to be soluble in water (Girouard 1969). Cofactor 2 was characterized as chlorogenic acid, isochlorogenic acid, and an unknown promoter P-254.

Interestingly, Hess' cofactor 1 and Kawase's most active fraction 1 had a similar Rf value of 0-0.1. Similarly, Thurman and Street (1960), Britton et al. (1956), and Audus and Gunning (1958) all found the strongest zone of growth promotion to be located at low Rf values of 0.1-0.2 (Table 15). In the present study, five rooting fractions (F_W sub-fractions) were found with rooting activity in the descending order F_{W1}, F_{W2}, F_{W5}, F_{W4}, F_{W3} (Fig. 14). The most active water-soluble sub-fraction F_{W1} was the first one to be eluted. This suggests that F_{W1} (Table 15), Kawase fraction 1, Hess cofactor 1, Thurman and Street Zone 1 and Britton et al. Zone X were similiar or related.

Thurman and Street (1960) also found that the substance responsible

Table 15. Characteristics of water-soluble fractions or zones from different studies.

Kawase (1964, 1970, 1971)	Hess (1963) Girouard (1969)	Thurman and Street (1960)	Britton et al. (1956)	Present Study
Fraction 1:*	**Cofactor 1:***	**Zone 1:***	**Zone X:**	F_{W1}:*
- UK	- UK	- tryptophane	- UK	- indole
- Rf 0-0.1	- Rf 0-0.13	- Rf 0.1-0.2	- Rf 0.1-0.2	- elution zone 100-508 mL
Fraction 2:	**Cofactor 2:**	**Zone 2:**	**Zone Y:***	F_{W2}:
- UK	- chlorogenic acid	- UK	- IAA	- UK
- Rf 0.3-0.4	- Rf 0.33-0.56	- Rf	- Rf 0.3	- eulution zone 508-548 mL
Fraction 3:	**Cofactor 3:**		**Zone Z:***	F_{W3}:
- UK	- chlorogenic acid		- indole (IAN)	- UK
- Rf 0.7-0.8	- isochlorogenic acid		- Rf near 1	- elution zone 548-579 mL
	- promoter P-254			
Fraction 4:				F_{W4}:
- UK				- UK
- Rf 0.9-1.0				- elution zone 579-612 mL
				F_{W5}:
				- UK
				- elution zone 612-700 mL

* Most active fraction
UK Unknown rooting substance(s)

for the Zone 1 activity was an indole compound which they characterized as tryptophane. Although in the present study, an indole compound was identified in the most active fraction (F_{W1}), this evidence does not prove that rooting activity is attributable to indole compound because further definitive tests were not conducted. Other researchers also detected the presence of indole compounds such as tryptophane, 3-indolepropionic acid, s-methyl indole, and 3-indoleacetonitrile in plant extracts and demonstrated their high root promoting activity (Aung 1972; Booth 1958; Gorter 1962; Zimmerman and Wilcoxon 1935). Housley and Bently (1956) detected three active zones (X, Y and Z) that contained an unknown substance, IAA, and an indole compound, respectively. The indole compound was tentatively identified as indoleacetonitrile (IAN), and thus could be considered as the precursor of auxin (Housley and Bently 1956). Gordon and Paleg (1961) showed that in the presence of some phenolic substances, phenolase yielded IAA from tryptophane, yet this same enzyme may also inactivate auxin. Since mung beans have been shown to be a rich source of phenolase (Gordon and Plaeg 1961), it is possible that the indole compound present in the water-soluble sub-fraction F_{W1} (Table 15) acted synergistically with phenols and(or) other rooting substances to enhance root initiation.

As a parallel to summary Table 15, Table 16 summarizes the results of some studies characterizing the fractions or zones present in nonwater soluble or lipophilic extracts. Lipophilic extracts or fractions were found to contain between one to three root promoting fractions, except in the present study where willow EToAc extract contained seven sub-fractions (Table 16). Hess' lipophilic cofactor 4 (Rf 0.8), an oxygenated terpenoid,

Table 16. Characteristics of nonwater-soluble fractions or zones from different studies.

Kawase (1964, 1970, 1971)	Hess (1963) Girouard (1969)	Thurman and Street (1960)	Britton et al. (1956)	Present Study
Fraction 1:* - UK(a) - Rf 0.35	Cofactor 1:* - oxygenated terpenoid - Rf 0.80-0.93	Zone 1:* - UK promoted - Rf 0.1-0.2	Zone X: - B inhibitor - Rf 0.1-0.2	F_{E1}:[c] - UK - elution zone 0-165 ml.
Fraction 2: - UK - Rf 0.7		Zone 2. - IAA - Rf	Zone Y:* - IAA - Rf	F_{E2}: - indole - elution zone 165-342 ml.
Fraction 3. - UK - Rf 0.95		Zone 3 - B inhibitor - Rf near 1	Zone Z:* - IAN - Rf near 1	F_{E3}: - IAA - elution zone 342-428 ml.
				F_{E4}: - IAA - elution zone 432-483 ml
				F_{E5}: - IAA elution zone 483-528 ml.
				F_{E6}: - IAA - elution zone 528-580 ml.
				F_{E7} IAA - elution zone 580-700 ml.

* Most active fraction

a Unknown rooting substance

c Root promoting activity dependent upon the concentration

was soluble in chloroform, ether, methanol, and ethyl acetate, and sparingly in water. Of the total of four cofactors found by Hess (hydrophilic cofactors 1, 2, 3 (Table 15), and lipophilic cofactor 4 (Table 16), cofactor 4 was the most active one. Since the rooting substances of the EToAc fractions and sub-fractions (Fig. 4) were soluble also in methanol and ethyl acetate, and insoluble (or slightly soluble) in water, this suggests that the EtoAc fractions and sub-fractions contained Hess' cofactor 4. However, the EToAc fractions and sub-fractions were not the most active ones (Fig. 10, 11, 14, 15, 17). In older trees as in this study (22-year-old), it is possible that there was a lack of or smaller quantity of these highly promoting rooting cofactors (Hess 1963). Heuser and Hess (1972) also purified three lipid-like root promoting substances from Hedera helix that were non-phenolic in nature and were soluble in methanol, chloroform, and ethyl acetate.

Unlike the hydrophilic fractions (Table 15), IAA was detected in the EToAc sub-fractions (F_{E3} - F_{E5}). The presence of IAA in EToAc was also identified by Thurman and Street (1960). Their fractions contained also an unknown promoter and at a similar relative position to the indole compound found in the F_{E2} sub-fraction of willow extract (Table 16). However the fraction of Thurman and Street (1960) contained an inhibitor at the B-position which was not detected in the present study. Apparently, inhibitors are found mainly in acidic fraction (Davis 1965; Thurman and Street 1960) whereas the EToAc sub-fractions in this study were close to being neutral (pH 6.5 - 6.8). Several authors have also identified the presence of IAA, IAN, and the B-inhibitor in their etheral acidic fraction

(Britton et al. 1957; Kennet-Clarck and Keffor; Housley and Bently 1956; Lexander 1953).

It is noteworthy that at the high concentration of the EtoAc sub-fractions (Fig. 14), a pattern of root promotion was observed at the indole group position (F_{E2}) but not at the IAA position. However, at the lower concentration (Fig. 16), the pattern was reversed, i.e. root promotion was observed at the IAA position (F_{E4}), but not at the indole group position (F_{E2}). This suggests that the rooting activity of the EToAc sub-fractions is concentration-dependent indicating that each sub-fraction has its own balance of rooting promoter:inhibitor and thus its own optimal concentration. This balance of rooting substances in the sub-fractions may also explain the unique behaviour of each EToAc sub-fraction when IAA-treated (Fig. 16, 18).

3. General Discussion

This study revealed large variations in the response of cuttings of diverse woody species to treatments with willow extracts, auxins, or both. A study of the current literature suggests that this is largely attributed to species difference (Childers and Snyder 1957; Hartmann and Kester 1975; Miller et al. 1982), the to presence of anatomical barriers (Beakbane 1961; Edwards and Thomas 1980; Nelson 1978), to the balance of endogenous growth promoters and inhibitors (Biran and Halevy 1973; Fadl and Hartmann 1967a; Hartmann and Kester 1975), and possibly to other nutritional or environmentally-related aspects associated both with the cuttings being rooted, and to the origin of the willow extracts. Var-

iation in IAA content as well as pronounced diurnal changes in the content of free (i.e. active IAA), but not of alkali-hydrolyzable IAA has been demonstrated (Sandberg et al. 1982). Thus it appears that the time and season when cuttings are treated with IAA or willow extracts has an influence on their rooting response and it is more likely that cuttings treated with willow extracts under long day conditions will be more beneficial (Richer-Leclerc et al. 1984).

Studies with mung bean rooting tests conducted under controlled environment conditions removed variability due to species being tested and thus results with regards to effect of extracts per se were more definitive. These investigations indicated greater root promoting activity in water soluble than in water-insoluble (methanol and EToAc) extracts, fractions or sub-fractions. Studies by Kawase (1964, 1970, 1971) showed that the major promoting substances in centrifugal diffusate and willow extracts was in the aqueous fraction. In no instance was the EToAc fractions or sub-fractions shown to cause greater response than the water counterparts (fig. 10-11, 14-18). Also the root promoting activity of their EToAc sub-fractions was found to be more variable and dependent upon their concentrations (Fig. 11-13, 15-18). These results re-emphasize the complexity of the growth factors involved in the rooting process (Vieitez and Pena 1968).

Since IAA was found to be present in the water soluble subfractions, this suggests that the root promoting activity of water-soluble willow extracts is not attributed to IAA, but rather to a non-IAA system. The evidence suggested that indole and water soluble phenolic compounds were

involved in the rooting activity of willow extracts. Gorter (1962) demonstrated that phenol nucleus acted synergistically with an indole nucleus to induce rooting. However, the lack of correlation between root number per mung bean cutting and total of dihydroxy phenol contents indicates the need for further research and more detailed analysis of the endogenous phenolic content of the willow as well as their role in the rooting activity of the extracts. Alternatively, the root promoting activity of the water sub-fractions (F_{W2}-F_{W5}, Fig. 14), shown to be devoid of IAA and any indole compounds, indicated that other rooting cofactors are involved.

The role of sugars in the rooting process also has been demonstrated (Nanda and Anand 1970). Richer-Leclerc (1982) found a correlation between rooting percentage of Philadelphus and soluble sugar content of willow extracts and between rooting percentage of Ribes and sugar/starch ratio of the extracts. Her study indicated the possibility that the presence of sugars was an important factor in willow extracts. However in the present study, no attempt was made to study sugar content in the water sub-fractions. Further research should aim to quantify, identify and elucidate the nature of their role in the rooting activity of willow extracts.

Substances such as minerals (Gorter 1958; Van Overbeek 1945), and vitamins (Hemberg 1953) have been shown to promote rooting. Since IAA, indole, phenols, sugars, vitamins, and minerals may all interrelate in the rooting process, the removal or change in concentration of any of these may result in a change in the rooting response caused by the willow extracts.

Thus it is possible that the willow rooting substance(s) is in effect a complexity of different substances which is likely altered in time by prevailing environmental factors. As such a study to define the nature of substances is a complex task.

SUMMARY

Plant extracts of willow (Salix spp.), a species known to root readily, and extracts of many other species, have been known to promote rooting of cuttings. This study investigated the use of willow extract as a rooting aid with cuttings and attempted to identify and characterize the nature of its root promoting activity using mung bean rooting tests under a controlled environment, fractionation techniques, and paper chromatography.

In 1982-1983, 13 woody nursery ornamental species were tested with crude willow extracts (1.6-10 g/mL distilled water), auxins (IAA, IBA and NAA) or both. Three of four Group 1 (shrubs) and one of five Group 3 (evergreens) species showed distinct positive response to crude willow extracts. None of four Group 2 (trees) showed any response. While all Group 3 species showed positive response to auxins, three out of four Group 1 species were adversely affected by auxin.

Although the results indicated the favorable use of plant extracts and its interaction with auxins for stimulating rooting of certain woody species, this study revealed large variation in the rooting response of cuttings to diverse woody species to treatments with willow extracts, auxins or both. It also emphasized the complexity of the willow rooting substances which appeared to be a delicate balance of promoters to inhibitors.

The influence of seasonal willow extracts (collected at intervals over

a one-year period) on rooting of mung bean cuttings was studied. The rooting activity of the extracts was greater in the winter months than in the summer months. Positive correlations were obtained between mean root number per mung bean cutting and total, dihydroxy and alkali-labile phenol contents in seasonal willow extracts. These results emphasized the possible role of water-soluble phenols as rooting cofactors in the rooting activity of the extracts.

In another study, the rooting activity of crude extract was compared with clarified extracts. Clarified extracts all increased the rooting response of mung bean cuttings in comparison with crude extract. The water-insoluble particles may decrease the efficacy of the absorption process or have a lower balance of root-promoter to inhibitor than the water-soluble substance(s).

Further experiments with water and methanol-water extracts and fractions or sub-fractions indicated greater rooting activity of water extracts or their fractions to those of methanol or ethyl acetate counterparts. IAA was detected in the ethyl acetate sub-fractions and an indole compound in both the ethyl acetate and water sub-fractions. This suggested a non-IAA system in the water extracts. The activity of the ethyl acetate sub-fractions was shown to be concentration-dependent which indicated that each sub-fraction has its own balance of rooting substances.

The results suggest that the willow rooting substance(s) is in effect a complexity of substances which is likely altered in time by prevailing environmental factors.

SUGGESTIONS FOR FUTURE RESEARCH

Further studies are required to identify and quantify the water-soluble hormones, as well as the phenolic, indole compounds, and carbohydrates found in willow extracts. Such studies should be performed with Phaseolus aureus bioassay and also with growth caleoptile tests on species such as wheat, pea and tomato. The association of plant extracts with other rooting substances such as minerals and vitamins should also be examined. The willow extracts should be partitioned with different solvents and the rooting cofactors of each fraction should be tested at different concentrations and in conjunction with IAA and specific indoles, phenols, and sugars. Similar studies of endogenous components should also be performed on the woody species to be rooted.

The seasonal variation of specific rooting compounds such as mono, dihydroxy and polyphenols, free and bound IAA, indole substances, and sugars should be investigated. Furthermore, the willow powder in cold storage should be examined each month to see if there are any changes in the endogenous rooting substances and also to observe the effect of such changes on the rooting activity of the extracts.

From a more practical point of view, the effect of centrifugation and(or) filtration, anti-oxidant, adjuvant, and pH of the extracts should be examined with mung bean bioassays and eventually on the rooting of woody species.

All these studies would increase our knowledge of the rooting ability of the willow extracts and would eventually broaden their use to the rooting of woody species especially the ones that are hard-to-root.

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