THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

DORMANCY AND GERMINATION IN FRUITS OF THE SUNFLOWER

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

FORREST EUGENE LANE

Norman, Oklahoma

1965

DORMANCY AND GERMINATION IN FRUITS OF THE SUNFLOWER

APPROVED BY

Lawrence W. Rabideaux

Norman Roth

Elroy L. Rice

[illegible]

Clyde E. Anderegg

DISSERTATION COMMITTEE

ACKNOWLEDGMENTS

I wish to express my sincere thanks to Dr. L. M. Rohrbaugh for his encouragement and many helpful suggestions during the course of these studies and for his many suggestions during the preparation of this manuscript.

I wish to thank Dr. E. L. Rice for his helpful suggestions and for supplying the isochlorogenic acid used as a reference compound in some of these studies.

I wish to thank the other members of my graduate committee for their suggestions on the preparation of this manuscript, and for their assistance on other occasions.

I wish to thank Dr. Edwin F. Alder of Eli Lilly and Co. at Greenfield, Indiana, for the gibberellic acid used in these studies and for other assistance given to me during my graduate career.

I wish to thank Dr. Lowell F. Bailey of the University of Arkansas for his help over many years in making this possible.

Thanks are due to the National Science Foundation for their support through an NSF Graduate Fellowship and two subsequent renewals.

TABLE OF CONTENTS

LIST OF TABLES

TABLE OF FIGURES

DORMANCY AND GERMINATION IN FRUITS OF THE SUNFLOWER

CHAPTER I

INTRODUCTION

For many years reports of dormancy in seeds[1] of many species have appeared in the literature. These seeds were reported to be dormant due to their failure to germinate when placed in environmental conditions usually considered favorable for germination. They were shown by other means to have viable embryos, and their failure to germinate was attributed to impermeability of the fruit or seed coats to water or gases, mechanically resistant coats, immature embryos, the presence of germination inhibitors, and special requirements on the part of the embryos. Germination inhibitors (8) and seed germination (5, 27, 42) have been reviewed recently.

Dormancy is usually more pronounced immediately after maturation of the seeds or fruits; and in most cases the

---

[1]Many structures other than seeds have been called "seeds" because of their physiologic behavior and their use as propagation units. These include achenes, caryopses, nutlets, and samaras, all of which are dry fruits.

depth of dormancy decreases with passing of time, with leaching, or with exposure to cold temperatures during the winter following their maturation. In temperate regions seed dormancy has survival value in species that are not winter hardy, because it prevents autumnal germination of the newly matured fruits.

Extended dormancy after maturation of the seeds seems to be widespread among native species, but mature seeds of most crop plants exhibit either no dormancy or only a short period of dormancy immediately after harvest. The lack of extended dormancy in these seeds probably has resulted from selection of seed for rapid and complete germination.

No reports have been found in which dormant seeds or fruits of a native species were compared physiologically and biochemically with non-dormant seeds of a domestic variety of the same species. The annual sunflower (Helianthus annuus L.), a common weed species throughout much of the United States, and the domestic "Russian Mammoth" sunflower which is considered to be a variety of the same species, presented the opportunity for such a study. The fruits of the native sunflower had been observed to have extended dormancy (E. L. Rice, personal communication), while those of the domestic variety, although reported to be dormant immediately after harvest, rapidly break dormancy at room temperatures and show rapid and complete germination within 10 weeks from harvest (51).

This paper characterizes dormancy in the achenes of the native sunflower and reports means of breaking that dormancy. It also reports the isolation of certain substances from extracts of dormant and non-dormant fruits of the native sunflower and from extracts of the non-dormant fruits of the Russian Mammoth variety. Paper chromatography was used to make these isolations and to make a comparison of the presence of these substances and the sequence of their appearance in the fruit extracts.

CHAPTER II

MATERIALS AND METHODS

Mature fruits of the native sunflower were collected on a road cut south of Norman, Oklahoma, at 1-week intervals from late September through October of 1963 and 1964. One small collection of fruits was made on May 7, 1965, from plants grown in the greenhouse. Unless indicated differently in the text, all studies reported were made using fruits collected October 19, 1963.

Fruits of the domestic variety were obtained from a commercial seed company (Burpee, Number 6111 "Russian Mammoth").

Preliminary tests were made to verify the existence of dormancy in the native sunflower fruits. Then dormancy was explored by water leaching to check for the presence of germination inhibitors, by constant and alternating temperatures on leached and non-leached fruits, by high oxygen concentrations, by mechanical scarification, by treatment with thiourea and gibberellic acid (GA), by moist stratification (2-5°), and by dissecting out the embryos for germination and inhibitor studies.

Extracts were prepared from both wild and domestic varieties by leaching in distilled water; by boiling in 50% methanol, 80% isopropanol, or 95% ethanol; and by Soxhlet extraction with 95% ethanol. Water leaching and Soxhlet extraction were used most frequently.

Water leachates were prepared in 2 ways. Leachates were prepared by placing a known quantity (10 or 100 fruits) on a 4.5-cm disc of Whatman No. 1 filter paper in a 5-cm petri dish and soaking them in 2 ml of 0.05 M phosphate buffer (pH 5.6) for 24, 48, or 72 hours. The fruits were then removed and the disc and water solution were bioassayed for inhibitor activity.

Water leachates from larger quantities of fruits (50 g) were prepared by gravity percolation of distilled water over the fruits in a leaching apparatus (fig 1) redesigned from that of LeCroix (23). It consists of 2 chambers made from 35 mm Pyrex tubing. Chamber B has an attached valve with a pointed moving part that was ground to make a tight seal when seated on closing of the valve. Chamber A has a similar valve, but it is separated from the chamber by a short length of Tygon tubing which is the closure point for a screw type pinchcock. The seeds are placed in chamber B with a plastic screen or glass wool covering the outlet in the bottom to prevent seeds from clogging the valve. The water used in leaching is placed in the beaker (C), and tube D is connected to an aspirator or other vacuum source.

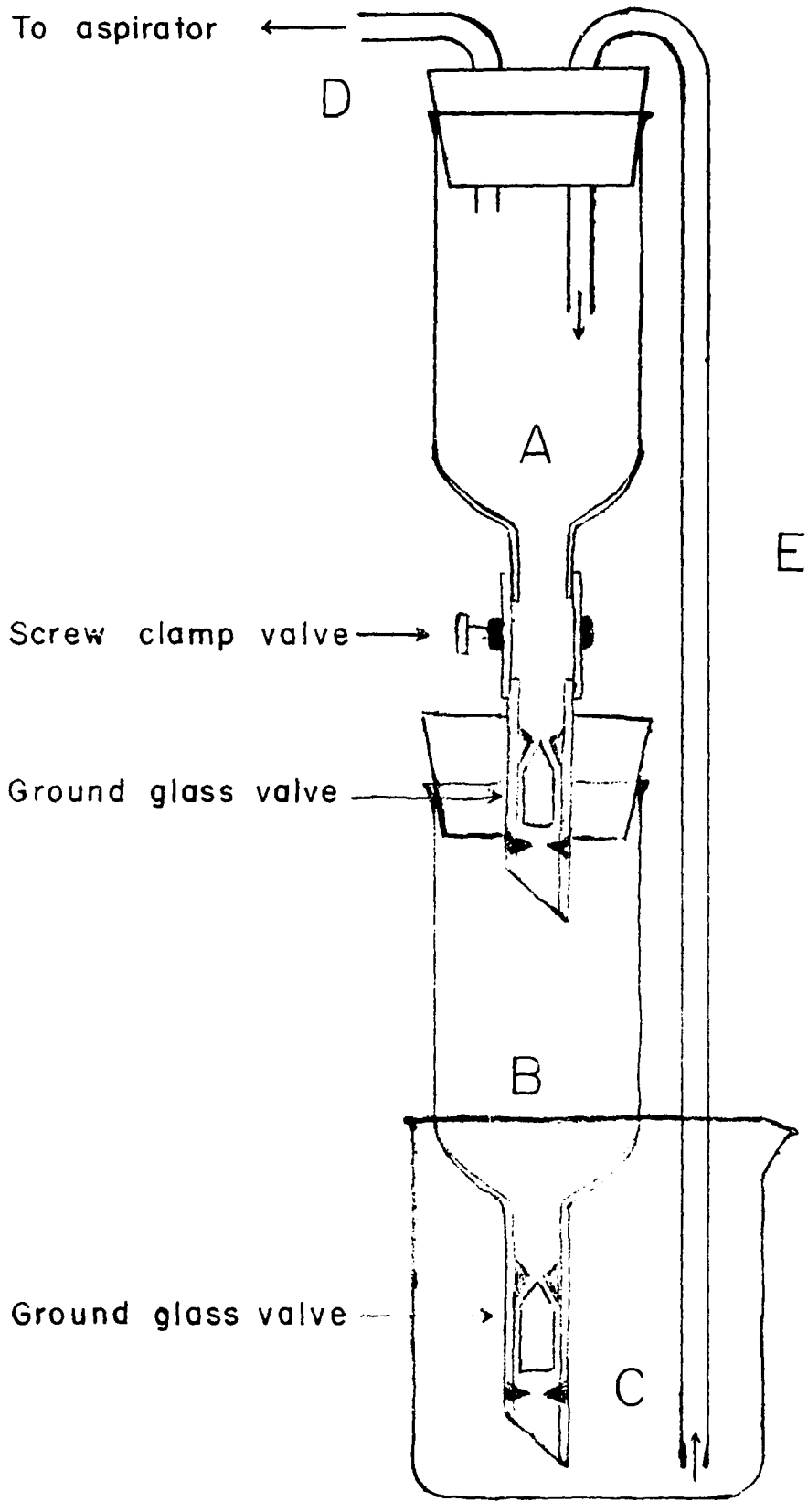

Fig. 1. Leaching apparatus for continuous leaching with a small volume of water.

When the aspirator is turned on, the 2 ground glass valves close due to the reduced pressure in chamber A, and the leaching water moves up tube E until the water level falls below the open end of the tube. The column of water then breaks, and the reduced pressure in chamber A is relieved as air enters tube E. This allows the glass valves to open due to the force of gravity, and water flows from chamber A into chamber B with the seeds. The rate of water flow from A into B can be controlled by the pinchcock valve between the chambers. When enough water seeps through the seed to raise the water level in the beaker to the end of tube E, the reduced pressure in chamber A initiates the refilling of that chamber. Because the water is aerated each time it empties into chamber A, seeds can be leached for long periods without anaerobiosis.

The leaching water was changed in the apparatus at 6-hour intervals. Distilled water (100 ml) was added to replace the leachate. This leaching procedure was continued for 72 hours (later 48, then 36 hours) for a total of 12 collections. During the leaching process, the leachate collections were separately labeled and stored at 2° until all collections had been made. At that time, a 1 ml aliquot of each collection was bioassayed for inhibitory activity. The remainders of all leachate collections showing inhibitory activity were then combined and reduced in volume in a rotary evaporator under reduced pressure at temperatures less

than 50°. An amount of methanol equal to one-fourth the volume of the extract was added to facilitate evaporation.

The concentrate was adjusted to pH 2.8 with 0.1 N HCl and extracted by shaking 5 times with equal volumes of diethyl ether. This acidic ether fraction was evaporated to dryness, taken up in a known volume of methanol, and measured aliquots equivalent to 0.25, 0.50, or 1.00 g of fruits (fresh weight) were streaked on paper for chromatography.

The extracted water was evaporated to dryness on the steam bath. The residue was taken up in methanol and streaked on paper for chromatography.

For Soxhlet extractions, the fruits were dropped into boiling 95% ethanol and boiled for 10 minutes. The boiled fruits were transferred to the Soxhlet apparatus along with the hot ethanol and extracted for 24 or 36 hours with the extraction chamber emptying every 45 minutes.

The ethanolic extract was reduced to dryness in a rotary evaporator under reduced pressure with the temperature maintained at less than 50°. The residue was taken up in distilled water (25 ml at 45-50°) and filtered to remove the insoluble material which was mostly lipid in nature. The filtrate was adjusted to pH 2.8 with 0.1 N HCl, and the subsequent treatment was the same as for the water leachate from large quantities of fruits.

Several solvent systems were utilized in chromatography. Isopropanol-acetic acid-water (IAW) (80:2:18, v/v/v)

was the most commonly used solvent system in preliminary separations. Other solvents used were n-butanol-acetic acid-water (BAW) (63:10:27 v/v/v) and 6% acetic acid (6 ml of glacial acetic acid diluted to 100 ml). Descending chromatography was used exclusively in the reported studies, using 19.5-cm wide strips of S&S 2043-b chromatography paper. The solvent was run the same direction on all papers with no prior equilibration.

Elution of materials from the chromatograms was accomplished by soaking the excised sections of the chromatograms in small volumes (ca. 25 ml) of absolute methanol for 10 minutes followed by shaking for 5 minutes, then decanting the methanol. This was repeated 3 times. The methanol extracts were combined, reduced to dryness, and either applied to another paper and rechromatographed, or hydrolyzed.

Hydrolysis of chlorogenic and isochlorogenic acids was accomplished by use of 10 ml of 0.5 N KOH at room temperature for 4 hours. The hydrolysate was acidified with 0.5 N HCl to pH 2.8 and extracted with diethyl ether as were the plant extracts. The ether solution was evaporated to dryness, taken up in absolute methanol, and applied to another paper for chromatography. The water residue from the hydrolysis was evaporated to dryness over the steam bath, and the dry residue was extracted with absolute methanol to eliminate the KCl. This methanol extract was reduced to dryness, taken up in a small volume of absolute methanol, and applied to paper.

Methods and reagents used in locating materials on chromatograms were:

1. Ultra violet light (2537 and 3660 A)
2. Ammonia vapor
3. Sodium picrate paper (1)
4. Ferric chloride-potassium ferricyanide (37)
5. Ehrlich's reagent (37)
6. Hoepfner's reagent (33)
7. Sodium metaperiodate-piperazine-sodium nitroferricyanide (3)
8. Diazotized p-nitroaniline (40)
9. Fast Red GG (stable diazo salt of p-nitroaniline) (29)
10. Fast Bordeaux BD (stable diazo salt of 4-amino-2,5-dimethoxybenzonitrile) (29)
11. Ethanolic $AlCl_3$, 1% (40)

Biological activity of the extracts and of materials on chromatograms was determined by the use of blond psyllium seed (Plantago ovata Forsk.) in germination tests run at 25° (24). Tests were made on the leachates by adding aliquots to petri dishes containing a 5-cm disc of Whatman No. 1 filter paper and adding 0.05 M phosphate buffer (ph 5.6) to give a total incubation volume of 2 ml. Tests on chromatograms were made by cutting out the zones of interest and placing them in petri dishes with 2 ml of the phosphate buffer.

Mechanical scarification was done by grinding a small hole in the flower scar of the fruits with a high speed

dental drill, or by making a small split in the coat by means of a scalpel.

Alternating temperature studies were run in a cold room thermostatically controlled at 15°. An incubator was wired into a time clock and installed in the cold room. When the time clock was in the "off" cycle, the temperature would drop to that of the room. When the time clock was in the "on" cycle, the temperature in the incubator would rise to 25°. There was a time lag of approximately 1.5 hours in changing from one temperature to the other. The clock was set for 8 hours at 25° and 16 hours at 15°.

Fruits were stratified by adding 50 g (approx 9000) of fruits and 400 ml of washed, air dried, white quartz sand to a 1 pint size polyethylene freezer bag. Distilled water (50 ml) was added, and the bag was closed. These packages were placed in cold storage at 2-5° for varying periods of time. Storage at this temperature in moist sand resulted in complete breaking of dormancy in 6 to 8 weeks. For small quantities (5-10g) of fruits, another technique was used. The fruits were placed in a petri dish on a disc of Whatman No. 1 filter paper, and an amount of distilled water was added that was equal to the weight of the fruits. The petri dish was then placed at 25° for 72 hours to obtain maximum imbibition, and then transferred to 2-5° for cold treatment.

Thiourea has been used to break dormancy in seeds of the peach (43), sugar maple (6), lettuce (41), Fraxinus

(50), and others (5, 27). Thiourea was tested for its effect on breaking dormancy in fruits of the native sunflower. They were allowed to imbibe for 2, 4, 8, 16, and 24 hours in the following 7 concentrations: 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, and 1.0 M. This gave a total of 35 different treatments.

Gibberellic acid[1] was also tested for its effect on breaking dormancy in fruits of the native sunflower. It had been successfully used in breaking the dormancy of potato seed (39), seed of some arctic circle plants (21), and others (27). Dry sunflower fruits were permitted to imbibe in and were continuously incubated in solutions of 0, 1, 10, 50, 100, 500, and 1000 mg per liter of GA. Each treatment consisted of 100 fruits in 2 ml of incubation solution and was run in triplicate.

Effects of high oxygen tension in breaking dormancy in these fruits were also checked. Concentrations of 20, 40, 60, 80, and 100% oxygen were used. The concentrations higher than in the normal atmosphere were prepared by adding the appropriate number of milliliters of water to a prescription bottle of known volume containing the fruits and a moistened piece of Whatman No. 1 filter paper. The water was then displaced by oxygen to give approximately the desired concentration. These experiments were set up in series of 5 replicates for each treatment.

---

[1]GA was supplied through the courtesy of Dr. Edwin F. Alder of Eli Lilly and Company, Greenfield, Indiana.

CHAPTER III

RESULTS

Inhibitor Studies

Effects of 1-ml aliquots of each of the 6-hour leachate collections on the germination of psyllium seed are shown in figure 2. All fractions collected during the first 36 hours of leaching showed inhibitory activity in these tests, while the later collections showed no activity. Germination of psyllium seed after 48 hours showed only slight inhibition in the 30 hour collection, indicating a low concentration of inhibitor(s) in that and subsequent collections. Germination inhibition was most pronounced in the leachate collections made during the first 18 hours. These data show the presence of a water soluble germination inhibitor in native sunflower fruits, and also show that it is readily leached.

This was confirmed by dilution studies (fig 3) in which a 10-fold dilution of the 6, 12, and 18 hour fractions showed considerably more inhibitory activity than the strongest assayed concentration (1:1) of the 30 hour fraction.

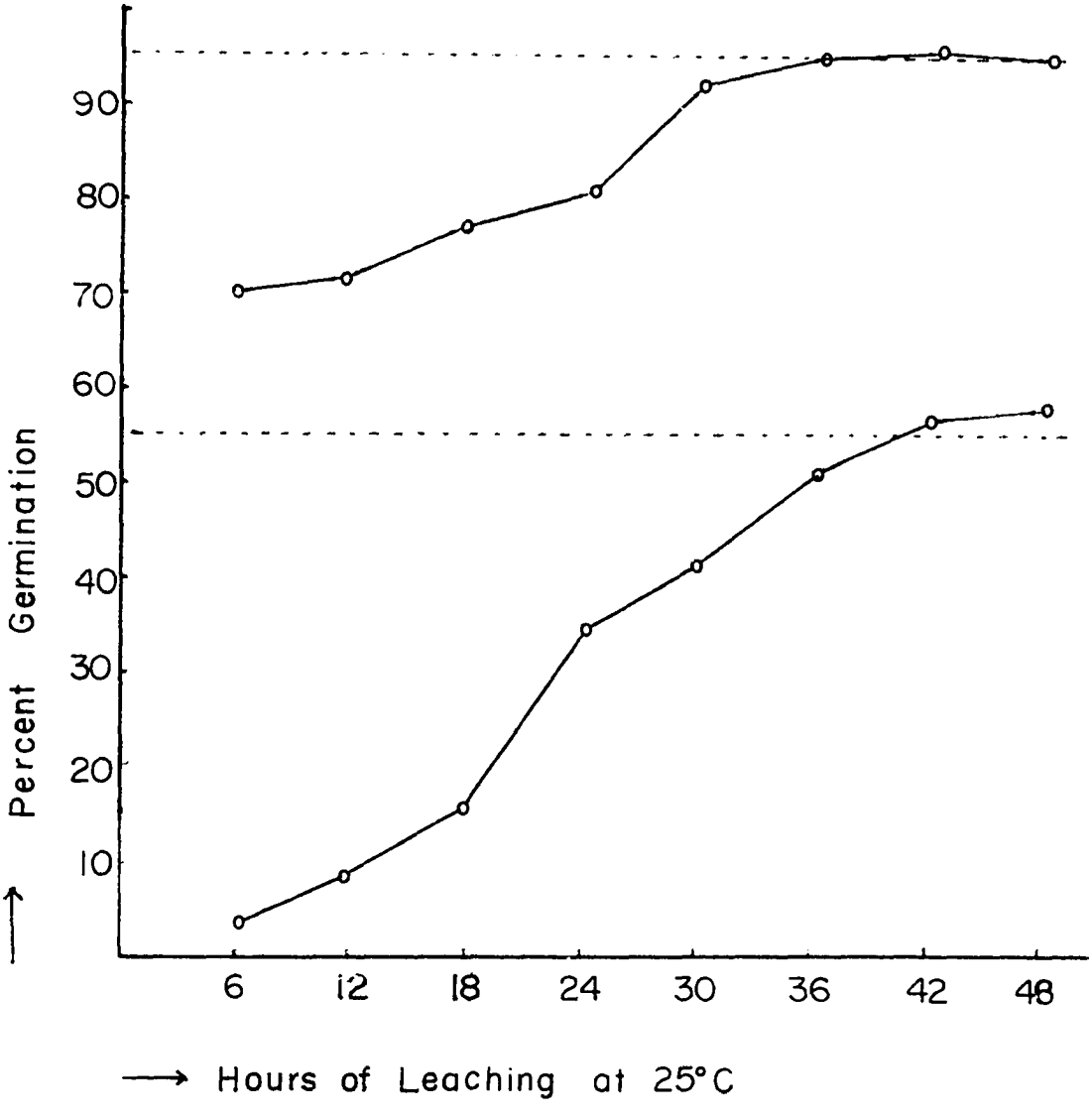

Fig. 2. Psyllium seed germination in 1:1 dilutions of leachates collected at 6-hour intervals from 50 gm of native sunflower fruits. The lower curve represents the number of seeds germinating in 24 hours; the upper curve, in 48 hours. Germination in the controls is shown by the broken line in each case.

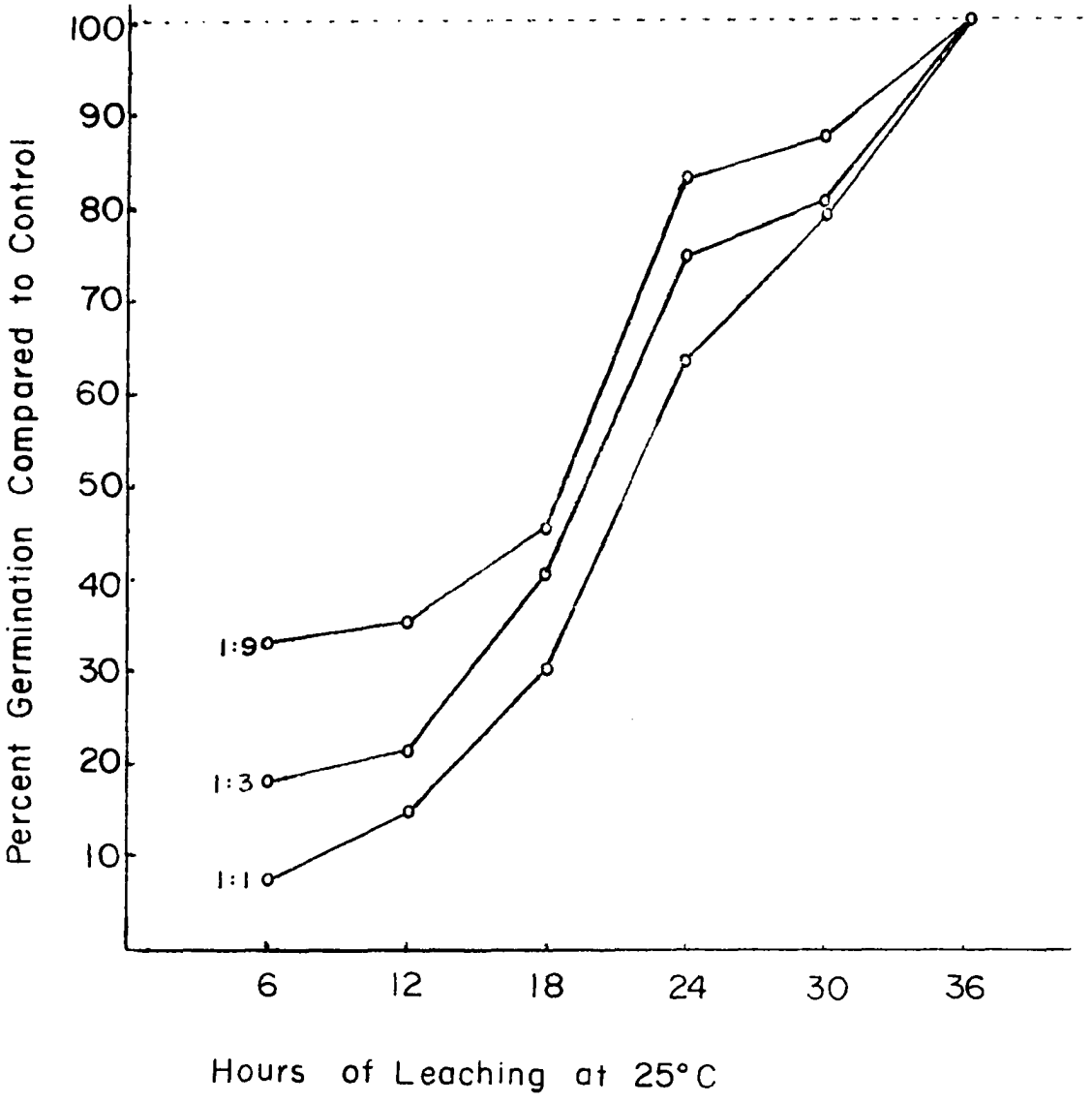

Fig. 3. Percentage germination of psyllium seeds in 1:1, 1:3, and 1:9 dilutions of leachates collected at 6-hour intervals from 50 g of native sunflower fruits. Values shown are based on the control as 100%, and represent germination after 48 hours of incubation.

The inhibitor was readily removed from the leachate by 4 hours of liquid-liquid extraction of the rotary evaporator concentrates using diethyl ether or ethyl acetate. Bioassay of the extract and the water residue showed that the inhibitor was present only in the extract. Extraction by shaking the acidified concentrate with diethyl ether was not as efficient as with the liquid-liquid extraction apparatus, but was used more frequently because less time was required.

Comparable levels of inhibition were obtained using extracts of equal weights of both the domestic and native varieties of sunflower. Psyllium seed germination was inhibited by water extracts of both the seed (embryo and membranous seed coat that was usually broken in excision) and the pericarp in both varieties, but was most inhibited by extracts of the pericarp (fig 4). These water extracts were obtained by incubating the separate fruit coats and embryos of 100 native sunflower fruits in phosphate buffer as described earlier. Seven Russian Mammoth fruits gave similar results when separated into embryos and coats and the water extract bioassayed.

Germination of hull-less oats, poppy, radish, and tomato seeds was inhibited by these leachates. After a few exploratory tests, psyllium seeds were used exclusively for bioassays.

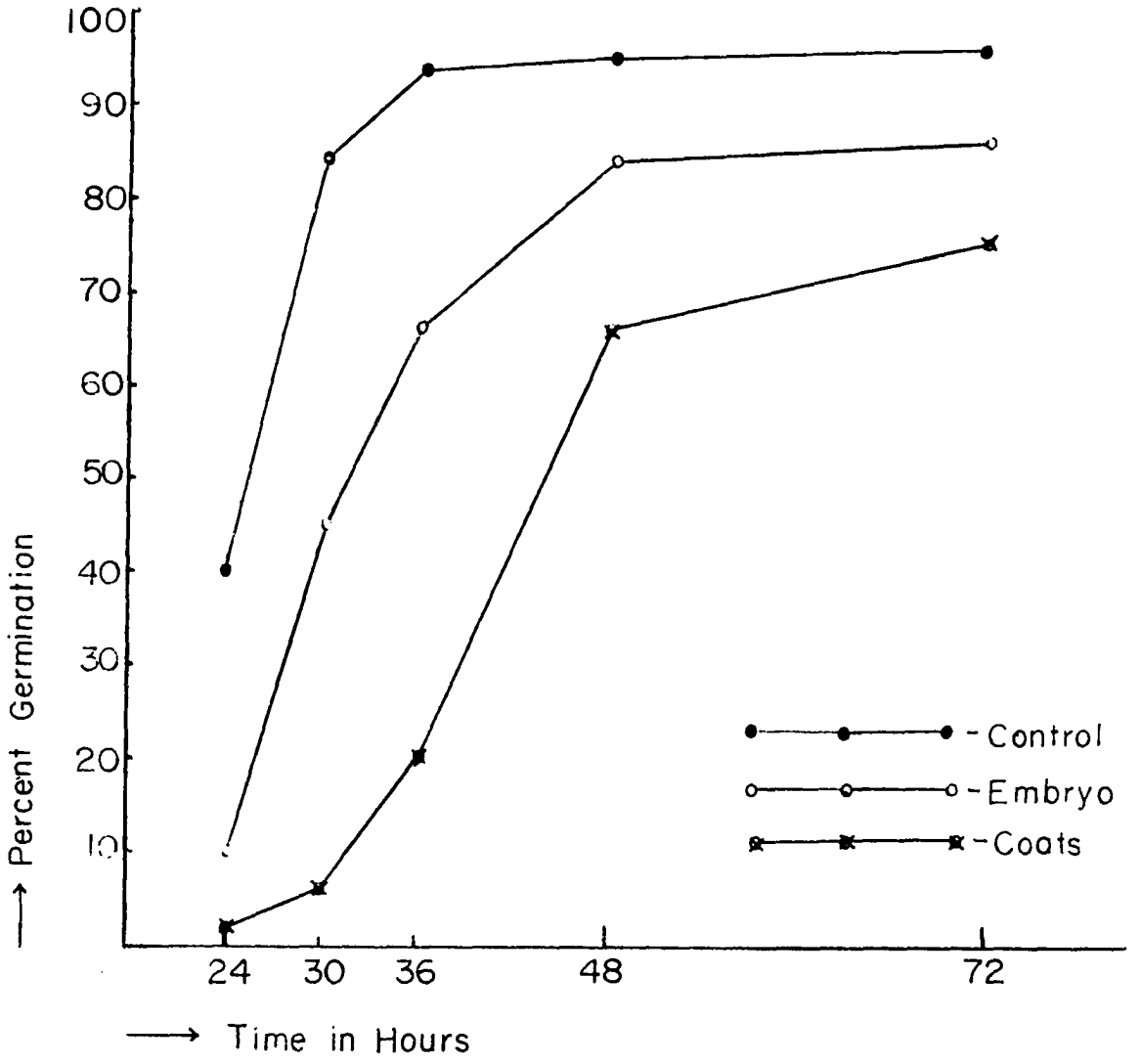

Fig. 4. Percent germination of psyllium seeds in water leachates obtained by incubation of 100 pericarps (coats) and 100 embryos in phosphate buffer for 48 hours. Phosphate buffer only was used in the control. Curve points are the means of 3 tests.

Presence of the inhibitor(s) from sunflower fruits retarded germination of all psyllium seeds and completely inhibited the germination of many of them, even when the amount present in the test dish was no more than that extractable from 0.25 g fresh weight of fruits. When these completely inhibited seeds were removed from the inhibitor solution after 6 days incubation, rinsed in distilled water, and placed in inhibitor-free phosphate buffer, germination began as usual and proceeded at a rate that was only slightly retarded. Thus inhibition of germination by the inhibitor(s) from sunflower fruits appears to be a reversible blocking of growth.

The inhibitor(s) in the leachate also was active against some molds and bacteria. Germination of psyllium seeds was inhibited 24 to 96 hours in low concentrations of the inhibitor; but deterioration of the seedlings, which usually started after 72 hours of incubation in the controls, was inhibited for 3 to 10 days in the presence of the inhibitor.

Fruits of the May 7, 1965, collection from greenhouse grown plants were compared with fruits collected on October 19, 1963, as to inhibitor content. The fresh fruits were collected before the head had dried sufficiently to allow the fruits to shatter from the heads and are called the non-matured fruits. The earlier collections were made after the

heads had dried, and the fruits would shatter when the heads were cut from the plant. These are called the matured fruits. Two-ml water leachates were prepared as described earlier from both the matured and non-matured fruits, using 10 embryos per dish and incubating them for 48 hours. Then the embryos were removed, and the leachate was bioassayed (fig 5). Psyllium seeds in all leachates were completely inhibited at 24 hours. At 36 hours, seeds in the leachate from the matured embryos showed only slight inhibition; and after 48 hours, no inhibition was present. After 36 hours, seeds in the leachate from the non-matured embryos showed strong inhibition that decreased at each examination but never equalled germination of the controls.

Additional leachates were prepared from the same embryos by transferring the embryos to fresh buffer and filter paper and incubating them for another 48-hour period. Leachates from matured embryos showed no further inhibition, but the leachates from the non-matured embryos continued to show a germination inhibitor in the leachates (fig 5, lower curve). Inhibitor production continued through fifteen 48-hour incubations of the same embryos. These embryos showed no signs of bacterial or mold contamination. Germination did not occur in the dark; but when transferred to light, 10% of the embryos showed some degree of germination (development of chlorophyll in isolated areas of the cotyledons and spreading

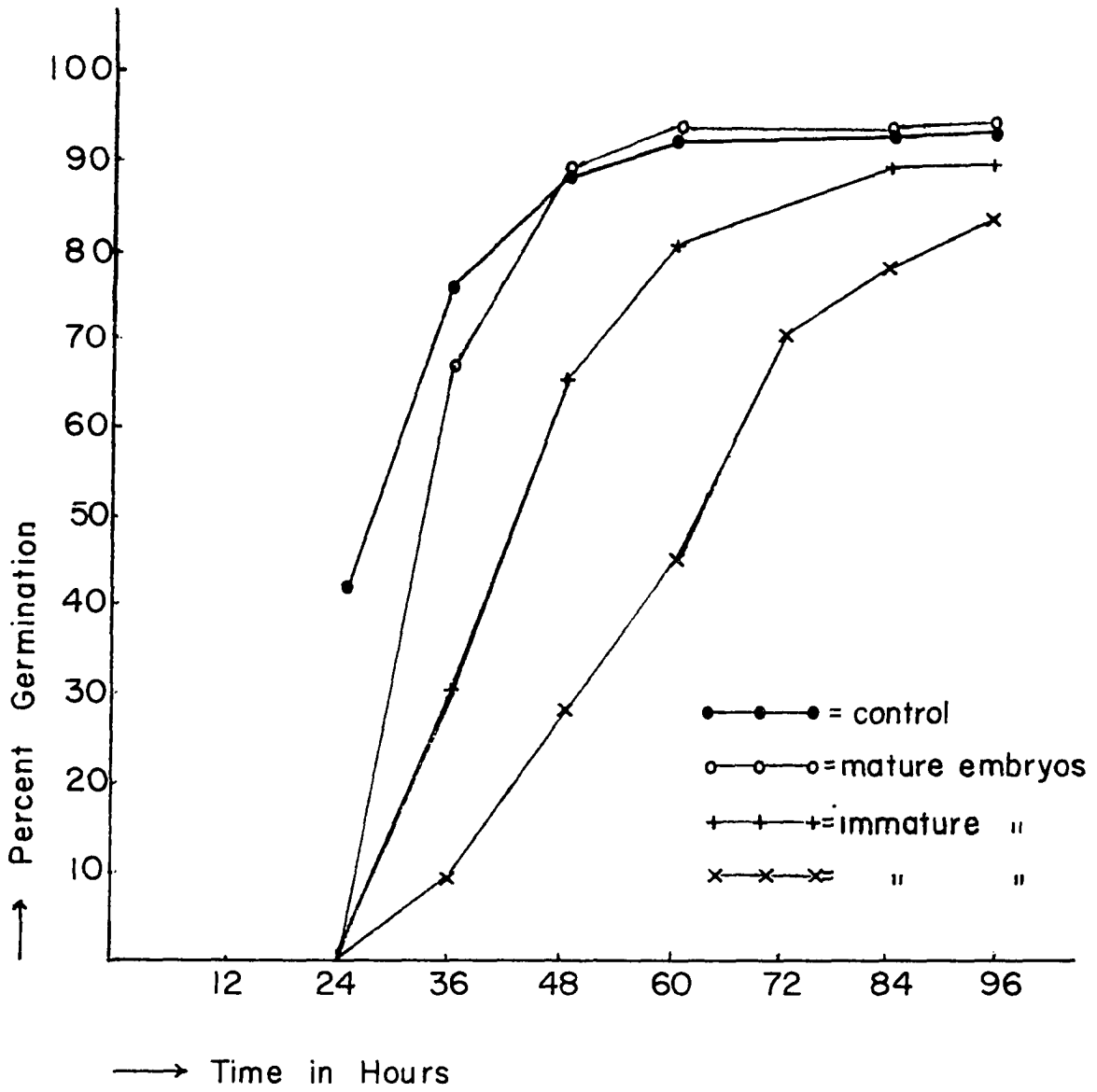

Fig. 5. Percent germination of psyllium seeds in extracts obtained by incubation of 10 matured and 10 non-matured embryos of native sunflower fruits in phosphate buffer for 48 hours in the case of the upper 2 curves. As a control, psyllium seeds were incubated in phosphate buffer alone. The lower curve shows germination of psyllium seeds in the third 48-hour extract from the same immature embryos. Each point is the mean of duplicate tests.

apart of the cotyledons, but no epicotyl of hypocotyl growth).

Leachates were similarly prepared from pericarps of 20 fruits from these 2 collections. The coats of the non-matured fruits were found to contain little inhibitor because germination of psyllium seed in these tests approximated that of the controls after the initial 24-hour reading. The coats of the matured fruits showed expected high levels of inhibitor (fig 6).

The inhibitor has been found to be soluble in water, diethyl ether, methanol, ethanol, isopropanol, and ethyl acetate, but insoluble in petroleum ether (bp 30-60°). It was extracted into diethyl ether at pH 2.8; thus it is probably acidic. It was found to be phenolic in nature by its reaction with the ferric chloride--potassium ferricyanide reagent and by its coupling reaction with diazotized reagents, including diazotized p-nitroaniline, the stable diazo salt of p-nitroaniline (Fast Red GG), and the stable diazo salt of 4-amino-2,5-dimethoxybenzonitrile (Fast Bordeaux BD). It does not fluoresce under either short (2537 A) or long (3660 A) wavelength UV light, either with or without ammonia vapor treatment. However, it does weakly absorb under short wavelength UV light.

Actual identification has not been made, but it is not a cyanogenic glycoside because all tests (1) for HCN were negative with extracts from both the native and domestic varieties of sunflower.

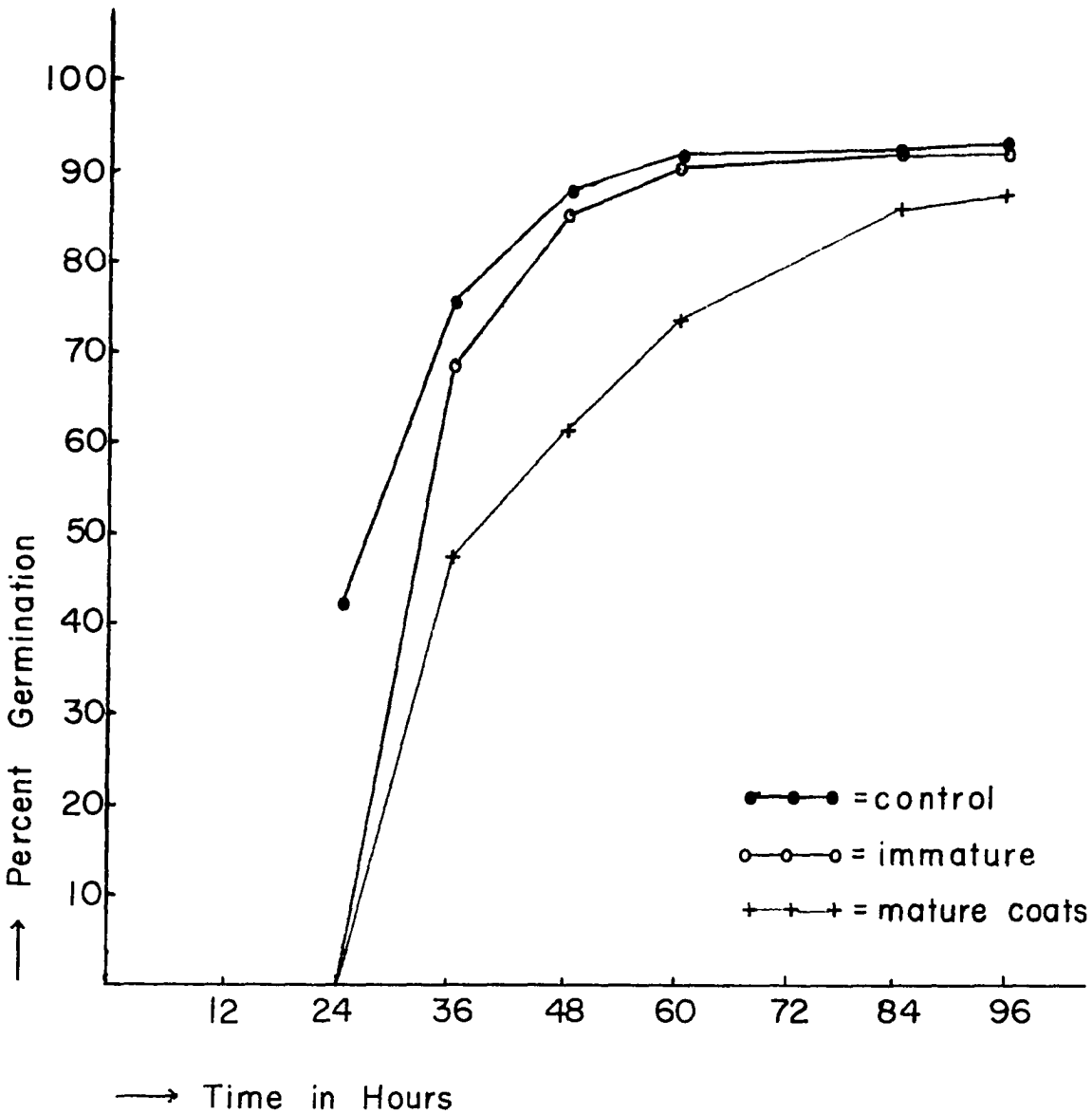

Fig. 6. Percent germination of psyllium seeds in extracts prepared by incubating the pericarps (coats) of 20 matured and 20 non-matured fruits of the native sunflower for 48 hours in phosphate buffer.

Germination Studies

Seeds in the leached fruits of the native sunflower were expected to germinate as a result of the loss of the inhibitor(s) during leaching. Germination tests at 25° resulted in poor germination with both leached and non-leached fruits. Germination in tests with leached fruits was usually about 0.25%, while that of the non-leached fruits was usually about 0.04%. In no case did germination exceed 3% in these tests.

Of the 35 thiourea treatments, 1 gave 3% germination, 8 gave 1% germination, and the remaining 26 treatments gave no germination. Germination that occurred was randomly distributed throughout the treatment series and showed no consistency.

During studies on the inhibitor in the embryos and pericarps, most of the excised embryos showed some development during incubation. This consisted of a slight geotropic curvature at the radicle apex. If these embryos were exposed to incandescent light, the cotyledons spread apart and developed chlorophyll. In a few embryos the hypocotyl slowly elongated, but epicotyl elongation did not occur. Table 1 shows the percentage distribution into size classes of seedlings developed from embryos of dormant, non-leached fruits and of fruits leached for 48 and 72 hours. The

Table I. Percentage distribution into size classes of seedlings showing arrested development following 96 hours growth.

Source of seedlings	Size classes		
	less than 1 cm	1-2 cm	more than 2 cm
Non-leached embryos	77	18	5
Leached for 48 hours	78	11	11
Leached for 72 hours	68	16	16

separation into size classes was made after 96 hours of development. At a similar age, 70% of the seedlings from fruits which had been cold treated for 4 weeks fell into the larger size class, and most of these had total lengths of 4 cm or more. None of the seedlings from embryos of dormant fruits (table I) exceeded 4 cm in length and 68% or more of them were in the smallest size class, indicating little development. However, these seedlings from the embryos of dormant fruits developed more normally following cold treatment at 2-5°. Decomposition by bacteria and molds began as early as the fifth day from the start of the test in the seedlings from leached fruits, but seedlings obtained from non-leached fruits did not begin deterioration until several days later.

The fact that there was some development of the embryo during incubation following excision from the fruit suggested lack of permeability in the fruit coat to either oxygen or water. Slight stimulation of germination was obtained with concentrations of oxygen above 60%; but germination was variable, and the slight differences did not prove significant.

Imbibition studies (fig 7) showed that fruits with split pericarps reach maximum inbibition after 36 hours, after which time a few seeds germinated. Maximum imbibition resulted in about a 40% increase over air-dry weight. Fruits with intact pericarps imbibed more slowly, but after 72 hours, they reached the same percent weight increase after

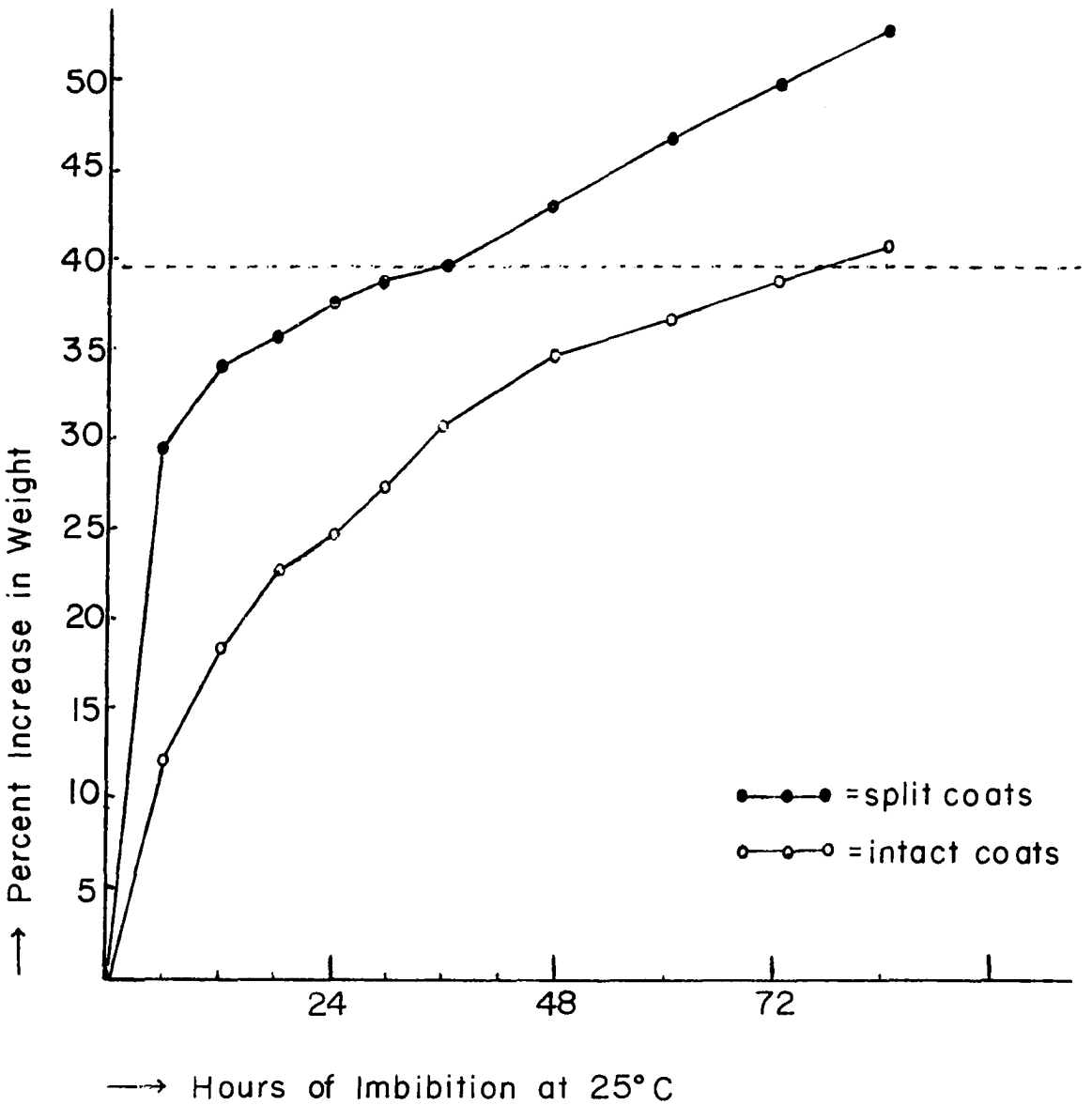

Fig. 7. Percent increase in weight during imbibition of sunflower fruits having split and intact pericarps (fruit coats).

which a few seeds germinated. Weight increases above the 40% level were due to germination and growth of the seedlings.

Figure 8 shows the effects of increasing concentrations of GA on germination in dormant sunflower fruits after 18 days incubation in the GA solution. GA treatment apparently stimulated germination at all concentrations used, but the stimulation attributed to 1 and 10 mg per liter of GA did not prove significant. Growth rates were not determined for seedlings obtained through GA treatments, but the seedlings did not show retarded growth like that in seedlings obtained from excised embryos.

Alternating temperatures between 15 and 25° stimulated germination in about 20% of the fruits of the native sunflower. After imbibition for 72 hours, one diurnal alternation stimulated germination in a few of the fruits. Six to 8 diurnal alternations were sufficient to give the maximum of 20% germination. Additional alternations gave no further increases in germination.

Effects of increasing lengths of cold treatment at 2-5° on breaking dormancy in fruits of the native sunflower are shown in figure 9. Following imbibition for 72 hours at 25°, all cold treatments used gave significant stimulation of germination. After 4 to 6 weeks of cold treatment, many seeds will germinate at 2° and show both root and hypocotyl elongation. After 6 weeks cold treatment, germination is

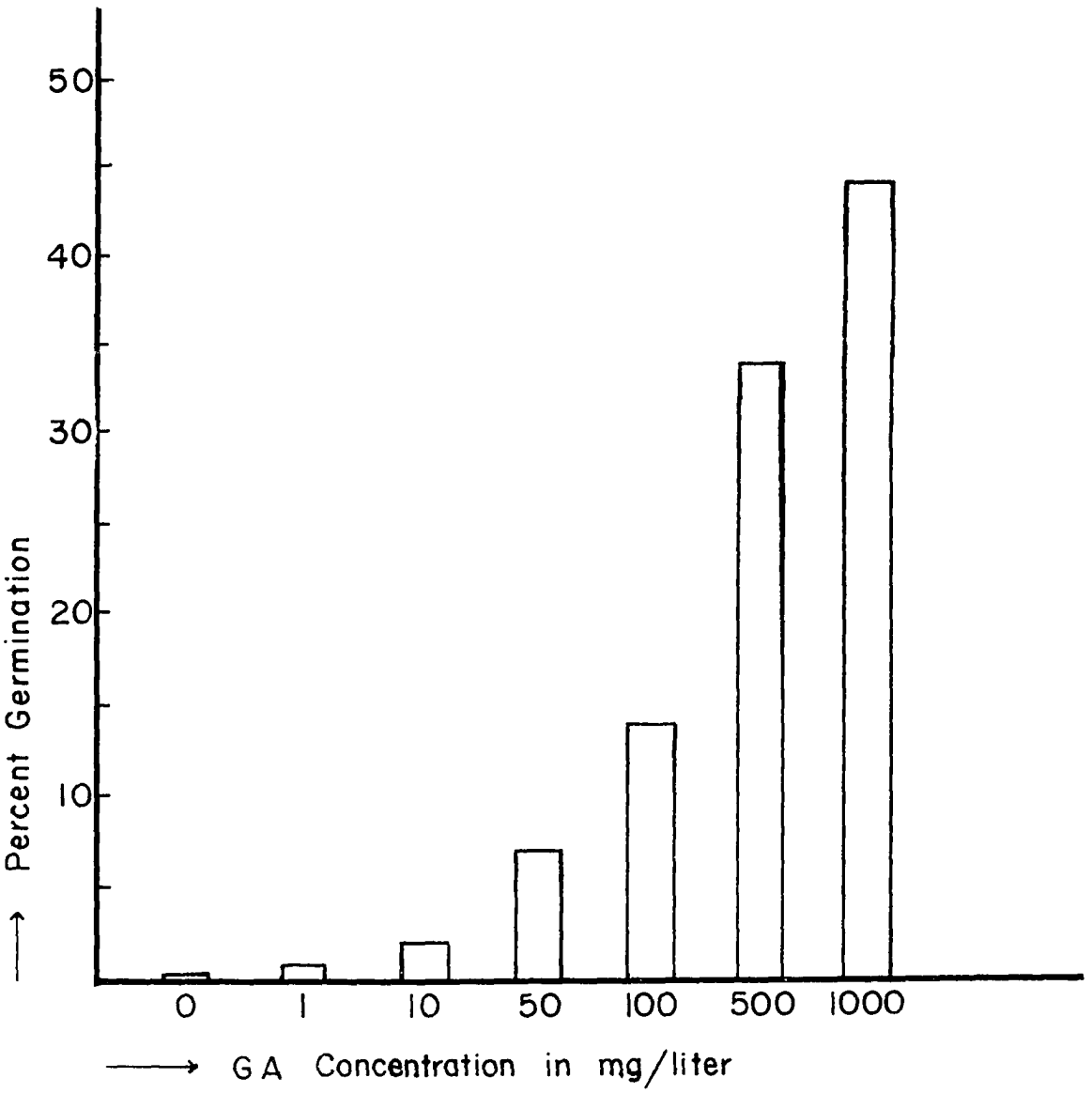

Fig. 8. Effect of incubation in gibberellic acid solutions on germination in dormant sunflower fruits. Data presented are the means of 3 replicates.

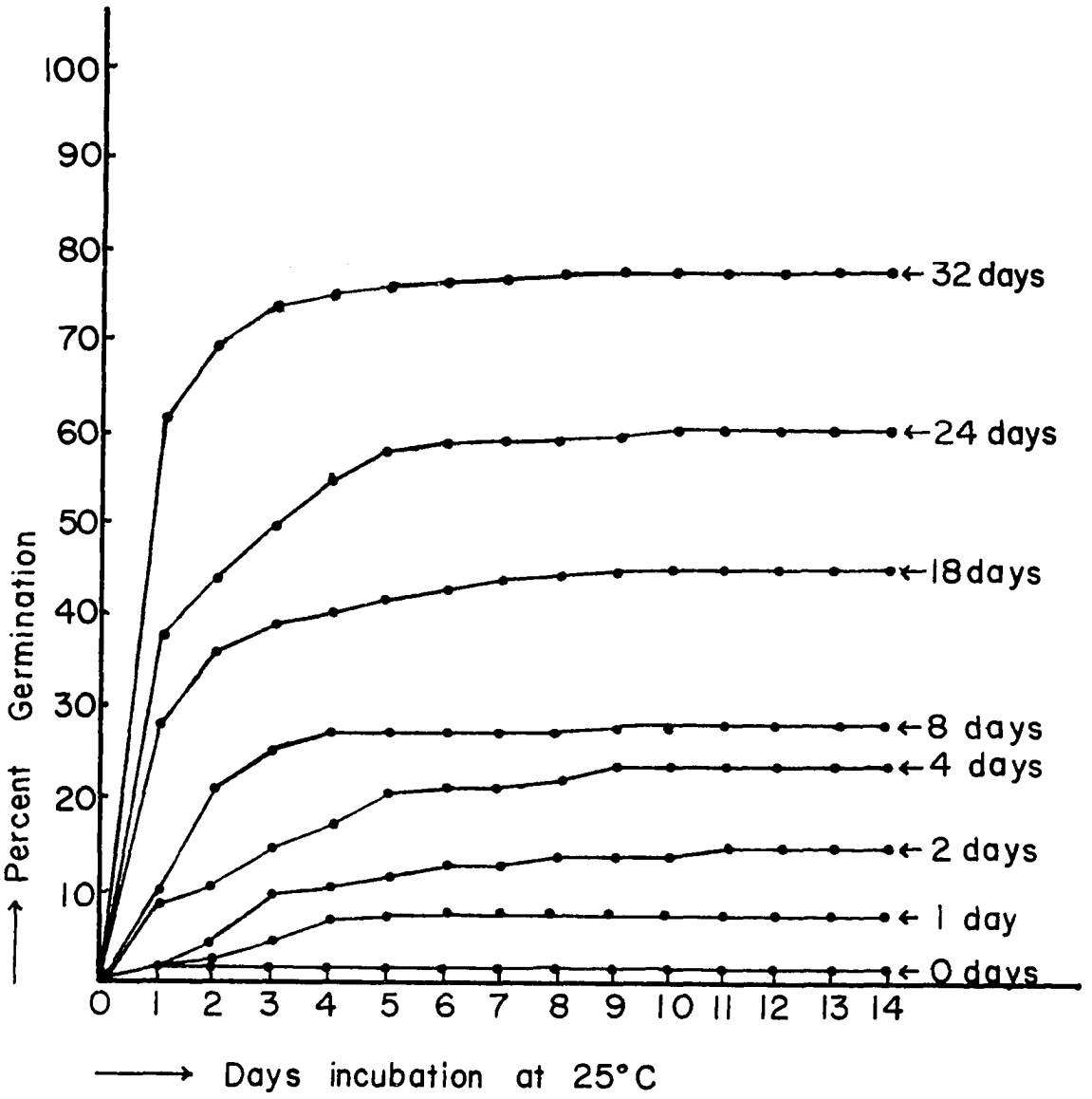

Fig. 9. Effect of stratification at 2-5° for 0 to 32 days on subsequent germination in the fruits of the native sunflower. Data presented are means of three determinations.

essentially complete following transfer to temperatures favorable for germination. Cold treatment was used to obtain all lots of non-dormant native sunflower fruits used for chromatography studies.

Changes in the inhibitor content of the fruits during stratification were followed using the psyllium seed bioassay on 2-ml water leachates of 100 fruits. Cold treatment of dormant fruits (fig 10) apparently increased the total inhibitor content of the water leachates. At the same time there was an increase in the acidity of the water and 95% ethanol extracts from stratified fruits. The reaction dropped from pH 7.6 in water solutions of 95% ethanol extracts of imbibed fruits to pH 6.6 in identically prepared extracts of fruits stratified for 16 days. This increase in acidity was reflected in an increase in the acidic ether fraction and in the presence of many more bands of fluorescent materials on chromatograms prepared from these extracts.

Soxhlet extracts were prepared from fruits of the native sunflower that had been stratified for 60 days. The acidic fraction was removed as previously described. Aliquots equivalent to the extract from 1 g fresh weight of fruits were streaked on paper and developed in IAW. The inhibitor was readily located on these chromatograms (fig 11) as shown by psyllium bioassay of one-fourth (longitudinal section) of one of these chromatograms. Values shown are the means of four determinations. Major inhibition after

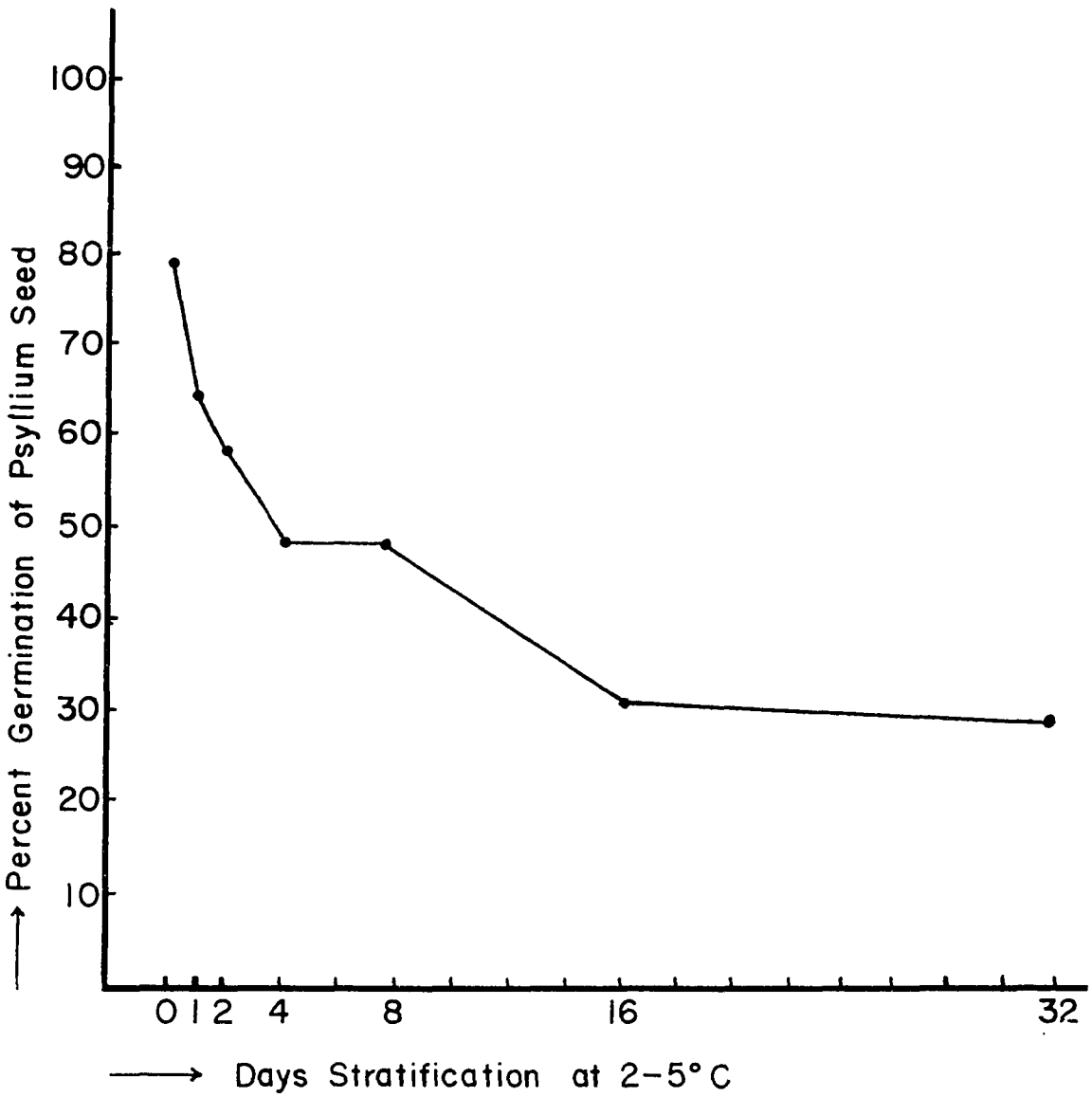

Fig. 10. Psyllium seed germination after 48 hours of incubation in the water leachate (2 ml) obtained from 100 sunflower fruits stratified for 32 days.

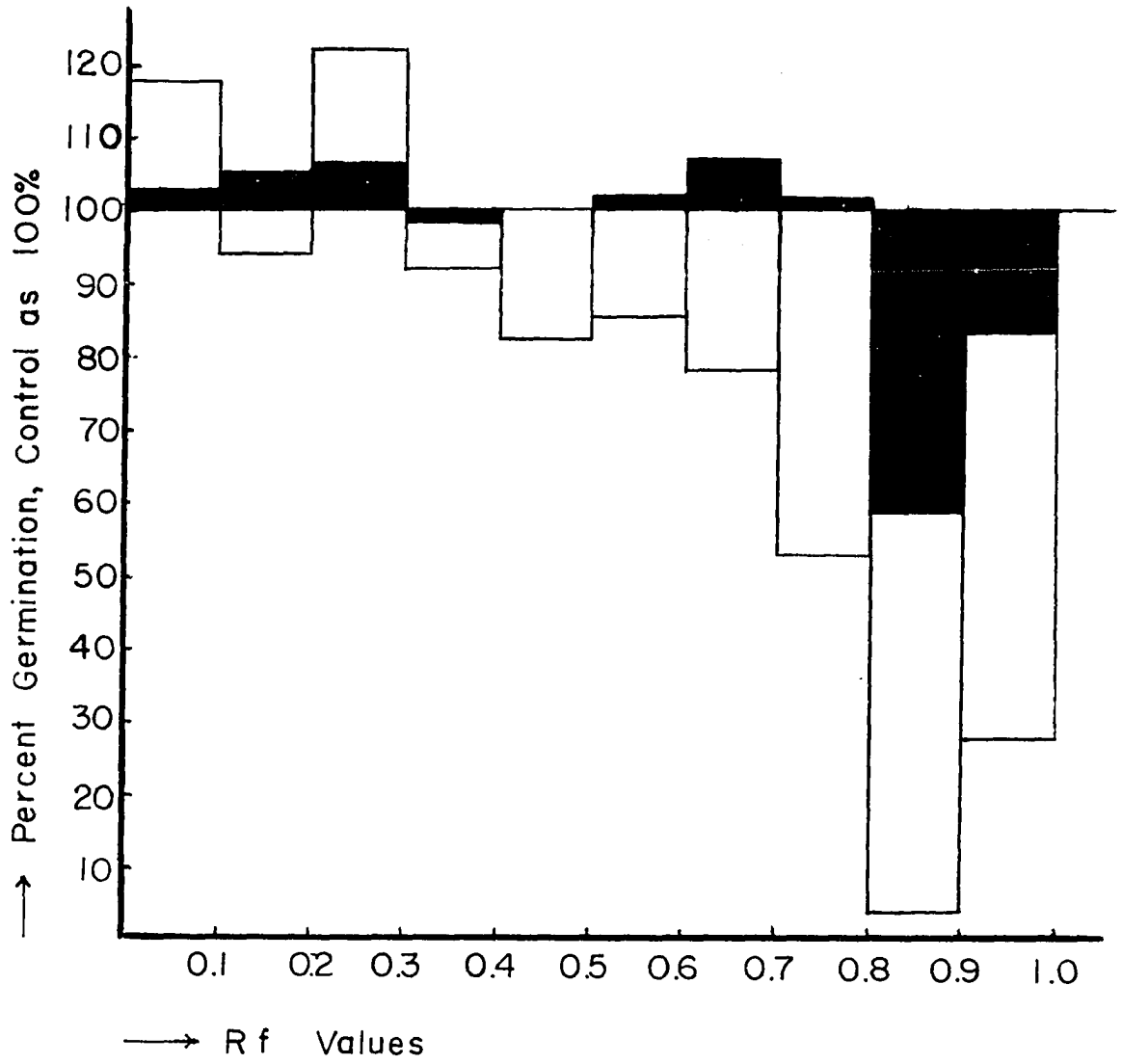

Fig. 11. Effects on psyllium seed germination produced by different Rf zones from chromatograms of the acidic fractions of extracts from native sunflower fruits stratified for 60 days. This histogram presents the mean percent germination in four tests as compared to the control, at the end of 24 hours (enclosed white) and 48 hours (black).

24 and 48 hours was found between Rf's 0.8-0.9 with lesser inhibition at 24 hours found between Rf's 0.7-0.8 and 0.9-1.0. The inhibition between Rf's 0.7-0.8 was possibly due to tailing from the inhibitor in the Rf 0.8-0.9 zone. The inhibition between Rf's 0.9-1.0 probably was not due to inhibitors in the chromatographed extract. A blank strip of S&S 2043-b chromatography paper was developed simultaneously with those carrying the extracts. This blank chromatogram was sectioned longitudinally into 4 parts. These 4 strips were then sectioned into 0.1 Rf units and germination tests made on these sections. The same level of germination inhibition was produced by the Rf 0.9-1.0 zone of this blank chromatogram (fig 12) as was produced by the same zone on chromatograms prepared from plant extracts (fig 11). The inhibition in this zone (Rf 0.9-1.0) seems to be due to impurities in the chromatography paper.

Inhibition due to the Rf 0.9-1.0 zone was associated with a light blue-white to cream-white fluorescent material that could be removed by pre-development of the paper by descending means and allowing the wash solvent to drip off the paper. Wash solvents used were 2% acetic acid and IAW (80:5:15 v/v/v). When the developing solvents resulted in overlapping of the impurities in the paper with the materials of interest in the chromatographed plant extracts, it was essential to wash the chromatography paper prior to its use. When developing solvents were used that left the plant materials

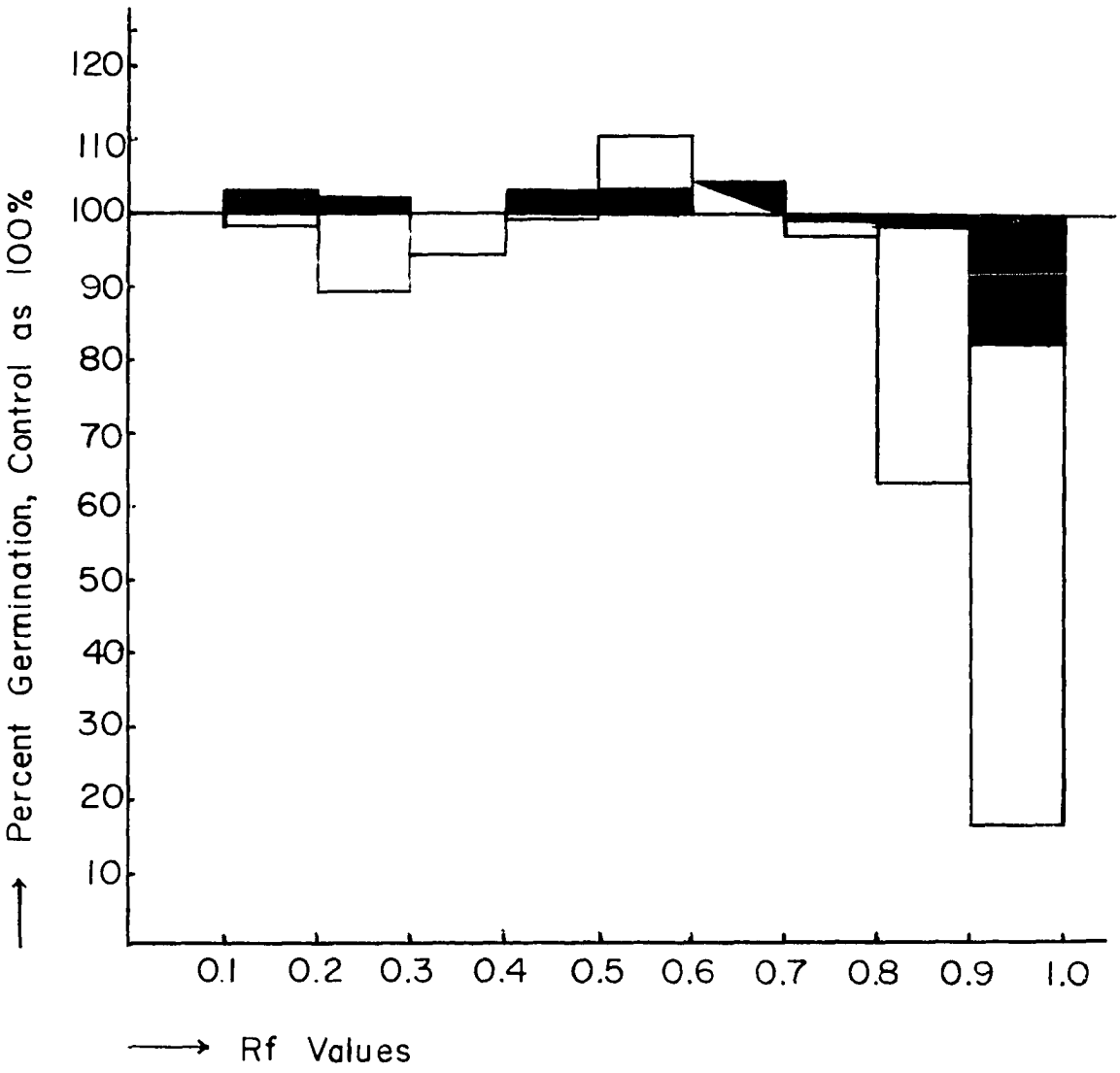

Fig. 12. Effects on germination of psyllium seeds produced by different zones of blank chromatograms developed simultaneously with the chromatograms used in the bioassay reported in figure 11. Values reported are the mean percent germination of four tests as compared to that of the control at the end of 24 hours (enclosed white) and 48 hours (black).

of interest widely separated from the front, no prior washing of the papers was necessary, and the paper impurities were ignored.

Stimulation of psyllium seed germination after 24 hours incubation was evident between Rf's 0.2-0.3 on the chromatograms represented by figure 11. On other chromatograms developed in the same solvent (IAW), the zone of stimulation appeared as high as Rf 0.45. It is not known whether only 1 or several substances are present in this zone. This zone shows no reaction with phenolic reagents.

Studies on the Isolation and Identification of Components of the Extracts

Chromatograms were prepared from the extracts of cold treated fruits previously used (fig 11), and were developed in IAW. Two distinctive bands were present on these chromatograms. Both of these zones fluoresced blue with both long and short wavelength UV light, fluoresced a brilliant yellow-green when exposed to ammonia vapors, and gave a dark blue color with the ferric chloride-potassium ferricyanide reagent for phenols. These data suggested that the constituents of the two bands were isomers of chlorogenic acid.

These bands (referred to as band "A" and band "B") were cut from the chromatograms and separately extracted from the paper. The extracts were reduced to dryness, taken up in absolute methanol, and spotted on other paper strips for further chromatography in BAW, IAW, and 6% acetic acid.

Table II. Comparison of known reference compounds with two compounds isolated from extracts of cold treated sunflower fruits.

Compound	Rf values			Fluorescence (UV)		Reactions with standard reagents		
	IAW	6% Acetic Acid	BAW	Without NH_3 vapor	With NH_3 vapor	Hoepfner's	$FeCl_3$-$K_3Fe(CN)_6$	Fast Bordeaux BD
Chlorogenic acid	0.56	0.65,0.78	0.60	lt blue	bril YG	+ (red-pink)	dk blue	bril Y
Band "A"	0.54	0.59,--	0.59	lt blue	bril YG	+ (red-pink)	dk blue	bril Y
Band "B"	0.70	0.21,0.30	0.73	lt blue	bril YG	+ (red-pink)	dk blue	bril Y
Isochlorogenic acid	0.70	0.17,0.33	0.73	lt blue	bril YG	+ (red-pink)	dk blue	bril Y

Abbreviations used in the table are: bril - brilliant, dk - dark, lt - light, Y - yellow, YG - yellow-green

Chlorogenic and isochlorogenic[1] acids were spotted on these same papers and developed in these solvents as reference standards. The two unknown substances from the cold treated fruits gave Rf values comparable to those of the reference compounds (table II), and both reacted positively to Hoepfner's reagent, which was reported to be specific for the isomers of chlorogenic acid (33). Thus band "A" and band "B" are apparently the same as the commercial preparations of chlorogenic and isochlorogenic acids, respectively. Chlorogenic acid has been known to be 3-O-caffeoyl-D-quinic acid for several years. Recently, isochlorogenic acid was shown to be a mixture of 2 (16) or 3 (36) dicaffeoyl depsides of Quinic acid. One of these has been identified tentatively as 3,5-O-dicaffeoyl-D-quinic acid (16).

The identities of these 2 bands with commercial chlorogenic and isochlorogenic acids were further confirmed by examination of the products of their alkaline hydrolysis. The ether soluble and ether insoluble fractions of the acidified hydrolysates were spotted on chromatography paper along with caffeic and D-quinic acids as reference compounds. The chromatograms were developed in IAW and 7% acetic acid, and gave good agreement between the Rf's of the hydrolysis products and those of caffeic and quinic acid (table III).

---

[1]The known isochlorogenic acid used in the early studies was supplied through the courtesy of Dr. E. L. Rice of the University of Oklahoma. The isochlorogenic acid used in later studies was a commercial preparation from Mann.

Table III. Comparison of reference compounds with the hydrolysis products of chlorogenic acid (band "A") and isochlorogenic acid (band "B") isolated from sunflower fruits.

Compound	Rf values on S&S 2043-b		UV Fluorescence		Quinic acid test
	IAW	6% Acetic	Without NH_3	With NH_3	
Caffeic acid	0.82	0.35	blue	lt blue	
Quinic acid	0.30	0.96	--	--	yellow
Hydrolysis products					
Acidic fraction of "A" (ether solubles)	0.80	0.34	blue	lt blue	
Water fraction of "A" (ether insolubles)	0.28	0.96	--	--	yellow
Acidic fraction of "B" (ether solubles)	0.84	0.34	blue	lt blue	
Water fraction of "B" (ether insolubles)	0.30	0.96	--	--	yellow

Chromatography of chlorogenic acid and other similar substances in predominantly aqueous acidic solvents separates the cis and trans forms of these compounds (38). When chromatographed in 6% acetic acid, chlorogenic acid in the plant extracts produced only 1 band which coincided with the band having the lower Rf (trans form) from reference chlorogenic acid. However, isochlorogenic acid produced 2 distinct bands when chromatographed in this solvent systems. These 2 bands coincided with those produced by the reference isochlorogenic acid.

While chlorogenic and isochlorogenic acids were both present in the extracts from stratified fruits of the native sunflower, neither could be detected on chromatograms prepared from equivalent quantities of extracts from dry fruits. The time and sequence of appearance of these acids during the breaking of dormancy was studied in a series of extracts from the fruits. The fruits were extracted with 95% ethanol in the Soxhlet apparatus. They were extracted dry; after 24, 48, and 72 hours of imbibition; and after 1, 2, 4, 8, 16, and 32 days of stratification at 2-5° following initial imbibition for 72 hours at 25°. Diagrams of the chromatograms prepared from these extracts (IAW solvent) are shown in figure 13a,b. The fluorescent color of each band is indicated on the diagram of the chromatogram on which it first appeared. Arrows connect equivalent bands on the diagrams.

Chlorogenic acid appeared first. It was not found

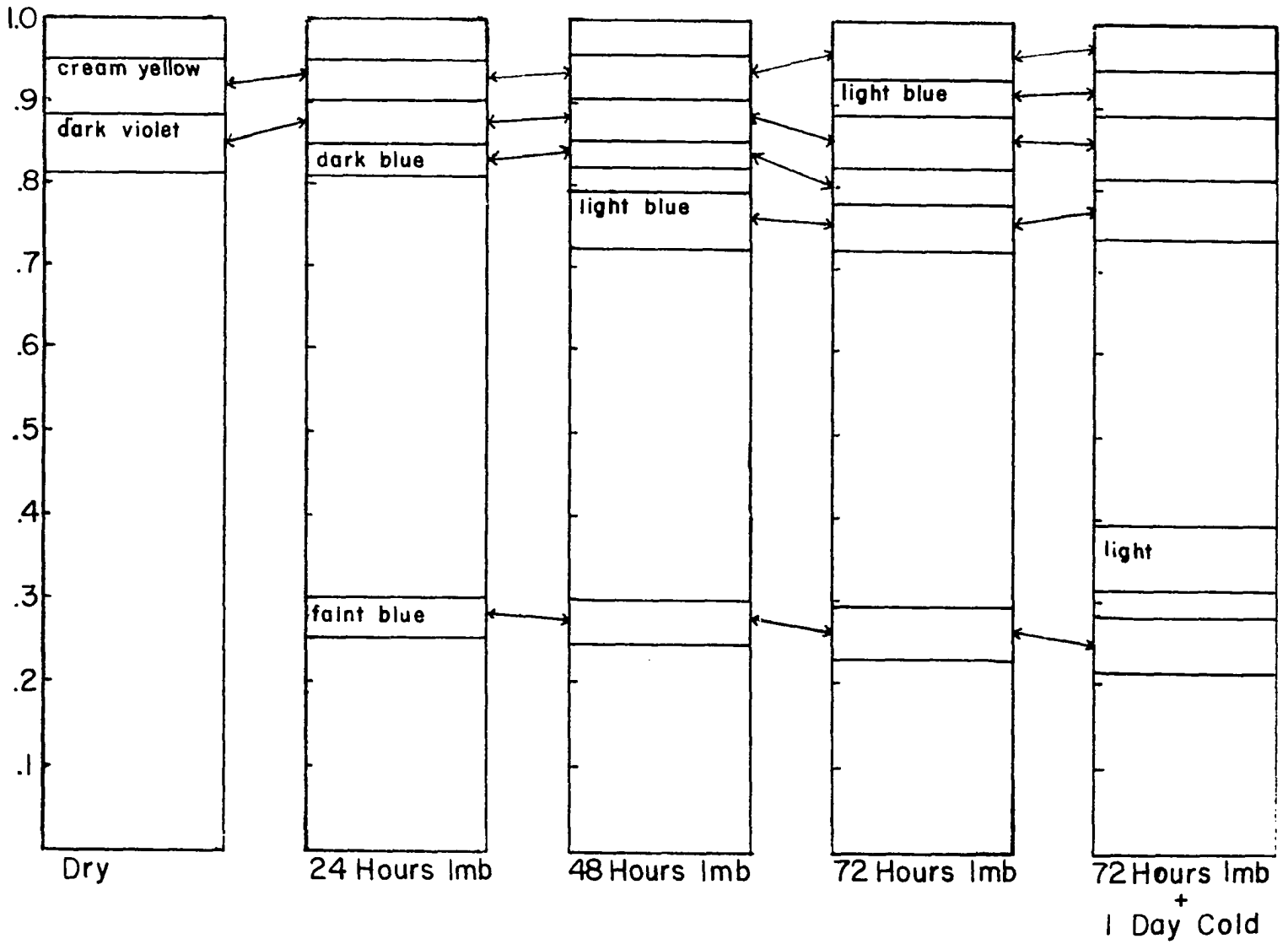

Fig. 13a. Diagrams of chromatograms prepared from extracts of wild sunflower fruits. Chromatograms were developed in IAW. Imb indicates imbibition.

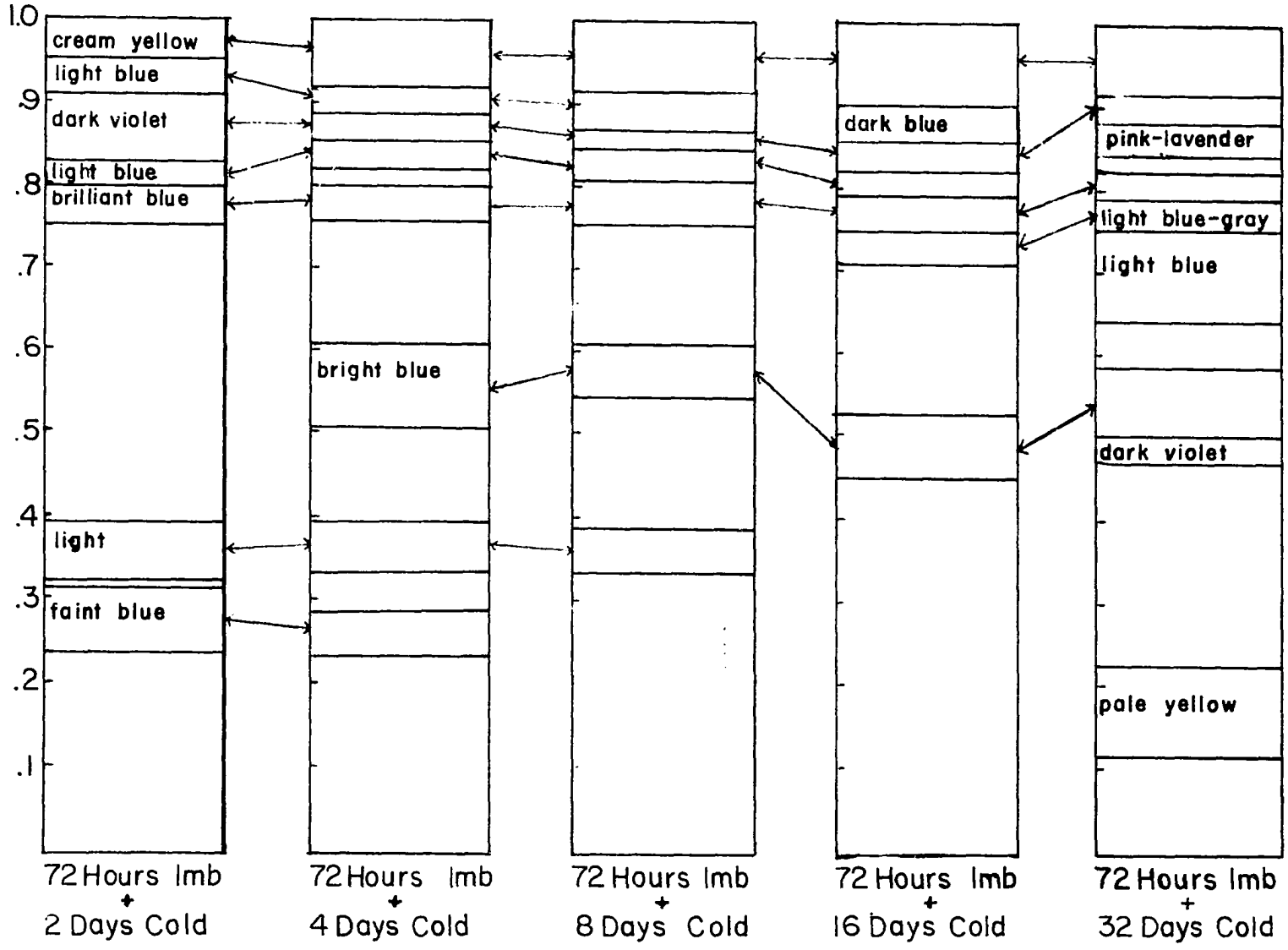
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
cream yellow
light blue
dark violet
light blue
brilliant blue
light
faint blue
bright blue
dark blue
pink-lavender
light blue-gray
light blue
dark violet
pale yellow
72 Hours Imb + 2 Days Cold
72 Hours Imb + 4 Days Cold
72 Hours Imb + 8 Days Cold
72 Hours Imb + 16 Days Cold
72 Hours Imb + 32 Days Cold

Fig. 13b.

in extracts of dry fruits, nor in extracts of fruits at different imbibition stages, but appeared between 48 and 96 hours after beginning stratification. It is identified in figure 13b as the bright blue band between Rf's 0.51 and 0.61. Isochlorogenic acid appeared between 16 and 32 days after beginning stratification. It is represented as the light blue band between Rf's 0.63 and 0.76 on the diagram of the chromatogram prepared from extracts of fruits receiving 32 days stratification. After it appears, isochlorogenic acid apparently becomes the predominant isomer in the non-dormant (stratified) fruits. The germination inhibitor present in these fruits is represented as the dark violet band between Rf's 0.8 and 0.9 on these diagrams. It is widely separated from either chlorogenic or isochlorogenic acid on chromatograms developed with IAW.

Fruits of the domestic variety "Russian Mammoth" were also examined for the presence of chlorogenic and isochlorogenic acids. Soxhlet extracts (95% ethanol) were made from dry fruits, and from fruits which had imbibed for 24, 48, and 96 hours. Diagrams of chromatograms prepared from these extracts are shown in figure 14. These chromatograms were run in IAW. Chlorogenic acid again was detected first, appearing during the first 24 hours of imbibition in these non-dormant fruits. Isochlorogenic acid appeared between 24 and 48 hours after beginning imbibition. From the Rf values, width, and intensity of the fluorescence of the

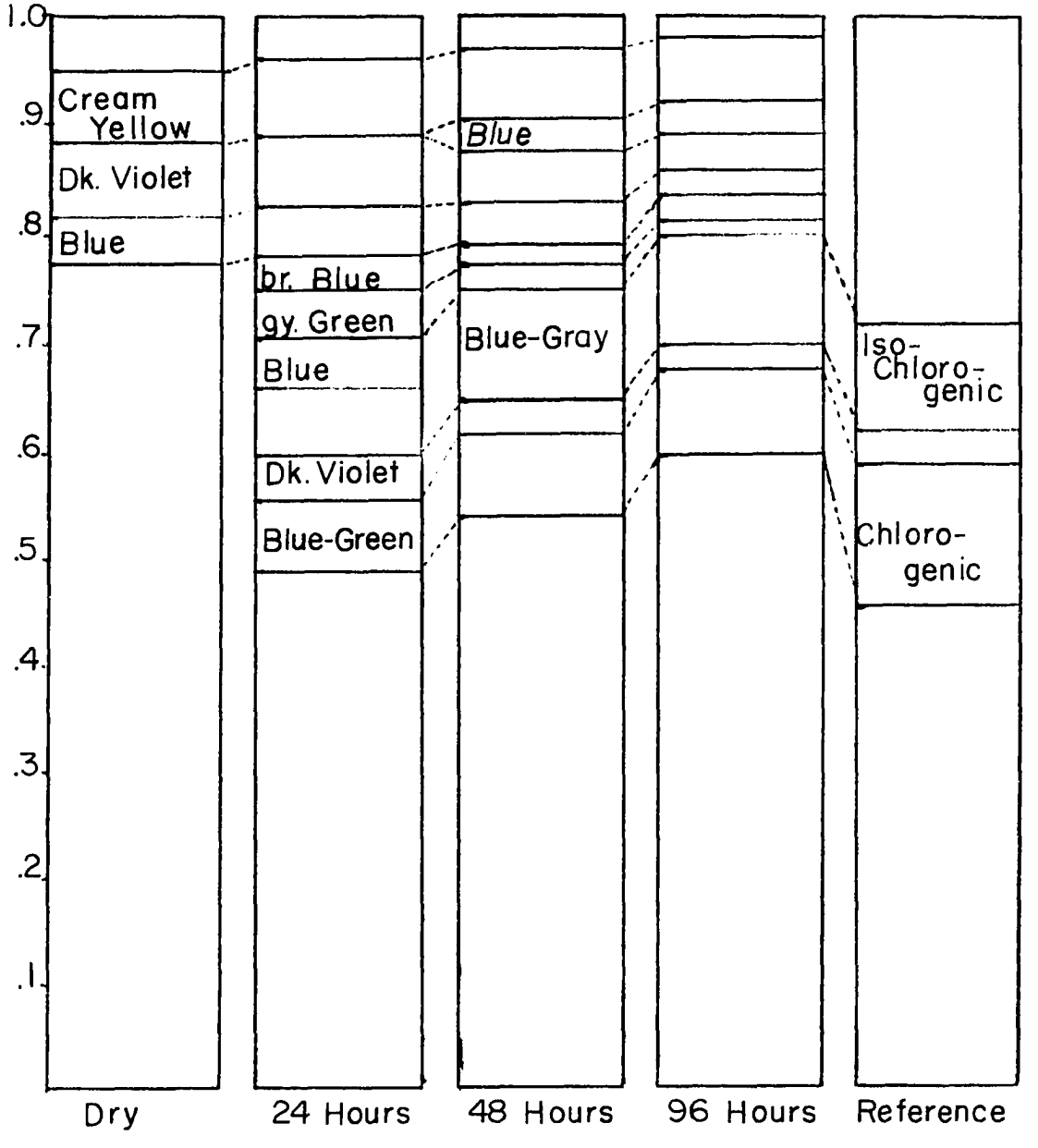

Fig. 14. Diagrams of chromatograms prepared from the acidic fraction of extracts of the domestic sunflower fruits. Chlorogenic and isochlorogenic acid are indicated on each chromatogram on which they appear.

bands of chlorogenic and isochlorogenic acid on chromatograms prepared from extracts of fruits imbibed 96 hours, isochlorogenic acid became the predominant isomer in the domestic sunflower also.

A material which reacts with Ehrlich's Reagent (indole test) appeared in the fruits of the native sunflower during cold treatment, and in the fruits of the domestic variety during imbibition. The Ehrlich positive material showed the same Rf in IAW (0.88) and BAW (0.90) as did IAA but reacted in this test more slowly than did IAA.

Almost all of the fluorescent bands present on the chromatograms diagrammed in figures 13 and 14 reacted with the ferric chloride-potassium ferricyanide reagent and with diazotized reagents, indicating that they are phenolic compounds. The presence of phenolic compounds other than chlorogenic acid has been reported in the stem, leaves, or roots of normal sunflower plants. These include caffeic acid (fig 15-C) (22, 44); p-hydroxybenzoic acid, p-coumaric acid (fig 15-D), ferulic acid (fig 15-G), and salicylic acid (22); neochlorogenic acid (53); scopolin (7, 52, 44); and isochlorogenic and 3-O-feruloyl-D-quinic acids (Dr. A. Zane and Dr. S. H. Wender- personal communication). Only chlorogenic and isochlorogenic acids have been definitely identified in this study.

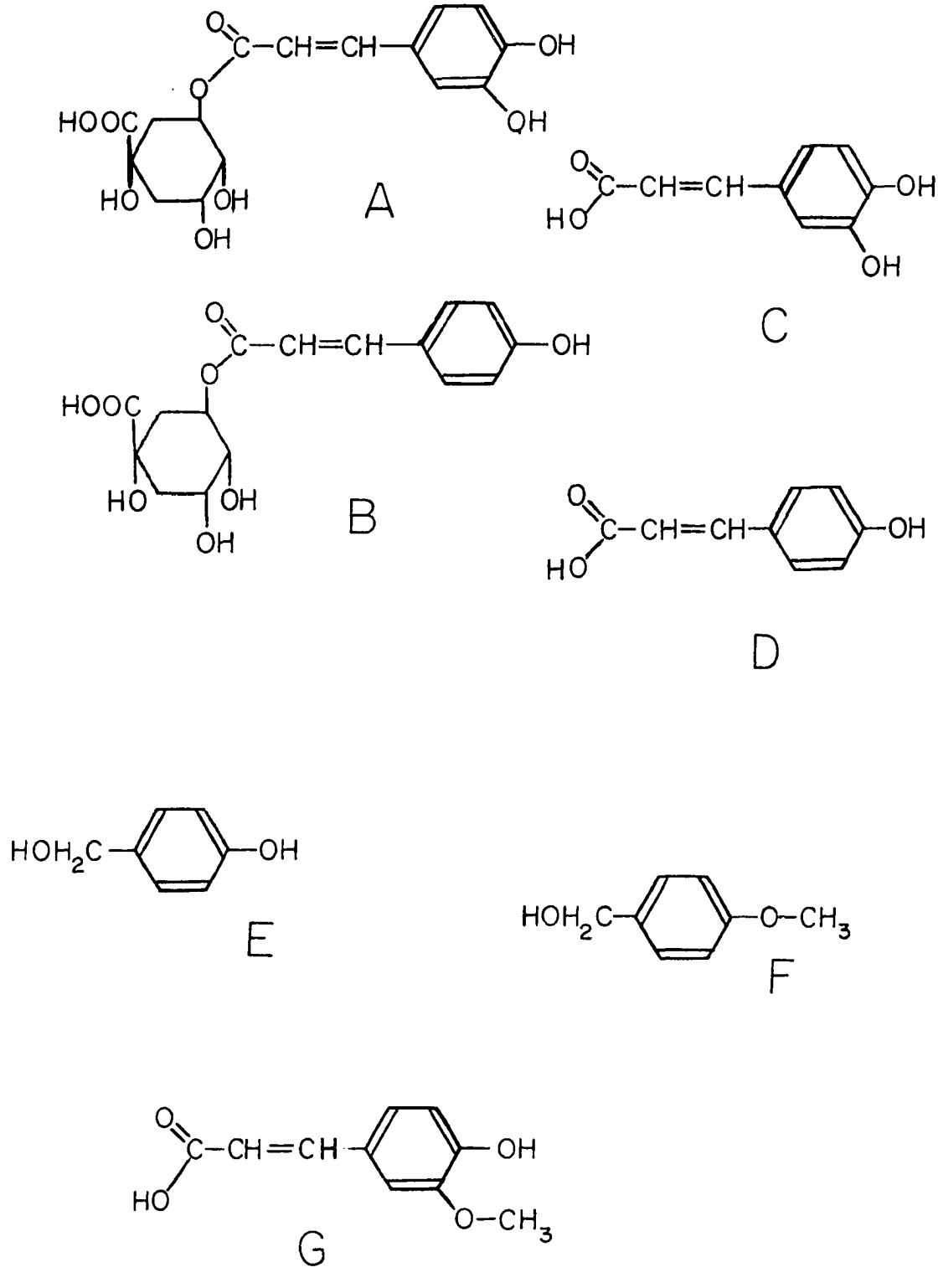

Fig. 15. Some important phenolic compounds and derivatives of interest in this study.

CHAPTER IV

DISCUSSION

Ruge (35) reported the presence of a germination inhibiting and a germination stimulating material in a black seeded variety of Helianthus annuus L. Identifications were not made but the compounds were proposed to be cyanogenic glycosides and thiocyanates, respectively. Köves and Varga (22) isolated inhibitors from the pericarps of sunflower fruits (no variety specified) and identified them as phenolic acids. The present study confirms the presence of germination inhibiting substances in the dry fruits of both the domestic variety "Russian Mammoth" and the native variety of Helianthus annuus L. The inhibitors (one or more) were not found to be cyanogenic glycosides, but were phenolic in nature. The specific identity of these substances was not determined, but chromatographic studies showed that the inhibitors were apparently the same in the two varieties.

The inhibitor content of dormant potato tubers (18), dormant buds (2, 19), and dormant apple seeds (26) has been reported to decline at the termination of dormancy or during stratification of the seeds. In Fraxinus excelsior L.

(50) the inhibitor content did not decline during stratification. In the present study, no decrease in the inhibitor content was observed. Instead, there was an increase in the total inhibitor content of water leachates from cold treated fruits, and also an increase in the acidity of the extracts. A germination stimulator appeared during cold treatment as was reported for sunflower (35), apple (26), and ash (50) seeds. Like in the apple (26), but unlike the ash (50), this stimulator could not be replaced by thiourea. GA was found to stimulate germination in non-stratified sunflower fruits.

Germination did not increase in the native sunflower fruits as a result of leaching. This apparent lack of control over germination in the sunflower and the increase in the inhibitor content during breaking of dormancy, suggests that the phenolic inhibitors may have some other function in the fruits. It was noticed in the psyllium seed bioassays and in germination tests with sunflower embryos that whenever the inhibitor was present, attack and decomposition of the seeds and embryos by bacteria and molds were retarded. This suggests that the inhibitor may have a positive survival value through protection of the embryos or young seedlings against microbial destruction. Ferenczy (9) reported antibacterial activity in the seeds or fruits of 52 species which did not include the sunflower. Rice (32) recently reported that chlorogenic and isochlorogenic acids, which were isolated from the leaves of the sunflower, are effective

inhibitors of the nitrogen-fixing and nitrifying bacteria. However, the seed germination inhibitor extracted from sunflower fruits is not chlorogenic or isochlorogenic acids. In the crude water leachates, these compounds may exert antibacterial activity or enhance the antibacterial activity of the actual germination inhibitor; but on chromatograms the germination inhibitor is widely separated from these acids.

It has been shown that pod tissue of the bean contains no inhibitor during early development; but as further development takes place, inhibitors appear and continue to increase throughout the maturation process (49). Likewise, the tissues surrounding the embryo of the apple seed contain more inhibitors at maturity than does the embryo (26). In this study the germination inhibitor was shown to be produced in the embryo and apparently translocated into the pericarp during the maturation and drying of the fruit.

Following excision from the pericarp, dormant native sunflower embryos were observed to undergo limited development when placed in conditions suitable for germination. This development consisted of a geotropic curvature in the tip of the radicle, and if they were exposed to light, formation of chlorophyll and spreading apart of the cotyledons. The same type of behavior has apparently been observed in apple (26) and in freshly harvested Russian Mammoth sunflower fruits (51). These seedlings are not dwarf in the same

sense as the "physiologic dwarfs" in Rhodotypos (11) or in peach (12) which have telescoped internodes and almost a rosette appearance. In the sunflower, no epicotyl growth was observed in these seedlings from dormant embryos and growth seems to be arrested at the cotyledon stage of seedling development.

Bioassays of blank chromatograms revealed an inhibitor zone between Rf's 0.9-1.0 that was due to impurities in the S.S 2043-b paper. Previous workers using this paper have reported inhibition of germination in bioassays due to materials chromatographing at the solvent front (10, 22, 45, 46, 47, 48, 49). These materials were designated the "Omega inhibitor" (10) and were thought to be due to the essential oils present in the plant extracts. Since these papers were not reported to have been washed prior to chromatography of the extracts, it is possible that the "Omega inhibitor" represents only the impurities in the chromatography paper.

Chlorogenic acid has been earlier reported in seeds and coats (34) and embryos (31) of sunflower fruits. These reports were not confirmed in this study with dry fruits of either the domestic or the native variety. However, chlorogenic acid did appear in fruits of the domestic variety during the first 24 hours of imbibition, but the length of time for it to first appear in detectable quantities is still unknown. Chlorogenic acid did not appear during imbibition in

the native variety, but appeared only after cold treatment had started. Isochlorogenic acid was not present in the dry fruits of either variety, but appeared in both after chlorogenic acid had appeared. In the domestic variety, it appeared between 24 and 48 hours after imbibition had started. In the native variety, isochlorogenic acid appeared between the 16th and 32nd days of stratification.

Chlorogenic and isochlorogenic acids are of interest because of their appearance during the breaking of dormancy in fruits of the native sunflower. Isochlorogenic acid, chlorogenic acid, other isomers of chlorogenic acid, caffeic acid, and other 3,4-dihydroxy phenolics have been reported to be inhibitors of indole-3-acetic acid oxidase (IAA oxidase) in vitro (13, 14, 15, 38). More recently, chlorogenic acid has been shown to increase the amount of readily extractable IAA (20, 30, 54), probably through in vivo inhibition of IAA oxidase activity.

The appearance of an Ehrlich positive material during cold treatment of the dormant fruits of the sunflower suggests that the chlorogenic acid appearing during stratification may be acting to inhibit IAA oxidase. If this is the case, cold treatment of these fruits may break their dormancy by stimulating the production (or favoring accumulation) of a substance which initiates production of chlorogenic acid. Chlorogenic acid then may inhibit IAA-oxidase and thus allow IAA to accumulate. With the increase in IAA,

growth resumes in the embryo.

Chlorogenic acid could even be produced from compounds that are cofactors for IAA-oxidase. p-Coumaric acid and 3-O-p-coumaroyl-D-quinic acid (fig 15 D and B respectively) have been shown to be very active cofactors (14) of IAA-oxidase from the pineapple, and have been shown to be precursors of chlorogenic acid (17, 25). Only a 3-hydroxyl group is different in 3-O-p-coumaroyl-D-quinic and chlorogenic acid, and the hydroxylation reaction forming chlorogenic acid may be reversible with p-coumaroyl-quinic acid being formed from chlorogenic acid. The regulation of growth may be controlled through the ratio of cofactor to inhibitor and the resulting activation or inactivation of IAA-oxidase. The predominant form present (cofactor or inhibitor) may possibly be controlled by the stimuli already known to affect the plant, such as light quality, temperature, and day length.

4-Hydroxybenzyl alcohol (fig 15 E) also has been shown to be a naturally occurring cofactor for IAA-oxidase from peas (28). It no longer exhibits cofactor activity following methylation of the hydroxy group (fig 15 F). If the activation-inactivation mechanism proposed above does operate in vivo, it may be the ratio of 4-monohydroxy to 3,4-dihydroxy or 4-methoxy phenolics that is important in determining IAA-oxidase activity.

CHAPTER V

SUMMARY

Fruits of the native variety of Helianthus annuus L. show extended periods of dormancy. Leaching experiments have shown the presence of one or more inhibitors in these fruits. The inhibitors were no longer detectable in the leachate obtained after 36 hours of continuous leaching. However, this leaching had little positive effect on germination in these fruits. The embryos from these leached fruits showed lessened resistance to attack and decomposition by bacteria and molds. The inhibitor was found to be active against other seeds and also retarded the decomposition of these assay seedlings by bacteria and molds.

The inhibitor was soluble in methanol, ethanol, isopropanol, diethyl ether, and ethyl acetate; but it was insoluble in petroleum ether. It was acidic and was found to be a phenolic compound. It did not fluoresce under UV light, but did show weak absorption under short UV. It was not a cyanogenic glycoside as was previously suggested.

The inhibitor was also found in fruits of the domestic sunflower, variety "Russian Mammoth". Paper chromatography has shown that the inhibitors in both these

varieties are apparently the same.

Dormancy in the fruits of the native variety was not due to impermeability of the pericarp or seed coat to water. High oxygen concentrations gave no significant increase in germination. Dormancy was attributed to conditions within the embryo because excised embryos either failed to germinate or showed only limited development consisting of a slight geotropic curvature of the radicle apex. If the embryos were exposed to light, the cotyledons spread apart and developed chlorophyll, but no epicotyl growth was observed in these embryos.

Gibberellic acid stimulated some germination at all concentrations used, but 500 and 1000 milligrams per liter concentrations were the most effective. Dormancy was broken in some fruits by alternating temperatures (8 hours at 25°, 16 hours at 15°), but moist stratification at 2-5° for 60 days resulted in germination of 100% of the viable fruits. The total inhibitor content and the acidity of the extracts both increased during stratification. A substance that stimulated germination of psyllium seeds appeared during stratification along with an Ehrlich positive material.

Chlorogenic and isochlorogenic acids appeared in that sequence during cold treatment of the fruits of the native sunflower and were isolated and identified by paper chromatography. These two compounds also appeared in the same sequence in fruits of the domestic variety during imbibition. Contrary to previous reports, chlorogenic acid was not found in dry fruits of either variety.

A germination inhibiting zone present on chromatograms at Rf's 0.9-1.0 was found to be an impurity in the S&S 2043-b paper.

LITERATURE CITED

1. Association of Official Agricultural Chemists: Official Methods of Analysis, 8th ed., page 379. 1955. Washington, D.C.

2. Blommaert, K. L. J. 1955. The significance of auxins and growth-inhibiting substances in relation to winter dormancy of the peach tree. W. P. Fruit Res. Sta., Union of South Africa. Sci. Bull. No. 368.

3. Cartwright, R. A., and E. A. H. Roberts. 1955. Theogallin as a galloyl ester of quinic acid. Chem. and Ind. (London): 230-31.

4. Corse, J., E. Sondheimer, and R. Lundin. 1962. 3-Feruloylquinic acid. A 3'-methyl ether of chlorogenic acid. Tetrahedron 18: 1207-10.

5. Crocker, W., and L. V. Barton. 1953. Physiology of Seeds. Chronica Botanica, Waltham, Mass. 267 pp.

6. Deubner, C. G. 1931. Chemical treatments to shorten the rest period of tree seeds. Science 73: 320-21.

7. Dieterman, L. J., C.-Y. Lin, L. M. Rohrbaugh, and S. H. Wender. 1964. Accumulation of ayapin and scopolin in sunflower plants treated with 2,4-dichlorophenoxyacetic acid. Arch. Biochem. Biophys. 105: 275-79.

8. Evenari, M. 1949. Germination inhibitors. Bot. Rev. 15: 153-94.

9. Ferenczy, L. 1956. Occurrence of antibacterial compounds in seeds and fruits. Acta Biol. Acad. Sci. Hung. 6: 317-23.

10. Ferenczy, L. 1957. Examination of ether extractable growth substances in grape and watermelon with paper chromatography. Phyton (Buenos Aires) 9: 47-52.

11. Flemion, F. 1933. Dwarf seedlings from non-after-ripened embryos of Rhodotypos kerrioides. Contrib. Boyce Thompson Inst. 5: 161-65.

12. Flemion, F. 1934. Dwarf seedlings from non-after-ripened embryos of peach, apple, and hawthorn. Contrib. Boyce Thompson Inst. 6: 205-09.

13. Gortner, W. A., and M. Kent. 1953. Indoleacetic acid oxidase and an inhibitor in pineapple tissue. J. Biol. Chem. 204: 593-603.

14. Gortner, W. A., and M. J. Kent. 1958. The coenzyme requirements and enzyme inhibitors of pineapple indoleacetic acid oxidase. J. Biol. Chem. 233: 731-35.

15. Griffin, D. H., and E. Sondheimer. 1960. Activation and inhibition of indoleacetic acid oxidase activity from peas. Science 131: 672.

16. Halsam, E., G. K. Makinson, M. O. Naumann, and J. Cunningham. 1964. Synthesis and properties of some hydroxycinnamoyl esters of quinic acid. J. Chem. Soc. (London) 2137-46.

17. Hanson, K. R., and M. Zucker. 1963. The biosynthesis of chlorogenic acid and related conjugates of the hydroxycinnamic acids. Chromatographic separation and characterization. J. Biol. Chem. 238: 1105-15.

18. Hemberg, T. 1949. Significance of growth-inhibiting substances and auxins for the rest period of the potato. Physiol. Plant. 2: 24-36.

19. Hendershott, C. H., and L. F. Bailey. 1955. Growth-inhibiting substances in extracts of dormant flower buds of peach. Proc. Amer. Soc. Hort. Sci. 65: 85-92.

20. Henderson, J. H. M., and J. P. Nitsch. 1962. Effect of certain phenolic acids on the elongation of Avena first internodes in the presence of auxins and tryptophan. Nature 195: 780-82.

21. Kallio, P., and P. Piiroinen. 1959. Effect of gibberellic acid on the termination of dormancy in some seeds. Nature 183: 1830-31.

22. Köves, E., and M. Varga. 1959. Comparative examination of water- and ether-soluble inhibiting substances in dry fruits. Phyton (Buenos Aires) 12: 93-99.

23. LaCroix, L. J. 1961. Seed dormancy in Abutilon theophrasti Medic. and Polygonum pensylvanicum L. Ph.D. Dissertation, Iowa State Univ., Ames.

24. Lane, F. E., and L. F. Bailey. 1964. Isolation and characterization studies on the β-inhibitor in dormant buds of the silver maple, Acer saccharinum L. Physiol. Plant. 17: 91-99.

25. Levy, C. C., and M. Zucker. 1960. Cinnamyl and p-coumaryl esters as intermediates in the biosynthesis of chlorogenic acid. J. Biol. Chem. 235: 2418-25.

26. Luckwill, L. C. 1952. Growth-inhibiting and growth-promoting substances in relation to the dormancy and after-ripening of apple seeds. J. Hort. Sci. (London) 27: 53-67.

27. Mayer, A. M., and A. Poljakoff-Mayber. 1963. The Germination of Seeds. Pergamon Press (Macmillan), New York. 236 pp.

28. Mumford, F. E., H. M. Stark, and D. H. Smith. 1963. 4-Hydroxybenzyl alcohol, a naturally occurring co-factor of indoleacetic acid oxidase. Phytochemistry 2: 215-20.

29. Pearl, I. A., and P. F. McCoy, 1960. Stable diazo salts for chromatographic spray reagents. Anal. Chem. 32: 1407-10.

30. Pilet, P. E. 1964. Effect of chlorogenic acid on the auxin catabolism and the auxin content of root tissues. Phytochemistry 3: 617-21.

31. Politis, J. 1949. Sur la distribution de l'acide chlorogenique dans la famille des Composees et dans les organes de ces plantes. Comp. Rend. Seances Acad. Sci. 228: 265-66.

32. Rice, E. L. 1965. Inhibition of nitrogen-fixing and nitrifying bacteria by seed plants. II. Characterization and identification of inhibitors. Physiol. Plant. 18: 255-68.

33. Roberts, E. A. H., and D. J. Wood. 1951. The Polyphenols and amino acids of the tobacco leaf. Arch. Biochem. Biophys. 33: 299-303.

34. Ruckenbrod, H. 1955. Untersuchungen über dem Umsatz der Chlorogensäure in höheren Pflanzen. Planta 46: 19-45.

35. Ruge, U. 1939. Zur Physiologie der genuinen keimungshemmenden und keimungsbeschleunigenden Stoffe von Helianthus annuus. Zeits. für Bot. 33: 529-71.

36. Scarpati, M. L., and P. Esposito. 1963. Neochlorogenic acid and "Band 510" structure. Tetrahedron Letters 18: 1147-50.

37. Smith, I. 1960. Chromatographic and Electrophoretic techniques. Volume I. Chromatography. Interscience Publishers, New York. 617 pp.

38. Sondheimer, E. 1964. Chlorogenic acids and related depsides. Bot. Rev. 30: 667-712.

39. Spicer, P. B., and L. A. Dionne. 1961. Use of gibberellin to hasten germination of Solanum seed. Nature 189: 327-28.

40. Swain, T. 1953. The identification of coumarins and related compounds by filter-paper chromatography. Biochem. J. 53: 200-08.

41. Thompson, R. C., and W. F. Kosar. 1939. Stimulation of germination of dormant lettuce seed by sulphur compounds. Plant Physiol. 14: 567-73.

42. Toole, E. H., S. B. Hendricks, H. A. Borthwick, and V. K. Toole. 1956. Physiology of seed germination. Ann. Rev. Plant Physiol. 7: 299-324.

43. Tukey, H. B., and R. F. Carlson. 1945. Breaking the dormancy of peach seed by treatment with thiourea. Plant Physiol. 20: 505-516.

44. Urban, R. 1958. Physiologische Untersuchungen uber einige Flavonoide und Oxyzimtsauren. I. Auswahl und Identifizierung der bearbeiteten Stoffkomponenten. Planta 52: 47-64.

45. Varga, M. 1957. Examination of growth-inhibiting substances separated by paper chromatography in fleshy fruits. I. Results of the bioassay of the chromatograms obtained from the ether extracts of the fruits. Acta Biol. Acad. Sci. Hung. 8: 41-47.

46. Varga, M. 1957. Examination of growth-inhibiting substances separated by paper chromatography in fleshy fruits. II. Identification of the substances of the growth-inhibiting zones on the chromatograms. Acta Biol. Szeged. 3: 213-23.

47. Varga, M. 1957. Examination of growth-inhibiting substances separated by paper chromatography in fleshy fruits. III. Changes in concentration of the growth-

inhibiting substances as a function of ripening. Acta Biol. Szeged. 3: 225-32.

48. Varga, M. 1957. Examination of growth-inhibiting substances separated by paper chromatography in fleshy fruits. IV. Paper chromatographic analysis of lemon juice containing germinated seeds. Acta Biol. Szeged. 3: 233-38.

49. Varga, M., and E. Köves. 1959. Distribution and quantitative changes in the β-inhibitor in the various organs of the bean plant during ontogeny. Acta Biol. Acad. Sci. Hung. 9: 369-78.

50. Villiers, T. A., and P. F. Wareing. 1960. Interaction of growth inhibitor and a natural germination stimulator in the dormancy of Fraxinus excelsior L. Nature 185: 112-14.

51. Wallace, R. H., and H. M. Haberman. 1958. Absence of seed dormancy in a white mutant strain of Helianthus annuus L. Plant Physiol. 33: 252-54.

52. Watanabe, R., W. Chorney, J. Skok, and S. H. Wender. 1964. Effect of boron deficiency on the polyphenol production in the sunflower. Phytochemistry 3: 391-93.

53. Zane, A. 1963. Isolation, identification, synthesis, pyrolysis of plant phenolic compounds. Ph.D. Dissertation, Univ. of Oklahoma, Norman.

54. Zenk, M. H., and G. Muller. 1963. In vivo destruction of exogenously applied indolyl-3-acetic acid as influenced by naturally occurring phenolic acids. Nature 200, 761-63.