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Hormonal Profiles in Some Insect and Mite-induced Plant Galls

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Biochemical analysis of insect and mite-induced plant gall tissues, namely, of *zizyphus mauritiana* stem galls induced by *Eriophyes cernuus*, *Achyranthes aspera* leaf galls induced by *Bemisia tabaci* and *Ficus mysorensis* leaf galls induced by an unknown insect vector of Psyllidae were carried out. Findings revealed: (i) an imbalance in the hormonal pattern of gall tissues; (ii) an increase in endogenous leaves of auxins, and (iii) inhibition of IAA-oxidase (the enzyme responsible for auxin destruction) activity by O-dihydroxyphenols, which are also increased in gall tissues. Protectors of phenolic nature bearing high molecular weight were separated on Sephadex columns. They are characterized and isolated in the form of Pr I, Pr II, Pr III. There was a considerable lag in the oxidation of peroxidase (HRP) catalysed oxidation of IAA in presence of protectors or crude gall tissues extract. The role of O-dihydroxy-phenols in IAA-oxidase inhibition and subsequent increase in auxin levels in gall tissues is considered responsible for abnormal growth.

Key Words: Hormonal profiles, Insect-induced plant galls, Mite-induced plant galls, Plant galls, FAA-oxidase inhibition

Introduction

There are large number of plant-feeding insects commonly referred as 'toxicogenic' which cause pathological disturbances of tissues not ascribable to mechanical injury or microbial contamination. Their effects on plants are manifested primarily as growth disorders and galls. The whole subject of insect galls was reviewed by Mani (1959, 1965) and Braun (1969). The plant aberrations induced in response to insect attack appear due to hormonal imbalance. The phenomenon of abnormal auxin and polyphenol metabolism was observed in various abnormal growths (Smith 1972, Shekhawat & Arya 1979, Purohit et al. 1979).

Auxin levels in plants are regulated both by auxin synthesizing and oxidising systems. The oxidation of Indoleacetic acid (IAA) can be inhibited by polyphenols (Schaeffer et al. 1967, Runkova et al. 1972).

The naturally occurring IAA-oxidase inhibitors of phenolic nature range from free low molecular weight compounds such as chlorogenic, ferulic and proto catechuic acid to the high molecular weight auxin protectors (Yoneda & Stonier 1967, Novak & Galston

1971, Atsumi & Hayashi 1978). The present report deals with levels of auxin, phenolics, IAA-oxidase and mechanism of IAA protection in normal and stem gall tissues of *Zizyphus mauritiana* induced by *Eriophyes cernuus* Massee, leaf gall of *Achyranthes aspera* caused by *Bemisia tabaci* and leaf gall of *Ficus mysorensis* incited by unknown insect vector Psyllidae.

Materials and Methods

Healthy and diseased leaves of equal size and age were collected from *Acryanthes aspera* L. plants growing near the Department of Botany, New Campus, Jodhpur.

The leaf and gall tissues of *Ficus mysorensis* were collected from fields of New Campus Jodhpur University in the month of December, 1979. Similarly healthy stem and gall tissues of *Zizyphus mauritiana* were collected from Botanical Garden, Jodhpur University, Jodhpur.

The materials were carefully washed in running tap water and dried in four layers of filter paper. Healthy and gall tissues (300 mg) were homogenized and centrifuged (5000 g) in a refrigerated centrifuge. The supernatant was used to determine IAA-oxidase activity.

IAA-oxidase activity: It was measured by the method of Srivastava and Van Huystee (1973) using salkowski reagent at 530 nm. Amount of IAA destroyed was calculated from Standard curve of IAA. The enzyme activity was expressed as μg IAA destroyed per gram of fresh tissues.

Extraction and determination of auxins: The extraction and purification of auxins were carried out by slightly modifying the method of Kamisaka and Larsen (1977). Plant materials, 100 g in fresh weight, were powdered by homogenizing them in a cold room using mortar and pestle. The paste was lyophilized (−30 °C) and dry powder used for further process. The powder was extracted with methanol and repeated 4 times. The methanol fraction was evaporated under reduced pressure at −30 °C. To the residue, 10 ml of 0.5 M K_2HPO_4 was added, the *p*H of the solution being 8.5 *ca*. Lipoidal substances in the solution were extracted thrice with cold light petroleum ether and twice with 10 ml ethyl ether. Then the *p*H of the solution was adjusted to 3.0 using an appropriate amount of 3M metaphosphoric acid. The acidic fraction was shaken with 10 ml cold ethyl ether. The ether franction containing IAA was again shaken with 10 ml of cold 0.05M K_2HPO_4 solution; then the aqueous fraction containing IAA was brought to *p*H 3.0 using 0.28M metaphosphoric acid solution. The acidic aqueous fraction was shaken with 10 ml of cold ethyl ether, and the acidic ethyl ether containing IAA was dried under reduced pressure at −30 °C. The residue dissolved in 5 ml of 30% metnanol was used for paper chromatographic estimation of auxins according to the method of Shekhawat and Arya (1979) with slight modification.

Aliquots of the methanol extract obtained as above were spotted on Whatman Paper No. 1 and substances were separated with isopropanol-Ammonia-water (8:1:1) solvent system. To detect indole compounds, chromatograms were sprayed with Salkowski (50 parts 5% $HClO_4$: 1 part of 0.5 M $FeCl_3$) reagent. Substances of Rf of IAA (0.45) were detected and colour density recorded. Quantitative estimation was done with the help of standard curve obtained by known amounts of IAA; chromatograms of which were also run parallel with unknown samples.

Extraction and quantitative estimation of phenolic compounds: Freeze-dried material was extracted in 90% ethanol in soxhlet apparatus. The alcohol was evaporated in vacuum and resuspended in 30% ethanol. Total phenols were estimated using Folin ciocalteu's reagent (Mahadevan 1975) and

O-dihydroxyphenols were determined by Sheen's (1971) method using Arnow's reagent.

Separation of auxin protectors in Zizyphus gall tissues and their assay: Extracts of healthy and gall tissues were obtained by grinding separately 5 g of each in 20 ml cold 0.02 M potassium phosphate buffer at *p*H 6.1, in a chilled mortar. The ground brei was homogenized thoroughly in cold. The ground brei strained through cheese cloth was centrifuged for 15 min at 6,000 g. The supernatants of 250 mg fresh wt./ml were stored at −15 °C.

Dextran gel filteration: Dextran gels, sephadex G-200, and G-50 were used to filter the extracts, as described by Tandon (1976). The column filterate was collected serially as 5 ml fractions. Dextran blue 2,000 and Pyronin G with a molecular weight about 2,000,00 and 300 respectively, were used as dye markers to delimit the molecular weights. One ml of the former was added to the extract as a 0.15% solution in buffer, the later was added only on a sephadex G-50 column as 1 ml of 0.005% solution as soon as the extract had entered the gel.

Protectors assay: The reaction mixture used to assay for auxin protection consisted of a mixture of Dichlorophenol (DCP), $MnCl_2$ and IAA each at a final concentration of 0.1 mM and HRP (Horseradish peroxidase enzyme) at a final concentration of 0.6, 0.2, and 0.1 μg per ml for Pr I, Pr II and Pr III (Protectors), respectively.

In the assay described above the samples were mixed with 2 ml Salkowski reagent, and following 1 hr wait, the absorbance of mixture was measured at 520 nm (Gordon & Weber 1951).

Results

Auxins: Biochemical analysis of normal and gall tissues revealed an identical pattern of auxin contents irrespective of the causal agent. There were hyperauxinic levels in gall tissues as compared to their normal counterparts. The values are given in μg/g fresh wt. of tissues. In *Zizyphus*, gall tissues auxin content was 5.85 while in normal tissues it was 4.00. In gall tissues of *Achyranthes* auxin content was 4.5 while healthy leaf tissues showed 2.2.

Ficus leaf gall tissues contained 5.2 while its normal counterpart had 1.3 auxin content (figure 1).

IAA-oxidase activity: All the gall tissues showed reduced auxin destroying capacity as compared to their normal counterparts. In *Zizyphus* gall tissue, the activity of IAA-oxidase was reduced to about half that of the normal. *Achyranthes* and *Ficus* leaf gall tissues also showed considerably reduced activity of this enzyme. The activity of IAA-oxidase corresponded with the amount of *O*-dihydroxyphenols present in the tissues. With the increase in *O*-dihydroxyphenol content activity of enzyme showed an inhibited pattern in all the gall tissues (figure 2).

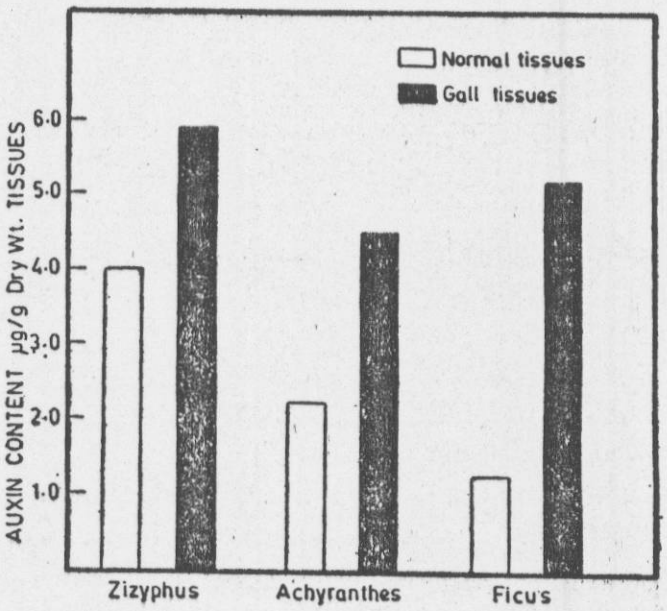

Figure 1 Auxin content (μg/g dry wt. tissues) in normal and gall tissues of *Zizyphus mauritianas*, *Achyranthes aspera* and *Ficus mysorensis*

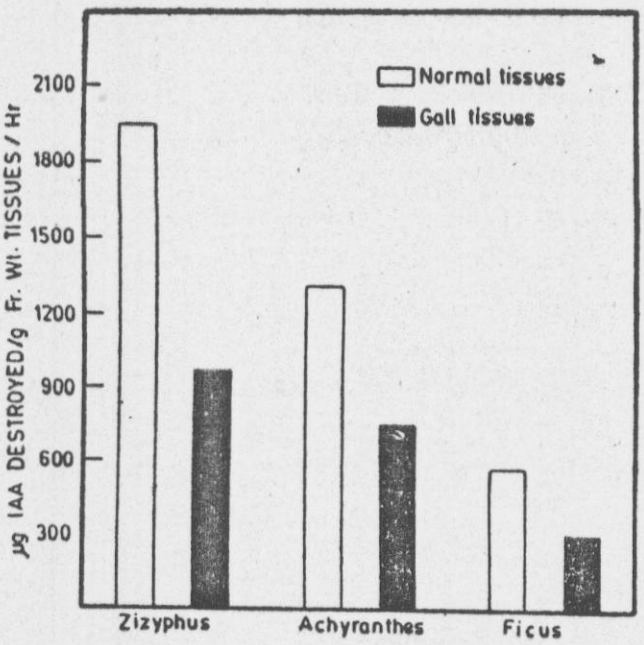

Figure 2 IAA-oxidase activity (µg IAA destroyed/g fresh wt. tissues/hr) in normal and gall tissues of *Zizyphus mauritiana*, *Achyranthes aspera* and *Ficus mysorensis*

Total and ortho-dihydroxy phenols: Increased levels of total and *O*-dihydroxyphenols were found associated with gall tissues. In total phenols, maximum contribution was that of *O*-dihydroxyphenols. The gall tissues of *Zizyphus*, *Achyranthes* and *Ficus* showed 24.2, 16.8 and 42.0 mg/g dry wt. of total phenols, respectively while the normal tissues showed 9.3, 10.5 and 18.6 mg/g dry wt. of total phenols (figure 3).

O-dihydroxyphenolic levels were 19.0, 10.4, and 28.6 mg/g dry wt. in gall tissues of *Zizyphus*, *Achyranthes* and *Ficus*, respectively while normal tissues showed 6.8, 7.3, and 11.3 mg/g dry wt. respectively (figure 3).

IAA destruction in tissue extracts: Zizyphus gall tissues extract produced a lag of several hours in IAA destruction by Horseradish peroxidase when the two were incubated together under necessary conditions. On the other hand, normal stem tissues extract allowed IAA destruction by Horseradish peroxidase. Various amounts of gall tissues extract from 0.01 to 0.5 mg were incubated with IAA in the reaction mixture to determine if destruction correlated directly with the quantity of tissues present. The results showed that greater the amount of gall tissues present, the longer was the lag prior to auxin destruction. Increased concentration of HRP did not reduce the lag. However, once destruction began, it proceeded at rates which correlated directly with the amount of tissues present (figure 4).

Separation of auxin protectors from Zizyphus gall tissue extract: By means of sephadex G-200 and G-50 it was possible to separate auxin-protecting substances. In sephadex G-200 eluates, the bulk of activity protecting destruction of IAA by HRP was recovered with two separate peaks of activity (figure 5). Pr I, the larger peak (around fraction 7), was light brown and weighed more than 2,00,000 daltons since it migrated as rapidly through sephadex G-200 column as the high molecular weight marker, Dextran blue 2,000 appeared in fraction 5 and 6. Colourless Pr II, the smaller peak (around fraction 18), on the other hand

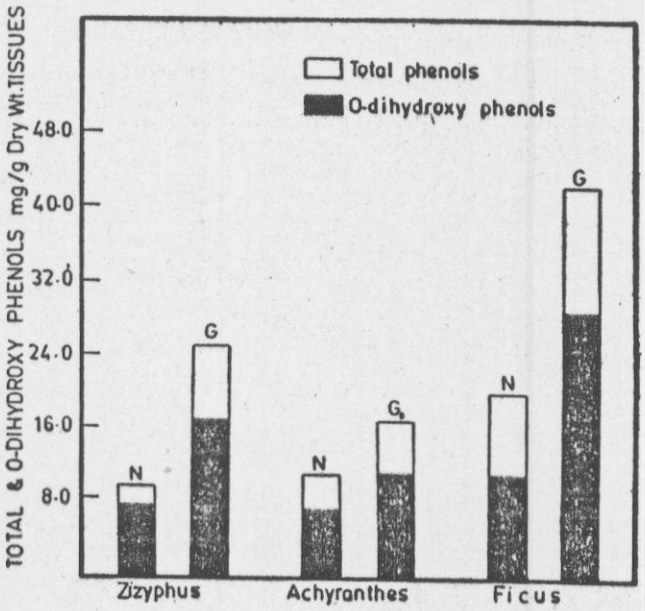

Figure 3 Total and *O*-dihydroxyphenol content (mg/g dry wt. tissues) in normal and gall tissues of *Zizyphus mauritiana*, *Achyranthes aspera* and *Ficus mysorensis*

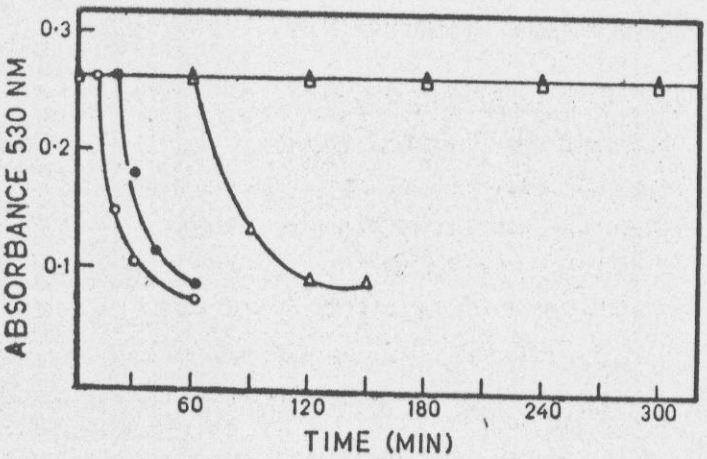

Figure 4 IAA destruction by HRP in presence of different concentrations of gall tissues. (O) : 0.01 mg; (●) : 0.05 mg (△) : 0.1 mg; (▲) : 0.3 mg; and (□) : 0.5 mg in 10 ml incubation mixture containing 0.1 mM each of IAA, Mn, DCP, and HRP (Final concentration 0.25 μg/ml) in 20 mM phosphate buffer *p*H 6.1

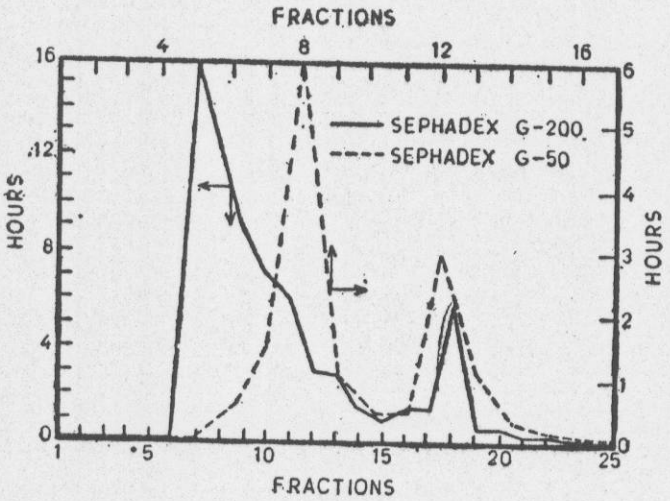

Figure 5 Inhibition of the destruction of IAA by HRP in the presence of various fractions of gall tissue extract filtered through sephadex G-200 and G-50 column 1.0 ml extract (250 mg equivalent fresh gall tissue) filtered through sephadex G-200 and G-50 with 5 ml fractions collected per tube. 0.2 ml of each fractions tested against 0.1 ml HRP (25 μg/ml) per 10 ml IAA reaction mixture

migrated more slowly in sephadex G-200, whereas in sephadex G-50, Pr II the larger peak (around fraction 8) moved as rapidly as the high molecular weight marker indicative of having molecular weight (*Ca* 10,000 daltons) (figure 5). In sephadex G-50 eluates, the smaller peak of Prs activity, Pr III (around fraction 12) was colourless and had a molecular weight *Ca* 2,000 daltons. The Dextran blue 2,000 appeared in fractions 5 and 6, and Pyronin G in fractions 14, 15 and 16 when passed through sephadex G-50 column.

Test for auxin protection in vitro: Isolated auxin protectors Prs I, II and III were incubated with auxin (IAA), enzyme (HRP) and cofactors. A lag of nearly 50 min was observed when Pr I was mixed with reaction mixture. Pr II and Pr III produced lag period of nearly 30 and 45 min, respectively. There was no lag in control without protectors. Concentrations of HRP did not have any effect on the lag (figure 6).

Discussion

It has remained a fact for about two decades that the physiological autonomy exhibited by the plant cancer cell reflects an abnormal growth hormone metabolism (Stonier 1972). Auxin levels are reported to be too high in such abnormal growths. There may be two explanations for hyperauxiny in gall tissues, viz., gall tissue synthesizes auxins in excess; or it destroys less. Excess auxins were found associated in various abnormal growths (Link & Eggers 1941, Shekhawat & Arya 1979, Purohit et al. 1979). Henderson and Bonner (1952) reported that normal sunflower tissues contained an inhibitor that prevented the conversion of tryptophan to IAA while it was absent in the tumor tissues. This favoured the theory of excessive auxin synthesis in gall tissues.

Table 5 *Frequencies (number) of induced morphological mutations recovered in the M_2 generation of sorghum following various mutagenic treatments*

Mutagen Treatments	Frequency of viable mutations(%)									Total viable mutations (%)	Ratio of viable/ chloro-phyll mutations
	Grain mutations				Glume type	Panicle type	Awned	Tall	White Midrib		
	Colour	Shape	Type	Size							
HZ	1.07 (12)*	0.67 (7)	—	0.45 (5)	—	—	—	—	—	2.14	0.07
EMS	0.36 (4)	—	—	0.27 (3)	0.18 (2)	—	—	—	—	0.81	0.04
Gamma rays	0.13 (8)	0.14 (8)	0.02 (1)	0.05 (3)	0.02 (1)	0.02 (1)	0.02 (1)	0.02 (1)	—	0.42	0.02
Gamma+HZ	0.88 (12)	0.74 (11)	0.45 (6)	0.15 (3)	0.60 (9)	0.15 (3)	0.10 (2)	0.05 (1)	0.10 (2)	3.22	0.13
EMS+HZ	0.09 (1)	—	—	—	—	—	—	—	—	0.09	0.03
HZ+EMS	—	—	—	—	—	—	—	—	—	—	—
HZ+CS	1.03 (7)	0.30 (2)	0.15 (1)	0.15 (1)	0.59 (4)	0.30 (2)	0.30 (2)	0.15 (1)	0.15 (1)	3.12	0.12
CS+HZ	2.06 (14)	1.48 (10)	1.04 (7)	0.44 (3)	0.74 (5)	0.60 (4)	1.04 (7)	—	—	7.40	0.25
Gamma+HZ	2.25 (43)	1.45 (29)	1.40 (28)	0.25 (5)	0.60 (12)	0.35 (7)	0.30 (6)	0.10 (2)	0.05 (1)	6.75	0.24

*Parenthetic numbers identify the number of independent mutations of each class which were recovered.

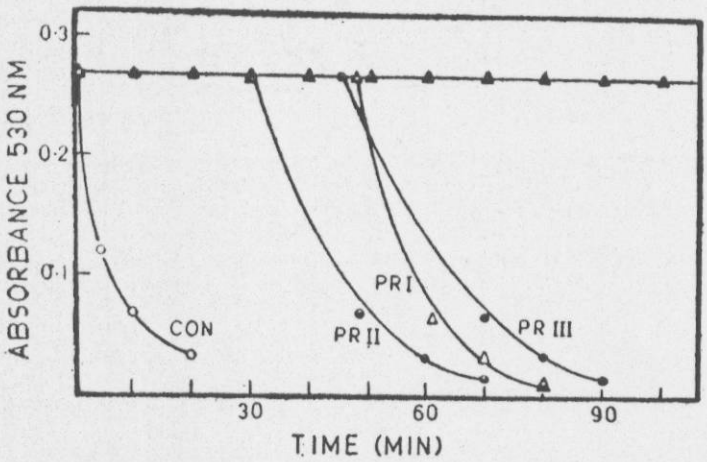

Figure 6 Induction of lag in HRP catalysed oxidation of IAA in presence of Pr I (△) Pr II (◒) Pr III (●) (isolated from *Zizyphus* gall tissue extract) and control (○). 10 ml incubation mixture containing 0.1 mM each of IAA, Mn, DCP, and HRP (Final concentration 0.25 μg/ml) in 20 mM phosphate buffer *p*H 6.1. (▲): only IAA without HRP

As far as the auxin catabolism is concerned, different points of view are expressed in literature. In some cases IAA-oxidase seems to play a role in establishing hyperauxiny in gall tissues. The activity of this enzyme was found less during gall induction processes (Bitancourt 1954, Platt 1954, Lipetz 1959, Sequeira 1963, Purohit et al. 1979) and more IAA-oxidase inhibitors were detected in gall tissues (Henderson & Bonner 1952, Witham & Gentile 1961, Rennert & Knypl 1967).

Polyphenols particularly *O*-dihydroxyphenols are reported potent IAA-sparing substances (Schaeffer et al. 1967). High levels of *O*-dihydroxyphenols inhibited IAA oxidation in all the gall tissues used as experimental material.

The finding that abnormally high levels of auxin protector are associated with gall tissues of *Zizyphus* is of interest as another proof to explain the anaplasia associated with the plant cancer state. High levels of protectors are normally encountered only in very young tissues (Stonier 1972). Pr I of the *Zizyphus* gall tissues exerted its protecting activity by acting as an anti-oxidant which interferred with the peroxidase catalysed oxidation of IAA. Similarly experiments indicated that this was also true with Prs II and III. All the protectors probably acted as poisers, stabilizing the redox state of chemical system not merely the oxidation of IAA. Possibly the induction of the plant cancer state involved primarily the activation of auxin protector synthesis in mature cells. The continued synthesis of the protective substances caused the afflicted cells to serve permanently to a juvenile state characterized among the other things by a reduced state and by high protection and hence high endogenous levels of IAA. The associated phenomenon of autonomy and anaplasia would be the inevitable consequences of such a metabolic alteration.

Prs acted as potent antioxidants which intervened in IAA oxidation in at least two, and probably more reactions. First the Prs interacted with manganese, keeping it in a reduced state (Stonier et al. 1968a, Tandon 1976); second, the Prs interacted with the hydrogen peroxide that was produced by peroxidase complex, thereby depriving the peroxidase of an electron accepter (Stonier et al. 1968b).

An important clue to the molecular mechanism of Pr I action was the shape of the curve that reflected the destruction of IAA by peroxidase in presence of the protector. The rate of the reaction was not appreciably changed. Instead, the presence of Pr I brought about a delay in the disappearance of Salkowski detectable IAA. This explained that Pr I did not act simply as an enzyme inhibitor, but it complexed with IAA thereby preventing enzymatic attack. Association of auxin-protecting substances was reported in young juvenile tissues of *Pharbitis* (Stonier & Yoneda 1967, Novak & Galston 1971), in sunflower gall tissues (Stonier 1969) inhabituated

auxin non-requiring tobacco cells (Syono & Furuya 1973) and in crown gall cell cultures (Atsumi & Hayashi 1978).

It is, therefore, concluded that *O*-dihydroxyphenols (which are abnormally high in gall tissues) acted as auxin-protecting substances which in turn inhibited the activity of IAA-oxidase resulting in hyperauxny and hence abnormal growth. The auxin protectors of higher molecular weight, namely Pr I, Pr II, Pr III were isolated using sephadex gel filteration. The protectors also produced lag in auxin oxidation by Horseradish peroxidase when tested *in vitro*.

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