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THE HISTOCHEMICAL LOCALIZATION OF PEROXIDASE IN ROOTS AND ITS INDUCTION BY INDOLEACETIC ACID¹

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Recent evidence (2) has indicated that the peroxidase activity of certain plant tissues rises significantly following the administration of indoleacetic acid (IAA). This phenomenon appears to be related to the induced formation of the IAA-oxidase system (4), of which peroxidase is one component (3). Since peroxidase has recently been shown to be involved in

the biosynthesis of lignin (1, 6), the induced formation of this enzyme would appear to have morphogenetic significance.

The purpose of the present paper is to localize in the root those cells and tissues that contain peroxidase, both induced by IAA and non-induced, and to investigate the physiological role of the induced peroxidase in the development of the cell.

Two different histochemical methods were employed. The first involved the use of freshly excised sections and the determination of peroxidase activity

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in vivo using conventional biochemical methods on a micro-scale. This approach, while yielding quantitative data, indicates only indirectly which cells participate in the reaction. The second method localizes the enzyme by employing a substrate which yields a colored, insoluble end product. This method, although it indicates the active cells, is only qualitative. Both methods have been used in the present work and their combination offers a quantitative approach at the cellular level to certain problems of cellular growth and differentiation.

METHODS

The seeds of *Vicia faba* var. broad Windsor were treated and grown as previously described (5). The seeds were soaked, seed coat removed, and the imbibed embryos placed in moist vermiculite. The growth rate of the primary root is dependent on a large number of factors. Therefore, selection of root tips for experimental work was not based on the age in terms of days after planting but rather on physiological age based on the growth pattern of the root. During the first stages of growth the growing tip of the primary root is relatively thick and somewhat stubby. After the root has attained a length of 2 to 3 cm the growing tip becomes smaller in diameter and then retains this thickness more or less constantly until a period of secondary root formation occurs. Under the growth conditions provided, secondary roots appeared when the primary root was from 8 to 10 cm long. The tips used, then, were from primary roots 3 to 8 cm in length or after initial decrease in diameter and before secondary root formation took place.

Roots were treated with IAA in three ways: 1) the roots of intact seedlings were immersed in an IAA solution in 0.02 M phosphate buffer, pH 6.1; 2) the first 2 cm of the tip was removed and placed in the IAA solution; and 3) the first 3 mm of the root were cut into 200-micron sections and these placed individually in IAA solutions. The duration of IAA treatment was 2 hours. At the end of this time the intact tips (procedures 1 and 2) were washed and cut into 200-micron sections by the method of Jensen (2).

The quantitative determination of peroxidase activity per section was made by placing the 200-micron sections in 0.5 ml of 0.02 M phosphate buffer, pH 4.5. To this was added 0.05 ml 0.1 M H_2O_2 and, at zero time, 0.05 ml of 0.01 M pyrogallol. The reaction was allowed to run 2 minutes. The solution was then rapidly removed from the tissue by a straight, drawn tip pipette and 0.5 ml 1% HCl-acetone added to the tissue. The absorption of the original solution and in the HCl-acetone was determined at 420 $m\mu$ in a Beckman spectrophotometer model DU with a micro-cuvette attachment. The results are expressed in micromoles purpurogallin produced per section divided by the number of cells in the section.

The localization of peroxidase activity in the sections was achieved by placing the 200-micron section in phosphate buffer at pH 4.5 containing 0.1 M H_2O_2

and 0.1 M guaiacol or benzidine. Activity was manifested as a red color with guaiacol and a blue color with benzidine substrate. Control experiments were performed in which either H_2O_2 or substrate were withheld or in which azide and heating the tissue at 90° C for 5 min were used to inhibit peroxidase activity. Such control sections produced no color over short periods and only little over long periods.

The localization procedures, although imperfect in the sense that the colored end products of peroxidase action are not completely insoluble (8) are adequate for localization of activities in particular cells. They are probably unreliable for intracellular localization, and therefore no attempts have been made in the present work to achieve localization within the cell.

RESULTS

MORPHOLOGICAL ANALYSIS: The first 3 mm of the primary root of *Vicia faba* have been previously analyzed in detail (2). The longitudinal diagram of the root in figure 1 indicates the main morphological features of the apex of the root. Six selected 200-micron sections were analyzed for number of cell types or tissues present; volume of each cell type or tissue; cross-sectional area, height, and volume of average cell in each cell type or tissue; number of cells of each cell type or tissue; and total number of cells in each 200-micron section. The complete method of analysis, and results, together with a detailed discussion of their meaning, have been previously presented (5). The number of cells in the various cell types or tissues is presented in figure 3. The decrease in number of cells in the more basal sections of some tissues is the result of increased elongation, decreasing the number of cells in a 200-micron section. The other data from the cell analysis can be summarized by noting that between the general meristem and about 1700 microns behind it the cells undergo rapid division, extensive radial enlargement and limited elongation. Above 1700 microns division and radial enlargement markedly decline while elongation increases rapidly.

QUANTITATIVE DETERMINATION OF PEROXIDASE ACTIVITY: The results of the quantitative determination of peroxidase activity are presented in figure 1. The first fifteen 200-micron sections of the tip were analyzed for peroxidase activity after the root had been treated with IAA by the three different methods. The results are expressed as activity per cell against distance from tip of the section analyzed. Each curve represents at least 4 sets of determinations. The experimental values from these determinations were normally a 10% range. Occasionally a set would be 20% higher but with the identical pattern so that when it was superimposed on the other values, without exception the experimental points fall within the 10% limits. When older roots which had begun formation of secondary roots were inadvertently used it was found they had a different pattern than the 3 to 8 cm roots normally used. It is felt that the roots below 3 cm in length would also show a different pattern. These changes in pattern were not investigated.

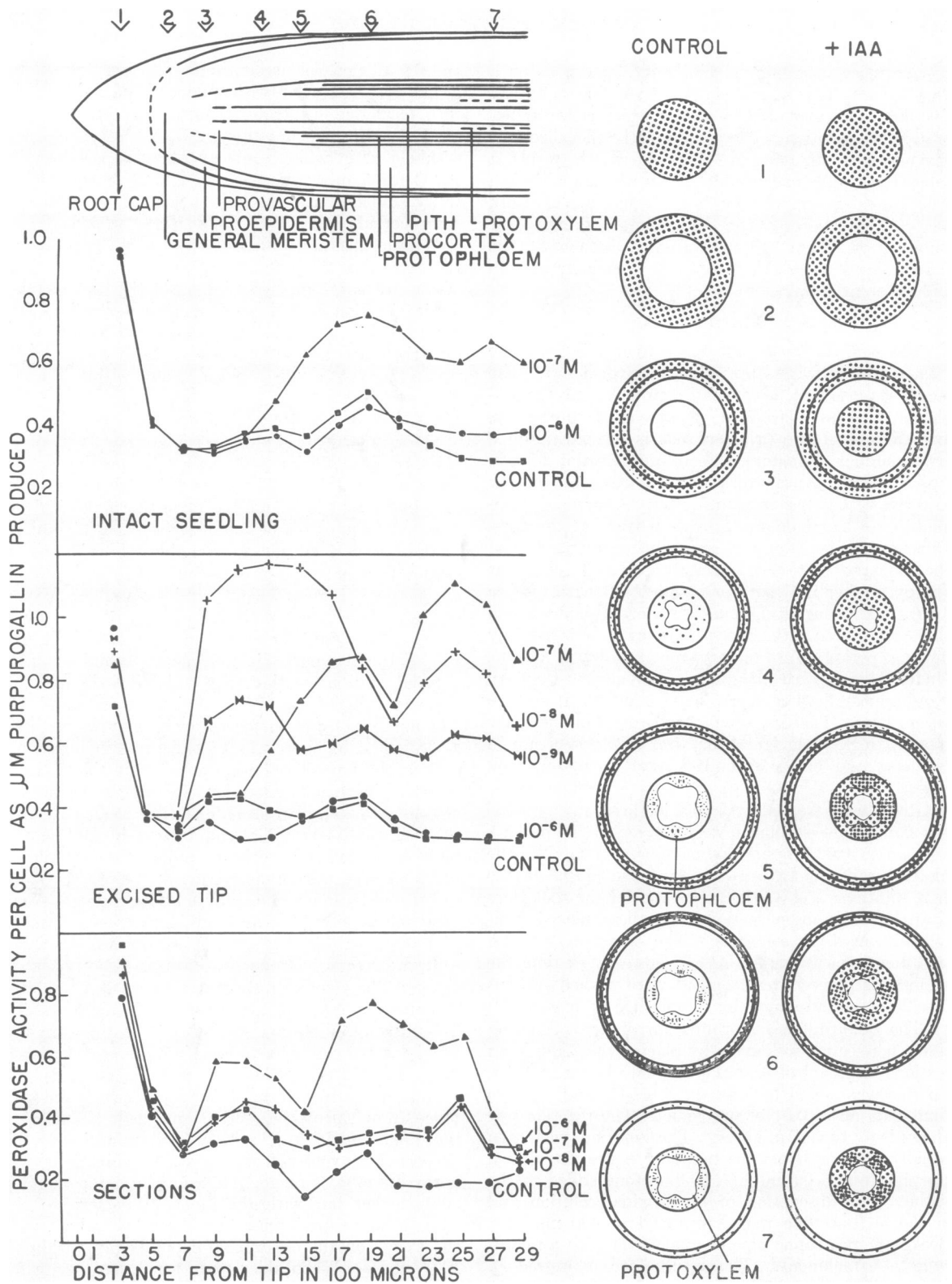


FIG. 1. *Left.* Peroxidase activity per cell as a function of distance from tip of section analyzed, after various treatments of the tips with IAA. See text for examination. Diagram of root on top same scale as graphs. *Right.* Localization of peroxidase in control and IAA $10^{-7}M$ treated roots. The shaded areas indicate a positive reaction with the intensity of the shading approximating the intensity of the reaction. The number of the sections corresponds to the number on the longitudinal diagram at left.

Considering first the data obtained when excised root tips were treated with IAA, it is clear that the region of the general meristem has little peroxidase activity and is unaffected by IAA. Further, while the cells of the root cap have high peroxidase activity, they similarly show no response to IAA. However, in all other areas of the root the cells show increase in peroxidase over the control after treatment with lower concentrations of IAA.

The control sections basal to the general meristem exhibit two maxima in peroxidase activity. The first corresponds with the appearance of the proepidermis and the provascular tissues, while the second corresponds with the formation of the protophloem. IAA at 10^{-6} M raises the first maximum but does nothing to the second. At 10^{-7} M IAA, the first maximum is increased above the control but not above that elicited by 10^{-6} M IAA, the second maximum is increased approximately 100% and a third maximum appears. This third maximum corresponds with the appearance of the first forming protoxylem and represents a 200% increase in activity over the control. Treatment with 10^{-8} M IAA causes a large increase in peroxidase activity directly behind the general meristem that obscures both the first two maxima. This large broad maximum corresponds with the early development of the provascular tissue and the protophloem. The additional maximum corresponds, as at 10^{-7} M, to the developing protoxylem. When 10^{-9} M IAA is used the large broad maximum becomes a double maximum, corresponding to provascular and proepidermis formation and to the development of the protophloem. Finally, the third maximum in the region of the protoxylem formation is present but smaller.

Thus, with changing IAA concentration there is a shift in maximum peroxidase induction from one region of the root to another. This shift can be better seen in figure 2 where a three-dimensional graph has been constructed from the excised tip data of figure 1. For clarity 5 sections are plotted instead of 15 and the drawing of the root indicates the regions in which these sections are located. The maximum in response to IAA treatment shifts from 10^{-8} M at the 900-micron level (area of provascular differentiation) to 10^{-7} M at the 2700-micron level (area of protoxylem differentiation). The region of protophloem development shows a slight maximum at 10^{-8} M IAA but both 10^{-7} and 10^{-8} M IAA elicit high response.

When the data from the sections treated with IAA (lowest graph, fig 1) are examined, a quite different relation of IAA to peroxidase is found. The control sections still have two maxima in peroxidase activity but the magnitude of these are shifted so that now the first is larger than the second. Thus, there is a trend in activity downward from the general meristem. This is interesting in the light of the process of self-induction discussed by Galston (2). When the root is sectioned and its continuity lost, the auxin producing site, which appears to be the general meristem, is detached from the basal areas. These areas

are now no longer supplied with native auxin and in 2 hours, as auxin content declines, peroxidase activity decreases.

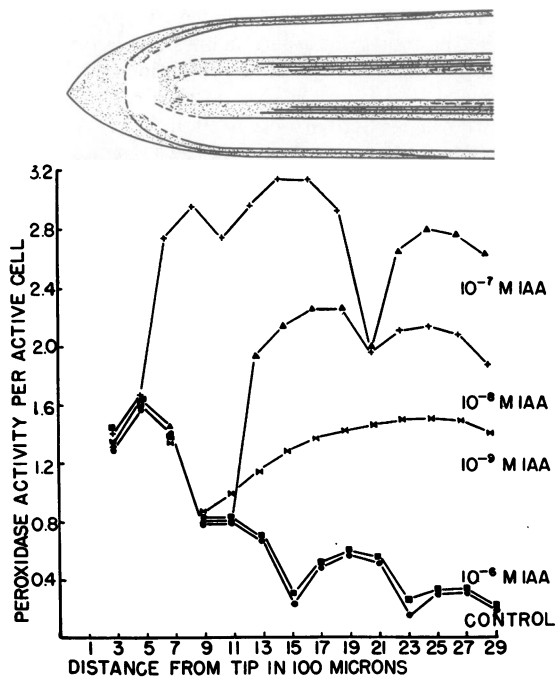
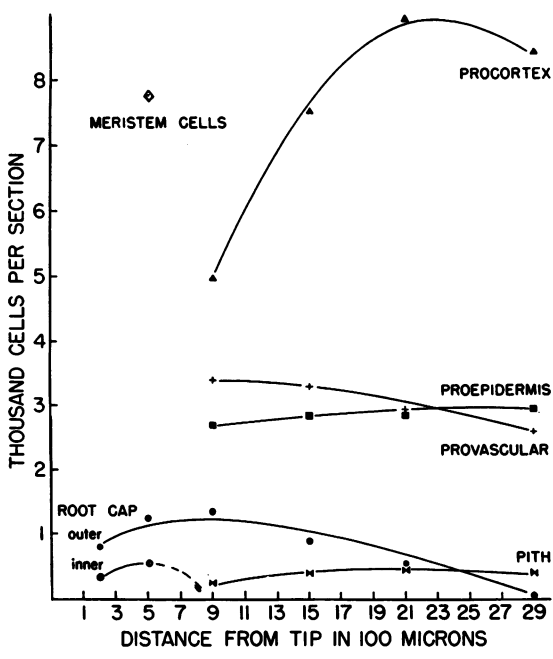
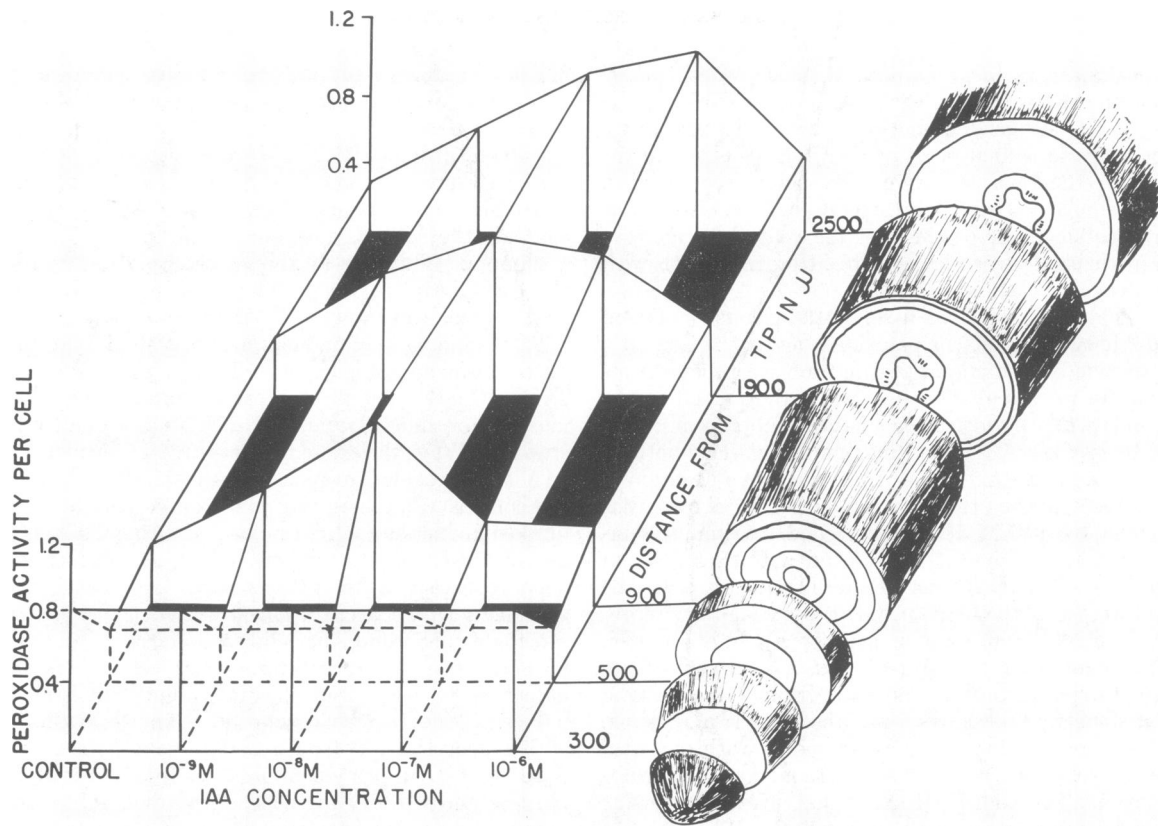
When 10^{-7} and 10^{-8} M IAA is supplied to these sections there is an increase in the first maximum, a general rise in activity that smooths and generally conceals the second, and the appearance of a third maximum. This last maximum corresponds, as with the excised tip, to the beginning of the protoxylem. In all cases, however, with these concentrations of IAA, the amount of induction is less than that produced with the excised roots. Treatment with 10^{-6} M IAA changes the picture drastically. The three maxima are much larger and induction is greatly increased. When the excised tip was treated with 10^{-6} M IAA the peroxidase response was little different from the control, while here the same concentration causes marked induction. One or both of two factors may be involved. First, the native auxin concentration may decrease in the sections and, therefore, higher exogenous IAA concentrations are needed to show the same effect. Second, the cells responding to induction may require substances from other cells to permit induction to occur but, lacking them, can still be induced if the IAA concentration is high enough.

To complete the picture, the roots of seedlings were treated (upper graph, fig 1). In a sense this is the least satisfactory method, as the root is receiving all of its nutrients from the cotyledons and any compound administered constitutes only a small addition. It is therefore not surprising that while the intact root responds at 10^{-7} M IAA, the response is less. The pattern of induction is roughly the same as in the other treatments except that the first maximum is very much lower.

LOCALIZATION OF PEROXIDASE ACTIVITY: The preceding analysis gave strong indications of which cells were active but by indirect rather than direct evidence. By employing localization procedures with guaiacol or benzidine as a substrate, one can visualize the cells that possess peroxidase activity. This has been done and the results are presented as the series of cross section diagrams in figure 1. The control and +IAA roots were treated as in the case of the excised root for quantitative determination up to the time of localization. The IAA concentrations were 10^{-7} and 10^{-8} M.

The results indicate that 1) not all cells at each area are reactive; 2) induction does not cause new tissues to become active but rather increases the activity and number of cells within the tissues that show some activity in the control; 3) those areas of the provascular tissue in the IAA treated roots that will form protophloem and protoxylem are distinguishable by their peroxidase activity before they are morphologically visible in the control.

With or without exogenous IAA the cells of the general meristem, procortex, and pith show no peroxidase activity. This does not completely exclude the possibility that they may have very low activity that is not apparent in these tests. Those cells that show



peroxidase activity compose the root cap, proepidermis, and provascular tissues, including the protophloem and the protoxylem. After IAA treatment the provascular tissues show more intense and extensive activity. The proepidermis may also be more active but not clearly so, as with the provascular tissue. Within the provascular tissue those cells that will compose the protophloem and protoxylem are much more reactive and, in both cases, are identifiable on the basis of their peroxidase activity closer to the tip than it is possible to identify them in the control. The cells that will form the endodermis are clearly delineated by their peroxidase activity approximately 2700 microns from the tip while the endodermis is not distinct morphologically for several more centimeters.

PEROXIDASE ACTIVITY PER ACTIVE CELL: In figure 1 the peroxidase activity is expressed on the basis of the total number of cells in the section. But from the localization work it is clear that not all the tissues in any section are active. Since we already know the number of cells in the different tissues in each section (fig 3), we may calculate peroxidase activity per active cell by dividing the total activity of the section by the number of cells in the active tissues. This has been done for the excised tip data and the results are presented in figure 4. The data now form a series of discontinuous curves rather than a single continuous one. The maxima of the various curves show a remarkable correlation with the appearance of certain cell types. A maximum in activity now appears at 500 microns where the other calculations indicate a minimum. This is a result of the cells of the general meristem being inactive and thus masking the activity of the root cap cells.

The conclusions from figure 4 are: 1) the cells of the root cap, proepidermis, and provascular tissues including the protophloem and protoxylem each have a period of maximum peroxidase activity, and 2) only the cells of the vascular tissues seem capable of induced peroxidase formation.

All the tissues mentioned above have cells with heavy cell walls, but only the vascular tissue, which is capable of induced peroxidase formation, has appreciable quantities of lignin in the mature cell wall. When mature portions of the root of *Vicia faba* are stained for lignin, elements of the phloem and xylem as well as the Casparian strip of the endodermis react.

PEROXIDASE ACTIVITY AND LIGNIN FORMATION: The correlation of the cells that show induced peroxidase formation with the cells that form lignin led to attempts to accomplish lignin synthesis in the sections. Siegel (6) has shown that lignin is biosynthesized by the action of peroxidase-H₂O₂ on hydroxy-

phenylpropane precursors such as eugenol. The excised root tips were therefore incubated for 2 hours with 10⁻⁷ M IAA or buffer at pH 6.1 and then cut into 200-micron sections. The sections were individually placed in 0.5 ml, pH 4.5 phosphate buffer that contained 0.1 M eugenol and 0.1 M H₂O₂. After one hour the sections were washed, chlorinated with acidified sodium hypochlorite, washed and placed in sodium sulfite solution. After a few minutes they were washed and either mounted on a slide in dark Karo syrup or dehydrated and mounted in Permount. With this staining procedure lignin should be colored red.

The sections thus treated clearly show that all the cells that have peroxidase activity form a brown compound that is associated with the cell wall. It is believed that this compound is the same as the intermediate in lignin synthesis discussed by Siegel (6). It has the same solubility characteristics as lignin and its formation is inhibited by cyanide and azide. Control tissue from which either eugenol or H₂O₂ was withheld did not produce the compound.

The vascular tissue of the control had a few cells that showed lignin formation, namely elements of the phloem, xylem, and endodermis. The vascular tissue of the IAA treated roots, on the other hand, showed lignin formation in almost all of the cells of the protophloem and protoxylem. The endodermis also showed intense activity.

DISCUSSION

The IAA-induced formation of peroxidase appears to be a possible factor of great importance in cellular differentiation, particularly of vascular tissue. It is significant that the cells respond very early in their development, often before there is any way of identifying them morphologically as different. That the induced peroxidase appears involved in lignin synthesis is of interest as it indicates a physiological role for the enzyme in a process that later manifests itself morphologically.

The observations on lignin formation help explain one of the most common morphological effects of IAA on roots, namely the occurrence of vascularization and lignification closer to the root meristem (7). The sequence of events would appear to be that the cell in the presence of increased IAA forms a system which destroys IAA; part of this system is a peroxidase that is also active in the formation of lignin. Hence, if a peroxide and eugenol type compound are present, lignin may be formed in cells previously exposed to auxin.

Several questions still remain unanswered. First, why is not mature lignin, i.e., the form that will give

FIG. 2 (top). Effect of various concentrations of IAA on the peroxidase activity in various regions of the root. The drawing of the root indicates the area of the root from which the sections analyzed were taken.

FIG. 3 (bottom, left). The number of cells in the various tissues as a function of distance from tip. The root cap cells, the proepidermis and the provascular cells show peroxidase activity.

FIG. 4 (bottom, right). Peroxidase activity per active cell as a function of distance from tip of the root of the section. Diagram of root at top is on the same scale and shading indicates those tissues showing peroxidase activity.

the typical lignin staining reaction, formed? It may be that another necessary enzyme is absent in this area of the root or that eugenol is not a completely satisfactory substrate for the reaction. Second, if the root cap and epidermis have the potentiality for lignin production why do they not make it normally? The answer may be the lack of either a satisfactory eugenol-type substrate, or a peroxide, or the presence of inhibitors.

Since the induction of any new peroxidase activity probably involves protein synthesis, it is instructive to examine available data on the locale of protein synthesis in the root. Protein nitrogen per cell has been measured in *Vicia faba* roots (5). When the protein nitrogen pattern is compared with the peroxidase pattern of the excised tip, the rise between 500 and 1100 microns in peroxidase activity at 10^{-8} M IAA closely parallels a rise in protein content per cell. Above 1100 microns, however, the protein nitrogen decreases per cell while the peroxidase activity shows an increase at 1700 and 2500 microns. This latter dissimilarity probably means that the content of protein per cell is a resultant of synthesis and degradation and that even while total protein per cell is declining, protein formation, some of which is peroxidase, is still continuing. The protein pattern does mean, however, that if peroxidase activity were expressed on a protein basis instead of a per cell basis, much of the induction pattern would be lost.

The results illustrate quite clearly the value of a histochemical approach based on a careful morphological study of the organ involved. Neither the quantitative determination of activity of the section nor the localization studies alone offer the information furnished by the combination of both. The application of these same methods to other enzymes and other organs promises to yield valuable data relating enzymatic activities to developmental patterns.

SUMMARY

Peroxidase activity was localized and measured in 200-micron sections of the primary root of *Vicia faba*

after treatment with IAA to elucidate the physiological role of the induced peroxidase in the cell. Only the first 3 mm of the root were used as this area had previously been morphologically analyzed in detail.

The cells of the root cap, proepidermis, and vascular tissue including the protofloem and protoxylem show peroxidase activity. However, only the cells of the vascular tissue clearly show the induced formation of peroxidase. The maximum response to IAA shifts from the early provascular tissue at 10^{-8} M to the developing protoxylem at 10^{-7} M.

The cells that contain peroxidase activity are capable of producing a compound when given eugenol and H_2O_2 which appears to be an intermediate in lignin biosynthesis. The vascular tissue that has high induced peroxidase is very active in lignin formation.

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SOME PHYSIOLOGICAL ASPECTS OF BUNT RESISTANCE IN WHEAT¹

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The very pronounced effect of temperature on the expression of bunt (caused by *Tilletia caries* (DC.) Tul.) in Baart wheat has been reported by Griffith, Zscheile, and Oswald (1). The usual final expression of bunt results in fruiting of the fungus (formation of chlamydospores) in the place normally occupied by the embryo developing into the wheat kernel. Under suitable conditions the resistant variety Baart 38 may permit very limited chlamydospore development (1) in the form of smaller bunt balls, partially bunted

kernels or heads, or small number of immature spores hidden within the interior of the normal-appearing kernels.

Since the experimental conditions reported (1) involved certain specific values for light quality and intensity and for the light-period, it is desirable that the relationships of these conditions be studied further in relation to race T-1 of *Tilletia caries*. Optimum values of these conditions for the more nearly complete expression of bunt susceptibility of for maximum differentiation between varieties with respect to bunt resistance, should be determined. These studies

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