ed in spring has filled an important gap in our companion crop program. This is particularly noteworthy because historically oats have been used as a fall protection crop in nursery production. The availability of new selective grass herbicides has opened a host of beneficial uses for this old "standard" companion crop.

CHARACTERIZATION OF THE ROOT-PROMOTING ACTIVITY IN WILLOW EXTRACTS[1]

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Abstract. Using the mung bean rooting test, fractionation, and chromatographic techniques, attempts were made to identify and characterize the nature of the root promoting substances in crude and partially purified willow extracts. Clarified extracts increased the rooting response in comparison to crude extracts. Rooting activity was greater in extracts from plant materials collected in winter months than in those of the summer months. There was a positive correlation between root number of mung bean cuttings and total phenol content in seasonal willow extracts. Water extracts or their fractions showed greater root promoting activity than those of ethylacetate counterparts. The results suggest that water soluble phenolic and indolic compounds are major root-promoting substances in willow extracts.

INTRODUCTION

Kawase (15) obtained strong root promoting activity on mung bean (*Phaseolus aureus*) by applying centrifugal diffusate of willow (*Salix alba*). The diffusate was strongly synergistic with indoleacetic acid (IAA) in inducing rooting of mung bean cuttings. Kawase (16) also extracted with water, rooting substances from *S. alba* similar to those found in the centrifugal diffusate. He suggested that the willow extracts contained large amounts of endogenous cofactors, as yet unidentified, and the right balance of hormone and rooting substances capable of improving rooting. Water-soluble substances from many

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woody plants were found to promote rooting when tested on mung bean (17). Root-promoting activity of willow extracts applied to cuttings of certain woody species also has been observed by other researchers (18,21,22).

This study attempted to identify the willow rooting substances and to elucidate the nature of their activity. In this context a systematic series of experiments using mung bean rooting tests under controlled environmental conditions were conducted with willow extracts. The willow extracts were clarified or subjected to progressive steps of greater purification and selected extracts or fractions were analyzed for the presence of phenolic and auxinic compounds.

MATERIALS AND METHODS

Mung bean test. The rooting test described by Kawase (15) was used. Mung bean cuttings, each 7 to 9 cm long with a 5 cm long hypocotyl, a 2 to 3 cm long epicotyl, and a pair of primary leaves, were obtained from seedlings germinated and grown in growth cabinets under 24°C and 18-hour photoperiod (400 lux of incandescent light). Cuttings were incubated for 7 days in 7 × 2.5 cm glass vials containing rooting test solutions or extracts that were maintained at 15 ml by daily addition of distilled water. Tests were conducted in growth cabinets supplied with 14,000 lux of light (25% incandescent and 75% fluorescent in wattage). Rooting activity was evaluated by recording the number of roots longer than 1 mm on each cutting (15). 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 with six cuttings.

Crude and clarified extracts. Crude extracts (aqueous slurry) were prepared by adding various amounts of ground, freeze-dried powder from willow (S. *alba* var. *tristis*) twigs, and the mixture shaken at 275 strokes per min for 1 h at 4°C to minimize possible enzyme reactions. The optimum concentration of crude extract was determined from a series of concentrations between 0 (distilled water) and 75 mg of willow powder per ml of distilled water. Concentrations of extract greater than 75 mg/ml were not tested because of the pasty consistency of such mixtures.

Crude extract (7.5 mg/ml distilled water) was clarified by filtration through Watman No. 1 filter paper (filtered extract) or by centifugation for 15 min at 4°C at 10,000 rpm in an automatic refrigerated centrifuge (supernatant extract). The residue of the supernatant extract was re-extracted with 10% methanol (residual extract) (13). The rooting activity of crude and clarified extracts were tested in comparison with distilled

water. Tests also were conducted on supernatant extracts derived from willow twigs collected at monthly intervals over a one-year period. The contents of total phenols were determined in these extracts using the colorimetric method of Swain and Hillis (24).

Fractionated extracts. Willow powder was extracted and partitioned with water, methanol, and ethylacetate (EToAc) according to the method of Jalal (14). These steps are outlined in Figure 1. When fractions F and G were each passed repeatedly through a 50.0 cm × 2.8 cm chromatographic column of Sephadex LH-20 at a flow rate of 5 ml/8 min at room temperature using methanol as eluent, five methanol-soluble water sub-fractions (F_{W1} – F_{W5}) and seven methanol-soluble EToAc sub-fractions (F_{E1} – F_{E7}) (Figure 1) were detected by spectrophotometry of the eluates (14). Each subfraction was subjected to ascending one- or two-dimensional thin layer chromatography (TLC) and subsequent qualitative tests for IAA, indole groups, and phenolic compounds (12,29). The EToAc sub-fractions also were analyzed for total phenols, as previously described. All extracts, fractions and sub-fractions (Figure 1) were evaporated to dryness *in vacuo*, taken up with distilled water to a concentration equivalent to 7.5 mg of willow powder per ml of distilled water, and subjected to mung bean rooting tests.

RESULTS

Crude and clarified extracts. Concentrations of crude willow extract between 1 and 75 mg/ml (Figure 2) increased the rooting response of mung bean cuttings in comparison with distilled water; the optimum concentration was 7.5 mg/ml. This result was confirmed using filtered extract at concentrations between 0 and 10 mg/ml (data not shown). In comparison with crude extract yielding 25.1 roots per mung bean cutting (Figure 3), clarified extracts (supernatant, filtered, or both) yielded 46 to 52% more roots; the 10% methanolic residual extract was slightly promotive in rooting activity.

The root-promoting activity of seasonal supernatant extracts fluctuated greatly during the one-year period (Figure 4), 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). Similar seasonal trends in mean root number and total phenols contents analyzed in the supernatant extracts (Figure 4) showed a correlation coefficient of $r = 0.658$ ($P<0.05$) between the two variables.

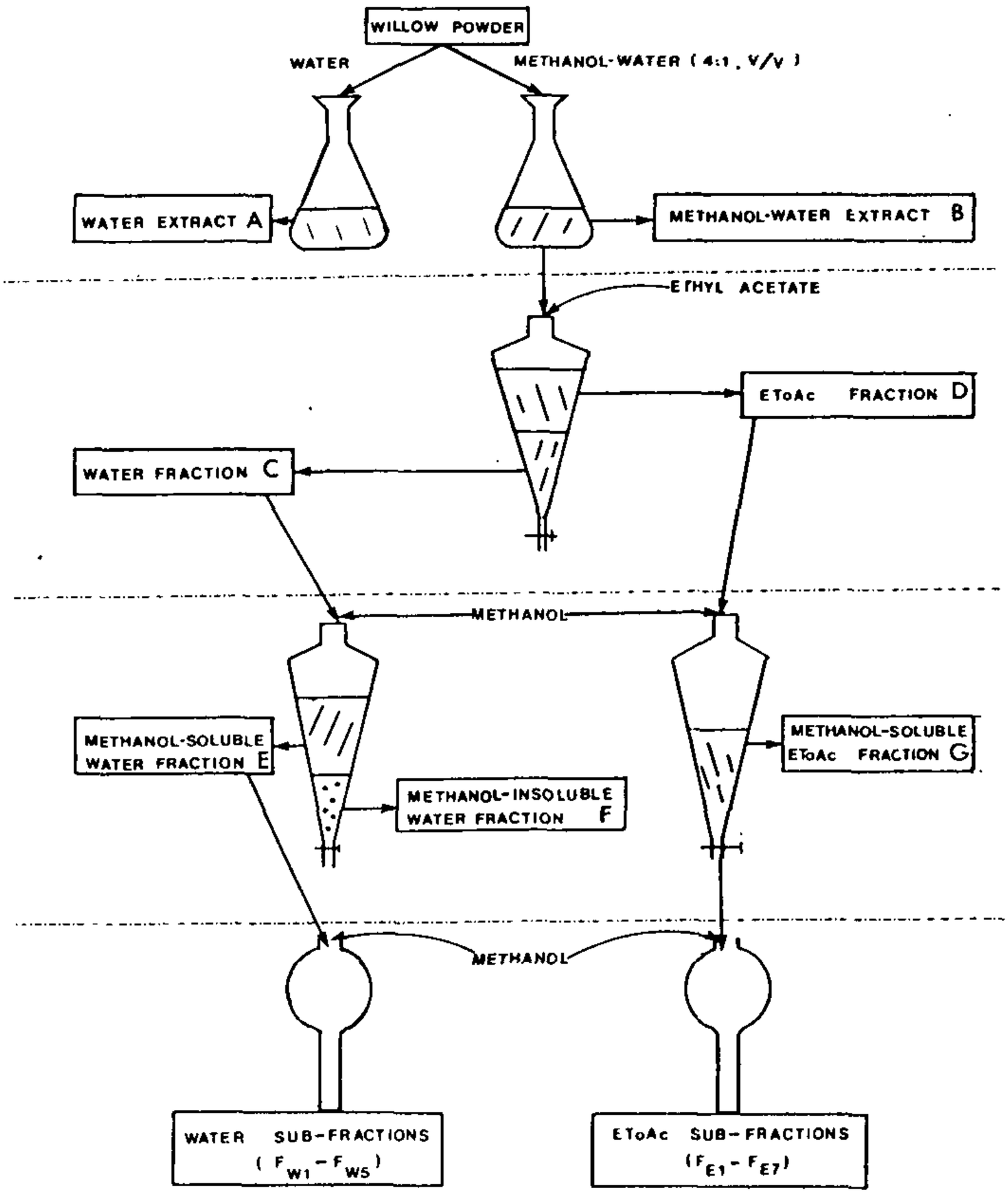

Figure 1. Steps in the extraction and fractionation of willow extract.

Fractionated extracts. All extracts and fractions (Figure 1) showed significant root promoting activity, except EToAc fraction D and methanol-soluble EToAc fraction G. The five methanol-soluble water sub-fractions (F_{W1} to F_{W5}) (Figure 1) showed consistently high root promoting activity with indole group detected in the F_{W1} sub-fraction only (Figure 5A). Of the seven methanol-soluble EToAc sub-fractions (F_{E1} to F_{E7}) (Figure 1), sub-fractions F_{E1}, F_{E2}, F_{E5} and F_{E6} showed low to moderate root promoting activity, while sub-fractions F_{E3}, F_{E4} and F_{E7} were slightly inhibitive (Figure 5B). Similar to the F_{W1} sub-fraction (Figure 5A), indole group also was detected in the F_{E2} sub-fraction (Figure 5B). IAA was detected in the F_{E3}, F_{E4} and F_{E5} sub-fractions (Figure 5B). Phenols were detected in all F_W and F_E sub-fractions but were not clearly separated by TLC to yield quantitative results.

DISCUSSION

Rooting cofactors have been found in many plant species (8,20,26). Using the mung bean bioassay, Hess (13) obtained

from methanolic extracts of easy-to-root, juvenile form of *Hedera helix* and red-flowering *Hibiscus rosa-sinensis*, four root-promoting substances, cofactors 1, 2, 3 and 4. Three of these cofactors were found to be soluble in water (10). Hess (13) also showed that chromatograms from hard-to-root, mature *Hedera* and white-flowering form of *Hibiscus* either lacked these cofactors or contained smaller quantities. Kawase (15,16,17) found four water soluble promotive fractions in willow extracts. Hess' cofactor 1 and Kawase's most active fraction 1 had a similar Rf value of 0. to 0.1. Similarly, Thurman and Street (25) and Britton *et al.* (5) found the strongest zone of growth promotion of other plant extracts to be located at low Rf values of 0.1 to 0.2. In the present study, water extracts or their fractions showed greater root-promoting activity than those of ethylacetate counterparts (Figure 5). The most active water-soluble sub-fraction, F_{W1}, was the first one to be eluted (Figure 5A), suggesting that F_{W1}, Kawase fraction 1, Hess cofactor 1, Thurman and Street Zone 1 and Britton *et al* Zone X are similar or closely related. Thurman and Street (1960) characterized the substance responsible for Zone 1 activity to be the indole compound, tryptophan. Other researchers also detected the presence of indole compounds, including tryptophan, 3-

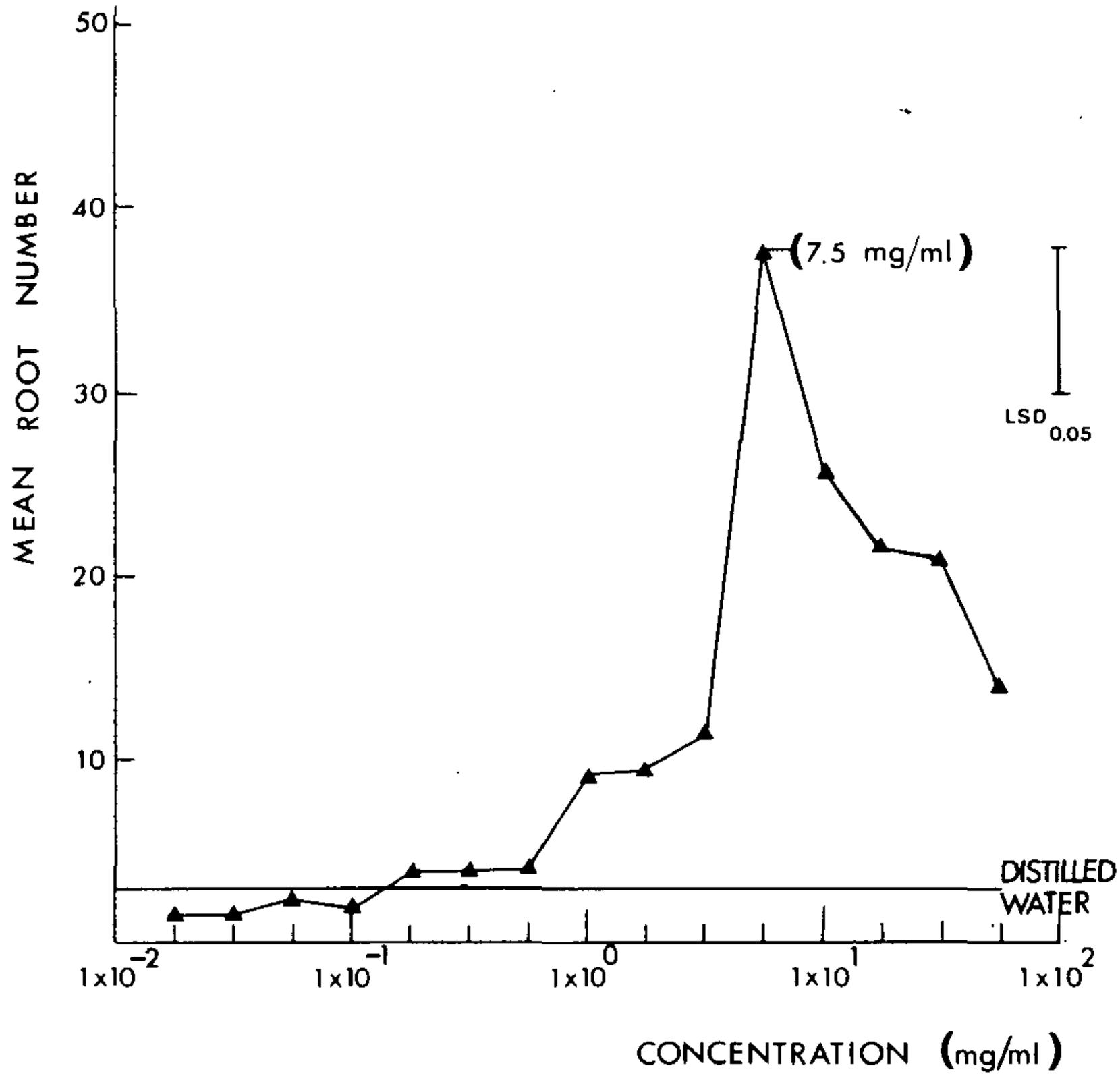

Figure 2. Mean root number for mung bean cutting in response to a series of 16 concentrations of crude willow extract.

indolepropionic acid, s-methyl indole, and 3-indoleacetonitrile in plant extracts and demonstrated their high root-promoting activity (2,11,28). Limited evidence showed that the rooting activity of the EToAc sub-fractions was concentration-dependent suggesting that each sub-fraction has its own balance of rooting promoter:inhibitor and thus its own optimal concentration.

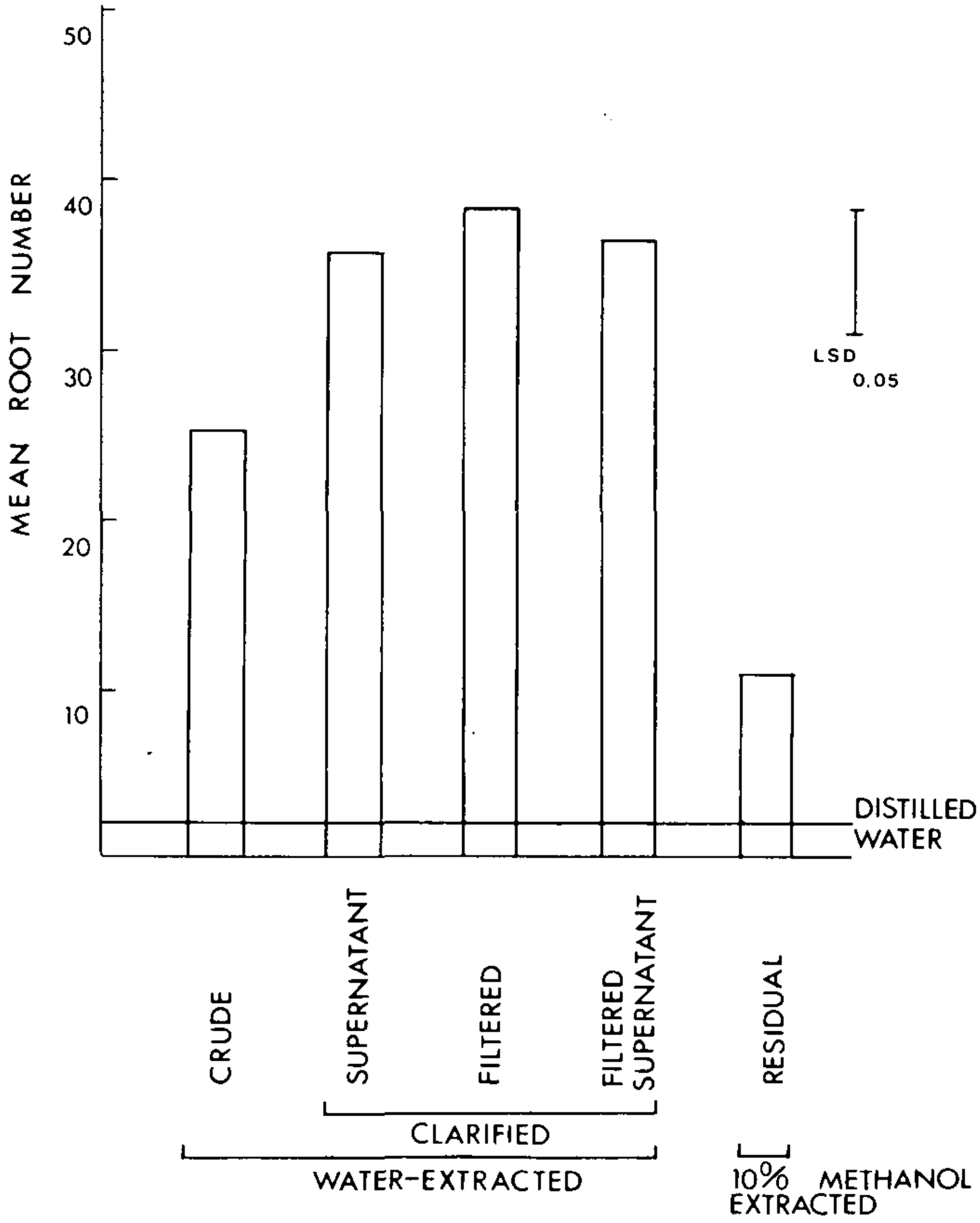

Figure 3. Mean root number per mung bean cutting in response to crude and clarified willow extracts.

Similar to the study of Richer-Leclerc and Chong (21), greater root-promoting activity in willow extract was observed during the winter months (Figure 4). Higher root-promoting activity in winter months has been attributed to the accumulation of rooting cofactors in the stem after leaf drop (23), or to the accumulation of inhibitors such as abscisic acid which interacted with endogenous auxin to promote rooting (1,6). Vieitez and Pena (27) found that rooting activity of acidic S. *cinerea* [syn. S. *atrocinerea*] extracts was lowest in the sum-

mer months (June to August) and related seasonal rooting activity of the extracts to amounts of endogenous IAA. Kikuchi *et al.* (18) and Lamphear and Meahl (19) reported no relationship between seasonal changes in rooting ability of cuttings of *S. kariyanagi*, *S. bakko*, *Taxus cuspidata* 'Nana', and *Juniperus horizontalis* 'Plumosa' and seasonal rooting activity of water-soluble substance(s) in extracts of these species.

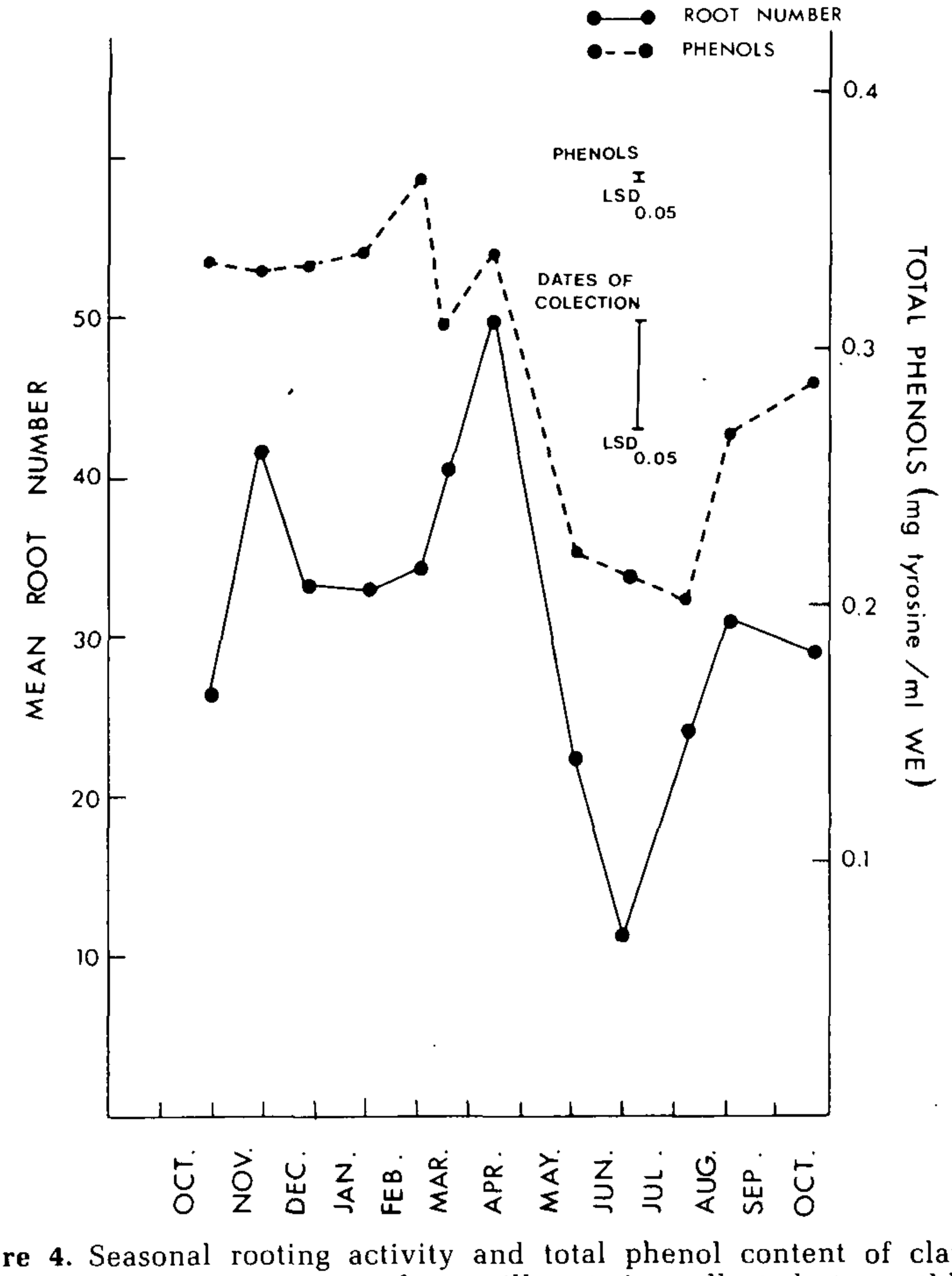

Figure 4. Seasonal rooting activity and total phenol content of clarified supernatant extracts from willow twigs collected at monthly intervals over a one-year period.

Bassuk and Howard (3) found a strong correlation between an abundant phenolic (phloridzin) and seasonal rooting of cuttings. Cortizo (1981) related a high level of phenols in stems in winter to low meristematic and hydrolytic enzyme activity of the plant. Forrest (9) observed similar seasonal trends in total and o-dihydroxy phenol content as in the present study and

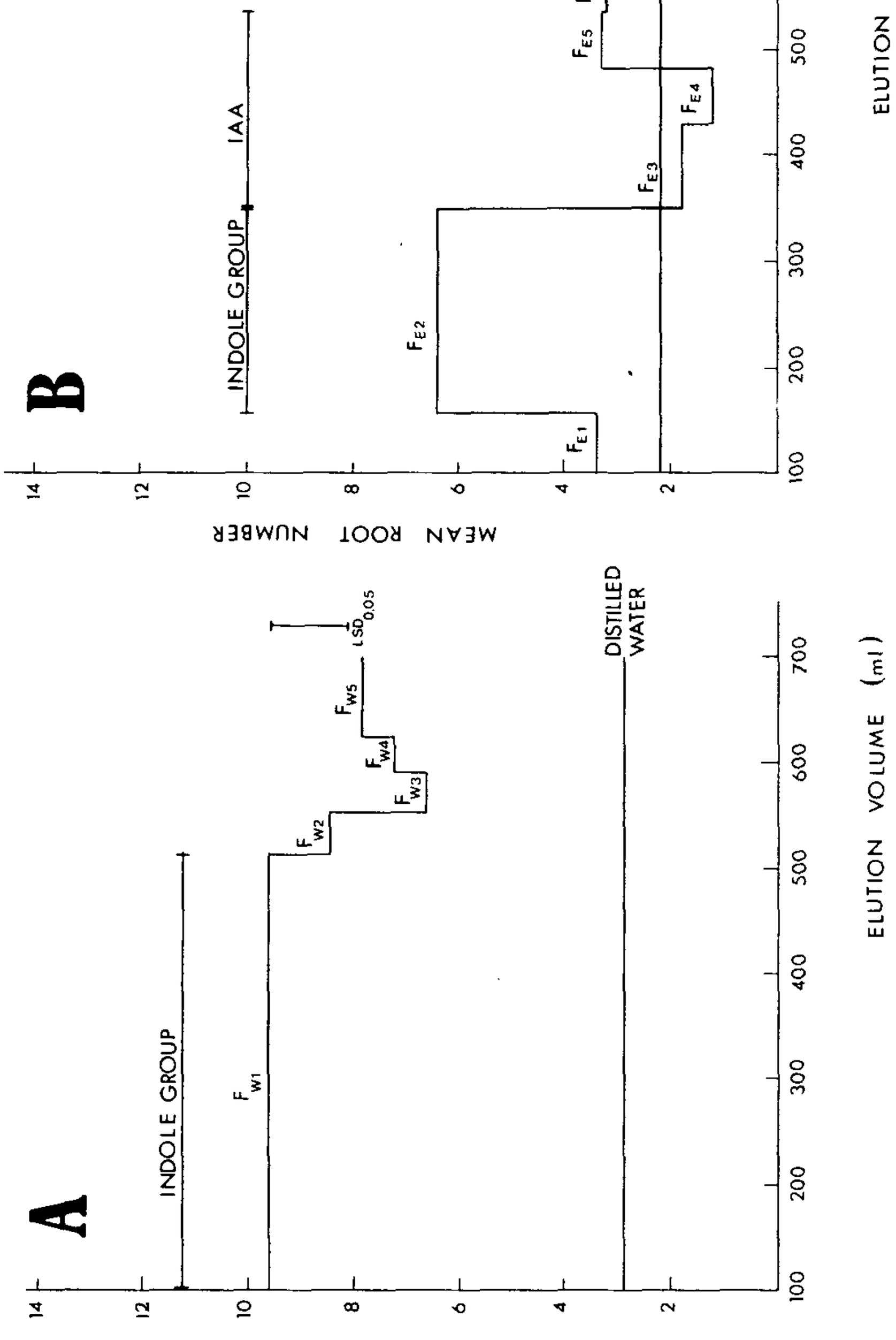

Figure 5. Mean root number per mung bean cutting in response to (A) five water sub-fractions, F_{W1} - F_{W5} and (B) seven EToAc sub-fractions, F_{E1} - F_{E7}.

related their presence to state of lignification of the tissue. Although the phenol nucleus has been reported to act synergistically with an indole nucleus to induce rooting (11), the role played by phenolic rooting cofactors remains controversial (4).

Evidence of the present study confirms that the willow rooting substance(s) is in effect a complexity of substances (21). The activity of these substances appears to be attributable primarily to the presence of phenolic and indolic compounds which are most prevalent in the water fraction; activity is altered by prevailing environmental and/or physiological conditions.

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