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Laccase-type phenoloxidase in salivary glands and watery saliva of the green rice leafhopper, *Nephotettix cincticeps*

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Abstract

The activity and composition of leafhopper saliva are important in interactions with the host rice plant, and it may play a physiological role in detoxifying toxic plant substances or ingesting sap. We have characterized diphenoloxidase in the salivary glands of *Nephotettix cincticeps*, its activity as a laccase, and its presence in the watery saliva with the objective of understanding its function in feeding on rice plants.

Nonreducing SDS-PAGE of salivary gland homogenates with staining by the typical laccase substrate 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), hydroquinone or syringaldazine revealed a band at a molecular mass of approximately 85 kDa at pH 5. A band also appeared at a molecular mass of approximately 200 kDa when the gels were treated with dopamine, L-3,4-dihydroxyphenylalanine (DOPA) or catechol at pH 7.

The ABTS-oxidizing activity of the homogenates was drastically inhibited by *N*-hydroxyglycine, a specific inhibitor of laccase. However, the dopamine-oxidizing activity was not inhibited by *N*-hydroxyglycine, while it was inhibited by phenylthiourea (PTU).

Thus, the salivary glands of *N. cincticeps* contain two types of phenoloxidases: a laccase (85 kDa) and a phenoloxidase (200 kDa). Laccase activity was detected in a holidic sucrose diet that was fed on for 16 h by two females, but only a trace of catechol oxidase activity was observed, suggesting that the laccase-type phenoloxidase was the predominant phenoloxidase secreted in watery saliva. The laccase exhibited an optimum pH of 4.75–5 in McIlvaine buffer and had a PI of 4.8. Enzyme activity was histochemically localized in V cells of the posterior lobe of the salivary glands. It remained at the same level throughout the adult stage from 2 days after eclosion. A possible function of *N. cincticeps* salivary laccase may be rapid oxidization of potentially toxic monolignols to nontoxic polymers during feeding on the rice plant. This is the first report proving that laccase occurs in the salivary glands of Hemiptera species and is secreted in the watery saliva.

Keywords: *p*-Diphenol:oxygen oxidoreductase; Salivary enzymes; *Nephotettix cincticeps*

1. Introduction

The green rice leafhopper, *Nephotettix cincticeps* (Uhler) (Homoptera: Cicadellidae) is one of the most important insect pests of rice in Japan. This species damages rice by ingesting sap and also transmits virus and phytoplasma diseases (Ōya and Sato, 1981; Kawabe, 1985; Nakashima and Hayashi, 1995). In the feeding process, leafhoppers eject two different types of salivary secretions, namely, coagulable and watery saliva (Sogawa, 1968). Coagulable saliva rapidly forms the salivary sheath surrounding the stylets that penetrate the host plant tissues, while watery saliva remains in the liquid form after being ejected from the stylets. It is considered that the saliva in aphids plays important physiological roles in detoxifying toxic substances or in continuous ingestion of the sieve element sap (Miles, 1999).

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The saliva of leafhoppers contains several enzymes, as this property has been reported in the case of other Hemiptera species (cf. Miles, 1972; Sogawa, 1968, 1971). Based on the reactions with various phenol compounds such as L-3,4-dihydroxyphenylalanine (DOPA) and *p*-phenylenediamine, it has been demonstrated that the salivary glands of *N. cincticeps* possess diphenoloxidase activity (Sogawa, 1968). However, it has not yet been proved whether the enzyme involved is indeed a laccase (EC 1.10.3.2) in a strict sense because the property of the enzyme has not been examined by using laccase-specific substrates or inhibitors. It also remained uncertain whether these activities are a result of the functioning of a mixture of laccase and catechol oxidase (EC 1.10.3.1) or of a single oxidase.

The phenoloxidase of *N. cincticeps* is inferred to be involved in the process of stylet sheath formation and undiffused out of the salivary sheath by an agar-gel test (Sogawa, 1968). On the other hand, the salivary oxidases in aphids are discharged in host plants and are considered to detoxify defensive phytochemicals (Miles, 1964; Peng and Miles, 1988; Urbanska et al., 1998). The electrical penetration graph of *N. cincticeps* that was feeding on rice phloem sap through a membrane recorded a distinct waveform that was indicative of salivation (Hattori, 1997).

In the present study, the phenoloxidases in the salivary glands of *N. cincticeps* were characterized and examined further to determine whether they were secreted in watery saliva for better understanding of their function in feeding on the rice plants.

2. Materials and methods

2.1. Insects

The green rice leafhopper, *N. cincticeps* was obtained from a stock colony successively reared on rice seedlings in our institute at $25\pm2\,^{\circ}C$ and $70\%\pm5\%$ rh with a L16:D8 photoperiod. Unless otherwise stated, 4- to 7-day-old adult females were used for the collection of salivary glands or watery saliva.

2.2. Chemicals

DOPA, 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid (ABTS), syringaldazine, 3-methyl-2-benzothiazolinone hydrazone (MBTH), Kojic acid and catalase (from bovine liver) were purchased from Sigma-Aldrich Co., St Louis, MO and hydroquinone, dopamine hydrochloride catechol and 1-phenyl 2-thiourea (PTU) were obtained from Wako Pure Chemical Industries Ltd., Osaka. *N*-Hydroxyglycine was synthesized by the method of Jahngen and Rossomando (1982).

2.3. Enzyme samples

After adult females were immobilized by placing them in a freezer ($-5\,^{\circ}C$) for 15 min, the salivary glands were dissected out from the insects with tweezers. Salivation of the enzyme was confirmed by measuring the enzyme activities of 5% sucrose solutions that were exposed to insect probing and sucking. For this purpose, glass cylinders (internal diameter, 28 mm; height, 24 mm) were used as feeding chambers. An 80-μl aliquot of 5% sucrose was dispensed between two membranes of a stretched Parafilm® M; these membranes were located at the upper end of the chamber. All membranes were immersed in 70% ethanol over 1 h and subsequently dried prior to use. The bottom of the chamber was enclosed with a piece of polyester net. Two adult females were confined within each of the 10 chambers, which were placed on a sheet of paper towel. "Unfed controls" were aliquots of 5% sucrose solution that were dispensed in the same manner but without any insects in the chambers. After 16 h, 50-μl aliquots were withdrawn per chamber and analyzed spectrophotometrically using a 96-well plate.

2.4. Electrophoretic analysis

Salivary glands were homogenized in Laemmli sample buffer (Laemmli, 1970), but 2-mercaptoethanol was omitted. After centrifugation at 15,000*g* for 10 min, each aliquot of the supernatant (equivalent to 15 pairs of salivary glands) was subjected to different lanes of 10% native polyacrylamide gel (PAGE) (NPU-10L, ATTO Co.) under denaturing conditions using SDS. After electrophoresis at $4\,^{\circ}C$, the gel was washed in 2.5% Triton X-100 for 15 min at room temperature, rinsed twice with distilled water and then immersed in individual buffer (pH 5 or 7) for 10 min. The gel was excised into individual lanes and then activity staining was separately performed with different substrates described in the next section.

Analytical isoelectric focusing (IEF) of the homogenates was performed on a 5.0% acrylamide gel slab in the pH range of 4.0–6.5 with a Multiphor electrophoresis system (Pharmacia Biotech) used in accordance with the manufacturer's instructions. The gels were stained with Coomassie brilliant blue (R250; Pharmacia) or with ABTS.

2.5. Visualization of enzyme activities within the gels

Laccase activity was detected by immersing the gels in 5 mM ABTS, hydroquinone, or syringaldazine, typical laccase substrates in 100 mM acetate buffer (pH 5) after electrophoresis. Catechol oxidase activity of the gels was also assayed by the procedure of Nellaiappan and Vinayagam (1986). This is based on the enzymatic oxidation of diphenolic substrates such as DOPA, dopamine and catechol into their quinone products and subsequent condensation with MBTH to form a brown product. The incubation mixture consisted of 4 ml of 10 mM of the substrates in either 100 mM sodium acetate buffer at pH 5 or McIlvaine citrate–phosphate buffer at pH 7 added to 1 ml of 0.3% MBTH in ethanol. Incubation was carried out for 60 min in the dark at $35\,^{\circ}C$, except for a gel that was treated with syringaldazine for 8 h.

2.6. Enzyme assay

The optimal pH range of salivary laccase was determined colorimetrically by using ABTS as the substrate. Homogenates of two pairs of salivary glands in 10 µl distilled water were placed in each well of a 96-well plate. Forty microliters of McIlvaine citrate–phosphate buffer with pH ranging from 3.5 to 8.0 was added. The reaction was initiated by the addition of 150 µl of 2 mM ABTS in distilled water. The increase in absorbance at 420 nm after 60 min at 28 °C was associated with the oxidation of the substrate and was measured on a microplate reader (Model 550; Biorad, USA).

In order to examine phenoloxidase activity in fed sucrose diet, 150 µl of ABTS in 100 mM sodium acetate buffer at pH 5 or 10 mM dopamine in McIlvaine buffer at pH 7 was added to every 50 µl of the fed diet (two females for 16 h) and unfed control diet in the well.

2.7. Inhibition of enzyme activity

The effect of various inhibitors on salivary phenoloxidase was evaluated by measuring the increase in the rate of absorbance (min^{-1}) at 420 nm during the enzyme reaction with ABTS or dopamine on a microplate reader at 28 °C. The reaction mixture was composed of 40 µl of salivary gland homogenates (two pairs), 150 µl of 2 mM ABTS in 100 mM acetate buffer (pH 5) or of 10 mM dopamine in McIlvaine buffer (pH 7) and 10 µl of various inhibitors in distilled water. *N*-Hydroxyglycine, a specific inhibitor of laccase (Murao et al., 1992) and other oxidase inhibitors with various concentrations were added to the salivary gland homogenates 30 min prior to the addition of the substrate. In order to verify the involvement of oxidation by peroxidase, catalase was added to the homogenates at 333 and 1000 units/ml to scavenge H_2O_2.

2.8. Laccase activity of salivary glands in adult stage

Newly emerged adult females were transferred from the rearing cage to another cage with rice seedlings 0–4 h before the light was turned off. Everyday or every 2 days for a period of 12 days, five females were taken from the latter cage and the laccase activity of salivary glands in each insect was examined. In 0-day-old females, the salivary glands were dissected within 1 h after eclosion.

2.9. Histochemical method

The salivary glands were fixed in 10% formalin in 0.8% sodium chloride solution for 15 min and incubated in 5 mM ABTS solution at 35 °C for 30 min.

3. Results

3.1. Electrophoretic analysis by activity staining

When the salivary gland homogenates of *N. cincticeps* were subjected to SDS-PAGE on a 10% gel under nonreducing and no-heating conditions, followed by activity staining with the laccase substrate ABTS at pH 5, a single band that was blue–green in color appeared in the gel at a molecular mass of approximately 85 kDa (Fig. 1A). When the gel was incubated in ABTS and H_2O_2, another band appeared at a molecular mass of approximately 15 kDa as a result of the peroxidase activity (data not shown).

The 85-kDa band was also visualized with hydroquinone, syringaldazine or dopamine at pH 5 and, DOPA and catechol at pH 5 and pH 7. On the other hand, a red or brown band appeared at a molecular mass of approximately 200 kDa when the gels were treated with dopamine, DOPA or catechol at pH 7 (Fig. 1A). No band appeared on reaction with ABTS, hydroquinone and syringaldazine at pH 7. IEF analysis with ABTS showed that laccase in the salivary glands yielded a broad band with an isoelectric point of approximately 4.8 (Fig. 1B).

3.2. Effect of inhibitors on two types of phenoloxidase activity

We then proceeded toward the characterization of salivary phenoloxidase reacting with ABTS at pH 5 or with dopamine at pH 7. The salivary gland homogenates were incubated in the presence of various types of phenoloxidase inhibitors (Table 1). The phenoloxidase activity detected with ABTS was completely inhibited by

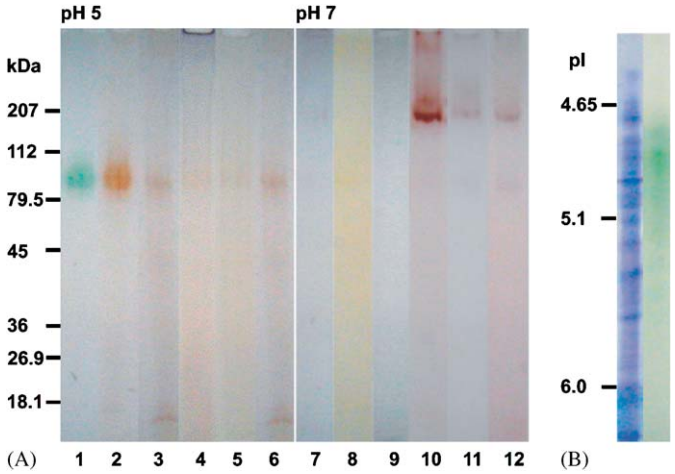

Fig. 1. (A) Nonreducing SDS-PAGE (10%) analysis of salivary gland homogenates of *N. cincticeps*. 1,7: activity staining with ABTS; 2,8: hydroquinone; 3,9: syringaldazine; 4,10: dopamine; 5,11: DOPA at pH 7; 6,12: catechol; 1–6: pH 5; 7–12: pH 7. (B) Isoelectric focusing (IEF) of the salivary gland homogenates on a 5.0% acrylamide gel slab in the pH range 4.0–6.5. Left: Coomassie blue R-250 staining, right: activity staining with ABTS at pH 5.

N-hydroxyglycine, a specific laccase inhibitor (Murao et al., 1992), at the 0.1 mM level, while dopamine-oxidizing activity was not inhibited even at the 10 mM level. In contrast, the ABTS-oxidizing activity was inhibited to a lesser extent by PTU, while dopamine-oxidizing activity was completely inhibited by PTU at the 1 mM level. Catalase reduced the ABTS-oxidizing activity by a meager 4.7% at a 333-U/ml concentration and did not perform the dopamine-oxidizing activity even at 1000 U/ml. Kojic acid, a general polyphenol oxidase inhibitor inactivated both oxidase activities at a similar level.

3.3. Optimum reaction pH of laccase

Salivary homogenates in distilled water were mixed with McIlvaine buffer ranging from pH 3.5 to pH 6.5. As shown in Fig. 2, the optimum reaction pH obtained by using ABTS as the substrate was estimated to be 4.75–5. The pH-activity curve is similar to that of cuticle laccase of *Bombyx mori* and *Schistocerca gregaria* with an optimum pH of 4.75–5 (Yamazaki, 1972; Anderson, 1978), but is distinct from polyphenol oxidase in the saliva of grain aphids, which is most active at a pH of 8.2–9.4 (Urbanska et al., 1998).

3.4. Salivary laccase activity at adult stage and phenoloxidase activities in sucrose diet exposed to leafhoppers

Laccase activity in the salivary glands was relatively low on 0 day after eclosion; however, it was constantly high for the period from 1 to 12 days after eclosion (Fig. 3). ABTS-oxidizing activity of the sucrose diet exposed to feeding of the leafhoppers was significantly higher than that of unfed sucrose diet (Table 2). No significant dopamine-oxidizing activity was recorded in the fed sucrose diet.

Table 1
Effect of various inhibitors on salivary phenoloxidase at different concentrations

Inhibitor	Concentration	Inhibition (%)	
		ABTS-oxidizing activity	Dopamine-oxidizing activity
p-Hydroxy glycine	0.1 mM	100.0	—
	1.0 mM	100.0	—
	10.0 mM	100.0	0.0
Kojic acid	1.0 mM	39.4	45.7
	10.0 mM	87.9	100.0
PTU	0.1 mM	2.8	52.0
	1.0 mM	50.0	100.0
Catalase	333 U/ml	4.7	—
	1000 U/ml	10.0	0.0

The assay was performed with ABTS at pH 5.0 or dopamine at pH 7.0 and the readings were taken at 420 nm or 470 nm, respectively. Activity was expressed relative to those obtained in the absence of the inhibitors.

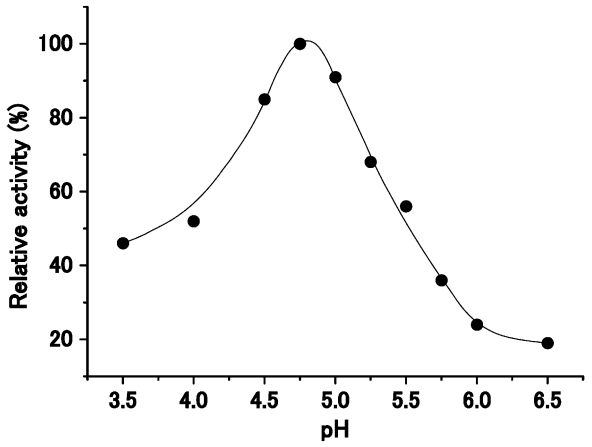

Fig. 2. Activity profile of salivary laccase; enzyme activity as a function of pH in McIlvaine buffer. The activity was assayed with ABTS, and readings were taken on a microplate reader at 420 nm. Each point represents Mean ± SE, $n = 10$.

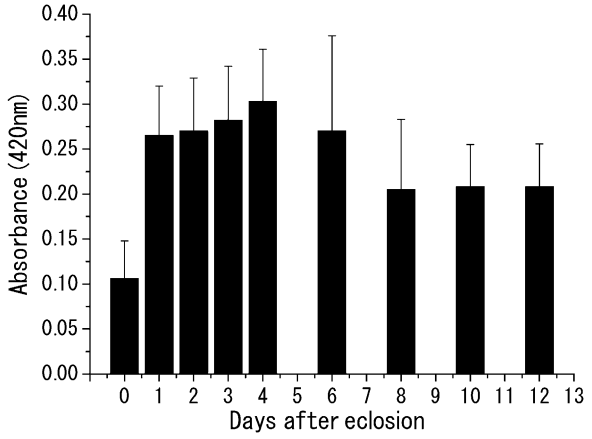

Fig. 3. Change in activity of laccase from the salivary glands of *N. cincticeps* females in adult stage. In 0-day-old adults, the activity was examined within 1 h after eclosion. Mean ± SE, $n = 5$.

3.5. Histochemical localization of laccase in the salivary glands and the salivary sheath materials

Laccase activity was detected in V-type cells of the posterior lobe of salivary glands in female adults by using ABTS as the substrate (Fig. 4). Catalase treatment did not affect the staining intensity in these cells (not shown). The V-type cells of the glands and the coagulated sheaths were stained with DOPA (Sogawa, 1968).

4. Discussion

The phenol-oxidizing enzymes have been classified into three groups based on their substrate specificity: monophenol oxidase (EC.1.14.18.1), catechol oxidase (EC.1.10.3.1) and laccase (EC.1.10.3.2). The phenoloxidase found in the salivary glands of *N. cincticeps* showed a capacity to oxidize *p*-phenylenediamine and *o*-diphenols

Table 2
Two types of phenoloxidase activities in sucrose diet exposed to feeding of *N. cincticeps*

Substrate (2 mM)	Absorbance at 420 nm		
	After feeding	Unfed control	
ABTS, pH 5	0.118 ± 0.009	0.061 ± 0.001	$P < 0.01$
	Absorbance at 470 nm		
	After feeding	Unfed control	
Dopamine, pH 7	0.136 ± 0.003	0.135 ± 0.002	ns

Eighty microliters of 5% sucrose was exposed to two adult females for 16 h, and subsequently, 50 μl was used for analysis with microplate reader at 28 °C for 60 min. Mean ± SE, $n = 10$.

Fig. 4. Localization of laccase in a pair of salivary glands of a *N. cincticeps* female. Blue–green colored parts are V cells in the posterior lobe showing laccase activity on ABTS staining. The bar in the figure represents 100 μm.

like catechol but not the monophenols such as tyrosine and *p*-cresol (Sogawa, 1968). Sogawa (1968) has described that this enzyme shows similar substrate specificity to a phenoloxidase isolated from the larvae of the blowfly, *Calliphora erythrocephala* (Karlson and Liebau, 1961), but differs from the one that was prepared from the colleterial gland of the cockroach *Periplaneta americana* (Whitehead et al., 1960). Catechol oxidase (tyrosinase) and laccase have been partially purified from the cuticle of larvae of *C. vicina* (Barrett and Anderson, 1981) and catechol oxidase from the colleterial gland of *P. americana* (Sugumaran and Nellaiappan, 1990).

In order to clarify the property of the salivary phenoloxidase of *N. cincticeps* and a possibility of a mixture of different oxidases, the salivary gland homogenates were reacted with several phenoloxidase-specific substrates after electrophoresis. It is well known that ABTS is specifically oxidized by laccase in the absence of hydrogen peroxide but not by catechol oxidase (Niku-Paavola et al., 1990). Nonreducing SDS-PAGE of the supernatant of salivary gland homogenates revealed a single laccase-positive band at 85 kDa by reacting with ABTS at pH 5; this band was also reacted with hydroquinone and syringaldazine, other diagnostic substrates for laccase, and dopamine, DOPA and catechol at pH 5. On the other hand, a band appeared at approximately 200 kDa on intense staining with dopamine, DOPA and catechol, substrates of catechol oxidase, at pH 7. Thus, two different types of phenoloxidases were detected from the salivary gland homogenates of *N. cincticeps* by using various substrates at different pH values.

ABTS-oxidizing activity of the salivary gland homogenates was barely affected by catalase treatment, thereby indicating little participation of peroxidase in oxidization. Furthermore, the ABTS-oxidizing activity was completely inhibited by *N*-hydroxyglycine at 0.1 mM, while dopamine-oxidizing activity was not inhibited even at 10 mM. On the other hand, the dopamine-oxidizing activity was distinctly inhibited by PTU, while the ABTS-oxidizing activity was inhibited to a lesser extent by PTU. *N*-Hydroxyglycine has been identified as a potent and specific inhibitor of laccase in *Penicillium citrinum*, but it did not inhibit other oxidases such as polyphenol oxidase and ascorbate oxidase (Murao et al., 1992); PTU are known as inhibitors of catechol oxidase (Barrett and Anderson, 1981; Barrett, 1987).

Thus, these results indicate that the ABTS-oxidizing enzyme with a molecular mass of 85 kDa is laccase-type phenoloxidase (EC 1.10.3.2, *p*-diphenol: oxygen oxidoreductase), and the dopamine oxidizing enzyme with a molecular mass of 200 kDa is catechol oxidase (EC 1.10.3.1, *o*-diphenol:oxygen oxidoreductase).

Thus far, insect laccase has been detected in the cuticles of many species (Barrett, 1987; Thomas et al., 1989). Recently, it was reported that a laccase gene was expressed in the midgut, malpighian tubules and fat body as well as in the epidermis of *Manduca sexta* (Dittmer et al., 2004). To our knowledge, *N. cincticeps* laccase is the first laccase found in the salivary glands, although catechol oxidase has been universally identified in the salivary glands of Hemiptera species (Miles, 1960; Miles, 1964; Peng and Miles, 1988; Madhusudhan and Miles, 1998; Urbanska et al., 1998).

Laccase activity in the salivary glands of *N. cincticeps* was constantly maintained throughout the adult stage from day 1 after eclosion—the period during which the leafhoppers feed actively. One of the proposed functions of the diphenoloxidase of *N. cincticeps* is the promotion of rapid oxidative gelling of the stylet sheath by the quinone tanning reaction because some polyphenol substance(s) has been detected in the salivary glands (Sogawa, 1971, 1973). This idea is conceivable because laccase is hypothesized to be involved in the insect cuticle sclerotization (Yamazaki,

1972; Barrett and Anderson, 1981; Barrett, 1987; Binnington and Barrett, 1988; Thomas et al., 1989; Ashida and Yamazaki, 1990; Sugumaran et al., 1992). In addition, Sogawa (1968) postulated that the salivary phenoloxidase of *N. cincticeps* was not ejected into the plant tissues by agar-gel test containing DOPA; no diffusion of darkened color was observed in the gel after feeding. However, in the present study, laccase activity was detected in the sucrose diet exposed to feeding of *N. cincticeps* through a membrane. Urbanska et al. (1998) reported that catechol oxidase was detected in sucrose syrup across a parafilm membrane, even though it was not detected in the sucrose–agar gel test. It may be possible that in *N. cincticeps* as well, laccase is secreted in watery saliva independently from the sheath materials similar to catechol oxidase in aphid saliva (Miles, 1964; Peng and Miles, 1988; Urbanska et al., 1998).

The stylets of *N. cincticeps* penetrate plant tissue intracellularly; they rupture the walls of the epidermal and mesophyll cells in order to access the phloem. Thus, saliva ejected from the stylets during probing would come in contact with the phenolic substances arising from the vacuoles in the cell, whose pH value is acidic (Taiz, 1992). The optimum pH for leafhopper laccase is 4.75–5.0, which differs from pH (8.2–9.4) for catechol oxidase in grain aphid saliva (Urbanska et al., 1998). The laccase ejected along with watery saliva may play a role in the oxidation of phenolic substances in plant tissues other than the sieve elements, since the phloem sap of rice is known to have a slightly alkaline pH of 8.0 (Fukumorita and Chino, 1982).

It was suggested that catechol oxidase may enable the insect to overcome the chemical defenses of the host plant (Miles, 1972, 1999; Gopalan, 1976). Urbanska et al. (1998) described that the phenolic compounds accumulate around an aphid's stylet sheath, where they are oxidized and polymerized by catechol oxidase. Catechol oxidase was not detected in the sucrose solution fed to *N. cincticeps*, at least under the present experimental conditions. Therefore, salivary laccase of *N. cincticeps* may oxidize some flavones such as carlinoside that are present in rice plants (Besson et al., 1985). Nevertheless, the fact that laccase may be able to promote the polymerization of monolignols in the absence of proteins would be of considerable significance in avoiding ingestion of quinone methides. Quinone methides are potent protein alkylating agents that can be toxic to insects (Felton and Gatehouse, 1996). These reactive intermediates are formed from monolignol by the peroxidase (and β-glucosidase) that is generated when the plant is wounded by stylet penetrations, since monolignol glucosides such as coniferin are thought to be present in rice plants (He and Terashima, 1989). Recently, laccase has also shown to oxidize monolignols like conifer alcohol to dehydrogenation polymers, lignin-like materials (Bao et al., 1993). Thus, rapid oxidation of monolignols by the salivary laccase may result in the formation of nontoxic polymer in wounded tissues around the stylet tip penetrating into the plants.

It is also known that laccase catalyzes the oxidation of phenolic lignin units as well as a wide variety of phenolic compounds and aromatic amines (Thurston, 1994). In this context, the possibility that laccase might play a role in lignin degradation is not ruled out.

Further knowledge regarding the biochemistry of the saliva, including laccase and plant injury response, will lead to a better understanding of leafhopper–rice plant interactions.

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