# Time of Naringin Production in Grapefruit[1]

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*Abstract.* From experiments conducted over several seasons it was found that naringin production in grapefruit takes place mainly during the early stages of fruit development. In the most definitive study, developing grapefruit and adjacent leaves were exposed to $^{14}C$ labeled carbon dioxide at weekly intervals following fruit set. The relative amount of $CO_2$ fixed and available for conversion at different treatment times was determined by measuring the $^{14}C$ activity in the soluble carbohydrate and organic acid fraction of the fruit. Activity was also followed in the naringenin rhamnoglucoside fraction. Carbon dioxide incorporation into the sugar-acids did not vary by greater than a factor of 10 for those exposures conducted from early April to mid-November. The ratio of activity in the naringenin rhamnoglucoside fraction to that in the sugar-acid fraction reached a pronounced maximum in mid-April and then declined rapidly by mid-June to 1/3000 of the peak value. A uniformly low ratio of incorporation into the naringenin rhamnoglucoside fraction is maintained to early maturity (October). Preliminary evidence suggests that minor subsequent rises in naringin production may coincide with periods of rapid vegetative growth.

## Introduction

The intensely bitter flavanone, naringin (naringenin-7-$\beta$-neohesperidoside) is the component whose presence in grapefruit is mainly responsible for the fruit's characteristic bitterness (4). Excessive amounts of naringin in grapefruit and processed grapefruit products can adversely affect their taste acceptability.

Kesterson and Hendrickson (5) employed a modified "Davis Test" (1) to follow the seasonal changes of naringenin rhamnoglucoside content in 5 varieties of grapefruit grown in Florida. They concluded that the different varieties all showed similar trends of total naringin per fruit during fruit development. They found that 'Duncan' grapefruit of ½ inch diameter, harvested around May 1, contained 250 mg of naringin, which represented 76% of the fruit's dry weight. The naringin content per fruit increased until the fruit reached a diameter of about 2 inches, which under Florida conditions is usually by June 1. Total naringin content did not show any significant change when the fruit continued to increase in size beyond the 2 inch diameter. Similar results were found by Maier (6) to be true for desert grown 'Marsh' grapefruit.

Fisher (2) has demonstrated that $^{14}C$-labeled naringin is produced in young grapefruit leaves fed phenylalanine-$^{14}C$ (U.L.). Older leaves showed a much lower rate of conversion. From this he concluded that the biosynthesis of naringin occurs mainly in the young, rapidly-metabolizing grapefruit leaves.

The purpose of the present study was to determine the time when naringenin rhamnoglucosides are most actively synthesized and later accumulated in the fruit, and when and if naringin synthesis and accumulation ceased.

## Materials and Methods

Grapefruit trees used in this study were 'Webb Redblush' grafted on sour orange rootstocks. All experiments were conducted on potted trees 4 to 5 years old, or on a mature grove tree growing at the Texas Agricultural Experiment Station, Substation No. 15, Weslaco.

*Treatment of trees with $^{14}CO_2$.* Slightly different methods of exposing leaves and fruit to $^{14}CO_2$ were used during different phases of this experiment, and markedly different methods of analyzing for $^{14}C$ activity were employed. These experimental conditions are summarized for each phase.

*1962–63–64 branch treatments.* Four exposures were made on the grove tree: Oct. 25, 1962 on a single mature fruit with adjacent leaves, and May 7, 1963, March 27 and April 7, 1964 on clusters of 2 or 3 young developing fruit with adjacent leaves. The branch to be treated was girdled just above its juncture with the main limb by removing a 2 cm strip of bark and cambium. The entire branch up to the point of the girdle was enclosed in an airtight plastic bag. A mixture of $Ba^{14}CO_3$-celite containing 25 μc of $^{14}C$ was placed in a small Erlenmeyer flask within the bag. Two milliliters of 3*N* $H_3PO_4$ acid was injected through the bag and into the flask with a hypodermic syringe. The puncture was sealed with tape. The walls of the bag were flexed to aid uniform mixing of the generated $CO_2$. The exposures were conducted for a 4 hr period in the morning while the bagged branch was in partial shade. At the end of the exposure time 5 ml of 4*N* NaOH was injected into the flask and the bag removed from the branch. The fruit were harvested after 10 days and the branch cut from the tree.

*1964 whole tree treatments.* The entire aerial portion of 4 different potted trees were exposed to $^{14}CO_2$ on May 12, August 25, October 9 and November 12. The treatment was similar to that described above except the entire tree above the bud union was bagged and the trunk was not girdled. Half the fruit from the May 12 exposure was harvested on August 17. The remaining fruit from all exposures was harvested on January 8, 1965.

*1967 branch treatments.* Numerous treatments of single fruits with 2 adjacent leaves of average proportions were conducted in a manner similar to that already described. These exposures were made with 50 μc of $Ba^{14}CO_3$ and exposures were conducted over a 24 hr period beginning at 5 hr before sunset. Individual branches on potted trees were treated at 2 week intervals from April 10 to August 16. Branches on the grove tree were treated on the alternate weeks from April 3 to May 31 and weekly thereafter

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to August 9, and again at approximately 2 week intervals from August 22 to November 13. The treated branch, leaves, and fruit were removed from the tree at the end of the 24 hr exposure period.

*Extraction of fruit.* The individual fruits were cut into longitudinal sections and blended at high speed in a Waring Blendor[3] with 4 ml methanol per g of fruit. Blending was done at the highest speed possible until the slurry appeared homogeneous. The contents of the blender were then filtered through a sintered glass Buchner funnel by application of vacuum.

At this stage in the isolation procedure samples obtained at different phases of the study were handled in different ways. For those fruit treated in 1962 and 1963 the aqueous methanol extract was concentrated in a rotary-film evaporator at 40°C to an amorphous solid residue. The residue was triturated with anhydrous methanol several times. The supernatant methanolic solutions were evaporated to dryness and the residue taken up in a minimum volume of hot water. Upon cooling, the aqueous solution deposited crystals of naringin. The naringin crystals were collected and their specific activity determined. Counts were made with a Nuclear-Chicago Model 47 Gas Flow Counter[3] on planchets containing a known weight of naringin deposited by evaporation of an aliquot portion of a methanolic solution of the crystals. Recrystallization was repeated until constant specific activity values were obtained.

All extracts from fruit exposed during 1964 were processed in the same way as discussed above except that portions of the supernatant methanolic solution obtained from trituration of the original concentration residue was chromatographed on thin layer plates of Woelm polyamide (Alupharm Chemicals, New Orleans, Louisiana[3]). Chromatographic development was with a solvent mixture of nitromethane-methanol (5:2, v/v). The $^{14}C$ activity of naringin separated by thin layer chromatography were ascertained in 2 ways: planchet counting of chromatographically pure naringin recovered by extraction with methanol from the thin layer polyamide resin or by autoradiography of the intact TLC plate bearing chromatographed aliquots of the different exposure extracts.

The fruit exposed in 1967 were treated so as to obtain complete recovery of the flavanone, soluble carbohydrate, and acid fractions. The isolation procedure was as follows:

After the initial blending of the fruit and filtration of the methanol-water slurry, the filter cake was rinsed twice with 1 ml anhydrous methanol per g of fruit. The methanolic solutions were combined and concentrated in a rotary-film evaporator at 40°C to approximately a single proportional volume. The concentrated solution was successively extracted with hexane to remove lipoidal material.

The filter cake remaining after the methanol rinses was washed with 2 successive proportional volumes of a 1:1 (v/v) mixture of acetone and hexane. The acetone-hexane was then extracted 5 times with 25 ml portions of water and the aqueous-acetone phases combined.

The pulp residue was then air dried and packed into a straight pass Soxhlet extractor. The pulp was extracted for 12 hr with refluxing anhydrous methanol.

The original concentrated aqueous extract, the aqueous-acetone extract, and methanolic Soxhlet extract were combined and concentrated to a small volume of syrup. The syrup was dissolved (sometimes with difficulty) in a methanol-water (4:1, v/v) mixture and brought to volume in a volumetric flask (50, 250, 500 ml capacity, depending on the size of fruit). After standing for about 1 week at 20°C, a small amount of pectin precipitated.

A 5 ml aliquot of the extract was then chromatographed on a column of polyvinylpyrrolidine resin (Polyclar AT, General Aniline and Film Corp., Grasselli, New Jersey[3]).

The column chromatographic procedure was essentially that described by Hagen, et al., (3) with the following modifications: the weight of resin employed was increased to 20 g and the diameter of the column was increased to 30 mm. The respective solvent volumes employed for elution and the volumes of the collected fractions were increased by a factor of 2.3 to compensate for the greater weight of resin and larger column diameter.

Five eluate fractions were evaporated to dryness in the rotary-film evaporator. Fraction 1 was dissolved in water-methanol (2:1, v/v), and fractions 2–5 were dissolved in methanol. All 5 fractions were made to volume in 10 ml volumetric flasks.

A 0.5 ml portion of fraction 1 and 1 ml portions of fractions 2–5 were placed in scintillation vials containing 10 ml of "scintillation cocktail" (5 g PPO/liter toluene). In addition, 10 ml of methanol was added to the vial containing fraction 1 to effect complete solubility.

Activity determinations of the samples were made with a liquid scintillation counter (Beckman Model 200B[3]). Counting efficiencies were determined from measure-

[3]It is not the policy of the Department to recommend the products of one company over those of any others engaged in the same business.

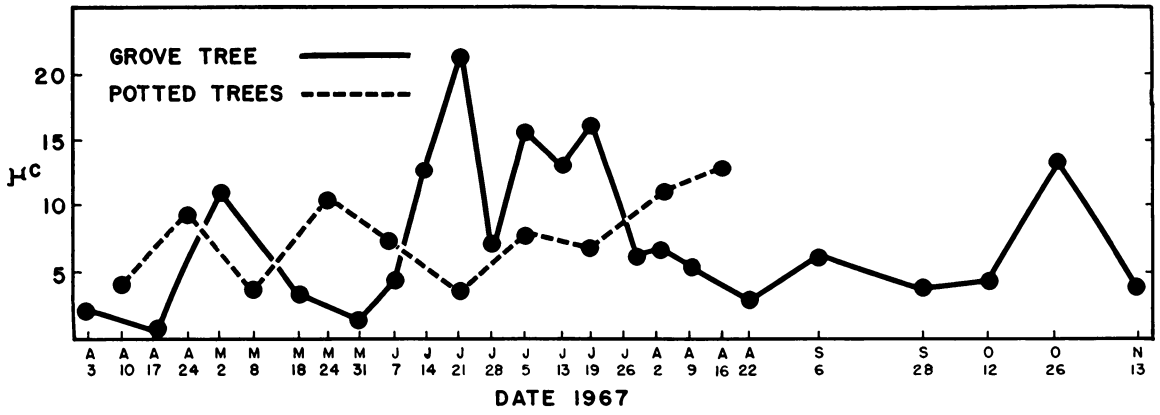


*Fig. 1.* $^{14}C$ activity (as microcuries) in carbohydrate-acid fraction of fruit at different exposure dates.

ments of external standard ratios and a calibration chart constructed with fraction aliquots and an internal $^{14}C$ toluene standard.

## Results

The preliminary exposures of fruited branches to $^{14}CO_2$ on Oct. 25, 1962 and May 7, 1963 and subsequent analyses of the fruit established that no $^{14}C$ was incorporated into the naringin isolated from the mature grapefruit from the October treatment, while the naringin isolated from the young developing fruit following the May treatment did show the presence of considerable activity after 4 recrystallizations to constant specific activity. When a sample of the recrystallized naringin was analyzed by thin layer chromatography on Woelm polyamide resin with the nitromethane-methanol developing solvent it was found that the naringin was still contaminated with very small quantities of 8 components, including naringenin-7-rutinoside, neohesperidin, poncirin and rhoifolin. This illustrates the difficulty of purifying polyphenolic compounds by crystallization and indicates the limitation of using a constancy of specific activity as a criterion for purity of low-activity samples.

Among the branch or potted-tree exposures made in 1964 only those made in March and April showed any $^{14}C$ activity in the naringenin rhamnoglucoside fraction of the fruit. The whole tree exposure made in the period from May 12 through November 12 produced mature fruit whose naringin contained no detectable activity. However, the sensitivity of the counting equipment available at that time was limited.

In the more definitive study conducted during 1967 each exposure to labeled $CO_2$ required fruit and leaves which could not be identical in all respects, nor was it possible to control the environmental factors to obtain uniform conditions throughout the term of the study. Because of this, the activity in the fruit's carbohydrate-acid fraction isolated following each exposure, was used as a basis of incorporation of label into the active metabolic pool of the fruit. The soluble carbohydrate and acid fraction was chosen since these groups of compounds include those first produced by the photosynthetic and dark fixation of $CO_2$. In addition, the sugar and A-ring moieties of naringin are believed to be derived directly from hexose phosphates and acetyl-CoA which are intimately associated with the metabolism of the carbohydrates and biosynthesis of many plant acids.

Fraction 1 was shown by thin layer chromatographic analysis to contain almost the entire carbohydrate-acid fraction. Occasionally insignificant amounts of carbohydrate may tail into fraction 2. Fig. 1 records the activity found in each carbohydrate-acid fraction after exposure. This figure illustrates the variations of $CO_2$ fixation expected under field conditions; yet the majority of the values are within a fairly narrow range and the most extreme values differ by no more than one order of magnitude.

Fraction 4 contained the 2 naringenin derivatives, naringin and the non-bitter naringenin-7-rutinoside. Very small amounts of neohesperidin and hesperidin were also present in the fraction; they constituted less than 2% of the total flavanones present. No other major components were detected. On occasion small quantities of naringin and naringenin-7-rutinoside may be found in fractions 3 or 5. The activity lost to fractions 3 and 5 by these small quantities did not significantly change the activity of fraction 4.

That the activity found in fraction 4 was due principally to the 2 naringenin derivatives and not to any other compounds was supported by activity determinations on the components of the fraction which had been purified by preparative-scale, multiple, thin layer chromatography.

Fig. 2 and 3 record, for the grove and potted trees respectively, the ratio of activity found in the fraction 4 (naringenin rhamnoglucoside fraction) to that found in fraction 1 (carbohydrate-acid fraction).

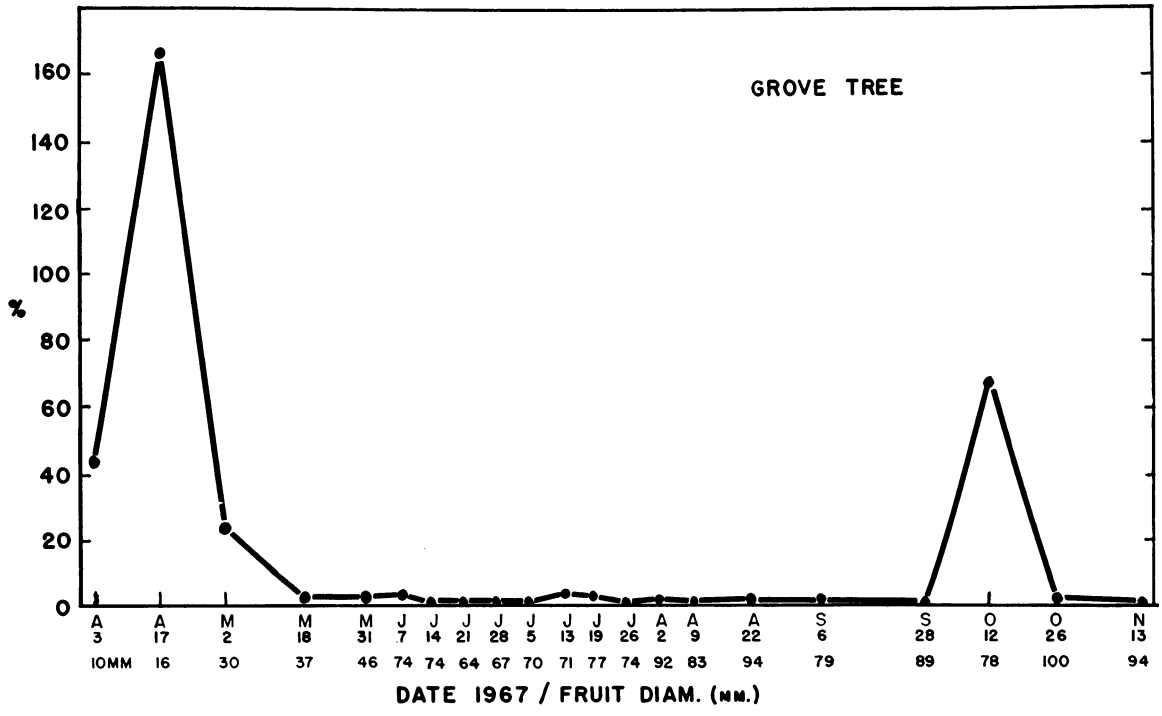


*Fig. 2.* Percentage of $^{14}C$ in naringenin rhamnoglucoside fraction to that in carbohydrate-acid fraction at different exposure times and fruit sizes for the grove tree.

## Discussion

Reference to Fig. 2 shows that during April a very large proportion of the $CO_2$ incorporated into the fruit of the grove tree is diverted into the production of naringin and naringenin-7-rutinoside. By mid-May when the fruit had reached a diameter of 37 mm the relative amount of label found in the naringenin rhamnoglucoside fraction had decreased to about 1/80 of the highest value found in mid-April. The general decline continued to a minimum in mid-June when the amount of label in the naringenin fraction was only 0.05% of that found in the carbohydrate-acid fraction. This is about 1/3000 of the value found at the April maximum. This large difference cannot be due entirely to any fundamental change in the amount of label in the carbohydrate-acid fractions since those amounts are randomly distributed about a rather narrow range of activities and there is no correlation between the absolute activity of these fractions and proportion of label in the naringenin fraction.

Fig. 3 illustrates the point that during April, fruit grown on the small potted trees underwent a similar maximum in the accumulation of label in the naringenin rhamnoglucoside fraction, as was true for fruit from the large grove tree. Also, a rapid, though less dramatic, decline in accumulation was found to occur after this time. A significantly smaller proportion of label was incorporated into the naringenin rhamnoglucoside fraction of the potted trees than was true of the grove tree. Whether this is true of young small trees in general or is due to the special environmental conditions experienced by the potted trees is not known. No significant differences existed in the amount of label accumulated by the carbohydrate-acid fraction from the fruit of the potted trees from that of the grove trees.

Although a dramatic decrease occurred in the rate of incorporation of label into the naringenin rhamnoglucoside fraction during late April and reached a very low value by mid-June, it does not appear that the naringenin rhamnoglucoside synthesis ever completely ceased during fruit development. Indeed, an increase in rate of incorporation into the fruit is seen to occur during July and again in October. Unfortunately, the rise in October is represented by only a single datum point and occurred after a hurricane partially defoliated the tree on September 20.

Citrus trees in the Lower Rio Grande Valley of Texas generally produce a relatively strong flush of growth during October; this, coupled with the hurricane defoliation, resulted in an especially vigorous flush of growth during October, 1967. The major growth flush appears in either February or March with a few minor flushes occurring during the summer. The time, number and intensity of these growth flushes is dependent on weather and soil moisture conditions.

It is not known whether the apparent temporal relationship between time of growth flushes and time of increased rate of naringenin rhamnoglucoside synthesis and accumulation is fortuitous or actually suggests a causal relationship. It is interesting to note, however, that Fisher (2) found that the rate of naringin production is greater in young leaves than in older leaves.

If, indeed, young rapidly-proliferating leaf-tissue is contributing to the accumulation of bitter naringenin flavanones in the fruit, then the variability of growth flushes between seasons, production areas, and individual groves may explain in some degree the observed differences in grapefruit naringin content and consequent bitterness that have been associated with these factors. Of more fundamental interest is the biochemical relationship between flavanone production and the breaking of dormancy and rapid tissue growth.

The periods of rapid naringin synthesis corresponds with those times of year when total naringin content of grapefruit is found to increase (Albach and Redman, unpublished data). Any decrease or cessation of naringin accumulation thus appears to be the result of restricted synthesis rather than due to any increase in the rate of naringin turnover or degradation.

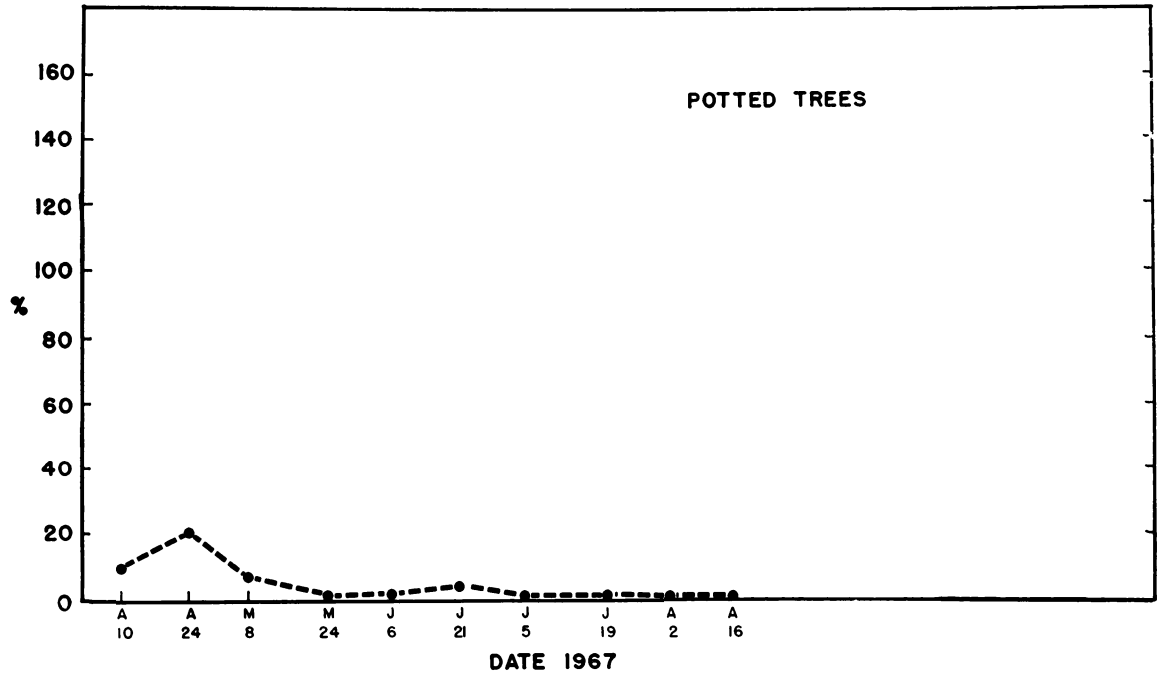


*Fig. 3.* Percentage of $^{14}C$ in naringenin rhamnoglucoside fraction to that in carbohydrate-acid fraction at different exposure times for the potted trees.

## Conclusions

The naringenin rhamnoglucosides of grapefruit are produced and accumulated in the fruit during the entire growth stage from ovary to maturity. The rate of production, however, appears to be several thousand times greater during the first month of fruit development than during subsequent months. Periodic increases in production may be associated with subsequent growth flushes. This knowledge could be utilized by the processing industry so as to allow them to anticipate forthcoming increases in fruit bitterness and make provisions for changing processing parameters so as to produce a product of more uniform quality. Cultural practices limiting naringin synthesis, if developed, could have optimum effectiveness during these periods of increased production.

The uniformity of the relative quantities of individual flavanones within the fruit during development and maturation (Albach and Redman, unpublished data) suggests that the other flavanone glycosides follow the same rate of production as the major flavanone components, the naringenin rhamnoglucosides.

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# Susceptibility of Evergreen Hosts to the Juniper Blight Fungus, *Phomopsis juniperovora,* Under Epidemic Conditions[1]


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*Abstract.* Relative damage due to juniper blight under field conditions was recorded on 188 species, varieties, and cultivars of juniper, cypress, false-cypress, and arborvitae during 2 out of 3 years of epidemic conditions in Illinois. Wide variation in relative susceptibility to blight was observed among the various hosts examined. No blight damage was recorded on 5 of 7 false-cypresses, 27 of 146 junipers, and 4 of 37 arborvitae examined in 1967 and 1968 at the Morton Arboretum, Lisle, Illinois, and at 3 large commercial nurseries in the state.


## Introduction

Juniper blight, caused by the fungus *Phomopsis juniperovora* Hahn, was reported from the Midwest as early as the late 19th century (2). *P. juniperovora* is widely distributed in Illinois and surrounding states, but seldom causes significant damage other than an occasional blighted shoot on highly susceptible evergreen hosts. The prolonged wet, cool springs of 1966, 1967, and 1968, however, were near optimum for disease development and spread of the pathogen, and extensive damage appeared during these 3 years.

It has long been known that evergreen hosts of *P. juniperovora* vary in disease susceptibility (1, 2, 3, 6), but valid data on the relative susceptibility of the various selections or cultivars grown on a commercial scale are very sparse. Since juniper blight was widespread and severe in Illinois for 3 consecutive years, it was felt that data collected on the relative amount of blight damage on the various hosts would be of value in the selection of evergreens for production and use in areas where juniper blight is a problem, and in the selection and breeding for blight resistance.


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[2]The author gratefully acknowledges the assistance of Mr. Floyd Swink, Plant Taxonomist at the Morton Arboretum, Lisle, Illinois, in checking plant names for conformity with the International Rules of Nomenclature.


## Materials and Methods

Surveys of juniper blight damage were made in 1967 and 1968 in the ornamental evergreen collections at the Morton Arboretum, Lisle, Illinois, and in stock block plantings at the Egyptian Nursery and Landscape Company, Farina, Illinois; the D. Hill Nursery Company, Dundee, Illinois; and the Matt Tures Sons Nursery, Roselle, Illinois. The surveys were conducted during June of both years, since damage from current season infection was most obvious at that time.

Uniform distribution of the disease was apparent in each of the plantings examined. Due to variations in disease severity between locations, however, damage estimates were based on comparisons within individual plantings. At least 3 specimens of each clone were rated as to relative blight damage. Each plant was examined for the presence of fruiting bodies of *P. juniperovora,* the most reliable indication of juniper blight.

Relative damage was divided into 4 categories based on visual observation: severe, moderate, light, and none. Since much inconsistency exists within the nursery industry in the use of scientific names for cultivars and varieties of ornamental evergreens, the nomenclature presented in Table 1 was checked by Plant Taxonomist Floyd Swink of the Morton Arboretum for conformity with the International Rules of Nomenclature. Names on tags or labels were accepted as the appropriate names for