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CUMULATIVE RADIATION EFFECTS ON SEXUAL REPRODUCTION IN PINE AND OAK[*]

by

François Mergen[**] and G. R. Stairs[†]

Yale University, School of Forestry, New Haven, Conn.

Biology Department, Brookhaven National Laboratory, Upton, N. Y.[‡]

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[†] The work on Quercus is part of a comprehensive study on radiation effects in oaks being carried out by Stairs in partial fulfillment of requirements for the Ph.D. degree.

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Abstract (Summary). The sexual reproductive ability of a forest exposed to low level ionizing radiation is dependent on the sensitivity of primordial differentiation, meiotic processes leading to the formation of gametes, fusion of the gametes, and subsequent growth of the sporophytic generations.

In this report it was shown that the initiation of reproductive primordia in *Pinus rigida*, *Quercus ilicifolia*, *Quercus alba*, and *Quercus coccinea* is not unusually sensitive to irradiation. Trees which had been severely damaged by chronic low level ionizing radiation (4 to 12 r/20 hr day) were able to differentiate floral structures and produce viable seed. However, flower phenology was retarded, and pronounced morphological aberrations were present. It is suggested that this delay may be the result of a reduction of endogenous growth substances.

During the meiotic processes in both pines and oaks, visual cytological evidence of chromosomal aberrations was obtained. Bridging and fragmentation of the chromosomes were observed at Anaphase I and Anaphase II.

Pollen abortion in both genera showed a significant increase with an increase in irradiation level; however, this damage was not as great in the oaks and occurred at a higher level of radiation. A decrease in pollen germination was observed in the pines, but the pollen tube lengths in both genera did not show a decrease.

The progeny from irradiated pine trees was more variable in height than the controls after one growing season. The seedlings also showed the following abnormalities: shorter and longer needles, twisted

needles, an irregular growth pattern, and lack of primordia on the leaders.

Introduction

A natural pitch pine-oak forest surrounds the gamma field at the Brookhaven National Laboratory. These trees have been exposed to varying amounts of low level chronic gamma radiation for several years and show diverse responses. These responses range from lethality to minute morphological changes, depending upon dose rate and species. There have been varied opinions on the effect of low level chronic ionizing conditions on plants, and one early opinion was that if no response was obtained within a few months, no serious damage would occur if the plants kept on growing under this low level of radiation (Gunckel, Sparrow, Morrow, and Christensen, 1953). However, the later observations by Sparrow have revealed that this is not the case for the forest trees surrounding the Brookhaven Gamma Field. The first published reports of these effects on *Pinus rigida* appeared in the 1959 Annual Report of the Brookhaven National Laboratory. An illustration was given of a large, dead pine tree that had been killed after an exposure of five years at the rate of not more than 8 r/day during 8 months of each year. Further observations on the effect on these forests were given by Mergen and Stairs (1962), by Mericle, Mericle, and Sparrow (1962), and Sparrow and Woodwell (1962). Mergen and Stairs (1962) reported on seed germination in *Pinus rigida*, and on embryo development, seed germination, and survival of seedlings in *Quercus*.

For pine there was a decrease in cone length and seed germination, that was associated with an increase in the exposure to gamma radiation;

in addition the irradiated seedlings had shorter hypocotyls. This radiation damage was related to the total dose accumulated by the individual trees prior to, and during, seed formation, rather than to the total dose accumulated in the seed. For Quercus alba, Quercus coccinea and Quercus ilicifolia there were internal abnormalities in the acorn morphology that were associated with radiation. These abnormalities were visible down to a level of 4 r/day. Sparrow and Woodwell (1962) reported detectable effects on the growth of mature Pinus rigida trees at average dose rates as low as 2 r/day during an exposure period of 9 years.

Mericle, et al. (1962) described the radiation damage to Quercus velutina and Quercus alba trees growing in this same forest, and effects included among others were: sparseness of foliage, distorted leaves, lack of meristematic tissue in the buds, and a reduction in vegetative and flower primordia. However, no lethality of entire oak trees had occurred at an average dose rate of 7 r/day.

In this report are discussed the cumulative effects of low level chronic gamma radiation on male and female flowers, pollen production and pollen germination on Pinus rigida Mill. and Quercus spp., as well as the results from a progeny test of Pinus rigida seedlings from irradiated mother trees.

Materials and Methods

The pine and oak trees studied were growing in a natural forest at the edge of the Gamma Field (Figure 1). These trees had been subjected to low level intensities of gamma radiation since the field went into operation in 1951. For further details of the design, operation, and radiation dosages of this field, the reader is referred to the description by Sparrow (1960). The pine trees that were studied were growing in an area that was exposed up to 5.5 r/20 hr day, and the oak trees were exposed to 1 to 15 r/20 hr day. All references to exposure levels of the pine and oak trees in this report are for the 1960-61, and 1962 seasons. The Cobalt 60 source was maintained at approximately the same level during this period. Cumulative dosages, as well as other pertinent details of the radiation at a representative level (13.18 r/20 hr day during 1960) have been compiled by Mericle, et al. (1962: Table 1).

Observations on flower phenology and morphology were done on fresh material during the spring of 1960, 1961, and 1962. Details of the microsporangia were obtained from microtome sections stained with hematoxylin and safranin. Prior to sectioning, the material was fixed in Craf III, and imbedded in Tissuemat. Cytological observations on microsporogenesis were done on material stained with propionic carmine. Scoring of fragmentation and bridging was done at Anaphase I.

For the pines, pollen was allowed to dehisce under natural conditions and was stored in a desiccator in a refrigerator until used. Abortion was scored after suspending the grains in Van Tieghem cells; estimates of pollen viability were made by incubation of pollen grains for five days in a 5% aqueous sucrose solution at 24° C, and by counting the number of grains that had germinated in a 200 grain sample. Pollen tube length was measured after five days with an ocular micometer at a magnification of 150 X.

Oak pollen was collected and stored in a similar manner to that of the pines. However, to evaluate abortion, catkins were fixed in Newcomber's solution at the time of dehiscence and the number of aborted grains were counted after staining with propionic carmine. At each level a total of 500 was scored on each of four slides; thus, the values given are based on 2000 grains. After several trial germination tests, it was decided to use an aqueous medium for the germination test. This medium consisted of double distilled water containing 20 ppm of boron. Incubation was in Van Tieghem cells at a temperature of 30° C for 24 hours. After this period, germination and tube length were evaluated in a similar method to that given for pine.

The progeny test consisted of collecting cones and acorns from the indicated trees, and for the pines, the cones were kept separate by tree and per year of formation. Progeny from seed formed during 1958, 1959, 1960, and 1961, but where the seed had remained in the cones on the trees until 1961, was available for the test. Germination and survival tests were done in the greenhouse; the details and results of these tests were given in a previous paper (Mergen and Stairs, 1962). After their initial growth the seedlings were moved to a nursery where the oaks were field-planted, and the pines were planted into individual pots and plunged into peat moss to minimize transplanting damages. Height measurements of the pines were made during late summer after height growth had ceased and the plants had formed terminal buds. Because the oak seedlings had completed their first flush of growth before they were outplanted, field evaluation could not be made for this report.

Results

Flower Phenology and Morphology

Throughout late winter and early spring the rate of development of the male catkins of the irradiated trees lagged behind that of the trees in the control forest. With the exception of the strobili on one tree (#61, 3.9 r/20 hr day) the irradiated ones were considerably shorter (6.5 mm vs. 12.0 mm). A representative cluster from an irradiated tree that had received 5.2 r/20 hr day is compared with a control in Figure 2. Because of their shorter length, these stobili had a squatty appearance. Longitudinal sections through the stobili showed a smaller number of grains per microsporangium, and the terminal scales were considerably larger. During the 1962 pollinating season, pollen dehisced naturally in the control forest on May 21, while in the irradiated trees pollen started to shed only on May 29. This 8-day delay was fairly uniform in the six trees studied; similar delays in pollen shedding were observed during two previous seasons.

Phenological observations on the three species of oaks showed a marked delay in the irradiated trees. To illustrate the stages of development, composite photographs were prepared for two dates for Quercus alba Linn., and one each for Quercus ilicifolia Wang. and Quercus coccinea Muench. Figure 3-1 illustrates the condition in Quercus alba, five days before pollen started to dehisce naturally in the controls. The delay of the male flowers of the trees exposed to 8 to 12 r/20 hr day is quite pronounced. At the time of pollen shedding, on May 26 (Figure 3-2), the delay was not as great, but pollen shedding was delayed by approximately one week. Although the irradiated catkins shed pollen, they were much shorter and did not appear to reach full vegetative maturity.

Natural pollen shedding in Quercus coccinea and Quercus ilicifolia preceded that of Quercus alba by approximately 14 days. There was a similar delay in the irradiated trees, and this delay was also paralleled by shorter catkins at the time of dehiscence (Figure 3-3; 3-4).

One pine tree (#61) that had been exposed to 3.9 r/20 hr day during 1960-61 had a large number of hermaphroditic microsporangiate strobili (Figure 4-1). Although hermaphroditic strobili were also observed in the control trees, the abundance in the irradiated tree was much greater than that observed in any of the control material. No statistical evaluation could be made because of relatively small sample. Besides the hermaphroditic strobili, there were many instances where the apical sporophylls did not contain microsporangia but were simple vegetative leaves (Figure 4-3). Deviations in the sexual expression of the oaks were also present. While hermaphroditism was not observed, there were, however, fusions of acorn stalks which resulted in fan-shaped structures. There was in addition a greater variability in the length of the acorn-bearing stalks (Figure 4-2). Internal structures were quite variable in many of the acorns that were examined (Figure 4-4).

For both pine and oak the phenology and size of the female flowers paralleled the conditions in the male flowers, and will thus not be described in detail.

Pollen

The cytological examination during meiosis in the pine revealed chromosomal aberrations, such as fragmentation and bridging at a level of 4.7 r/20 hr day (Figure 5-1; 5-2). Of the cells examined at this level during Anaphase I, 29% showed fragmentation with a frequency of approximately one fragment per cell. This value may be contrasted with an abortion rate of 79.5% for pollen from the same tree. In addition to the fragmentation that was observed at Anaphase I, there were bridges at Anaphase I and Anaphase II (Figure 5-3). These might account in part for the high abortion rateof the pollen. However, besides these visual aberrations there are genetic as well as physiological disturbances. Micronuclei were present not only in the sporogenous tissue and microspores, but also in the vegetative cells of the surrounding layer (Figure 5-4). These micronuclei probably are the result of lagging chromosomes or chromatic fragments excluded from the nuclei during reformation following division.

Similar observations were made in the oaks at somewhat higher levels (12 r/day), but these aberrations were not as frequent and not as well defined. A comparison of the size of the chromosomes of pine (Figure 5-5) and oak (Figure 5-6) shows that the oak chromosomes are considerably smaller, although both genera have a somatic number of 2n = 24. Due to the smaller size of the chromosomes in the oaks, a larger sample than was available would be necessary to obtain definitive statistics of these abnormal conditions.

When pollen from irradiated trees was compared with that from the control trees, there was a highly significant increase in the percent of aborted pollen grains. The percent abortion increased with an increase of the exposure of the parent trees. For the regression analysis the percentage values were subjected to an arcsin transformation to stabilize the variance, and also partially to improve the fit. The slope for the regression was highly significant and is illustrated in Figure 6A.

Percent germination of the control pollen was also greater than in the irradiated pollen. The data were also transformed to arcsin for the regression analysis (Figure 6B), and the value for the slope "b" approached significance at the 5 percent level (F = 5.96 vs. 5.99). The germination percentage was expressed on the basis of filled grains present in a slide, rather than on the total number of grains that were present. If aborted grains had been included in the count, and the germination capacity had been expressed on the basis of total number of grains present, a stronger relationship would have been obtained. This indicates that as a result of cumulative gamma radiation, not only are fewer apparently normal pollen grains produced, but that those grains that appear to be normal under a microscope also have a lower germination capacity. One interesting observation, however, was the fact that those grains that did germinate produced normal pollen tubes with no reduction in tube length (Figure 6C). Nevertheless, the tube sizes from the irradiated pollen were more variable, and one tree produced considerably longer tubes.

Pollen abortion in the oaks showed a similar response to cumulative radiation as was described for the pine trees; however, the over-all damage was less and occurred at a higher exposure. The data for the three species were kept separate for the analysis, and tests of significance between the regression lines of the transformed data (arcsin) showed that there was no significant difference between the three species in either the "Y" intercept (a), or in the slope (b). Therefore, a common slope was calculated for the three species, and this regression is illustrated in Figure 6D. Different symbols are used in the graph to distinguish each species. For this regression the values for the controls, although they are indicated on the ordinate of the graph, were not used to calculate the regression. Most of the values were close to the calculated regression with the exception of the pollen from one white oak tree. This tree was exposed to approximately 9 r/20 hr day and had a considerably higher percentage of aborted grains. This particular tree also shows greater morphological aberrations and is in general in poorer physiological condition.

Pollen germination techniques are not too satisfactory an estimate of pollen viability in the oaks and considerable normal variations are present. For the irradiated lots, the variation between trees was more variable than in the controls, and there was also considerable variation in pollen tube length. Similar to the pines the pollen tubes produced from the irradiated oak appeared normal and in some instances the tubes were longer than the controls.

Progeny Tests

Although radiation damage has been observed in the various stages of sexual reproduction, pine cones with viable seed have been obtained from severely damaged trees; including some trees that were dying or are dead.

The length of the hypocotyl in the pine seedlings from the irradiated trees was significantly shorter when compared to the controls (Mergen and Stairs, 1962). As was pointed out in that report, the length of the hypocotyl reflects the vigor of the female parent, and the initial height growth of a pine seedling is the result of cell elongation, rather than of cell division. This reduced initial height may be a somatic radiation response rather than a permanent genetic effect. However, subsequent growth of the seedlings, and their ontogeny, are caused by mitotic cell divisions, and at this time genetic mutations can become apparent.

In Figure 7 the height measurements at the end of one growing season are illustrated for the various progeny groups where the means (± one standard error) are given for each group. From this graph it can be seen that the progeny from the irradiated trees is much more variable both within, and between, groups. For three of the years (1958, 1959, and 1960) the seedlings were taller after one growing season, while for the 1961 collection there were some lots that were shorter and some had particularly tall seedlings. Besides the radiation effects, the effect of the age of the seed was still apparent after one growing season.

Within the irradiated group there was a greater number of "abnormal" seedlings. The range of variability within the control seedlings, and within the irradiated seedlings is illustrated in Figure 8. These abnormalities included shorter and longer needles, an irregular apical growth pattern, lack of primordia on the leader, and twisted needles.

Even under natural conditions oaks as a group are quite variable in morphology during the juvenile stage. Although some abnormal behaviors were present in the progeny test with the irradiated acorns, their evaluation will need to be delayed until the seedlings have passed the juvenile stage.

Discussion

The cumulative radiation effects on the sexual reproduction of pine and oak that were described are probably a combination of somatic effect and permanent genetic changes. The delay in the over-all phenology in the reproductive structures was well pronounced for both species and was a clinal response to the altered environment. This suggests a reduction in endogenous growth substances, either *in situ* at the meristematic areas, or in their synthesis at the molecular level. Gordon (1956) pointed out the relative sensitivity of auxin metabolism to radiation effects, and he found drastic reduction in auxin level in irradiated plants at dosages of less than 100 r. The application of TIBA to *Nicotiana* hybrids by Sparrow and Schairer (1958) resulted in effects similar to those of radiation treatments. It is planned to collect scions from the irradiated trees, and these shoots will be grafted on normal stock plants outside the exposure area of the gamma field, to separate the genetic effects from the somatic ones.

However, the evidence given on aberrations during meiosis, and post meiotic divisions, is proof that genetic damage in the nature of visual and chromosomal changes is taking place at as low a level as 4.7

r/20 hr day in the Pinus rigida. With this high incidence of visible aberrations during meiosis, it can be assumed that the number of submicroscopic changes must be very high also. Micronuclei have been observed in mature pollen grains of irradiated pitch pine trees, and this would indicate that the abortion of pollen does not present an effective block against the perpetuation of these genetic changes. Controlled pollinations with pollen from the irradiated trees will be made, including selfings of the irradiated trees, to obtain statistical information on the mutations that have occurred.

The greater variation in the progeny from the irradiated trees suggests that there are considerable mutations as a result of cumulative low level chronic radiation. These phenotypic expressions until now are limited to their juvenile ontogeny, and as these seedlings develop, additional abnormalities will probably occur in both their mature vegetative and sexual expressions.

It is of interest to note that while the variations as a result of cumulative chronic gamma radiation that were observed ranged from near normal to lethal conditions, no complete block to sexual reproduction in the living tree was interposed by the gamma irradiation. Basically the effect of low level radiation as herein investigated acts to diminish steadily the meristematic base which will initiate the annual tree growth. The exact nature of differentiation of this meristematic base in the trees into somatic and reproductive components is today a largely unsolved problem in Forest Biology. Results of this and previous studies in the area under investigation indicate that the processes involved in formation of sexual primordia in pine and oak do not present any unusual sensitivity to cumulative gamma radiation. Their further development, however, is greatly influenced. Despite the greater sensitivity of meiosis the capacity for sexual reproduction in both genera parallels that for vegetative survival under these low level conditions.

Acknowledgments

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Captions for Illustrations

Figure 1. Oblique aerial view of Co^{60} Gamma Field at the Brookhaven National Laboratory. Pine and oak trees inside marked area were used for this study. The photograph was taken during the dormant season, after the oak trees had lost their leaves.

Figure 2. Comparison between outward morphology and anatomy of microsporangiate strobili from a control and irradiated *Pinus rigida*. Scale for macrophotographs is in mm. The terminal scale in the cross-section of the irradiated microsporophyll (4) is in evidence.

Figure 3. Effect of radiation on phenology of male flowers in *Quercus*. (1) *Quercus alba* collected on May 21, 1962; A = Control, B = 5.2 r, C = 6.0 r, D = 10.5 r, E = 11.5 r, F = 12.0 r. (2) *Quercus alba* collected on May 26, 1962; A = Control, B = 3.4 r, C = 5.2 r, D = 6.0 r, E = 9.4 r, F = 10.5 r, G = 11.5 r. (3) *Quercus ilicifolia*. A = Control, B = 9.0 r, C = 13.5 r. (4) *Quercus coccinea*. A = Control, B = 7.4 r, C = 11.5 r. Scale is in mm.

Figure 4. Effect of radiation on female reproductive structures. (1) Hermaphroditic strobili of *Pinus rigida*, some with well defined ovulate sporophylls at the apex. Short dark tips on strobili are vegetative in character and bear sterile sporophylls. A cross-section through this type of structure is given in (3) where the transition from sporophylls, filled with pollen

grains, to non-reproductive leaves is well illustrated. (2) Aberrant acorn bearing structure of *Quercus alba* showing fusion of acorn stalks on left, and excessive elongation of stalks on right. (4) Cross-section through acorn during early development. Carpellate structure is irregular, and shows a greater number of carpels (7) than in the controls (6). Scale in macrophotographs is in mm.

Figure 5. (1-3) Chromosomal aberrations during microsporogenesis of *Pinus rigida*. (1) Bridge formed at Anaphase I. (2) Micronuclei forming from fragments of chromosomes at Telophase I. (3) Bridge and micronucleus at Telophase II. (4) Micronucleus in vegetative cell. (5) Comparison of size of chromosomes in *Pinus rigida* and (6) *Quercus alba*. (5 and 6 are at the same magnification).

Figure 6. Effect of radiation on the behavior of pollen. *Pinus rigida* A. Percent abortion; B. Percent germination; C. Tube length. *Quercus* sp.; D. Percent abortion.

Figure 7. Average height measurements (± one standard error) or irradiated *Pinus rigida* seedlings after one growing season. The seedlings are kept separate by year during which seed formed. All seed was collected during the fall of 1961.

Figure 8. Extent of variation in control and irradiated seedlings of *Pinus rigida* at the end of one growing season. All seedlings are in 4" pots.

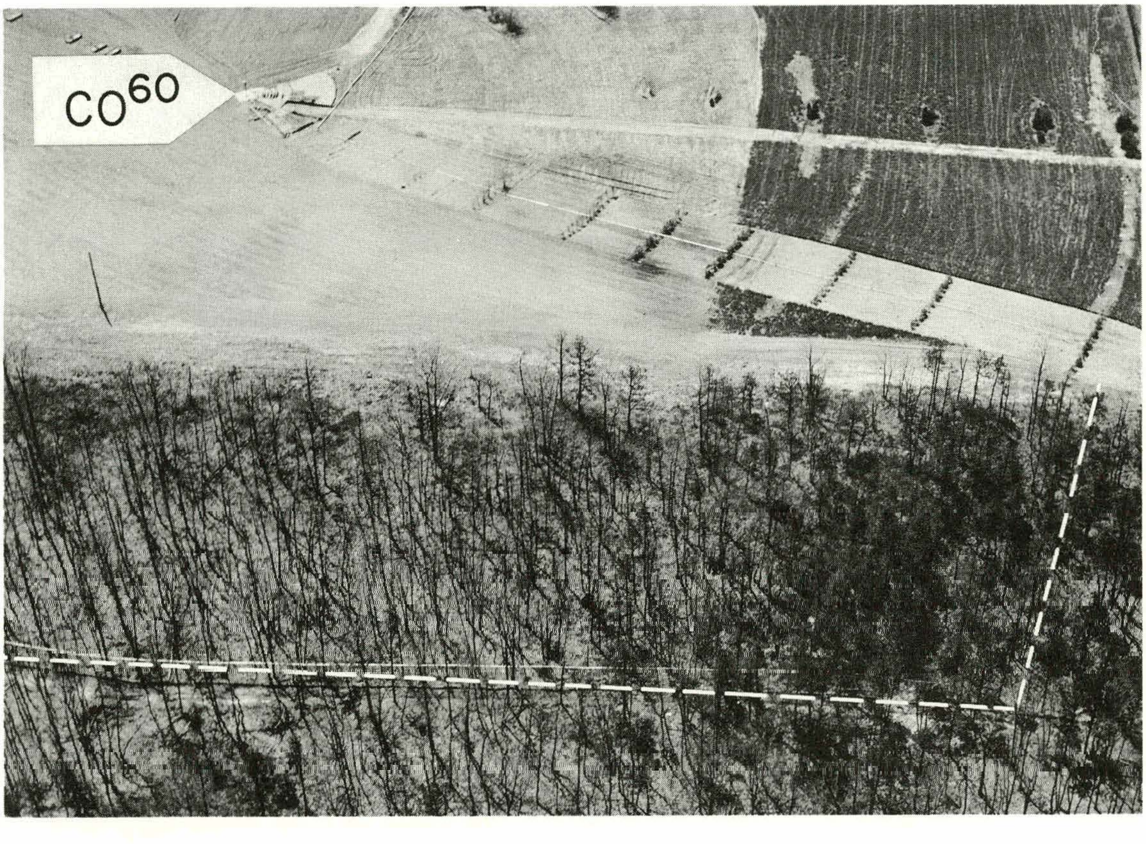

FIGURE 1

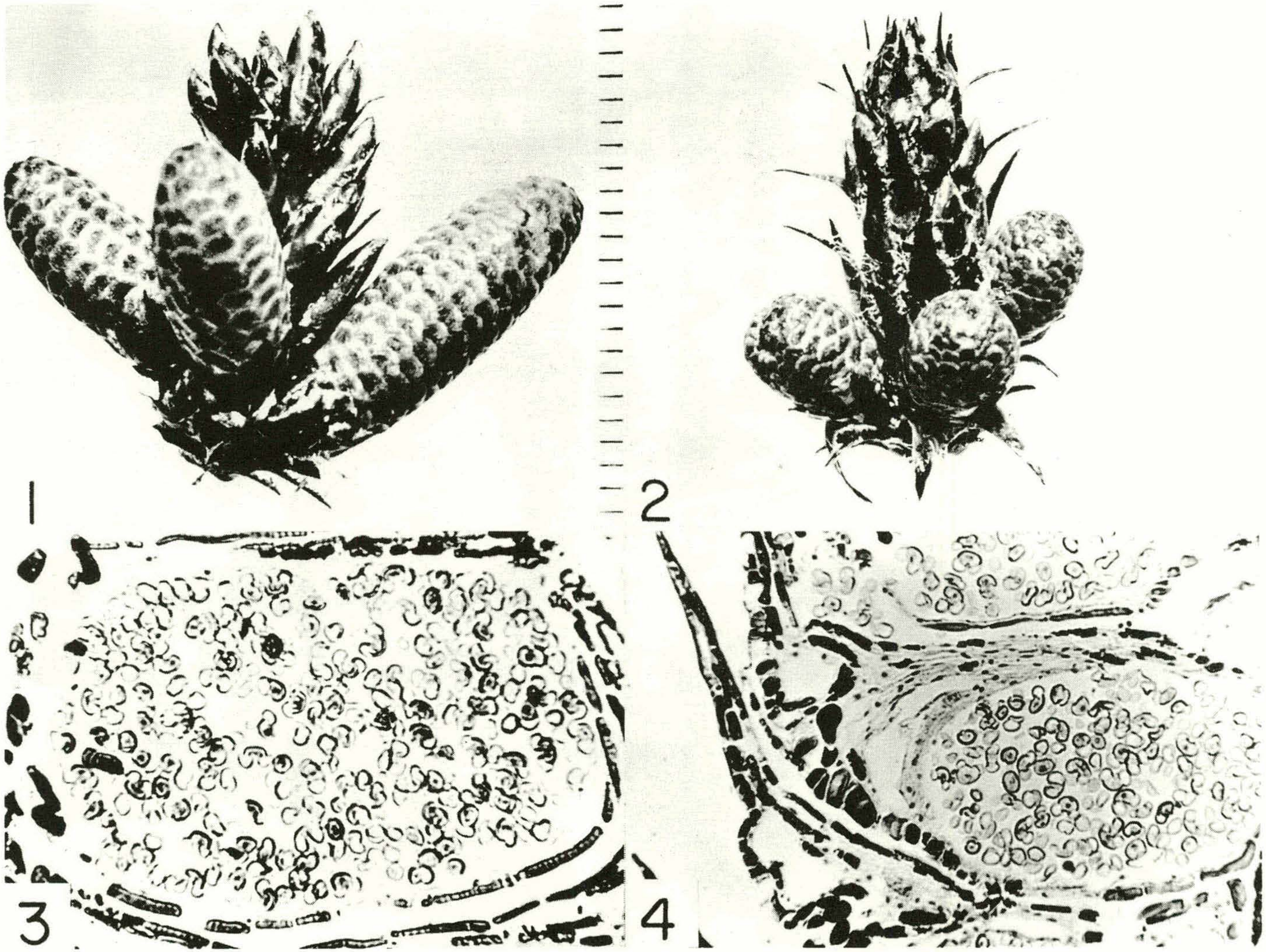

FIGURE 2

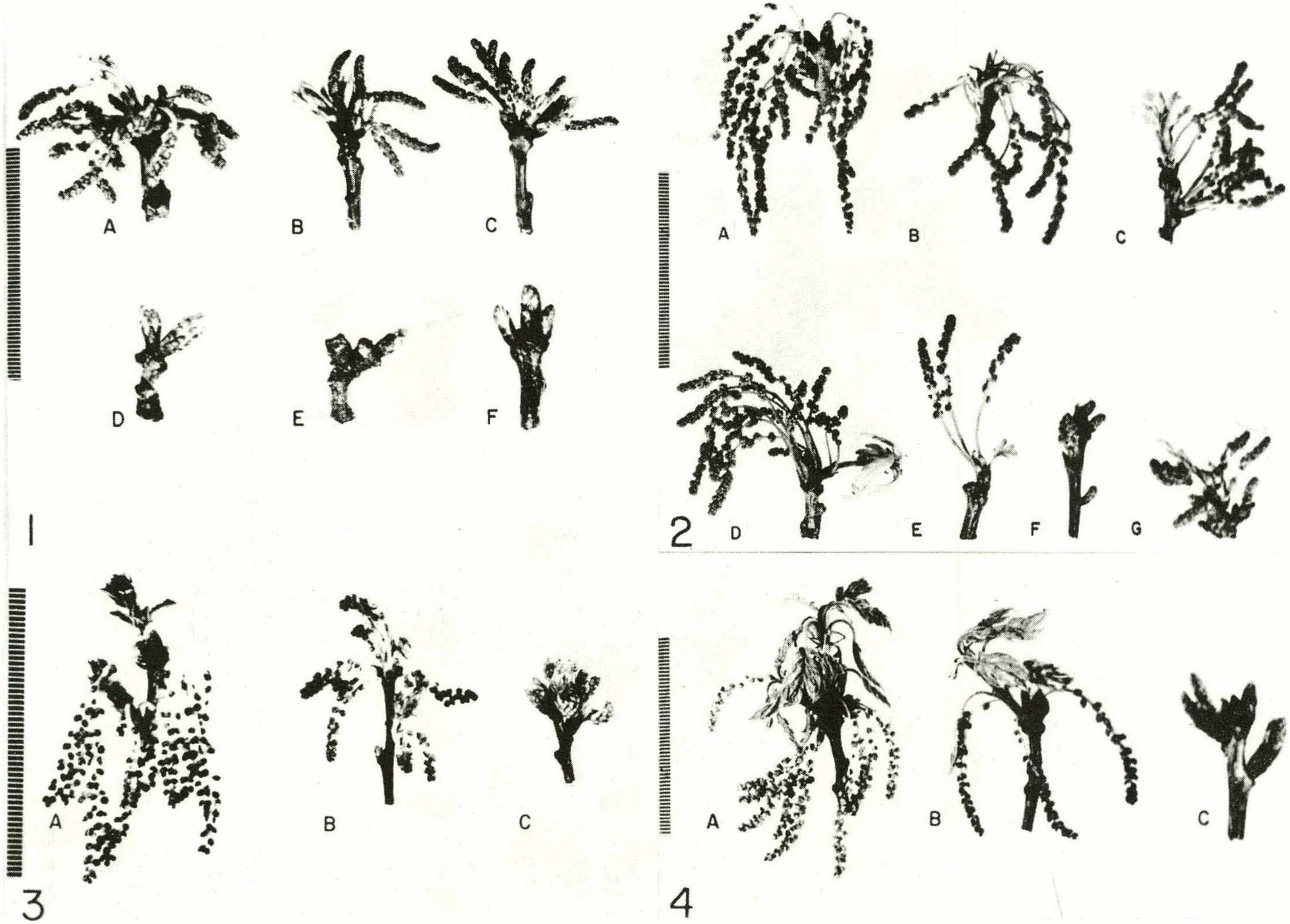

FIGURE 3

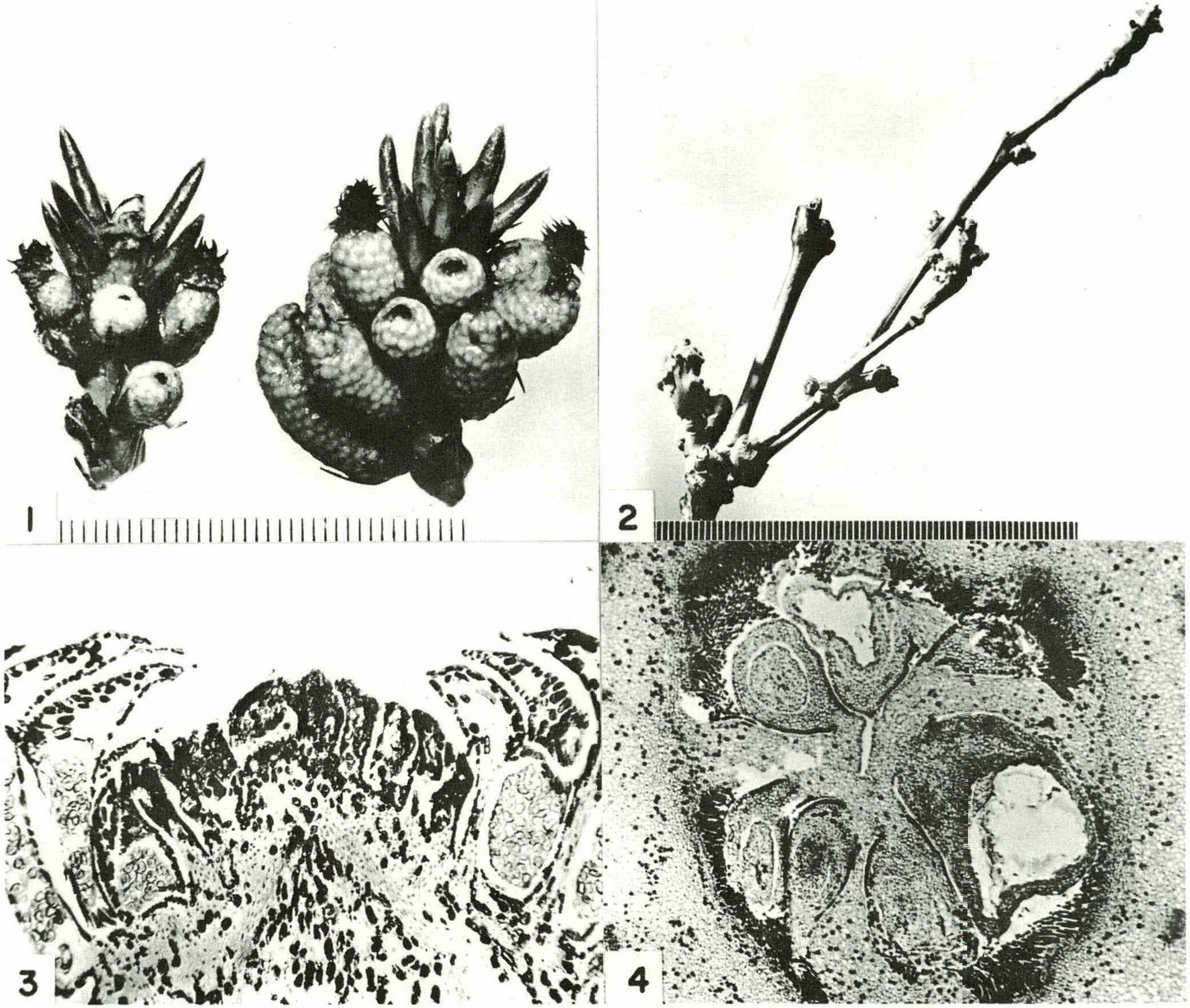

FIGURE 4

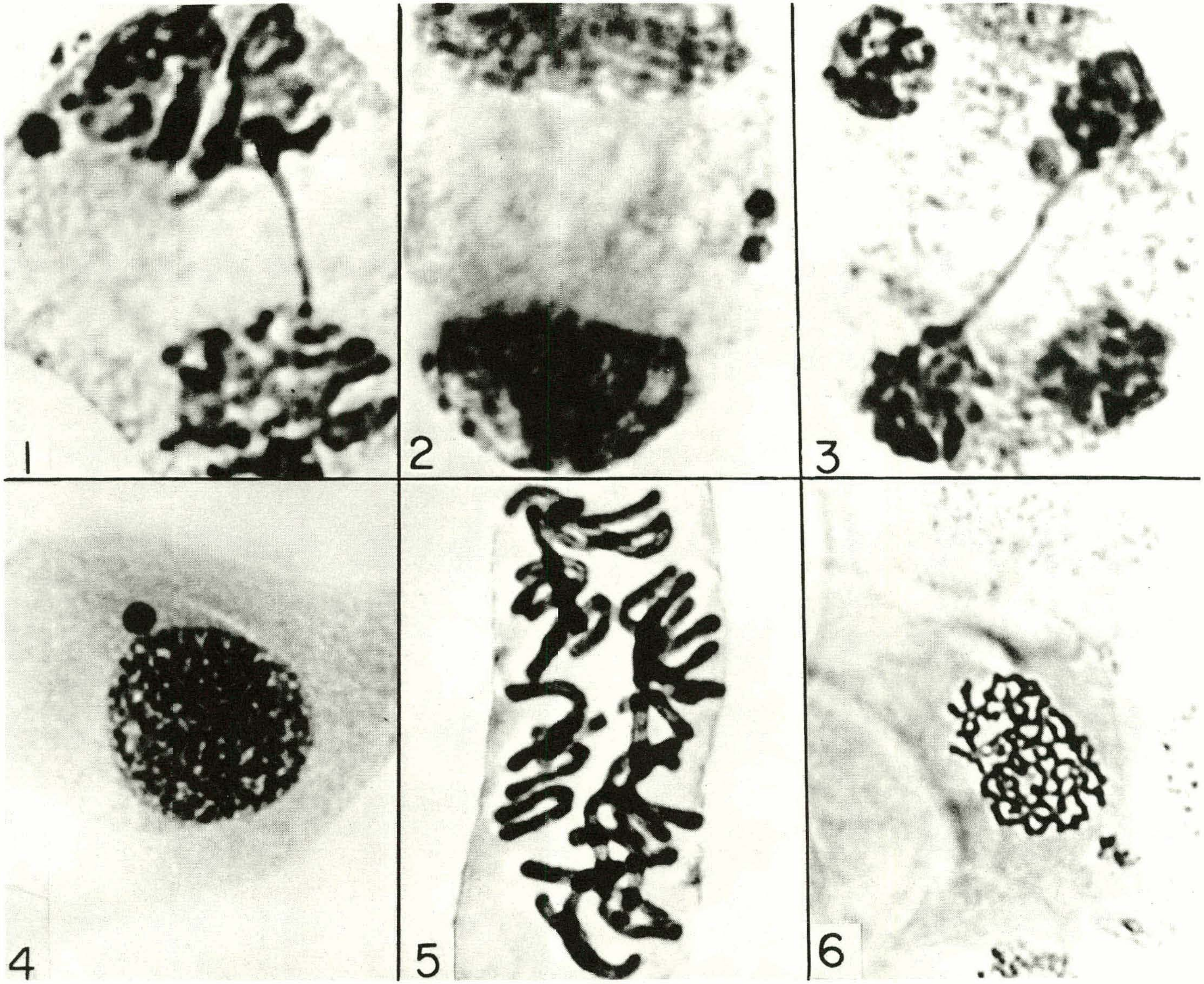

FIGURE 5

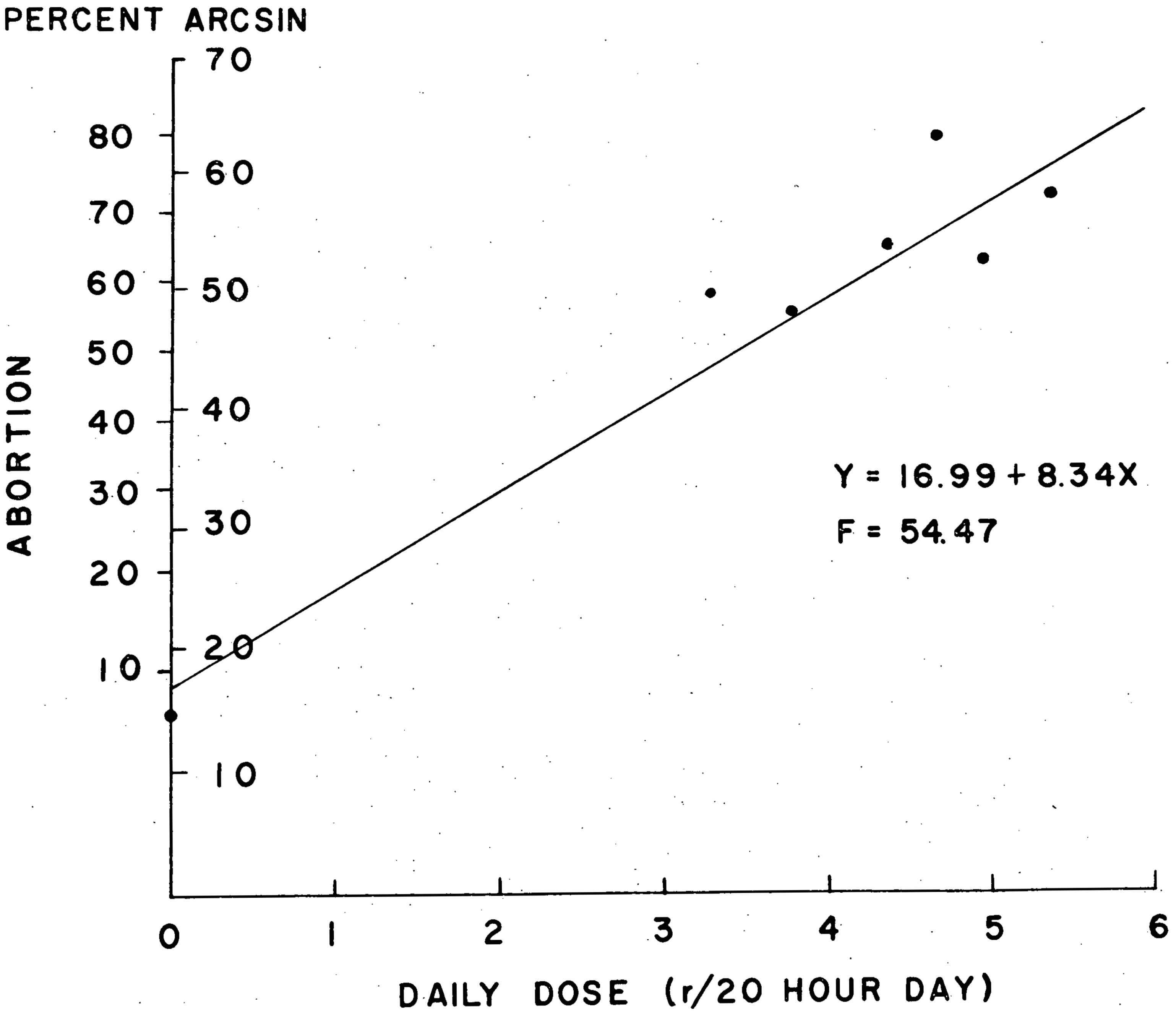
PERCENT ARCSIN
ABORTION
80
70
60
50
40
30
20
10
70
60
50
40
30
20
10
Y = 16.99 + 8.34X
F = 54.47
0
1
2
3
4
5
6
DAILY DOSE (r/20 HOUR DAY)

FIGURE 6A

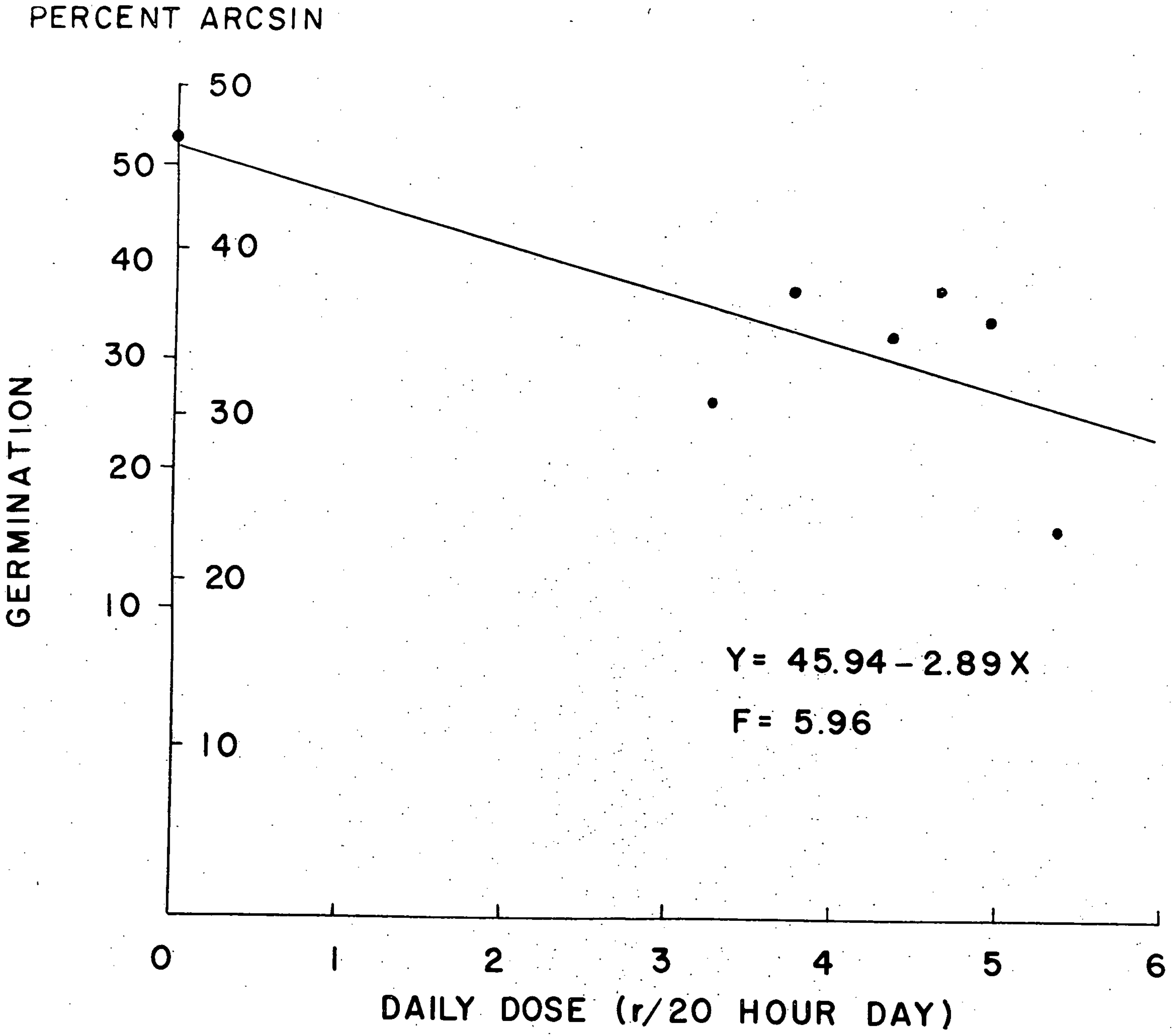
PERCENT ARCSIN
GERMINATION
50
40
30
20
10
50
40
30
20
10
Y= 45.94 - 2.89 X
F= 5.96
0
1
2
3
4
5
6
DAILY DOSE (r/20 HOUR DAY)

FIGURE 6B

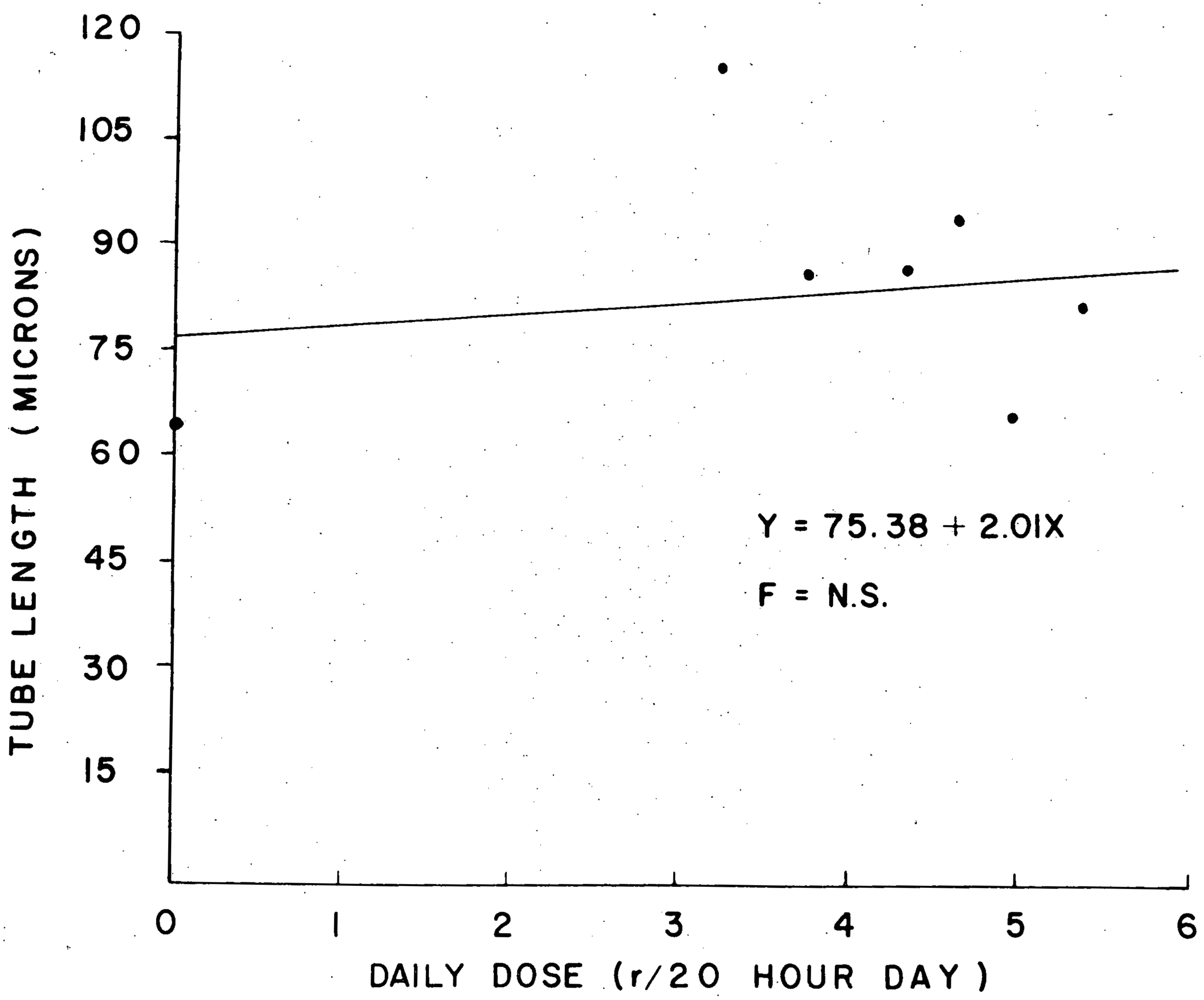
120
105
90
75
60
45
30
15
TUBE LENGTH (MICRONS)
0
1
2
3
4
5
6
DAILY DOSE (r/20 HOUR DAY)
Y = 75.38 + 2.01X
F = N.S.

FIGURE 6C

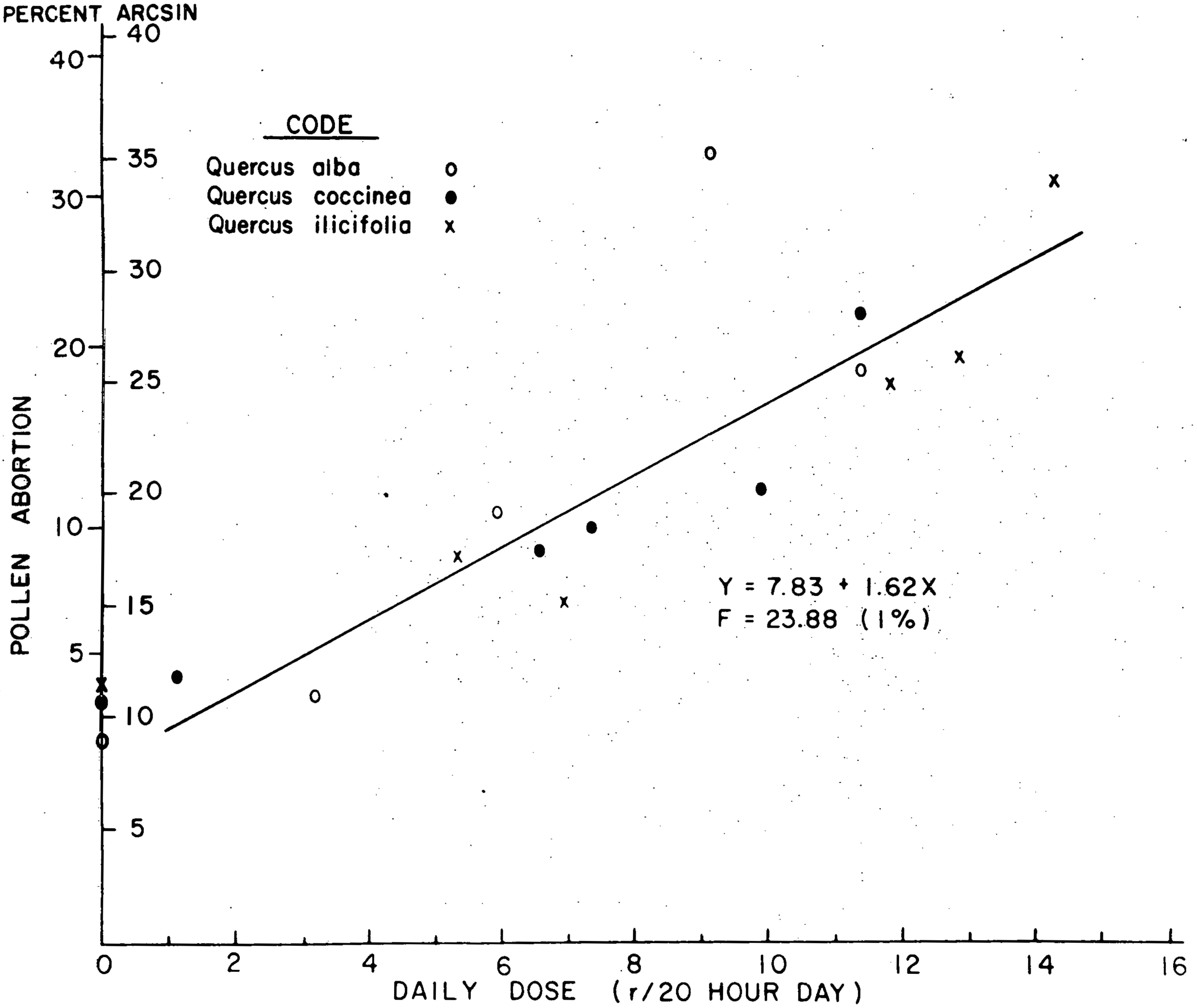
PERCENT
ARCSIN
40
35
30
25
20
15
10
5
40
30
20
10
5
POLLEN ABORTION
CODE
Quercus alba o
Quercus coccinea ●
Quercus ilicifolia x
Y = 7.83 + 1.62X
F = 23.88 (1%)
0
2
4
6
8
10
12
14
16
DAILY DOSE (r/20 HOUR DAY)

FIGURE 6D

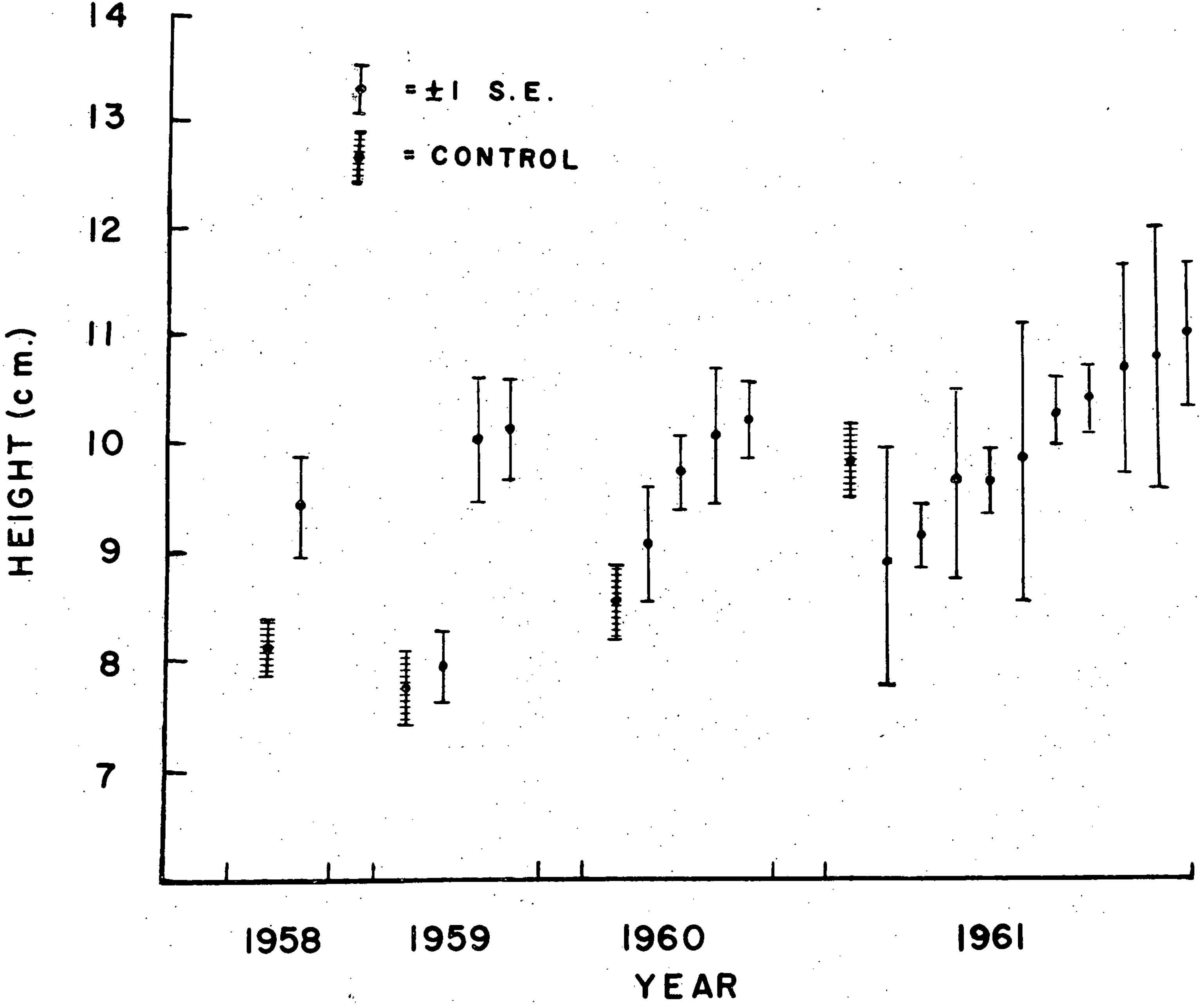
14
13
12
11
10
9
8
7
HEIGHT (cm.)
= ±1 S.E.
= CONTROL
1958
1959
1960
1961
YEAR

FIGURE 7

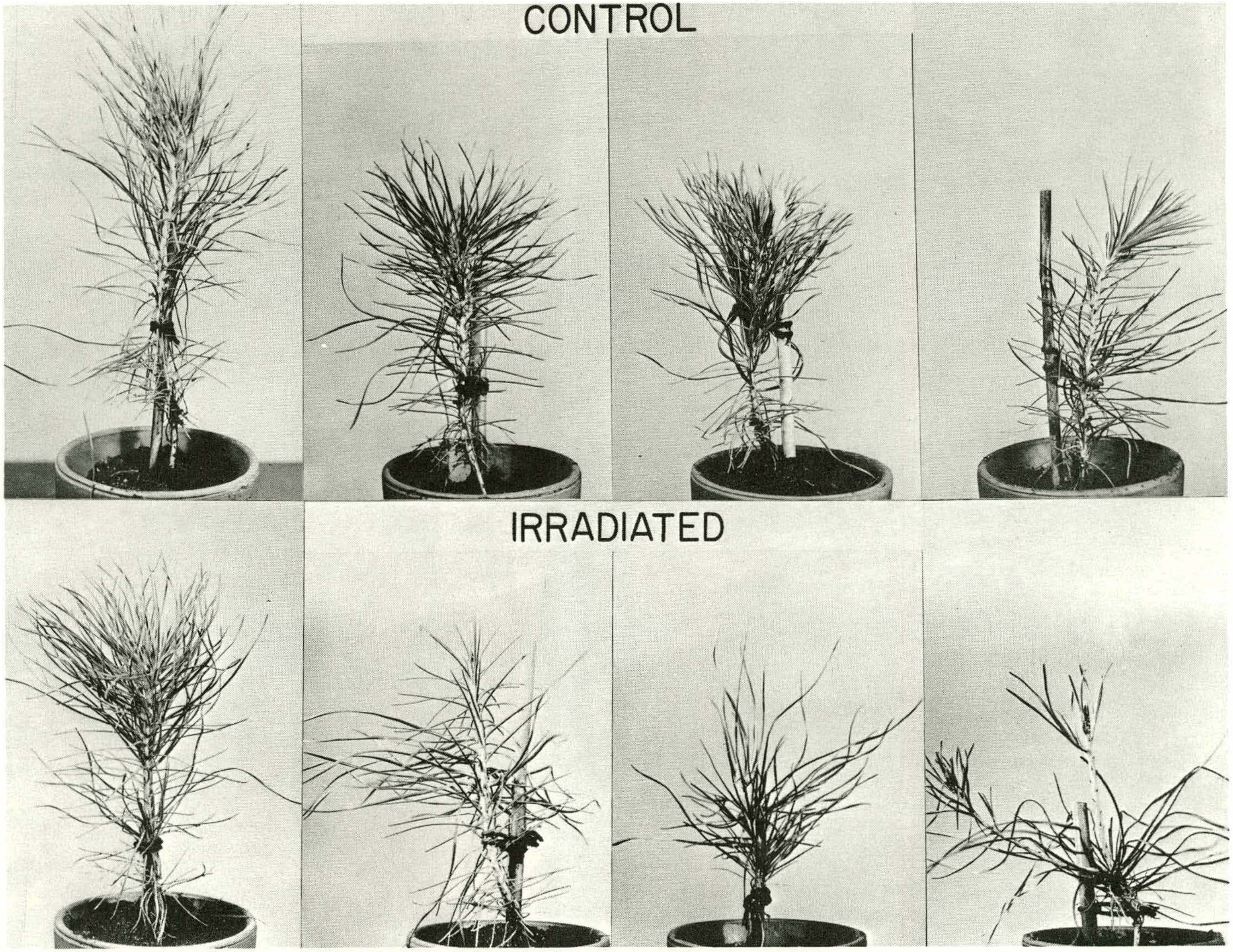

FIGURE 8