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Histological responses of azalea to Off-shoot-O and Dikegulac as chemical pinching agents

Susan Tai-An Peng

To the Graduate Council:

I am submitting herewith a thesis written by Susan Tai-An Peng entitled "Histological responses of azalea to Off-shoot-O and Dikegulac as chemical pinching agents." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Landscape Architecture.

Effin T. Graham, Major Professor

We have read this thesis and recommend its acceptance:

John W. Day, Donald B. Williams

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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We have read this thesis
and recommend its acceptance:

J B Williams

John W Hay

Accepted for the Council:

L Evans Roth

Vice Chancellor
Graduate Studies and Research

HISTOLOGICAL RESPONSES OF AZALEA TO OFF-SHOOT-O AND DIKEGULAC AS CHEMICAL PINCHING AGENTS

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Susan Tai-An Peng

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ABSTRACT

Histological responses to chemical pinching agents, Off-Shoot-O and Dikegulac, applied as foliar sprays, were observed in shoot apices of Rhododendron obtusum 'Hinodegiri.' Tissues for paraffin and plastic embedding were fixed in buffered glutaraldehyde, and for plastic were further fixed in osmium tetroxide.

The non-treated control shoot tips were characterized by large populations of cytoplasmic globules in a great range of sizes, some larger than nuclei. These globules were especially prominent in the trichomes of the differentiating primordial leaves, but were also present in the differentiating young stem tissue. The observation of these unique cellular inclusions was apparently the result of non-coagulant fixation, since they have not been reported from other studies in which coagulant fixation was employed.

The large number of globules suggests that they may have an important role in actively growing tissues. The composition of the globules has not been identified. In this experiment, however, their color reaction with methylene blue-azure A in plastic sections suggests a phenolic composition, possibly a precursor to lignin in the globules.

In the treatment with Off-Shoot-O, the globules in the trichomes disappeared three minutes after spraying. Dikegulac did not destroy the globules or other cellular inclusions, but the cells in the young primordial leaves and apical meristems of the shoot tips taken two weeks after treatment showed some changes compared with the non-treated

shoot tips. Changes were observed in the color of the stained cells and there was increased vacuolization. Therefore, Dikegulac could interfere with the activities of plant hormones, especially auxin and cytokinin, in relation to apical dominance and side branching.

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CHAPTER I

INTRODUCTION

Azalea is a popular florist pot plant for holidays as well as a major ornamental plant for landscape design in the United States. A major expense in the management of an azalea crop is the labor required for pinching the terminal shoot tips. Pinching promotes branching and a more compact plant with more flower buds results. Chemical pinching agents are less costly than hand pinching. However, some pinching agents are difficult to use and plant damage may result because response varies according to cultivars, environmental conditions, and concentration of the chemical.

Off-Shoot-O and Dikegulac are different kinds of chemical pinching agents. Both induce side branching but their modes of action are different. Off-Shoot-O kills the terminal shoot tips but Dikegulac causes side branching without destroying the shoot tips. There must be physical contact of Off-Shoot-O with the tips because the chemical is not translocated (33). Dikegulac, on the other hand, has systemic activity (2, 30, 41). The exact modes of action of both chemicals are still unknown. Some researchers have proposed that the nucleus, cytoplasm, cell wall, plasmalemma and the tonoplast membrane were in various states of disintegration after application of Off-Shoot-O and methyl decanoate (1, 25, 39). The physiological effect of Dikegulac may interfere with the activity of auxin and cytokinin (30).

Continued improvement of chemical pinching would be enhanced by any new information about the anatomical, biochemical, and physiological responses of plants to the treatment. The purpose of this research is to study the histological responses of the azalea shoot apex to Off-Shoot-O and Dikegulac.

CHAPTER II

LITERATURE REVIEW

The special cultural requirements of azalea must be recognized in any system of crop management. Pinching must be carefully timed within the general management program to produce a well-shaped, heavily budded plant. It usually must be done two or three times before the desired size of plant is obtained and the flower buds develop (2, 4). Chemical pinching should be repeated as soon as the new shoots grow one to two inches long (2, 26). Brabson and Lindstrom (8) reported that temperature was the major environmental factor that influenced chemical pinching. Plants were more sensitive to chemical pinching agents when preconditioned at low temperature rather than at high temperature. Setting of flower buds requires 18°C to 21°C for approximately eight weeks. This is followed by a cold storage treatment at 2°C to 10°C for four to eight weeks to break the dormancy of the buds and induce bloom (11, 13, 23). Criley (12) reported that a short-day period induced flower initiation, and that gibberellin could partially or completely substitute for cold storage treatment when the buds were well developed. Some other growth regulatory chemicals such as Cycocel, B-Nine, and Phosphon can induce bud setting (15, 23).

A number of chemicals have been investigated to substitute for hand pinching. Schoene and Hoffman (31) reported that maleic hydrazide would inhibit the growth of terminal shoot tips and induce

lateral shoots on tomato plants. Maleic hydrazide was the first chemical tested to achieve the effect of chemical pinching (5). Tso and McMurtrey (37) reported that methyl ester fatty acids (MEFA) would inhibit axillary bud growth on tobacco plants. They also reported that mature cells were not damaged under most conditions (38). Cathey and associates (9) reported that the lower alkyl esters of fatty acids selectively destroyed or inhibited the apical meristem in many plants. Furuta (17) mentioned that the effectiveness of MEFA varied with the carbon chain length and a C_{10} chain length was the most effective. Stuart (35) reported that MEFA containing eight to twelve carbons pruned azalea shoot tips, and those with fewer carbons were not only less active pinching agents but also caused more damage to the plants.

Off-Shoot-O was the first commercial chemical pinching agent (26). Its growth regulatory ingredients are the methyl esters of C_6-C_{12} fatty acids (18, 22, 26). A chemical structure of a methyl ester of fatty acid is shown below.

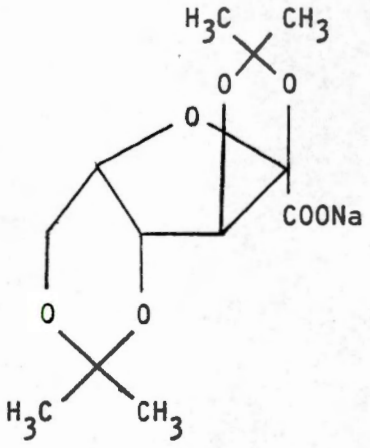
There must be physical contact of Off-Shoot-O with the shoot tips because the chemical is not translocated (33). In about one hour after it is applied as a foliar spray, the younger unexpanded leaves and shoot tips darken to a brown color (1, 26). Nelson and Poteet (25) indicated that the plasmalemma (cell membrane) and tonoplast (vacuolar) membrane were destroyed by methyl decanoate, one of the

active ingredients in Off-Shoot-O, in some plasmolysis experiments. Arisumi (1) reported that the nucleus, cytoplasm and cell wall were damaged after application of Off-Shoot-O. Uhring (39) histologically observed chrysanthemum treated with methyl deconate and stated that it was a destructive agent of the nucleus, but did not explain the mode of action.

The concentration, surfactant, variety of plant, maturity of shoot tips, and environmental factors must be considered (8, 9, 22, 25, 26, 33). Concentrations that are too high can kill the whole plant while lower concentrations may not affect the shoot tips at all (9, 22). MEFA are insoluble in water so that a surfactant (wetting agent) is needed to emulsify the chemicals (8, 25, 26). Selecting a suitable variety of plant is also important because the cuticles and the trichomes might affect the penetration of the chemicals, and these factors are quite variable among different varieties of azaleas (25, 33). The results also depend on the state of growth of shoot tips since older leaves and dormant buds do not respond to the chemicals (25, 33).

Dikegulac was developed by Hoffman-La Roche Inc. in 1975 (2). It is an intermediate in the commercial synthesis of L-ascorbic acid (7). The chemical identity is a Sodium salt of 2,3:4,6 Bis-O-(1-Methylethylidene)-α-L-Xylo-2-Hexulofuranosonic acid (2, 7, 15). The chemical structure is shown on page 6.

Dikegulac has been shown to have systemic activity, causing side branching without destroying the shoot tips (2, 30, 41). One to two weeks after application the upper leaves turned yellow with temporary chlorosis, and in two to four weeks the axillary buds broke and



elongated (15, 28, 29). Four to eight weeks after treatment, the growth appeared normal and chlorosis disappeared (15, 29). Bocion and deSilva (7) stated that Dikegulac was very effective in the inhibition of apical dominance. Dikegulac may interfere with the activity of auxin and cytokinin which are related to apical dominance and growth of axillary buds (30).

CHAPTER III

MATERIAL AND METHODS

Samples of shoot tips were obtained from Rhododendron obtusum 'Hinodegiri'[1] which responded to Dikegulac (30). The treatments were applied when the new vegetative shoots were one to two inches long and were in a vigorous state of growth after blooming in the greenhouse in February, 1978. Off-Shoot-O at five percent v/v aqueous solution was applied on March 21, 1978. Dikegulac in 25 ml/liter aqueous solution was sprayed on March 15, 1978. These two applications were sprayed by a one liter hand sprayer in the greenhouse at a temperature of 18°C. Off-Shoot-O reacts with the plant tissue quickly (1, 38). Therefore, the samples were taken at intervals of 3, 6, 10, 15, 20, and 30 minutes and 1, 2, 3, 4, 5, 6, 24, and 48 hours after treatment. There is no information about the histology of plants treated with Dikegulac. According to the physiological responses of Dikegulac in other researchers' studies (2, 15, 28, 29, 30), the sample intervals were at 3, 6, 12, 24, and 36 hours and 2, 3, 5, 7, 9, 11, 13, 16, 19, 22, 25, and 28 days after treatment. Six treated samples were obtained for each interval, three for paraffin embedding and three for plastic embedding. Four control samples were obtained for each interval, two

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[1]Plants were obtained from R. A. Schnall's experiments (30). They had been sprayed with Dikegulac on July 29, 1977. The plants were originally provided by Arnell's Nursery, Mobile, Alabama.

for paraffin embedding and two for plastic embedding. The controls for the Off-Shoot-0 experiment were taken at 30 minutes, 5 and 24 hours intervals.

Shoot tip samples (one to two millimeters long) were excised and fixed 24 hours in a solution of 2.5 percent glutaraldehyde in phosphate buffer at pH 7.0. To prevent the tissues from floating in the dehydrating alcohols and paraffin, the shoot tips in the fixing solution were subjected to 25 psi negative pressure in a vacuum chamber for several minutes until the shoot tips sank to the bottom, indicating the air in the tissues was evacuated.

The tissue was dehydrated by solvent replacement of water, using increasing concentrations of alcohol terminated with tertiary butyl alcohol and paraffin at 60°C. The infiltration alcohol was eliminated by slow evaporation and the tissue was embedded in paraffin. The paraffin blocks were sectioned five microns thick on a rotary microtome, and the sections were mounted on bare glass slids by flotation on water followed by drying on a slide-warming plate.

The mounted sections were washed in xylene three times to dissolve the paraffin, rinsed in absolute alcohol three times, and rehydrated in a descending series of ethyl alcohol concentrations to water. Then, the sections were ready to stain. They were stained two minutes in 0.1 percent aqueous safranin 0 and rinsed in water quickly, followed by 10 minutes in aniline blue-orange G in citrate buffer at pH 3.5 (20). Excess stain was removed from sections by a very quick rinse in distilled water followed by rinsing in tertiary butyl alcohol and xylene. Finally, the sections were coverglassed and sealed with resin.

The shoot tips for embedding in plastic were fixed in 2.5 percent glutaraldehyde in phosphate buffer at pH 7.0. After rinsing in distilled water three times, they were post-fixed for one hour in 2 percent aqueous osmium tetroxide (OsO_4). The samples were rinsed again and solvent-dehydrated in the tertiary butyl alcohol series, and then embedded in gelatin capsules containing Spurr low-viscosity medium (34) which was hardened overnight at 70°C. The plastic samples were trimmed and sectioned at three to four microns thick on an ultramicrotome. The sections were stained by floating them on methylene blue-azure A in 0.1 M phosphate buffer at pH 6.9 (40) in capped polyethylene capsules in an oven at 70°-80°C for one hour. Thus, the sections were simultaneously stained as well as heat-expanded to eliminate wrinkles. The stained sections were washed in distilled water in a microchemical spot plate and then floated onto glass slides, which were coated with chrome alum-gelatin (20). The purpose of the special gelatin coating was to facilitate the arrangement of the sections on individual water droplets. The slides were placed on a hot plate at 80°C and as the water evaporated the sections settled onto the surface and were bonded tightly to the glass. The cover-glasses were applied with immersion oil.

The slides were observed on a Wild brightfield microscope, and photographed with a Nikon 35 mm photomicrographic light meter and camera unit.

CHAPTER IV

RESULTS AND DISCUSSION

Azalea shoots responded to Off-Shoot-O very quickly. Approximately six minutes after application the older plant tissues did not appear damaged but the younger leaves and petioles were discolored. The brown color in the younger expanded and unexpanded leaves was more obvious one hour later. The younger expanded leaves, most of the unexpanded leaves, and some of the trichomes were removed before fixation. The shoot tips darkened increasingly and became brittle so that the excision of the shoot tips was more difficult, and the remaining leaf primordia and trichomes were broken and lost easily during dehydration and embedding.

The sectioned materials of the untreated shoot tips of azalea had many round globules throughout the shoot tips, including the trichomes, primordial leaves, apical meristem, and vascular bundles (Fig. 1). The composition of the globules has not yet been identified by histochemical tests for the major groups of the cellular biochemicals, such as polysaccharides, protein, nucleic acid, or lipid. However, the globules were stained bright blue-green after non-coagulant fixation in glutaraldehyde-OsO_4 in plastic sections. The blue-green color suggests a phenolic composition, possibly a precursor to lignin in the globules. Jensen (20) has emphasized that a blue-green metachromatic peak of azure B can be indicative of lignin in plant tissues.

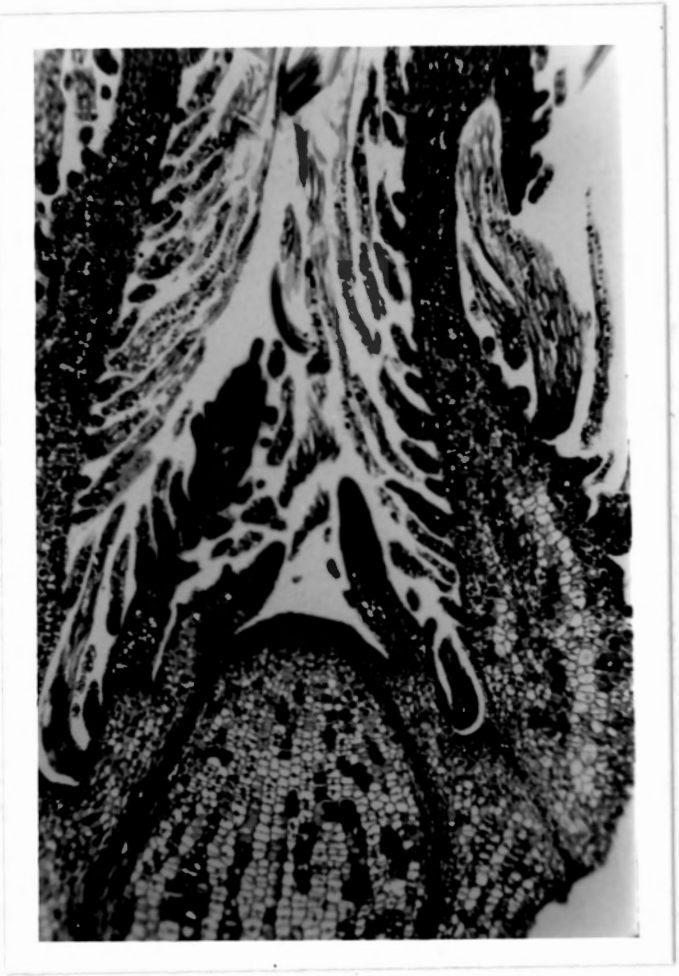

Fig. 1. Globules throughout shoot tip of untreated azalea bud embedded in paraffin. (Magnification, x31.25 enlarged x3)

Azure B and methylene blue-azure A used in this investigation are similar types of dyes.

The globules were numerous and occurred in a wide range of sizes in the shoot tips of azalea. Many were much larger than nuclei, while others descended in size to the limit of resolution of the light microscope. The large number of globules, compared with other cellular components, suggests that they may have an important role in the total energy balance of actively growing tissues.

The discrepancy between the observation of the globular cellular inclusions in this investigation, and the apparent absence of these structures in previous investigations, is probably the result of different methods of fixation. For example, a histological study in Kalanchoe has shown that cytoplasmic globules similar to those reported in this thesis were preserved in non-coagulant fixation but not in coagulant fixation (42). Arisumi (1) did not mention the globules in his histological report on azalea shoot tips fixed in FAA (formalin-acetic acid-alcohol). FAA is a very strong coagulant fixative as defined by Baker (3). It is apparently the aqueous non-coagulant fixation which effectively preserves the globules, while strong alcoholic coagulant fixation does not protect these structures. The absence of the globules in tissued fixed in FAA could be due to physical dissolution, chemical decomposition, or structural collapse. However, there is no available evidence on this point.

In the sectioned tissues taken three minutes after treatment the globules from the trichomes had disappeared (Fig. 2, 3). However, the globules in the other parts of the tissues still existed (Fig. 4).

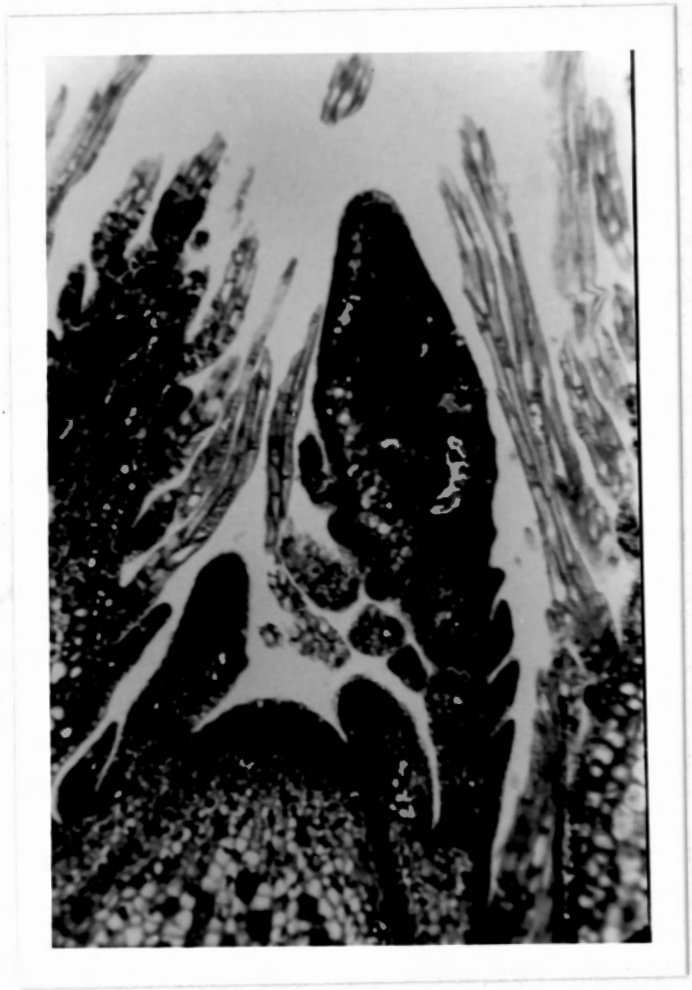

Fig. 2. Azalea shoot tip taken three minutes after treatment with Off-Shoot-O and embedded in paraffin, showing the absence of globules in the trichomes. (Magnification, x62.5 enlarged x3)

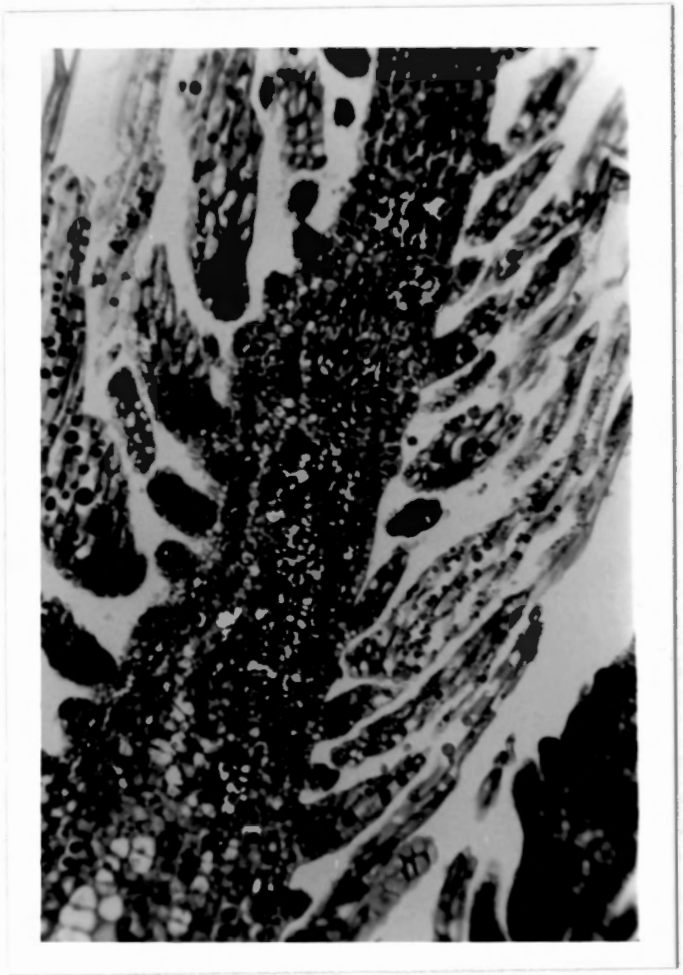

Fig. 3. Globules in trichomes of untreated azalea shoot tip embedded in paraffin. (Magnification, x62.5 enlarged x3)

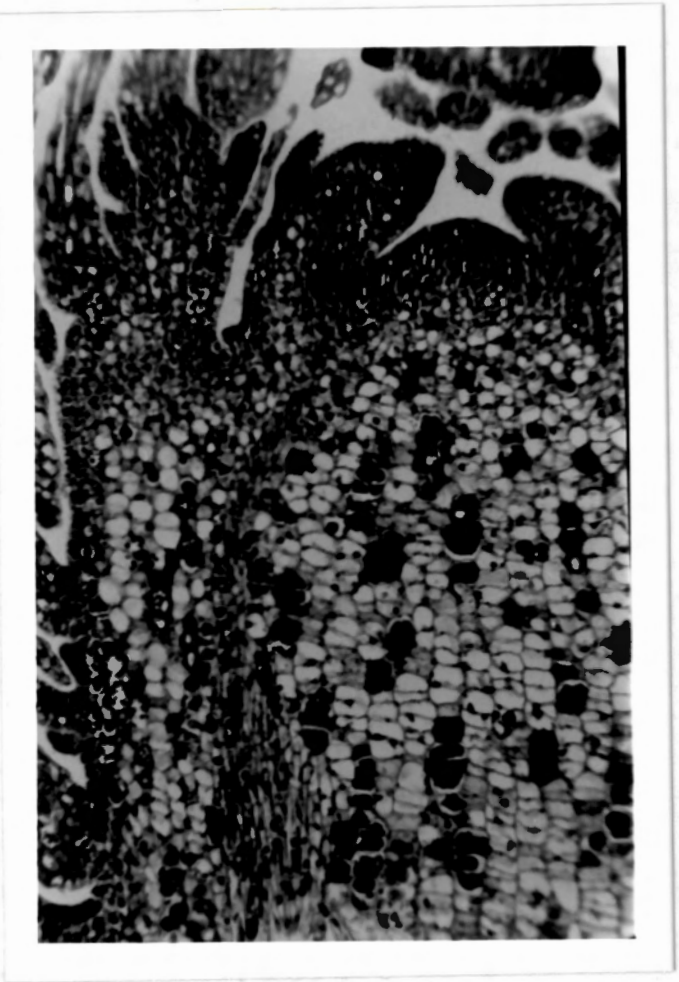

Fig. 4. Azalea shoot tip taken one hour after treatment with Off-Shoot-O and embedded in paraffin, showing globules in the other parts of the shoot tip except the trichomes. (Magnification, x62.5 enlarged x3)

Figures 5 and 6 show that there are numerous orange-red round globules in the trichomes of the control sample, and there are none in the trichomes sampled three minutes after treatment. After the globules disappeared from the trichomes, many hollow round globule shapes were apparent in the trichomes (Fig. 7). It seemed that the contents in the globules dissolved and flowed out, and the hollow shapes remained.

The outer leaf primordia were damaged in the treated samples taken one hour after treatment and later, but the apical meristem itself appeared normal because the chemical had not penetrated there (Fig. 8). The injured tissues were heavily stained, and the outline of the cells and the tissues were completely transformed. Most of the treated samples taken at three to five hours showed damaged areas on the younger leaf primordia and apical meristems (Fig. 9). All the treated shoot tips samples after 6, 24, and 48 hours showed complete discoloration and obliteration of the apical meristem and the younger leaf primordia, but the portion immediately subjacent to the apical meristem appeared normal (Fig. 10). This indicates that Off-Shoot-O was not translocated and the pinching effect required direct physical contact. Therefore, the presence of the cuticles and the trichomes are important factors which prevent the penetration of the chemical. This also explains why the older plant tissues and flower buds usually are not affected by Off-Shoot-O. This has also been observed by Nelson and Poteet (25). Since the apical meristem is protected by tightly furled primordial leaves, with cuticles and trichomes already formed, the amount of the chemical and its effectiveness for pinching is inevitably reduced after it moves across those barriers. Therefore,

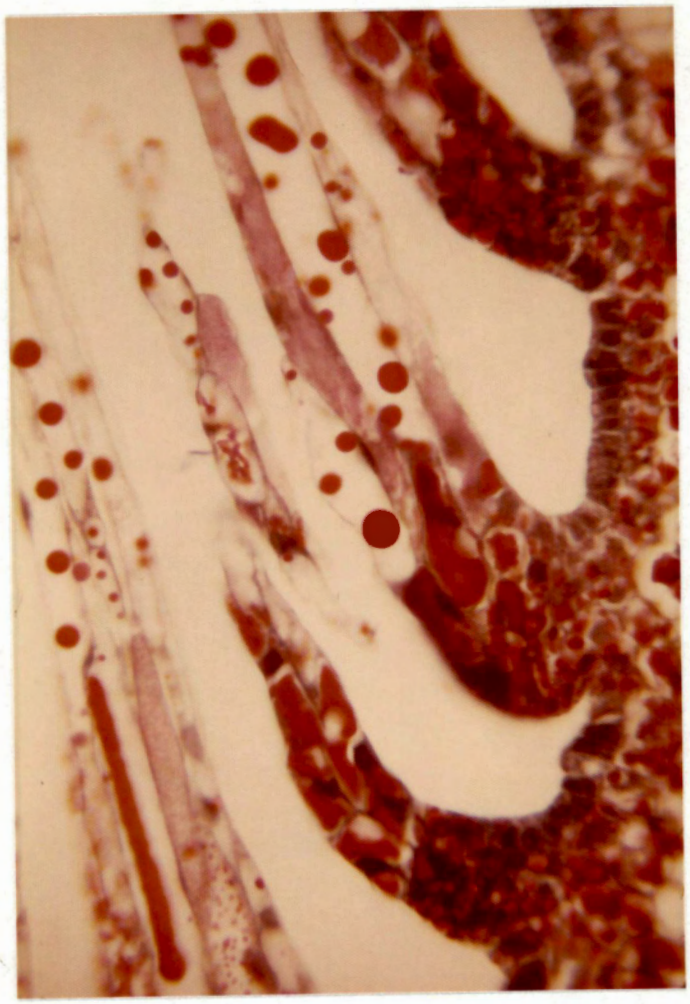

Fig. 5. The orange-red round globules in the trichomes of untreated azalea shoot tip embedded in paraffin. (Magnification, x156.25 enlarged x3)

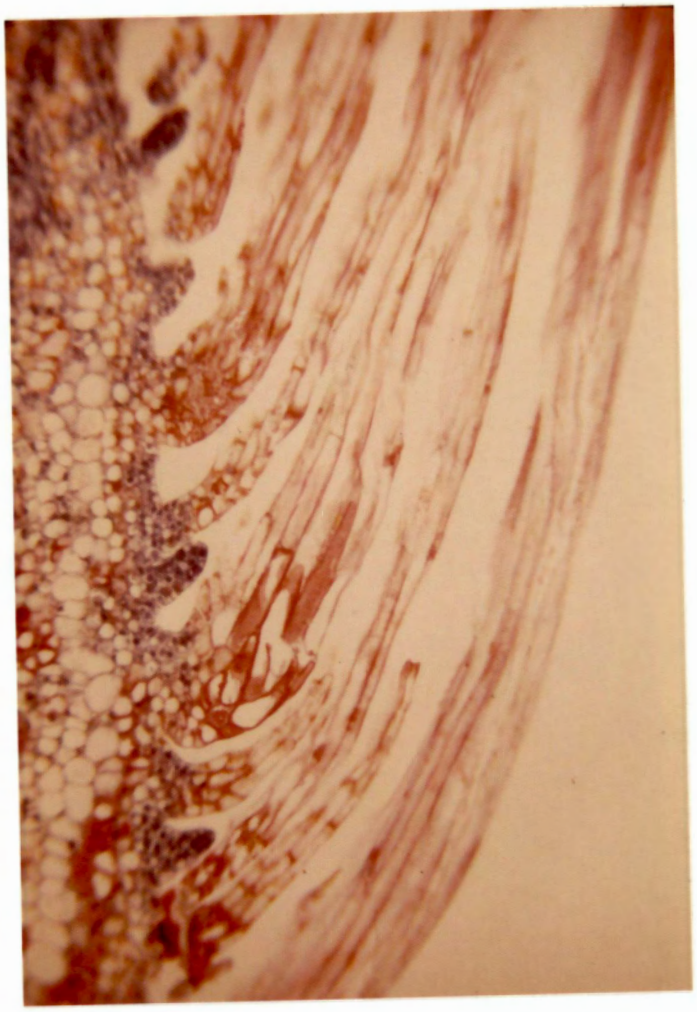

Fig. 6. Azalea shoot tip taken three minutes after treatment with Off-Shoot-O and embedded in paraffin, showing the absence of globules in the trichomes. (Magnification, x62.5 enlarged x3)

Fig. 8. Damaged outer leaf primordia of azalea shoot tip taken one hour after treatment with Off-Shoot-O and embedded in paraffin; the apical meristem appeared normal. (Magnification, x31.25 enlarged x3)

Fig. 9. Azalea shoot tip taken four hours after treatment with Off-Shoot-O and embedded in paraffin, showing the injured apical meristem heavily stained and obliterated. (Magnification, x62.5 enlarged x3)

Fig. 10. Azalea shoot tip taken 24 hours after treatment with Off-Shoot-O and embedded in paraffin; the portion immediately subjacent to the apical meristem appeared normal. (Magnification, x62.5 enlarged x3)

the decision as to what concentration of the chemical to use is dependent on the variety of plant, since the densities of cuticles and trichomes would be expected to vary among varieties.

Within ten days after application of Dikegulac, temporary chlorosis was found on the upper leaves of the shoots. The growth of axillary buds was observed about 14 days after spraying. The growth rate of the treated shoots was slow. The untreated shoots grew about one inch more than the treated shoots during four weeks. Dikegulac did not injure the shoot tips like Off-Shoot-O. Therefore, Dikegulac actually did not "pinch" the shoot tips to cause side branching.

There was no histologically observable difference between Dikegulac-treated and non-treated shoot tips taken before two weeks after treatment. The globules remained throughout the treated shoot tips, and did not disappear after treatment (Fig. 11). The younger leaf primordia, trichomes, and apical meristem in the treated shoot tips were not damaged, i.e. they were not heavily stained or obliterated, and their cellular integrity seemed unchanged. However, in the photographs of the treated samples taken on the 13th day and later, there were some differences in the cells of leaf primordia and apical meristem. Figure 12 shows the diagrams of a meristematic cell, undifferentiated and unspecialized plant cell and a differentiated parenchymatous cell. "The meristematic cells are usually small and thin-walled with a relatively large nucleus and no prominent central vacuole" (19). In observations of both the treated and control shoot tips taken before the 13th day, most of the cells in the apical meristem and leaf primordia were undifferentiated and unspecialized.

Fig. 11. Azalea shoot tip taken 12 hours after treatment with Dikegulac and embedded in plastic, showing appearance of globules after treatment. (Magnification, x62.5 enlarged x3)

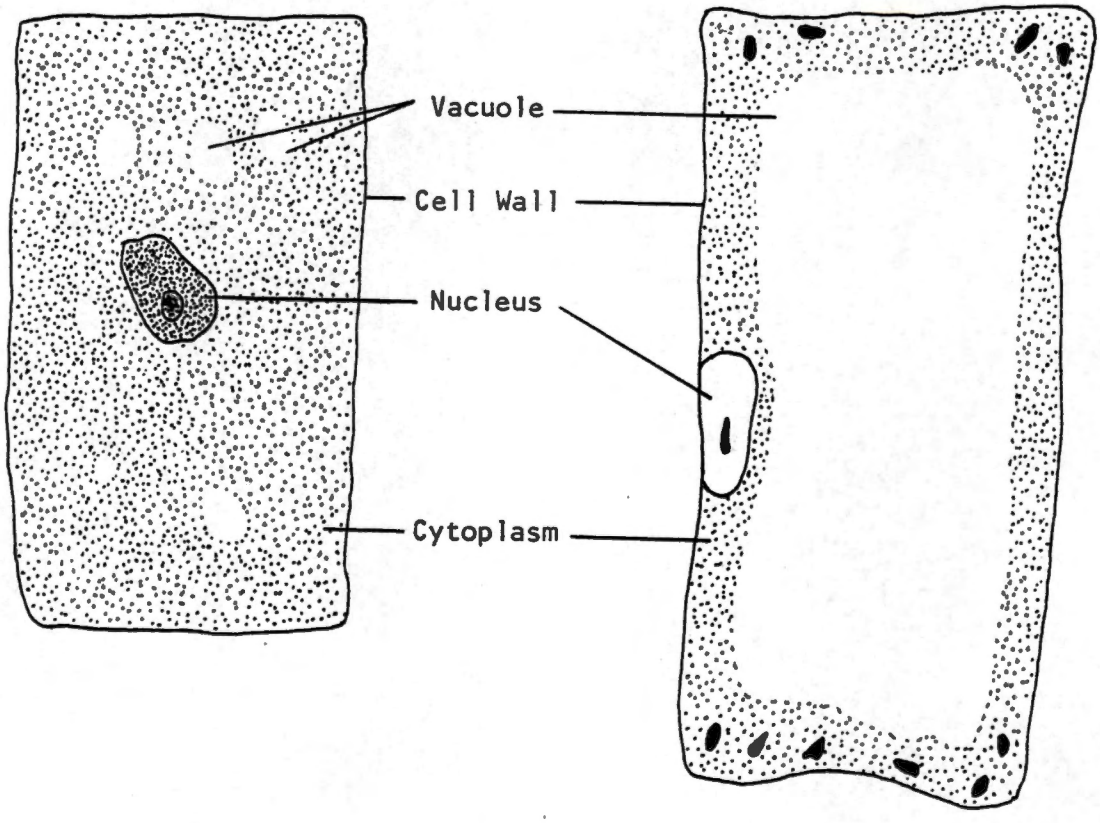

Fig. 12. Diagrams of a meristematic cell and a parenchymatous cell. (Not drawn to scale)

Thirteen days after treatment with Dikegulac, the undifferentiated cells in the primordial leaves and apical meristem seemed to become differentiated; the size of the cells, the vacuole, and the cytoplasm had changed. This consistent pattern was very apparent in the 19th, 22nd, 25th, and 28th day samples (Fig. 13, 14, 15). The cells included blue cytoplasm, darkened nuclei, and colorless vacuoles in the plastic sections stained by methylene blue-azure A. Therefore, the primordial leaves in the treated samples taken 18, 22, 25, and 28 days after treatment seemed to have increased vacuolization since the vacuoles became the largest inclusions or constituents in the cells (Fig. 16, 17). A possible relationship between Dikegulac activity and apical dominance has been suggested by others (2, 7, 30). Therefore, it could be proposed that Dikegulac reduced apical dominance and induced growth of axillary buds by interfering with the activities of plant hormones, particularly auxin and cytokinin which are related to cell differentiation and cell division (24).

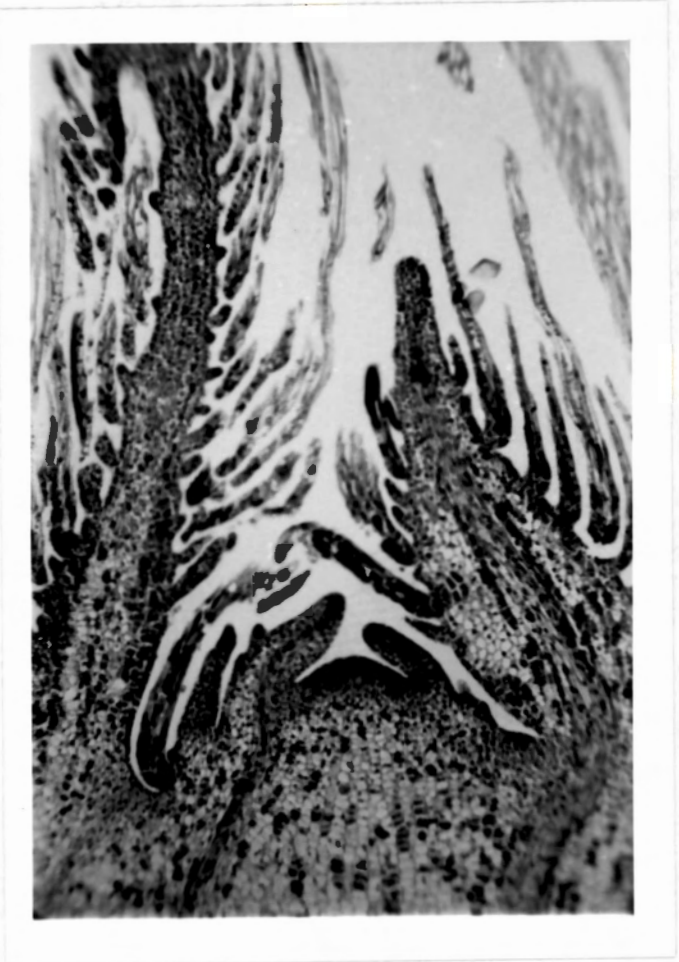

Fig. 13. The control shoot tip of azalea taken at the interval of the 25th day and embedded in paraffin for the comparison with Dikegulac-treated shoot tip. (Magnification, x31.25 enlarged x3)

Fig. 14. Dikegulac-treated shoot tip of azalea taken 19 days after treatment and embedded in plastic, showing the changes in vacuole and cytoplasm in cells of leaf primordia and apical meristem compared with the control sample. (Magnification, x31.25 enlarged x3)

Fig. 15. Dikegulac-treated shoot tip of azalea taken 28 days after treatment and embedded in plastic, showing the larger cells and vacuolization of cells in leaf primordia and apical meristem compared with the control sample. (Magnification, x31.25 enlarged x3)

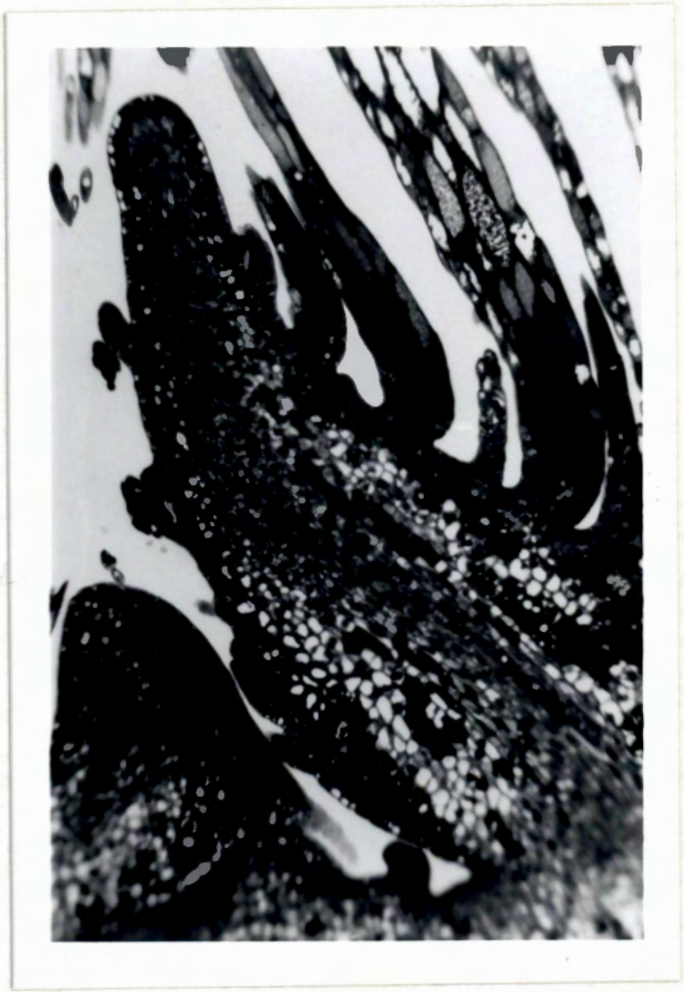

Fig. 16. The leaf primordia of the control sample taken at the interval of the 25th day and embedded in paraffin for comparison with Dikegulac-treated leaf primordia of azalea shoot tip. (Magnification, x62.5 enlarged x3)

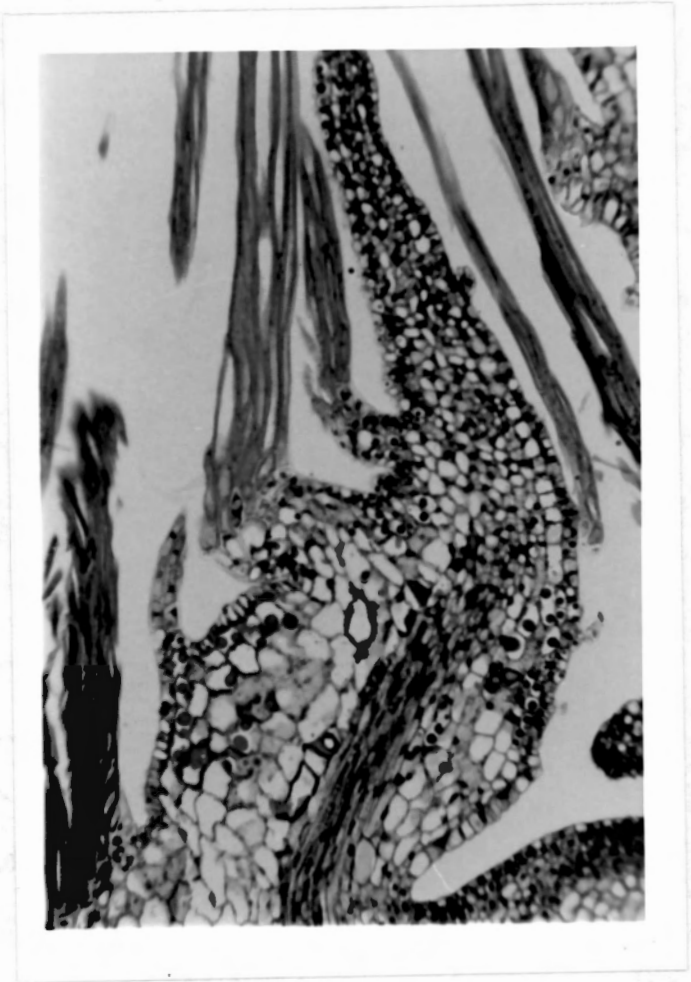

Fig. 17. Dikegulac-treated shoot tip taken 22 days after treatment and embedded in paraffin, showing the vacuolization in the cells of leaf primordia. (Magnification, x62.5 enlarged x3)

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