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32

Association of auxin protectors in *Cinnamomum tamala* Fr. Nees leaf gall formation

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A gradient of auxin protection activity was observed in *C. tamala* leaf-gall from young to brown stage. Three auxin protectors, protector-I (mol wt approx. 200,000 daltons), protector-II (mol wt approx. 8,000 daltons) and protector-III (mol wt approx. 2,000 daltons), were isolated from gall tissues using Sephadex gel filtration. Protectors appeared to be oligomers or polymers of lower molecular weight phenolic substances. A possible role of these protectors in gall formation is discussed.

One feature that is common to almost all plant tumors is their abnormal growth hormone metabolism[1]. Elevated levels of growth hormones, particularly auxin, as compared to their normal counterparts have been reported in several tumor tissues[2-4]. On the other hand, in certain cases higher levels of growth hormones were not essential for the unorganized growth of plant tumors[5,6]. Bouckaert-Urban and Vendrig[7] suggested that growth regulators possibly affect a metabolic pathway that leads to the synthesis of other (co) factor(s) which may be involved in the tumor formation. The discovery of auxin protector substances in sunflower internode inoculated with a virulent strain of *Agrobacterium tumefaciens* by Stonier[8] has given a new direction in the field of auxin metabolism in tumor tissues. These substances prevent the peroxidase-catalysed oxidation of indole-3-acetic acid (IAA) by inducing a lag period prior to IAA oxidation.

The induction of a lag period by *Cinnamomum tamala* leaf-gall tissue extract prior to IAA oxidation by horse radish peroxidase (HRP) and normal tissue enzyme led us to investigate the presence of protectors in the gall tissues.

Materials and Methods

Fresh tissues, from *Cinnamomum tamala* Fr. Nees leaf-galls (different developmental stages, viz. young gall, green mature gall, completely transformed leaf and brown gall) incited by a mite and normal leaf, were used as experimental material.

Tissue extraction—Tissue extracts were obtained by grinding separately 5 g each of the tissues mentioned above in 20 ml cold potassium phosphate buffer (20 m*M*; *p*H 6.1) in a chilled mortar and pestle. Homogenates were filtered through cheese cloth and centrifuged at 12,000 rpm for 15 min at 4°C.

Destruction of IAA by crude extracts—Crude extracts of normal (0.3 and 0.5 mg fresh wt equivalent) and gall tissue (0.3 mg fresh wt equivalent) were added separately in IAA oxidation mixture to study destruction of IAA. HRP (SISCO) was added at 1.25 µg/ml final concentration of the reaction mixture in order to speed up the reaction. Gall tissue extract (0.3 mg) was also added in assay mixture of IAA destruction in presence of normal tissue extract. Gall tissue extracts from different stages of its development in concentration ranging from 0.01-0.5 mg fresh weight equivalent were added in IAA incubation mixture.

IAA oxidation mixture contained dichlorophenol (DCP), manganese chloride ($MnCl_2$) and IAA, each at a final concentration of 0.1 m*M* in 20 ml reaction mixture. All reactions were buffered at *p*H 6.1 with 20 m*M* potassium phosphate. Oxidation of IAA was followed by removing 1 ml of sample from reaction mixture at various time intervals, mixed with 4 ml of Salkowski reagent, and following 1 hr wait, the absorbance of mixture was measured at 530 nm[9].

Separation of auxin protectors and assay—The separation of auxin protectors has been described earlier[10] following basically the method of Yondeda and Stonier[11]. Dextran gels, Sephadex G-200 and G-50 were used to filter gall tissue extracts using Pharmacia Frac-100 fraction collector. Dextran blue 2000 and Pyronin G with a mol wt of about 2000,000 and 300 respectively were used as dye markers to delimit the mol wt. For assay of auxin

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protection, reaction mixture as described above for destruction of IAA was used except for HRP which was used at a final concentration of 0.375, 0.25, and 0.10 µg/ml for protector-I, protector-II and protector-III, respectively in 20 ml reaction mixture. Reaction mixture was shaken in 25 × 200 mm test tubes in a water bath at 30°C. To prevent IAA destruction, 0.2 ml protector was added. Oxidation of IAA was followed by removing samples from reaction mixture at various time intervals and assaying by means of Salkowski reagent. The presence of protectors manifested as a lag period prior to IAA oxidation. Absorbance of fractions collected were also taken at 280 nm.

Absorption spectra of protectors – UV and visible scanning (200 to 700 nm) of diluted protectors were conducted using Hitachi 220 spectrophotometer. Some phenolics like caffeic acid, catechol, chlorogenic acid, protocatechuic acid, pyrogallol and *p*-coumaric acid were also scanned.

Effect of protectors and o-dihydroxyphenols on peroxidase catalysed IAA oxidation – IAA incubation mixture of 3 ml containing 0.3 m*M* IAA, 50 µM each of DCP and $MnCl_2$, 50 m*M* potassium phosphate buffer (*p*H 6.0) and 50 µg HRP at final concentrations was used to study the effect of protectors and *o*-dihydroxyphenols (caffeic acid, catechol and chlorogenic acid) on the time course oxidation of IAA at 261 nm. Protectors and phenolics were added prior to addition of HRP.

For all experiments AR grade chemicals were used.

Results

Destruction of IAA by crude extracts – Cinnamomum normal leaf tissue extract destroyed IAA when the two were incubated together (Fig. 1).

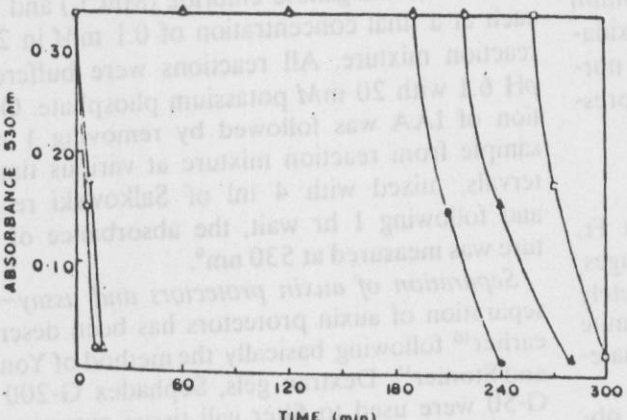

Fig. 1 – Destruction of IAA by normal and gall tissue extracts: 0.3 mg normal (○); 0.5 mg normal (△); 0.3 mg gall (□) closed symbols represent normal tissue extract along with 0.3 mg gall tissue extract

On the other hand, with gall tissue extract, IAA destruction occurred only following a lag of several hours. Also, if gall tissue extract was added in incubation mixture of IAA destruction by normal leaf tissue extract, a lag was observed prior to IAA oxidation (Fig. 1). Young gall tissue showed maximum auxin protection activity followed by green mature gall, completely transformed leaf and brown gall (Table 1).

Separation of auxin protectors – By means of Sephadex G-200 and G-50 it was possible to separate auxin protecting substances. In Sephadex G-200 eluates, the peak of activity protecting the destruction of IAA by HRP was recovered in fraction 6-10 (volume of effluent 30-50 ml). Protector-I, the larger peak around fraction 8, was light brown in colour and its weight exceeded 200,000 daltons since it migrated as rapidly through Sephadex G-200 column as the high mol wt marker (dextran blue 2,000). Dextran blue appeared in fraction 3-5 (Fig. 2A). In Sephadex G-50, the bulk of protector activity was recovered in two separate peaks (Fig. 2B). Protector-II, the larger peak (fractions 6-9; volume of effluent 30-45 ml) moved as rapidly as the high mol wt markers, indicative of having a mol wt approximately 8,000 daltons. Protector-III, the smaller peak around fraction[11] (volume of effluent 50-55 ml), was colourless and had a mol wt of approximately 2,000 daltons. Dextran blue appeared in fractions 3-5, and pyronin G in fractions 14-17 when passed through Sephadex G-50 column. These peaks were comparable with the absorption curves recorded at 280 nm (Fig. 2).

Absorption spectra of protectors – Protectors did not show any absorption in the visible range. Therefore, the results of scanning between 200 and 400 nm only are given in Fig. 3. All protectors showed two absorption maxima, one at 235

Table 1 – Effect of various concentrations of different stages of gall tissue extracts on IAA oxidation

Tissue	Concentration (mg)				
	0.01	0.05	0.1	0.3	0.5
	Length of lag period (min)				
*Control	< 1				
Young gall	6	26	70	260	> 300
Green mature gall	4	10	20	120	240
Completely transformed leaf	< 1	1	5	60	120
Brown gall	< 1	< 1	1	40	100

*No gall tissue extract

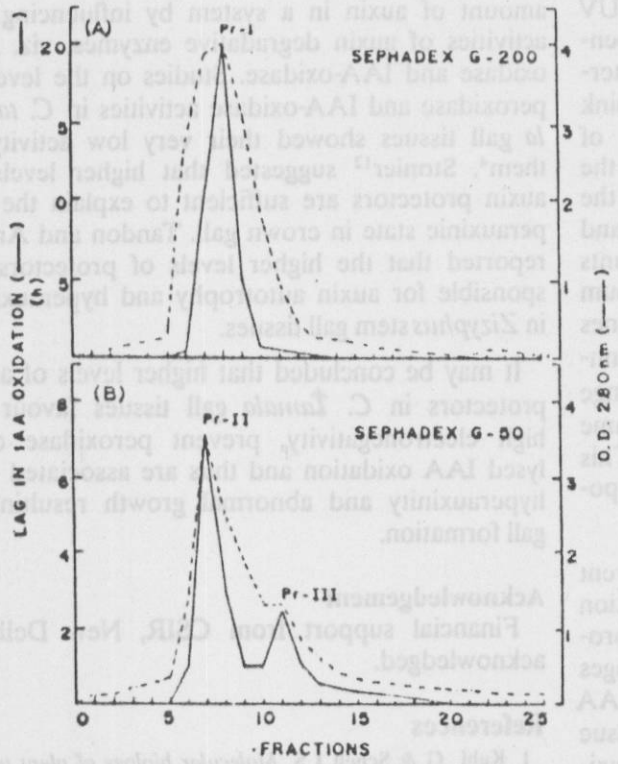

Fig. 2 – Protection of IAA destruction by various fractions (5 ml each) obtained by filtration of 1 ml of gall tissue extract through (A) Sephadex G-200 column and (B) Sephadex G-50 column. For composition of reaction mixture, see Materials & Methods section. Dotted line represents absorbance of fractions at 280 nm

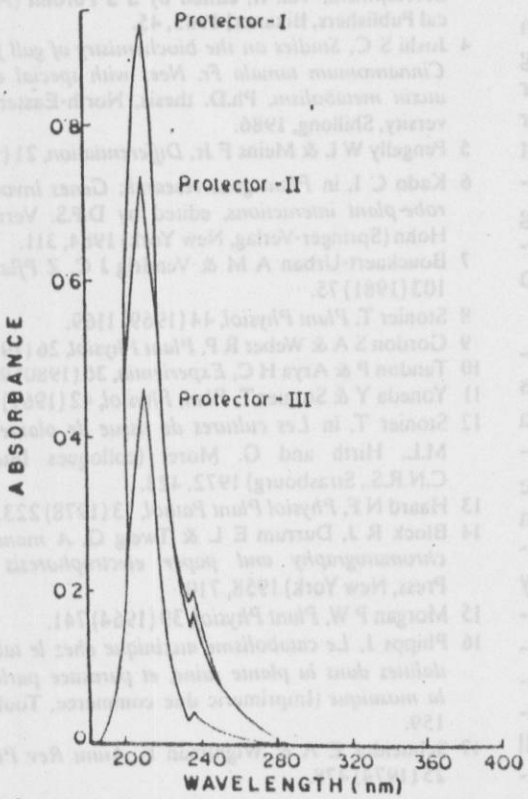

Fig. 3 – Absorption spectra of protector-I, protector-II and protector-III in UV light

nm (a smaller peak) and another at 210 nm (larger peak). The phenolics tested also showed absorption maxima in the range of 200-290 nm (not shown here).

Effect of protectors and o-dihydroxyphenols on peroxidase catalysed IAA oxidation – Data obtained for protector-I only are given here because during experimentation it became evident that protectors are oligomers or polymers of the same sub-units. Similarly only the effect of catechol on IAA oxidation is given here. In both cases the height of curves was identical, confirming that inhibition of IAA oxidation was only temporary. The lag was directly proportional to the concentration of protectors/dihydroxyphenols (Fig. 4).

Discussion

The results presented here imply the existence of protectors of IAA destruction by enzymes normally found in the normal tissue and commercially prepared peroxidase (HRP). Using Sephadex G-50 and G-200, three protector substances were isolated from the *C. tamala* leaf gall tissue. Earlier studies on protectors from morning glory[12], fungal incited tumors on potato[13], and mite-incited stem galls on *Zizyphus*[10] indicated that the active sites on the protector molecules were probably o-dihydroxyphenolic in nature. In case of *C. tamala* protector molecules also appeared to be o-dihydrox-

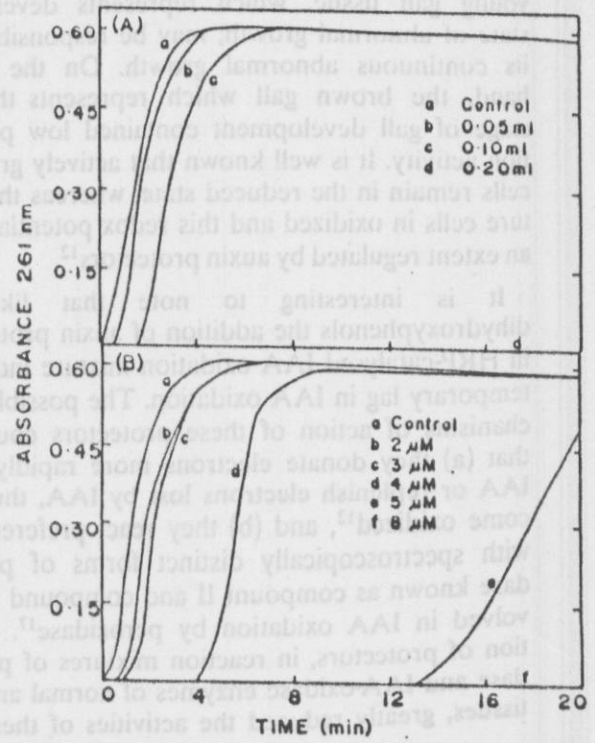

Fig. 4 – Inhibition of IAA oxidation by different concentrations of (A) protector-I and (B) catechol

yphenols based on: (i) paper chromatography, protectors showed blue fluorescing spots in UV light. On spraying the spots with sucrose suspension the spots gave a light brown color characteristic of diphenols[14]; (ii) protectors showed pink colour with Arnow's reagent (characteristic of o-dihydroxyphenols); (iii) protectors affected the rate of *in vitro* enzymic IAA oxidation in the same fashion as did the diphenol (catechol); and (iv) the absorption spectra of gel filtration eluants of *C. tamala* gall tissue extract showed maximum absorption in the UV light resembling the ones obtained with dihydroxyphenols. Further, scanning of equally diluted protectors in the UV range (200-400 nm) clearly indicate that they are same substances having similar basic units (Fig. 4). This suggests that the protectors are oligomers or polymers of lower mol wt phenolic substances.

The addition of various amounts of different stages of gall tissue extracts in IAA oxidation reaction mixture showed that (i) a gradient of protection activity existed amongst the various stages of gall tissues, and (ii) the destruction of IAA correlated directly with the quantity of the tissue extract. Young gall tissue extract showed maximum auxin protection activity which diminished as the gall tissue matured. Higher protector activity has also been reported in juvenile tissues of cotton plants[15], tobacco leaves[16] and Japanese morning glory[12] as compared to their mature tissues. Presence of more auxin protecting activity in young gall tissue, which represents developing state of abnormal growth, may be responsible for its continuous abnormal growth. On the other hand, the brown gall which represents the last stage of gall development contained low protection activity. It is well known that actively growing cells remain in the reduced state, whereas the mature cells in oxidized and this redox potential is to an extent regulated by auxin protectors[12].

It is interesting to note that like *o*-dihydroxyphenols the addition of auxin protectors in HRP-catalysed IAA oxidation mixture induce a temporary lag in IAA oxidation. The possible mechanisms of action of these protectors could be that (a) they donate electrons more rapidly than IAA or replenish electrons lost by IAA, thus become oxidized[12], and (b) they react preferentially with spectroscopically distinct forms of peroxidase known as compound II and compound III involved in IAA oxidation by peroxidase[17]. Addition of protectors, in reaction mixtures of peroxidase and IAA-oxidase enzymes of normal and gall tissues, greatly reduced the activities of these enzymes[4]. Thus, protectors can influence the amount of auxin in a system by influencing the activities of auxin degradative enzymes, viz. peroxidase and IAA-oxidase. Studies on the level of peroxidase and IAA-oxidase activities in *C. tamala* gall tissues showed their very low activity in them[4]. Stonier[12] suggested that higher levels of auxin protectors are sufficient to explain the hyperauxinic state in crown gall. Tandon and Arya[10] reported that the higher levels of protectors responsible for auxin autotrophy and hyperauxinity in *Zizyphus* stem gall tissues.

It may be concluded that higher levels of auxin protectors in *C. tamala* gall tissues favour the high electronegativity, prevent peroxidase catalysed IAA oxidation and thus are associated with hyperauxinity and abnormal growth resulting in gall formation.

Acknowledgement

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