AUXIN INACTIVATION AND ITS RELATION TO LEAF DROP CAUSED BY THE FUNGUS OMPHALIA FLAVIDA [1]

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Omphalia flavida Maubl. & Rang. is the agent of the most destructive disease of coffee in the American tropics. Even conservative estimates by field workers indicate that the damages caused by this fungus in several Central and South American countries amount to an annual loss in coffee production valued at several million dollars. *O. flavida* is a member of the family Agaricaceae of the Basidiomycetes. Comprehensive modern surveys of the biology and pathological importance of this fungus have been given by Buller (1), by Wellman (14) and most recently by Sequeira (9).

The disease is characterized by the development, on the leaves of coffee and a wide range of other host plants, of round or slightly ovoid spots, 0.4 to 1.0 cm in diameter (fig. 1). The number of lesions per leaf varies from 1 to well over 100. In general, the main pathogenic effect of the fungus is the severe premature defoliation which it causes.

It has frequently been reported that in most cases the attacked coffee leaves which have fallen bear a lesion at the base of the leaf blade close to the petiole or on the petiole, whereas the leaves that remain attached to the tree may have a large number of lesions but none at the base of the blade. In general, a single lesion located at the point where the leaf blade begins to widen is enough to cause the leaf to drop. This effect has been generally interpreted, without experimental proof, as the result of mechanical destruction of the vascular tissue of the leaf by the fungus.

Our preliminary work on experimental defoliation of coffee in the field and, for comparison, of Coleus in the greenhouse, indicates that the defoliation caused by this fungus cannot be explained simply as a result of mechanical injury to the leaf. Further investigation has shown that the inactivation of plant growth hormones by *O. flavida* may be offered as a possible explanation of the leaf drop caused by this fungus. It is the purpose of this paper to present the evidence obtained from several lines of investigation which have led to this interpretation of the mechanism of the main pathogenic effect of the fungus.

EXPERIMENTAL RESULTS

EXPERIMENTAL DEFOLIATION: Extensive gross inoculations of coffee trees in plantations at Turrialba, Costa Rica, with the gemmae of *O. flavida* indicated that whenever the fungus caused a lesion at the base of a leaf, or on the petiole, the leaf always dropped within 5 days to 1 week from the time of inoculation. Similar experiments on *Coleus Blumei* in the greenhouse at Harvard, in which a large number of leaves were inoculated at the base of the blade close to the petiole, showed that the fungus readily attacked the leaves of this plant and that many infected leaves began to fall within 3 days, defoliation being completed within 6 to 9 days. The mature leaves always fell off before the younger ones. In both coffee and Coleus our controls indicated that intact leaves remained attached to the plants for 3 weeks or more after all of the inoculated leaves had fallen.

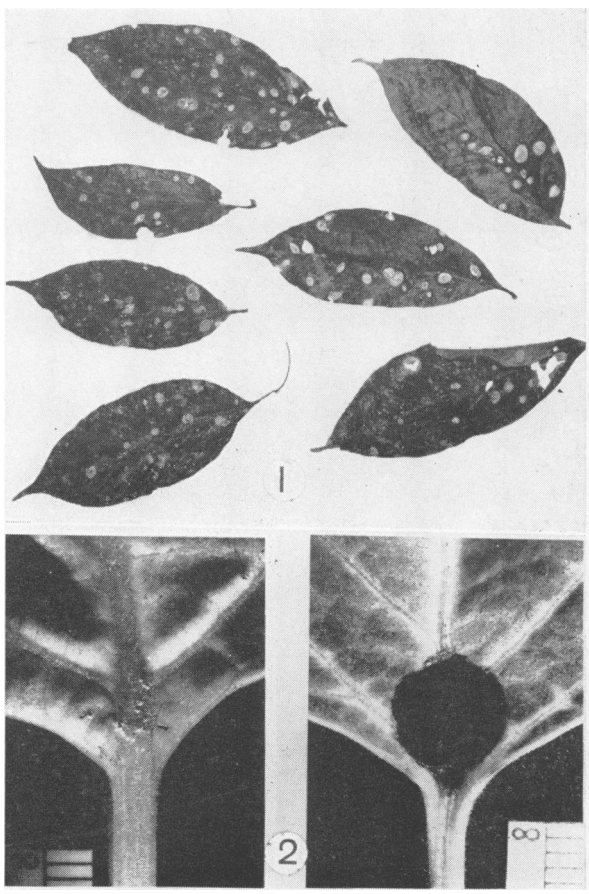

FIG. 1. Leaves of *Coffea arabica* L. collected at Turrialba, C. R., showing lesions resulting from the growth of *Omphalia flavida*.

FIG. 2. Comparison of the extent of injury in Coleus leaves produced by the growth of *Omphalia flavida* (left) and by mechanical perforation (right). The fungus-infected leaf fell 5 days after inoculation. The perforated leaf was still attached 18 days after treatment. (Scale in millimeters.)

In order to determine the effect of mechanical injuries similar in extent and location to the Omphalia

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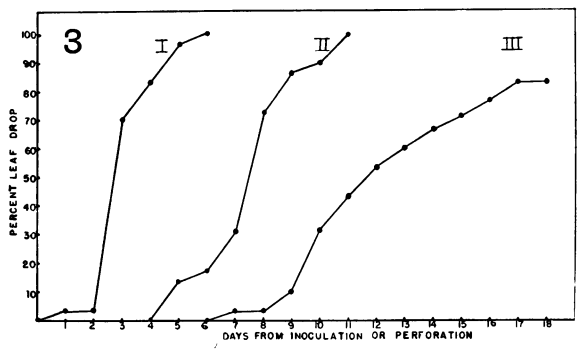
lesions which cause shedding of coffee and Coleus leaves, a large number of leaves of these plants were perforated at the base of the leaf blade by the use of a paper-punch. Figure 2 compares a punched leaf of Coleus with a leaf bearing a fungus lesion which caused the leaf to drop. In all cases, both in coffee and in Coleus, the perforated leaves remained attached for a period considerably longer than that for



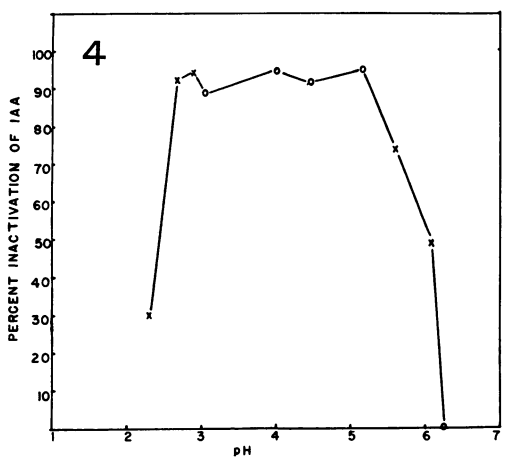


FIG. 3. Comparison of the rate of leaf abscission in Coleus induced by growth of *Omphalia flavida* at the base of the blade (I), growth by *Omphalia flavida* at the base of the blade with IAA-lanolin paste applied to the petiole below the lesion (II), and mechanical perforation at the base of the blade (III). In each group 30 leaves of comparable age were treated.

FIG. 4. The pH sensitivity of the auxin inactivation system in culture filtrate of *Omphalia flavida*. The curve combines the data of 2 separate experiments. The points obtained in each experiment are designated by a characteristic symbol.

the inoculated leaves. This is shown in figure 3 which represents the effect of these two treatments on groups of 30 Coleus leaves of comparable age. It is clear that no perforated leaves had fallen by the time all of the inoculated leaves had been shed, even though the perforation removed all, or almost all, of the vascular tissue and only a thin bridge of leaf tissue was left to provide a connection between the leaf blade and the petiole (fig. 2).

The work of LaRue (6), Myers (7), Gardner and Cooper (3) and others has shown that when the leaf blades of Coleus are removed, the petioles begin to fall off within 3 days, the process of abscission being complete for all petioles very soon afterwards. We have repeated and confirmed these observations under our greenhouse conditions. Similarly, Miss Lucy Hastings, pathologist at the Inter-American Institute of Agricultural Sciences, Turrialba, Costa Rica, has reported to us that in coffee, drastically debladed leaves fall off much more rapidly than intact leaves. In one experiment more than 57% of the debladed leaves fell within 5 days as compared with 100% retention of the intact leaves at the end of the same period.

It would appear, therefore, that in the rapidity of the resulting abscission, the effect of the fungus lesion resembles the effect of complete removal of the leaf blade. Laibach (4, 5) has shown that orchid pollinia, and an auxin paste prepared from orchid pollinia, are able to retard leaf abscission in debladed leaves of Coleus, and LaRue (6), Myers (7), and Gardner and Cooper (3) have obtained similar results with synthetic growth hormones. Myers (7) has cited further evidence indicating that leaf abscission is normally retarded in intact leaves of Coleus by plant growth hormones which diffuse down from the leaf blade. He has also demonstrated that these substances can move through the cells of the petiole even when the vascular system is severed. In a study of the abscission of leaflets of bean, Shoji, Addicott and Swets (10) have shown a correlation between a fall of the auxin concentration in the leaflet and the approach of abscission, and have indicated that an auxin gradient across the abscission zone, rather than the absolute amount of auxin present, controls abscission. Wetmore and Jacobs (16), working with Coleus, have concluded that the diffusible auxin from the leaf blade is the factor which normally controls the pattern of leaf abscission; but they favor the interpretation that auxin exerts an indirect effect upon the abscission layer.

We have been able to show that if an additional source of growth hormone in the form of a 0.5% indoleacetic acid in lanolin is applied to the petioles of leaves inoculated with *O. flavida*, below the lesion caused by the fungus, leaf drop can be delayed considerably. This is shown in figure 2, which represents the effect of this treatment on 30 Coleus leaves. In many cases the petioles of leaves treated in this way remained attached for some time after the leaf blades had dropped off as the result of extended decay in the region of the fungus lesion.

These preliminary experiments on defoliation clearly suggested that *O. flavida* caused leaf drop by interrupting, in other than a purely mechanical way, the normal flow of auxin from the leaf blade to the petiole.

AUXIN INACTIVATION IN VITRO BY OMPHALIA FLAVIDA: Two possibilities for preventing the passage of auxin to the petiole immediately suggested themselves: 1. that the fungus utilizes the growth hormone as a substrate and therefore metabolically destroys it and 2. that the fungus produces a substance which

in some way inactivates the auxin thereby preventing it from inhibiting the abscission process. We shall describe the experiments which we have performed in attempting to determine if either of these possibilities is indeed the correct explanation.

All experiments were carried out with strain T_4 of *O. flavida,* originally isolated from lesions on leaves of *Coffea excelsa* growing at Atirro (Turrialba), Costa Rica (9). The fungus was cultured in all experiments on a synthetic liquid medium which previous nutritional experiments (9) had indicated to be suitable for good growth. This medium contained:

Glucose	20.0 gm
Ammonium tartrate	9.2 gm
Potassium dihydrogen phosphate	5.8 gm
Magnesium sulfate	2.5 gm
Ferric chloride (1% sol.)	0.6 ml
Thiamine hydrochloride	100 μg
Distilled water	1000 ml

Each culture consisted of 40 ml of this medium in a 250-ml Erlenmeyer flask, inoculated with a number of gemmae of *O. flavida,* collected from young potato-dextrose agar cultures. The cultures were grown at room temperature for a period of 10 to 12 days on a reciprocating shake machine, at approximately 75 cycles per minute. The growth of the fungus under these conditions produces numerous spherical masses of mycelium dispersed in the medium.

In all experiments testing the capacity of the fungus to utilize growth hormones metabolically, or to inactivate them, synthetic indoleacetic acid (IAA) was used as the substrate. The determination of growth hormone in test solutions was carried out by means of the standard Avena test as described by Went and Thimann (15). On each occasion the curvature of a sensitivity standard of known concentration (25 μg IAA per l of agar) was determined, and these standards were used as a basis for the corrections necessary in comparing data from different experiments and in calculating absolute concentrations of growth substance in test solutions.

At the outset of these experiments it was shown (table I) that neither the culture medium (column A) nor a filtrate of medium in which fungus has grown (column B) possess any significant growth promoting activity. In order to test for auxin inactivation by fungus growth, IAA was added to the medium before inoculation to give a concentration of 250 μg/l. When this culture filtrate was tested for growth hormone activity 10 days after inoculation it gave no significant curvature (column D, table I), indicating a complete disappearance of the IAA. If IAA in the same concentration was maintained for 10 days in the liquid medium under the same conditions but not inoculated with *Omphalia flavida,* the medium showed a high degree of activity at the end of this period, as is shown in column C of table I.

Further experiments not reported in detail have indicated that the inactivation of IAA by the fungus probably occurs within 2 to 3 days. The growth of the fungus for a period of 10 days changes the pH of the medium from approximately 5.0 to approximately 3.75. It was shown that this increase of acidity is not responsible for the inactivation by adjusting medium containing IAA to pH 3.5 with 1 *N* HCl, autoclaving and allowing to stand on the shake machine for 5 days. A test at the end of this time showed no loss of auxin activity.

In order to determine the effect of metabolic products of the fungus on IAA in the absence of the fungus, the mycelia of 12-day-old cultures were carefully filtered out; to the remaining liquid IAA was added, and the mixture was incubated at room temperature for 1 hour in most cases. At the end of this period the preparation was heated to 95–100°C for 10 minutes, or autoclaved at 15 pounds pressure for an equal length of time to inactivate the enzymes.

TABLE I

AUXIN CONTENT, EXPRESSED IN DEGREES OF CURVATURE OF AVENA PLANTS, OF CULTURE MEDIUM, CULTURE MEDIUM IN WHICH *O. flavida* HAS GROWN FOR 10 DAYS (CULTURE FILTRATE), CULTURE MEDIUM CONTAINING 250 μG/L IAA,* CULTURE MEDIUM CONTAINING 250 μG/L IAA IN WHICH *O. flavida* HAS GROWN FOR 10 DAYS

TEST NO.	CULTURE MEDIUM (A)	CULTURE FILTRATE (B)	CULTURE MEDIUM WITH IAA ADDED (C)	CULTURE FILTRATE WITH IAA ADDED (D)
1	0.0 ± 0.0	1.0 ± 0.5	23.3 ± 2.1	1.1 ± 0.4
2	0.6 ± 0.3	0.5 ± 0.3	21.2 ± 1.3	0.9 ± 0.5
3	0.7 ± 0.4	0.7 ± 0.4	21.0 ± 1.3	0.3 ± 0.2
4	0.9 ± 0.5	3.3 ± 1.2	23.6 ± 1.5	0.8 ± 0.3
5	0.9 ± 0.3	1.9 ± 0.7	25.5 ± 2.4	1.5 ± 0.5
6	.....	.....	35.2 ± 2.5	0.4 ± 0.3
7	.....	.....	.....	0.7 ± 0.5
Average †	0.6	1.5	26.1	0.8

* The concentration in the test blocks used in the Avena test is one-half that of the original solution because of dilution with 3% agar in the preparation of the blocks.

† In computing averages all curvatures were corrected on the basis of a sensitivity standard of 12.0 degrees.

The results, shown in column A of table II, indicate that the culture filtrate can inactivate up to 500 μg/l of indoleacetic acid completely in the time allotted. The small remaining amounts of auxin in the table are not significant, since concentrations of less than 10 μg/l (5 μg/l in final test dilution) are below the effective sensitivity of the Avena test.

The cytoplasmic contents of the mycelium of *O. flavida* show the same auxin-inactivating properties as those described for the culture filtrates. A water extract of carefully washed mycelium, ground in a Waring microblender, was as effective as the culture filtrates in the inactivation of IAA. When IAA (50 μg/l) was added to the mycelium extract and this mixture was assayed for auxin activity at the end of 1 hour, a remaining concentration of 3 μg/l was found.

The inactivation of IAA by Omphalia culture filtrate can be completely prevented if this filtrate is

TABLE II

INACTIVATION OF IAA BY CULTURE FILTRATE OF *O. flavida* AND THE EFFECT OF HEAT UPON THIS INACTIVATION. THE INCUBATION PERIOD IS ONE HOUR EXCEPT WHERE NOTED

TEST NO.	CONCENTRATION OF ADDED IAA IN μG/L	CONCENTRATION OF IAA IN μG/L	
		FILTRATE INCUBATED WITH IAA	HEATED FILTRATE INCUBATED WITH IAA
		A	B
1	50	6	54
2	50	4	57
3	50	3	50
4	50	7	...
5	50	7	...
6	250 *	1	146 †
7	250	3	78 †
8	250	2	...
9	250 *	7	...
10	500	7	270 †

* Incubation period twelve hours.
† Represents maximum or nearly maximum curvature of Avena plants for the day and therefore does not necessarily indicate partial inactivation.

heated to 95–100°C for 10 minutes or autoclaved at 15 pounds pressure for an equal length of time, before adding the growth hormone. Some typical results of this treatment are shown in column B of table II and may be compared with the results obtained from unheated filtrate (column A) on each occasion.

Culture filtrates dialyzed in cellophane tubing for 18 hours, under constant agitation, are as effective as the undialyzed filtrates in the inactivation of IAA. For example, in two experiments in which IAA at an initial concentration of 50 μg/l was incubated for 1 hour with dialyzed fungus filtrate, the final concentrations measured were 5 μg/l and 2 μg/l, indicating essentially complete inactivation.

It seemed desirable to determine whether this heat-labile, nondialyzable system is pH sensitive. Inactivation tests, using 2-hour incubation periods, were carried out in a pH range of 2.3 to 7.2 in citric acid–disodium phosphate buffers. In each test equal volumes of dialyzed culture filtrate and buffer of the desired pH were mixed and IAA was added to a final concentration of 250 μg/l. The actual pH was then determined. The results of these studies are shown in figure 4. While it is not possible to determine a pH optimum for the system, it is clear that the system is pH sensitive, showing a high degree of activity between pH 2.65 and pH 5.60, and a rather striking decrease of activity on either side of these values.

EFFECT OF ENZYME INHIBITORS: Since the foregoing experiments have strongly suggested that the auxin-inactivating system in Omphalia culture filtrate is enzymatic in nature, an attempt was made to obtain a preliminary characterization of the system by testing the effects of several enzyme inhibitors upon the ability of the system to inactivate IAA. The results of these experiments are shown in table III in which the percentage of the original IAA concentration which was inactivated in the presence of the inhibitor is shown. In each experiment the inhibitor was added to the culture filtrate before the addition of IAA. Some difficulty was encountered in these experiments because of an unexplained variation in auxin-inactivating ability between different batches of culture filtrate. It was consequently difficult to select suitable concentrations of IAA or of inhibitor to show the effect of the inhibitor to best advantage. In addition certain of the inhibitors exerted a depressing effect upon the curvature of the Avena plants.

The most striking inhibition effect noted in these experiments was that produced by the reducing agents ascorbic acid and cysteine at a concentration of 1 gram per liter (table III, exps. 2, 3, and 4). These two substances afforded essentially complete protection for IAA and suggest that an oxidative enzyme is responsible for auxin inactivation by the culture filtrate. Moreover, if nitrogen gas is bubbled through the reaction mixture during the incubation period, the destruction of the growth hormone is considerably reduced (table III, exps. 5 and 6).

Potassium cyanide gives a rather variable degree of inhibition of the enzyme, but seems to afford at least partial protection to the growth substance. The results are somewhat complicated by the fact that potassium cyanide reduces the curvature of the Avena test even though ferrous sulfate is added to the reaction mixture after incubation to remove excess cyanide ions. Hydroxylamine also partially inhibits the activity of the enzyme, but is somewhat harmful to the Avena plants so that its full effectiveness cannot be evaluated.

These preliminary experiments with enzyme inhibitors would seem to indicate that auxin inactivation by Omphalia culture filtrate is carried out by an

TABLE III

THE INFLUENCE OF SEVERAL ENZYME INHIBITORS ON THE INACTIVATION OF IAA BY CULTURE FILTRATE OF *O. flavida*. THE INCUBATION PERIOD IS ONE HOUR EXCEPT WHERE NOTED

TEST NO.	INHIBITOR	CONCENTRATION OF IAA IN μG/L	PERCENTAGE INACTIVATION OF IAA
1	None	50–500	ca. 100 *
2	Ascorbic acid 1 gm/l	50	0.0
3	Cysteine 1 gm/l	50	2.6
4	Cysteine 1 gm/l	50	0.0
5	N_2 †	250	36.4
6	N_2 †	250	40.9
7	KCN .005 M	50	60.0
8	KCN .01 M	50	76.8
9	KCN .01 M	250 ‡	24.5
10	$NH_2OH \cdot HCl$ .005 M	50	45.0
11	$NH_2OH \cdot HCl$ .01 M	250 ‡	51.0

* Based on the experiments recorded in Table II.
† Water pumped nitrogen, 99.6% N_2.
‡ Incubation period two hours.

oxidative enzyme system which is only partly inhibited by cyanide and hydroxylamine.

Discussion

The inability of mechanical injury to duplicate the abscission-promoting effect of *Omphalia flavida* and the effectiveness of added growth hormone in preventing abscission of infected leaves of Coleus strongly suggest that the fungus prevents the normal flow of auxin from the blade into the petiole by other than mechanical means. An experimental demonstration of the ability of the fungus grown *in vitro* to inactivate IAA has been given. A similar action of the fungus in the leaf of the host would provide a reasonable explanation of its abscission-promoting properties, and it is suggested that this is the mechanism of the main pathogenic effect of the fungus.

Several attempts were made to provide further evidence for this conclusion by demonstrating auxin inactivation by *O. flavida* in Coleus leaves in comparative diffusions from infected and uninfected leaves. The results of these experiments were inconclusive, however, because the yields of auxin obtained even from uninfected leaves were below the effective sensitivity of the Avena test. Myers (7) had obtained similar low yields from Coleus leaves at certain seasons of the year. Since such low auxin yields are sufficient to retard abscission of Coleus leaves, a direct demonstration that *O. flavida* does promote abscission by inactivating auxin in the leaf will be difficult to obtain.

Previous work on the relationship between fungi and plant hormones has dealt, as far as we are aware, with the production of auxin by these organisms, or with the response of the organisms to added auxin, rather than with auxin inactivation. It has been shown that numerous fungi and bacteria produce auxin in culture (see review by Went and Thimann (15), pp. 71–72). Recently Wolf (17) has identified IAA as a metabolic product of *Ustilago zeae* and has correlated its production with the tumor formation in the host induced by the fungus. Synthetic growth hormones are also reported to produce both stimulatory and inhibitory effects upon the growth of various microorganisms, the specific response depending upon the organism tested and the concentration used (8).

Since the filtrate of a culture in which *O. flavida* has grown is capable of inactivating added IAA, it is clear that the auxin is not directly metabolized by the fungus but rather that the organism produces and releases into the substrate, a substance which is capable of inactivating the auxin. The substance is also present within the cells of the fungus and we have not established whether it is actively secreted into the medium or is released through the process of autolysis.

Since the IAA inactivating substance is heat-labile, non-dialyzable, and pH-sensitive, we have concluded that it is an enzyme. Its pronounced inhibition by the reducing agents ascorbic acid and cysteine, and also by a nitrogen atmosphere strongly suggest that it is an oxidative enzyme. The sensitivity of the enzyme to the inhibitors potassium cyanide and hydroxylamine, which seems reasonably well established, might suggest that the enzyme involved is a peroxidase, but the pyrogallol test of Sumner and Gjessing (11) showed no peroxidase activity in the culture filtrate. Since pyrogallol is not oxidized by the culture filtrate, it is unlikely that the enzyme present is a polyphenol oxidase.

Auxin-inactivating enzymes have thus far been described only for the higher plants. There have been many reports of enzymatic auxin inactivation at cut surfaces and in crushed tissue extracts, but relatively few thorough studies of the actual systems involved. Tang and Bonner (12) have described an "indoleacetic acid oxidase" from etiolated seedlings of pea which they believe to be an iron-containing protein. Wagenknecht and Burris (13), on the other hand, have concluded that this same enzyme is a copper-containing protein, and they have reported a similar enzyme from the root sap of beans. The exact nature of the enzyme is apparently still in dispute. More recently Galston (2), in connection with an investigation of the biological significance of riboflavin, has re-examined Tang and Bonner's "indoleacetic acid oxidase" and believes it to be composed not of a single enzyme but of a light activated flavoprotein enzyme and a peroxidase. The flavoprotein is believed to produce hydrogen peroxide which is then utilized by the peroxidase in the oxidation of IAA.[4] It was considered likely that IAA is also a substrate for the flavoprotein.

Our study of the auxin-inactivating enzyme produced by *O. flavida* is not sufficient to permit any detailed comparison with the enzymes described from higher plants. It may be noted, however, that the pH sensitivity range of 2.65–5.60 for the inactivating system of the fungus differs from the sharp optimum between 6.2 and 6.7 described by Tang and Bonner (12) and 6.0 and 6.5 described by Wagenknecht and Burris (13). It seems unlikely that it could be a system of the sort described by Galston (2) because we were unable to obtain any evidence of peroxidase activity in the culture filtrate. It is not unreasonable to expect the enzyme system of *O. flavida* to differ from those of the higher plants in view of the vastly different relationships of the source organisms and the very different mode of action. Work is now in progress to obtain a more definite characterization of the enzyme involved.

Summary

Omphalia flavida exerts its pathogenic effect on coffee and other hosts by bringing about severe premature defoliation. In many cases rapid leaf drop results from the presence of a single lesion at the base of the blade or on the petiole. Mechanical injury of comparable extent does not accelerate leaf abscission in coffee or in Coleus to the same degree. Abscission of Coleus leaves inoculated with the fungus can be

[4] In a further report, published since the present paper was submitted (Galston, A. W., J. Bonner and R. S. Baker, Arch. Biochem. 42: 456–470. 1953), this interpretation has been extended and confirmed.

delayed by application of synthetic IAA to the petiole below the lesion. This evidence suggests that the fungus accelerates abscission by preventing the normal flow of auxin from the blade to the petiole.

O. flavida inactivates IAA included in its culture medium; and culture filtrate, in the absence of mycelium, has been found to inactivate up to 500 μg/l of IAA in an hour. Extracts of ground mycelium possess the same auxin-inactivating properties.

Preliminary studies on the auxin-inactivating substance from Omphalia show that it is heat-labile, non-dialyzable, and pH-sensitive. It is strongly inhibited by ascorbic acid, cysteine, and a nitrogen atmosphere, and appears to be somewhat sensitive to potassium cyanide and hydroxylamine. It is concluded that it is an oxidative enzyme, but probably not a peroxidase or polyphenol oxidase. It is not clear whether the enzyme is actively secreted or is released by autolysis.

It is suggested that this auxin-inactivating enzyme is responsible for the main pathogenic effect of *Omphalia flavida*.

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DIRECT CHEMICAL PROOF OF ETHYLENE PRODUCTION BY DETACHED LEAVES[1]

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Ethylene production by leaves has long been suspected. Using potato leaf epinasty as a qualitative bioassay, Denny and Miller (3) showed production of an active emanation, presumably ethylene, by detached leaves of dandelion, rhubarb, calla, hollyhock and leafy stalks of peony. This work was extended by Denny (2), using chopped leaves of rhubarb, lettuce, potato, tomato, onion, Virginia creeper and rose. Nelson and Harvey (8) showed that chopped stalks and leaves of Golden Self-Blanching celery caused tomato leaf epinasty, while those of Winter Queen (a green celery) did not. Hall (5) used a non-specific chemical test as evidence for ethylene production by healthy detached leaves of rose and cotton.

Similar studies have been made with leaves affected by various agents. Williamson (13) showed that various kinds of leaves suffering from mechanical injury and leaves infected with various fungi produced appreciably more active emanation than did healthy intact leaves. Ross and Williamson (11) got similar results with leaves from virus-infected plants and with leaves injured by phytotoxic chemicals. Jackson (7) and Hall (6) observed increased production of ethylene by cotton leaves treated with defoliating agents. Gawadi and Avery (4), after confusing ethylene and ethylene chlorohydrin, suggested that ethyl-

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