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Regulation of Indole Acetic Acid Oxidase Activities in Hevea *Leaves by Naturally Occurring Phenolics*

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Indole acetic acid (IAA) oxidase activities of preparations from Hevea *leaves were stimulated by 2,4-dichlorophenol as well as by naturally occurring phenolics, p-coumaric acid, scopoletin, 4-methylumbelliferone and chlorogenic acid. Kaempferol and quercetin which have both been associated with resistance to the South American leaf blight (SALB) disease of* Hevea *were shown to function both as cofactors and as competitive inhibitors of* Hevea *leaf IAA oxidase. The contribution of these findings to the understanding of pathogenicity in SALB is briefly discussed.*

Naturally occurring phenolics are known both to stimulate and to inhibit indole acetic acid (IAA) oxidase from different plant sources. Thus, p-coumaric acid[1] and 4-methyl umbelliferone[2] have been described as co-factors for IAA oxidase and chlorogenic acid[3] as a competitive inhibitor of the enzyme. Moreover, Imbert and Wilson[4,5,6] showed that scopoletin, chlorogenic and caffeic acids functioned both as stimulators and competitive inhibitors of sweet potato root IAA oxidases. Further, conjugates of kaempferol and quercetin, which have been shown to function as promoters and inhibitors, respectively, of pea IAA oxidase at physiological concentrations[7] have also been associated with resistance to the South American leaf blight (SALB) disease of *Hevea*[8,9], caused by the fungus *Microcyclus ulei* (P. Henn) Arx. Symptoms of this disease include development of necrotic lesions followed by premature abscission in young leaflets[10]. Since other phenolics, e.g. scopoletin[11] and chlorogenic acid[12] have been involved in the mechanisms both of leaf abscission[13] as well as of fungal disease resistance[14], it was decided to investigate the control of the activity of IAA oxidase from young *Hevea* leaves by naturally occurring phenolics, with particular reference to kaempferol and quercetin, as a first step in the exploration of possible contributions of these phenolics to pathogenicity in SALB.

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MATERIALS AND METHODS

Extraction of Enzyme

Young (seven-day old) leaves of *Hevea brasiliensis* Muell. Arg. (clone F 351) were harvested from experimental plots at the University of the West Indies Field Station, Trinidad, before 0800 h, washed in distilled water and immediately placed in the deep freeze at −10°C. Frozen leaves were processed into an acetone powder[15], and the dry powder suspended overnight in phosphate-citrate buffer pH 5.0 in the refrigerator at 4°C. Enzyme preparations were obtained from the centrifuged acetone powder extract, by acetone precipitation[16], separation of the residue by centrifugation for 10 min at 0°C and redissolving it in phosphate citrate buffer, pH 5.0, or as otherwise described.

Assay of Indole Acetic Acid Oxidase Activity

Enzyme activity was assayed as previously described[4,5,6]. The reaction mixture included $10^{-3}M$ IAA (0.5 ml); 0.2 *M* phosphate-citrate buffer at specified pH (2.2 ml);

cofactors as described (0.8 ml) and enzyme preparation (0.5 ml) in a total volume of 4.0 millilitres. Incubation was at 30°C in a shaking incubator for periods varying from 15 min to 60 min depending on enzyme activity. Indole acetic acid oxidase activity was expressed as micromoles IAA destroyed per hour per microgramme protein or per millilitre enzyme. Protein contents of enzyme preparations were determined by the Folin method[17].

RESULTS

Although IAA oxidase activity could not be demonstrated in young *Hevea* leaf preparations made directly by the acetone precipitate procedure[16], active enzymes were obtained when leaf acetone powders[15] were extracted overnight in the refrigerator at 4°C with phosphate-citrate buffer in the pH range 4 – 8 and the resulting extract subjected to the acetone precipitation procedure. Preparations extracted at pH 5 had the highest activities. Further shaking of acetone powders for 1 – 3 h after overnight suspension in buffer did not increase enzyme activity. Indole acetic acid oxidase activity was low in the absence of cofactors, but preparations showed considerable (up to 0.1 μmole IAA destroyed per hour per millilitre enzyme) 2,4 dichlorophenol (DCP) – stimulated enzyme activity, which was enhanced by Mn in the concentration range 0.25 μmole to 1.0 μmole per millilitre reaction mixture.

There were increases in p-coumaric acid-stimulated IAA oxidase activity with increasing enzyme concentration in the range 10 μg to 60 μg per millilitre reaction mixture. Also, increasing the substrate concentration (0.25 – 0.50 μmole IAA per millilitre) at an enzyme concentration of 13.2 μg protein per millilitre increased enzyme activity but at higher substrate concentrations (0.50 – 1.25 μmole IAA per millilitre enzyme activity was inhibited. When the enzyme concentration was increased to 66 μg protein per millilitre, there was a linear increase in enzyme activity with IAA concentration up to 0.75 μmole per millilitre but activity remained constant thereafter *(Figure 1)*. The *Km* value for p-coumaric acid-stimulated IAA oxidase activity was calculated at 6.92×10^{-3} *M* compared with 3.70×10^{-3} *M* for DCP-stimulated enzyme activity.

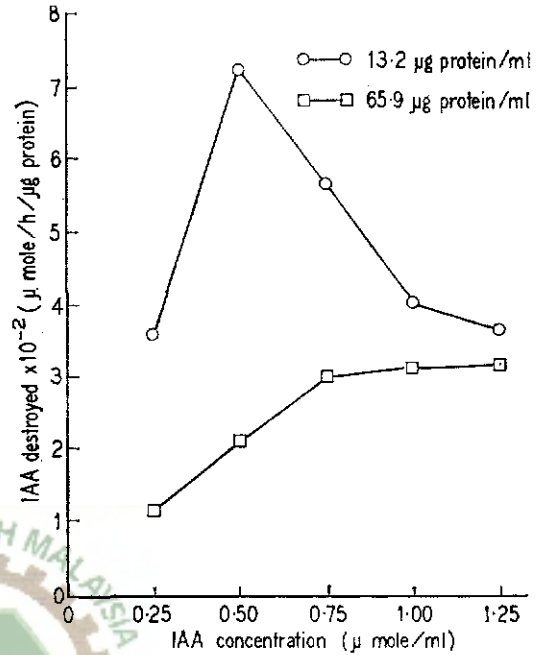

Figure 1. Effect of IAA concentration on p-coumaric acid-induced Hevea *leaf IAA oxidase activity at two enzyme concentrations.*

Naturally occurring phenolics tested were all found to stimulate IAA oxidase preparations from young *Hevea* leaves, with maximal activity at pH 3.5 for quercetin, 4-methyl umbelliferone and p-coumaric acid, at pH 4.0 for kaempferol and scopoletin and pH 4.5 for chlorogenic acid. Kaempferol-stimulated enzyme activity was most sensitive to changes in pH, there being a 98% decrease in activity at pH 6.0 compared with maximal IAA oxidase activity at pH 4.0 *(Figure 2)*. Thus, although kaempferol and quercetin-induced

enzyme activities were similar at pH 3.5, there was a 30% decrease in quercetin activity compared with kaempferol-induced activity at pH 4.0 and at pH 5.0, the quercetin-stimulated enzyme had higher activity. When assayed at the above mentioned pH optima, cofactor concentrations for maximal stimulation of activities of enzymes containing 20 – 60 μg protein per millilitre occurred at 0.2×10^{-2} μmole per millilitre reaction mixture for p-coumaric acid and scopoletin, 2×10^{-2} μmole per millilitre for 4-methyl umbelliferone and 1×10^{-2} μmole per millilitre for kaempferol and 2×10^{-5} μmole per millilitre for chlorogenic acid.

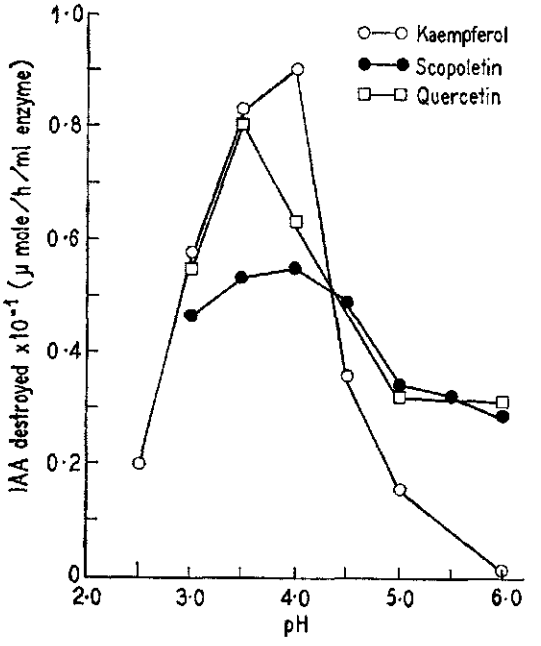

Figure 2. Effect of pH on kaempferol-, scopoletin- and quercetin-induced Hevea *leaf IAA oxidase activities.*

Kaempferol was a more potent cofactor and quercetin the more effective inhibitor of rubber leaf IAA oxidase. Thus, in an enzyme preparation containing 42 μg protein per millilitre, 40% stimulation of IAA oxidase activity could be achieved by 1×10^{-2} μmole per millilitre kaempferol but 50% inhibition of enzyme activity resulted with 2×10^{-4} μmole per millilitre quercetin *(Table 1)*. Moreover, both stimulation and inhibition of IAA oxidase activities could be induced with either of these phenolics by manipulation of enzyme, substrate and cofactor concentrations. Accordingly, increasing enzyme concentration from 14 μg to 42 μg per millilitre reaction mixture reversed a 53% kaempferol-induced inhibition of IAA oxidase activity to a 40% stimulation of enzyme activity *(Table 1)*. Further, complete inhibition of IAA oxidase activity of an enzyme preparation containing 71 μg protein per millilitre by 2×10^{-2} μmole per millilitre quercetin in the presence of 0.125 μmole per millilitre IAA was reversed to a 50% stimulation over the control without quercetin, when substrate concentration was increased to 0.625 μmole per millilitre, but further increase in IAA concentration to 1.25 μmole per millilitre induced a 10% inhibition of IAA oxidase activity *(Figure 3)*. Thus, both flavonols functioned as competitive inhibitors but quercetin appeared to be the stronger competitive inhibitor since it had a smaller molar concentration requirement (per unit of enzyme protein) than kaempferol for inhibition of enzyme activity. A kaempferol concentration of 7.14×10^{-4} and quercetin concentration of 4.76×10^{-6} μmole per microgramme protein induced 47% and 50% inhibition of IAA oxidase activity respectively in the same rubber leaf preparation *(Table 1)*.

TABLE 1. INHIBITION/STIMULATION OF INDOLE ACETIC ACID OXIDASE ACTIVITIES BY QUERCETIN AND KAEMPFEROL AT THREE ENZYME CONCENTRATIONS

Enzyme concentration (μg protein/ml)	IAA oxidase activity [% stimulation (+) or inhibition (−) over controls without cofactors]	
	Kaempferol (10^{-2} μmole/ml)	Quercetin (2×10^{-4} μmole/ml)
14	−53	−100
28	00	−100
42	+40	− 50

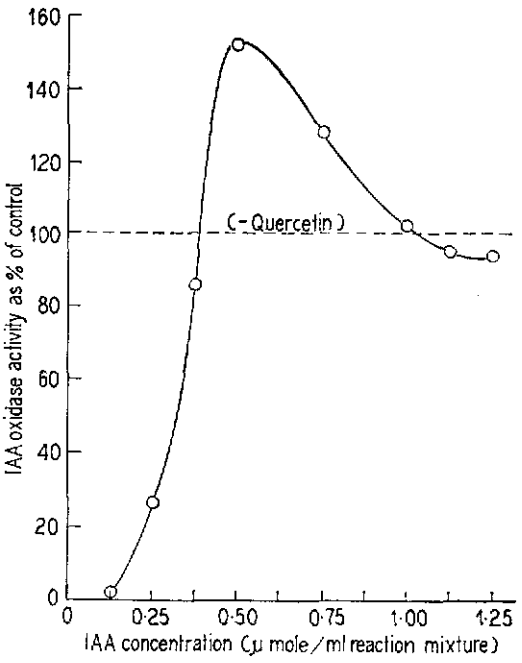

Figure 3. Stimulation/inhibition of quercetin-induced Hevea *leaf IAA oxidase activity as affected by IAA concentration.*

Quercetin-induced stimulation/inhibition of IAA oxidase activity also depended on the pH. Levels of quercetin concentration which were inhibitory at low pH stimulated IAA oxidase activities at higher pH. Thus at the optimum pH (3.5), with an enzyme concentration of 20 μg protein per millilitre, and substrate concentration at 0.125 μmole per millilitre, quercetin (2×10^{-6} μmole per millilitre) induced maximal (23%) stimulation of enzyme activity over controls but at 2×10^{-2} μmole per millilitre quercetin, an 86% inhibition of IAA oxidase activity resulted. Increasing the enzyme concentration to 61 μg per millilitre increased the optimal quercetin concentration for maximal enzyme activity to 2×10^{-3} μmole per millilitre, with 77% inhibition of control activity occurring at 2×10^{-2} μmole per millilitre quercetin. At pH 6.0, maximal IAA oxidase activity occurred with 2×10^{-2} μmole quercetin even at the low enzyme concentration of 26 μg per millilitre *(Figure 4)*. Similar quercetin-induced stimulations and inhibitions of IAA oxidase activity with changes in pH and quercetin concentration were demonstrated in several experiments.

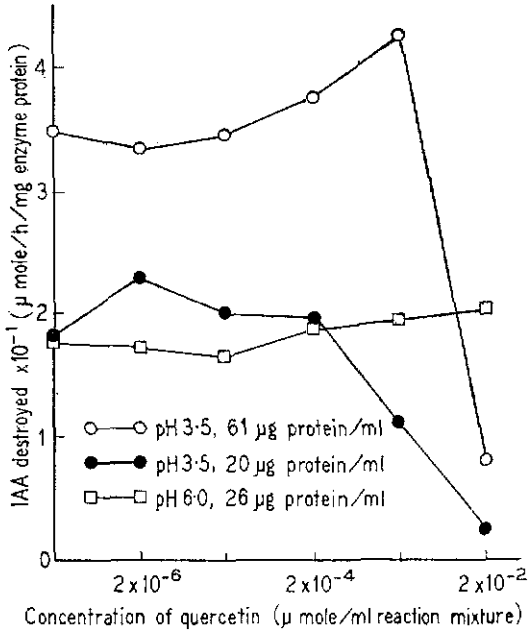

Figure 4. Stimulation/inhibition of Hevea *leaf IAA oxidase activity as affected by quercetin and enzyme concentrations and pH.*

DISCUSSION

Indole acetic acid oxidase preparations from young *Hevea* leaves were similar to those from other plant sources in that they were stimulated by DCP and MN^{++} as well as by naturally occurring phenolics, p-coumaric acid, scopoletin, 4-methyl umbelliferone and chlorogenic acid, but different from pea epitcotyl preparations in the cofactor activity of the flavonols, kaempferol and quercetin. Thus, while kaempferol was shown to be an inhibitor of the pea enzyme by Mumford *et al.*[18] and later conjugates of kaempferol and quercetin were suggested as promoters and inhibitors respectively of an enzyme from a similar source by Furuya *et al.*[7], it is

now shown that both kaempferol and quercetin stimulated IAA oxidase from *Hevea* leaf preparations, but that kaempferol was the more potent cofactor. Both flavonols functioned as competitive inhibitors in that inhibition of IAA oxidase activity could be reversed by changing relative concentrations of substrate and enzyme, but quercetin appeared to be the more effective inhibitor.

Our results indicated that kaempferol had the more profound stimulatory effect on *Hevea* leaf IAA oxidase activity as measured by sensitivity of the enzyme both to concentration changes of the flavonol and pH changes of the reaction medium and the lower aqueous solubility of kaempferol (5×10^{-2} μmole per millilitre) limited its capacity for inhibition of IAA oxidase activity at high enzyme concentration. Moreover, although quercetin was the more effective inhibitor of *Hevea* leaf IAA oxidase activity, such inhibition could be reversed to stimulation at high pH (6.0). It is worth noting here that earlier, scopoletin, chlorogenic and caffeic acids[4,5,6] and now kaempferol and quercetin have been shown to function both as cofactors and competitive inhibitors of IAA oxidase activity and hence the dual capacity of these and other molecules for competitive inhibition/stimulation of this enzyme seems to warrant further investigation. The physiological role of these naturally occurring phenolics in regulating *in vivo* IAA oxidase activity may also be of greater significance than hitherto suspected.

Interest in the role of phenolics in the pathogenicity of SALB was first generated by Blasquez and Owen[19] who demonstrated the presence of tannins in the yellowish-brown colouration which developed above the arrested lesions in leaves from resistant rubber clones after inoculation with *M. ulei* conidia. Later, Figari[20] showed that chlorogenic and caffeic acids, catechol as well as *Hevea* leaf extracts believed to contain a flavonol, all inhibited germination of *M. ulei* conidia. The effective substance in such extracts was identified by Martains *et al.*[8] as kaempferol 3-rhamnodiglucoside but Chee and Seaforth[9] found quercetin, rather than kaempferol in leaf extracts from the same and several other *Hevea* clones. These findings suggested that phenolics and particularly the flavonols kaempferol and/or quercetin might be involved in mechanisms of resistance to SALB.

Disease resistance in crop plants has previously been related to IAA, IAA oxidase and to phenolics both through direct toxic effects on fungal spore germination and hyphal growth as well as regulation of IAA oxidase activity. Accordingly, auxin changes including hyper-[21,22] and hypo-[23,24] auxiny have been observed in many host-parasite relationships. Resistance to wheat rust was associated with high IAA oxidase activity leading in turn to control of auxin levels[14]. Moreover, symptoms of stunting and increase in the necrotic area of rust-infected sunflower leaves were ascribed to stimulation/inhibition of IAA oxidase activity by scopoletin, depending on the concentration[25]. As mentioned earlier, similar stimulation/inhibition of *Hevea* leaf IAA oxidase was here induced by both kaempferol and quercetin and these flavonols are known to increase in concentration in virus-infected cherry leaves[26]. Toxic effects of kaempferol and/or quercetin[8,9] or their glucosides on germination of *M. ulei* conidia might also be similar to those of phloridzin/phloretin in the resistance of apple leaves to *Venturia inaequalis* (Cooks) Wint. infection[27,28]. Further, premature leaf fall in SALB could be effected in a manner analogous to leaf fall in coffee[23] and rose[24] plants infected with *Omphalia flavida* Maubl. & Rang. and *Diplocarpon rosea* Wolf respectively, where it was shown that IAA oxidase degraded IAA and thereby reduced the normal flow of auxin from lamina to petiole. Since it was demonstrated that there were significant differences in the effects of pH on kaempferol

and quercetin-induced rubber leaf IAA oxidase activities and mature rubber leaves are known to develop resistance to *M. ulei* infection, such resistance might well be associated with changes in leaf pH during ontogeny as shown to occur in apple leaves[28].

Therefore, naturally occurring phenolics, including the flavonols, kaempferol and quercetin, might play either a direct role in pathogenicity in SALB e.g. through inhibition of fungal spread or an indirect role through their capacity for modification of IAA oxidase activity and thereby regulating auxin levels. However, since profound difference in the effects of kaempferol and quercetin on IAA oxidase activity from young rubber leaves have been demonstrated, it is suggested that the presence and relative concentrations of these flavonols and their metabolism in a range of *Hevea* clones of different susceptibilities to SALB must be unequivocably established before mechanisms for the involvement of these compounds in pathogenicity could be adumbrated and their possible participation in SALB disease resistance fully explored. The proposition that IAA oxidase has something to do with SALB-induced leaf fall and resistance to infection appears highly simplistic at this stage however work on abscission of SALB infected leaflets and post-infection changes of IAA oxidase as well as peroxidase published elsewhere[29,30] indicated the merits for further investigation.

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