Biochemical Assay Detects Feeding Damage to Loblolly Pine Seeds Caused by the Leaffooted Pine Seed Bug (Hemiptera: Coreidae)[1]

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Abstract A large number of proteins in salivary gland extracts of the leaffooted pine seed bug, *Leptoglossus corculus* Say, were strongly recognized by a polyclonal antibody-based assay developed for detecting saliva of the western conifer seed bug. *Leptoglossus occidentalis* Heidemann, in lodgepole pine, *Pinus contorta* var. *latifolia* Engelmann, seeds. An average of approximately 85% of loblolly pine, *Pinus taeda* L., seeds exposed to feeding by *L. corculus* for 1 to 4 weeks in the laboratory contained detectable amounts of salivary proteins when the antibody assays were performed weekly on samples (n = 10) of seed. In comparison, radiography of exposed seed detected an average of approximately 63% damaged seed over the same 4-wk period, indicating that the antibody assay increased sensitivity of damage detection by approximately one-third. Depletion of insoluble polypeptides and proliferation of soluble polypeptides ≤23.5 kDa was apparent after SDS-PAGE and quantitative assays were performed on proteins extracted from seeds that were damaged by exposure to *L. corculus* feeding. Our data suggest that the antibody-based test could be used to obtain accurate estimates of seed losses attributable to *L. corculus* feeding in southern pine seed orchards.

Key Words Seed bug, *Pinus taeda*, antibody, immunodetection, damage

The leaffooted pine seed bug, *Leptoglossus corculus* Say (Hemiptera: Coreidae), first observed in southern pine seed orchards in 1966 (DeBarr 1967), causes seed losses of 50 to 90% in commercial pine seed orchards throughout the southern USA (Ebel et al. 1980). Seed bugs penetrate cone scales with their stylets and remove the major storage reserves from individual seeds, leaving little obvious damage to the exterior of cones or seeds (Koerber 1963, DeBarr 1970, Ebel et al. 1980). Suspected feeding activity of seed bugs can be detected by close examination of chemically-stained cone scales for evidence of minute stylet wounds (Campbell and Shea 1990). However, the extent of damage to individual seeds within wounded cones cannot be determined by visual inspection alone.

Radiographic analyses of seeds from 54 southern pine seed orchards from 1971-1973 indicated that 10% of loblolly pine, *Pinus taeda* L., and 15% of other pine seeds

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showed signs of damage attributable to seed bugs as evidenced by partially filled seeds (Belcher and DeBarr 1975). However, empty seeds accounted for 18% of loblolly and 15% of slash pine, *Pinus elliottii* Engelmann, seed crops, some of which could have resulted from complete removal of the contents by seed bugs.

Feeding damage to seeds of lodgepole pine, *Pinus contorta* var. *latifolia* Engelmann, and six other conifers, caused by the western conifer seed bug; *Leptoglossus occidentalis* Heidemann, has been identified using an antibody-based assay. The assay detects minor traces of *L. occidentalis* salivary protein remaining in individual seeds after feeding has occurred (Lait et al. 2001a, b, Bates et al. 2002).

A similar reliable method of detecting feeding damage by *L. corculus* could greatly enhance the assessment of seed bug impact on pine seed production in the southeastern USA. As a first step toward that goal, our objective was to test whether the polyclonal antibody developed for detecting salivary proteins of *L. occidentalis* could be used to detect evidence of feeding by *L. corculus.*

Materials and Methods

Seed feeding exposure and radiographic analysis. On 23 Aug 2000, 10 male and 10 female adult *L. corculus* were added to each of five paper buckets (4.88 L) (Sweetheart Cup Co. Inc., Chicago, IL), containing several fresh branches of loblolly pine in small cups of water (236 mL). The seed bugs came from a laboratory colony originating with adults collected from mature loblolly pine near Milledgeville, GA, in the summer of 2000. The bugs were kept at 28 to 33°C, with 65 to 70% RH and a 16L:8D photoperiod. The top of each bucket was covered with mesh screening (mesh size = 1.4 mm). Mature loblolly pine seeds (mean ± SE seed weight = 44.4 ± 1.0 mg) were obtained from the Weyerhaeuser Seed Orchard near Lyons, GA. After radiographic examination, a set of 250 filled seeds was randomly selected and 50 seeds were retained as controls and stored at 6°C. The remaining 200 seeds were divided equally and placed on the top of screens covering each of the five buckets, allowing bugs to feed on individual seeds through the mesh. Ten seeds were randomly collected from each bucket at weekly intervals for 4 wks, resulting in progressively more intense feeding pressure on the seeds remaining on the screen. All seeds exposed to feeding and control seeds were x-rayed on the day of collection with a Hewlett Packard X-ray machine (Model 4380SN) and Kodak Industrex Instant 620 radiography film, exposed for 90 sec at 15 kV. Seeds were then classified as filled (>95% of tissue remaining), partially-filled (20% to 95% of tissue remaining), and empty (<20% of tissue remaining) (Fig. 1). Seeds were stored at 6°C for further analysis.

Protein analysis. Salivary glands (10 pairs) were excised from adult *L. corculus* and homogenized in polypropylene microcentrifuge tubes using disposable plastic pestles (Fisher Scientific, Pittsburgh, PA) with 0.1 mL of isotonic saline consisting of 0.02 M Tris-HCl, pH 7.0, PMSF (1 mM), leupeptin (5 μM), and benzamidine (5 μM). The homogenate was centrifuged at 14000 × g for 2 min to pellet debris, and the supernatant (extract) containing salivary gland proteins was stored at −20°C until analysis. The same procedure was repeated with glands from adult *L. occidentalis.*

Seed protein analyses were conducted blind (without knowledge of radiography results). Ten seeds were randomly selected from the 50 exposed seeds removed each week from the buckets containing seed bugs. After removing the seed coat, individual seeds were homogenized in 0.3 mL of chilled buffer-A, consisting of 0.05 M $NaPO_4$ pH 7.5 with phenylmethylsulfonylfluoride (PMSF) (1 mM), leupeptin (5 μM)

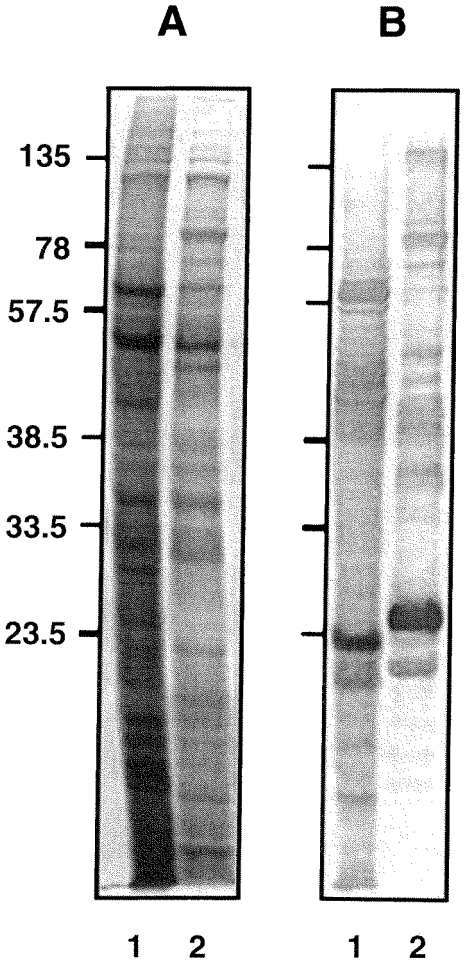

Fig. 1. Reduced SDS-PAGE gel of total proteins in salivary gland extracts stained with silver (A), and Western blot of a duplicate gel immunostained with affinity-adsorbed polyclonal antibody raised against *L. occidentalis* salivary gland extracts (B). Lane 1 = *L. occidentalis,* lane 2 = *L. corculus.* Equal quantity of protein (10 μg) was loaded in each lane. Numbers on left indicate approximate molecular masses in kDa of polypeptides.

and benzamidine (5 μM). The homogenate was centrifuged at 14000 × g for 30 min and the supernatant containing buffer soluble proteins was transferred to a new microcentrifuge tube and an equal volume of buffer-B, consisting of 62.5 mM Tris-HCl pH 6.8, 2% SDS (w/v) and 10% glycerol (v/v) was added. The lipid residue was removed from the tube containing the pellet using a tissue paper, and the pellet was washed and centrifuged, as above, three times using 1 mL of buffer-A each time and

discarding the supernatant. The pellet was re-suspended in 0.25 mL of buffer-B, boiled for 5 min and cooled. Following centrifugation at 14000 × g for 15 min, the supernatant containing buffer insoluble proteins was transferred to a new microcentrifuge tube.

Protein quantities extracted from salivary glands and seeds were determined (prior to the addition of β-mercaptoethanol) using the Bio-Rad *DC* Protein Assay (Alam 1992) with bovine serum albumin fraction V as a standard.

Salivary gland protein extracts were prepared for SDS-PAGE by adding an equal volume (0.1 mL) of buffer containing 0.125 M Tris-HCl pH 6.8, 8% (w/v) SDS, 20% (v/v) glycerol, and 1% (v/v) β-mercaptoethanol. Seed protein extracts were prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by adding 2 μL of buffer containing 60 mM Tris-HCl pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, and 5% (v/v) β-mercaptoethanol. The protein extracts (2-10 μl) were subjected to SDS-PAGE (Laemmli 1970), with 15% acrylamide resolving gels. Following electrophoresis, proteins on SDS-PAGE gels were stained with Coomassie Brilliant Blue R-250, silver stain, or were Western blotted to nitrocellulose for polyclonal antibody assays.

Polyclonal antibody assays. Polyclonal antibodies were raised against SDS-denatured antigens, from the salivary gland extracts of fourth-instar *L. occidentalis* (Lait et al. 2001a). We relied on the characteristic of antibody cross-reactivity to allow detection of *L. corculus* salivary proteins with the antibody prepared against salivary proteins of the related seed bug *L. occidentalis.* Antibodies were subjected to affinity adsorption against undamaged (non-hydrolyzed) total loblolly pine seed proteins extracted with 62.5 mM Tris-HCl pH 6.8, 2% SDS (w/v). Total seed protein extract (20 mg) was added to 5 mL of Affi-Gel 10 (Bio-Rad Laboratories, USA) and mixed on a rocking platform for 4 h at 4°C. Next, 500 μL of 0.1 M ethanolamine-HCl pH 8.0 was added to the gel, and mixing was continued for 1 h. A 1.0 × 10 cm chromatography column was packed with the affinity gel and equilibrated with 5 volumes of phosphate-buffered saline pH 7.5 (PBS). A 1.5-mL aliquot of antisera was loaded onto the column and the flow stopped. After 3 h of exposure, the column was rinsed with 5 column volumes of PBS and the first 3 mL of eluted antibodies were kept.

SDS-PAGE gels containing the insoluble protein fractions of 10 individual seeds representing each duration of laboratory exposure to *L. corculus* feeding, as well as those containing salivary gland proteins, were Western blotted to nitrocellulose membranes (Towbin et al. 1979) using a Trans-Blot SD semi-dry transfer cell (Bio-Rad Laboratories, USA). Nitrocellulose membranes were blocked overnight in Tris-buffered saline (TBS) containing 5% (w/v) non-fat milk powder and then incubated at room temperature with affinity-adsorbed primary polyclonal antibody (1:5000) raised against denatured *L. occidentalis* salivary gland protein extracts. Secondary antibody conjugated to alkaline phosphatase (1:10000) (Sigma Chemical Company, St. Louis, MO) was applied to show positive immunostaining with primary antibodies using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) as developing reagents. All primary and secondary antibodies were diluted in Tris-buffered saline containing 1% (w/v) non-fat milk powder and 0.05% Tween 20 (TTBS). Molecular weights were estimated using Bio-Rad low range pre-stained standards; phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme. Seed samples that contained immunoreactive polypeptides were designated as antibody positive.

Statistical analyses. The amount of protein remaining in seeds following each

exposure period was analyzed by a one-way analysis of variance (ANOVA). Means were compared by the Tukey Kramer HSD test, $\alpha = 0.05$ (Day and Quinn 1989). Data analyses were performed using JMP in software, version 4.0.3 (SAS institute, Cary, NC).

Results and Discussion

The silver-stained protein profiles of the salivary gland extracts, separated on SDS-PAGE gels, differed slightly between *L. corculus* and *L. occidentalis* (Fig. 1A). However, the polyclonal antibody raised against *L. occidentalis* salivary gland extract strongly recognized a large number of cross-reacting proteins from *L. corculus* salivary gland extracts on Western blots (Fig. 1B) indicating that antigens found in salivary gland extracts of the two related seed bugs are similar. The polyclonal antibody also recognized salivary proteins on Western blots of insoluble protein extracts from loblolly pine seeds that were partially filled or empty following exposure to *L. corculus* feeding, but did not detect any proteins in filled control seed that was not exposed to seed bugs or seed that was full (presumably not fed on) after exposure to seed bugs (Fig. 2). These results indicate that the antibody raised against *L. occidentalis* could be used to detect the presence of *L. corculus* salivary proteins in damaged seeds.

The major insoluble proteins of filled loblolly pine seeds resolved on reduced SDS-PAGE gels as two main groups of polypeptides from 22.5 to 24 kDa and 35.5 to 39.5 kDa (Fig. 2A). Similar protein profiles have been reported for several other *Pinus* spp. (Gifford 1988). There was substantial hydrolysis of these two main groups of insoluble polypeptides in seeds that were partially filled or empty following exposure to *L. corculus* feeding (Fig. 2B). An increased abundance of polypeptides $\leq$23.5 kDa on reduced SDS-PAGE gels also was observed in the soluble protein fraction of seeds that were partially filled or empty following exposure to *L. corculus* feeding (Fig. 2B). In contrast, seeds that sustained no feeding damage retained all of their major insoluble proteins and did not have increased amounts of these small soluble polypeptides (Fig. 2A).

Following 1 wk of laboratory exposure to feeding by *L. corculus*, the mean quantity of insoluble protein in loblolly pine seeds fell to about 55% of that in undamaged control seeds. Significant depletion of insoluble protein occurred only after 3 (63%) and 4 (89%) wks of increasingly intense feeding pressure (Fig 3). In contrast, the mean quantity of soluble protein rose to >2000 μg per seed after 1 wk of exposure, and then fell significantly after 3 and 4 wks. Although feeding pressure must have increased as the number of seeds was reduced over the 4-wk period, the hydrolysis and subsequent depletion of loblolly pine seed protein reserves caused by *L. corculus* was strikingly similar to the depletion of protein reserves in Douglas-fir seeds that sustained different levels of feeding damage after exposure to *L. occidentalis* for only 4 to 7 days (Bates et al. 2000). As in Douglas-fir, the generation of soluble peptides derived from hydrolysis of insoluble seed proteins (Lait et al. 2001a) likely accounts for the initial increase in soluble protein observed in loblolly pine seeds after 1 wk of exposure to feeding by *L. corculus* (Fig. 3).

Radiography (Fig. 4) and the antibody assay produced different damage estimates (Table 1). Both methods disclosed that extensive damage occurred after 1 wk of feeding, but the radiographic estimates over the 4-wk period were generally lower than the antibody determinations. Although radiography cannot be used to discriminate between abortion and seed bug feeding in field-damaged seeds, it may still be

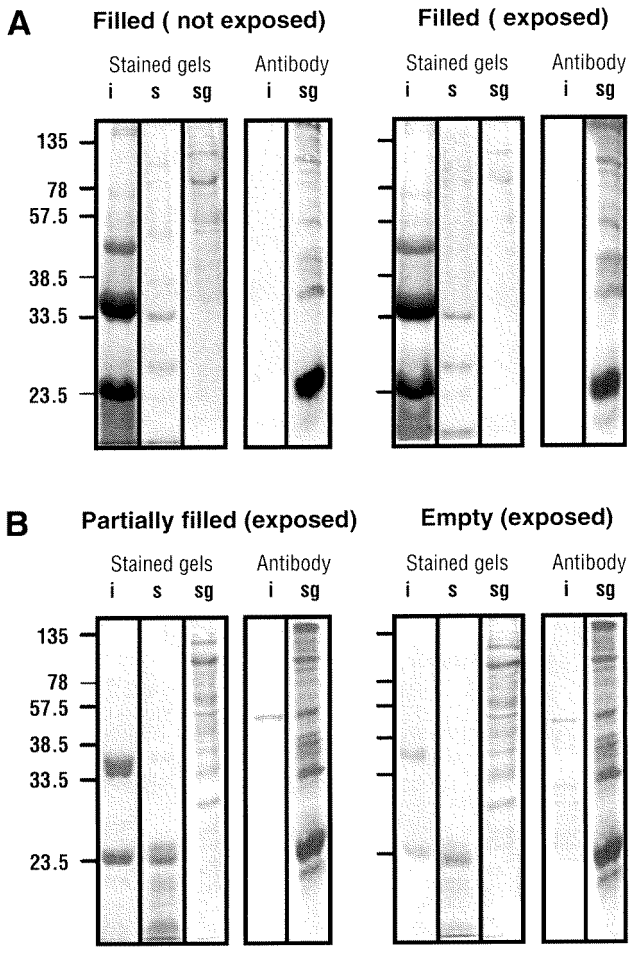

Fig. 2. Examples of insoluble and soluble protein profiles (SDS-PAGE and Western blots) from individual loblolly pine seeds that were: filled (not exposed to *L. corculus*) or filled following 2 wks of laboratory exposure to *L. corculus* (A); and partially filled or empty after 2 and 3 wks of laboratory exposure to *L. corculus*, respectively (B). SDS-PAGE gels were stained with Coomassie Brilliant Blue R-250 (Stained gels) and Western blots were immunostained with affinity-adsorbed polyclonal antibody raised against *L. occidentalis* salivary gland extract (Antibody). Lane identities: i = insoluble protein extract from seeds, s = soluble protein extract from seeds, sg = salivary gland extract from *L. corculus*. Numbers on left indicate approximate molecular masses in kDa. Sample volumes loaded in each lane were: 5 µL of insoluble proteins, 10 µL of soluble proteins, and 2 µL of salivary gland extract.

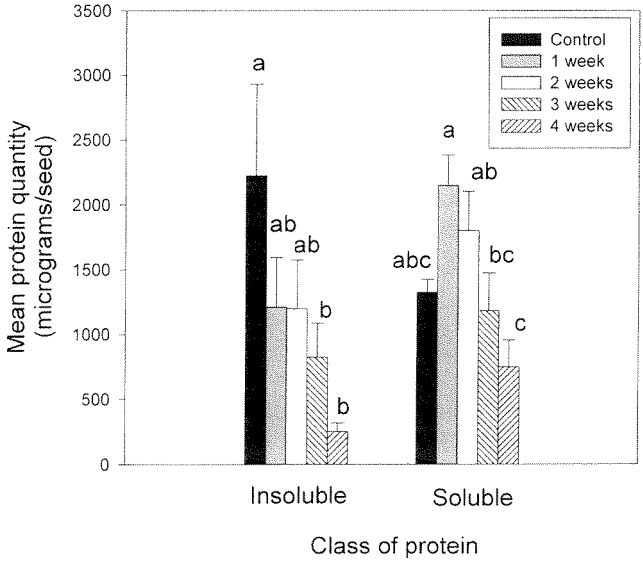

Fig. 3. Comparison of mean quantities (+SE, n = 10) of insoluble and soluble proteins in undamaged control loblolly pine seeds, and seeds that were exposed to continuous feeding by *L. corculus* in the laboratory for 1, 2, 3, or 4 wks, respectively. Bars for insoluble or soluble proteins with the same letter are not significantly different, Tukey-Kramer HSD test, $P < 0.05$.

useful with conifer species that produce large seeds, such as loblolly pine, to assess the degree of damage in partially depleted seeds (Bates et al. 2000). However, to assess seed bug impact in species that produce small seeds, which are more likely to be completely emptied during insect feeding, it is critical to have an accurate diagnostic tool such as the antibody assay (Bates et al. 2002). The antibody-based assay and radiography can only provide partial estimates of *L. corculus* feeding damage in loblolly pine because both methods use samples of mature seed. While the antibody-based assay is very specific and represents an improvement over radiography alone, neither technique accounts for the substantial pre-harvest losses of first year cones caused by second instar *L. corculus* nymphs or seed losses prior to seed extraction due to first and second-year ovule abortion (DeBarr and Ebel 1973, 1974, DeBarr and Kormanik 1975). In addition, it is possible that larger seeds such as loblolly pine may sustain minor amounts of damage without loss of seed viability. To accurately correlate feeding damage with loss of seed viability, germination studies must first be performed on seeds that have sustained varying degrees of physical damage.

The antibody assay is more sensitive than radiography (Table 1) and provides an accurate measure of feeding damage, including discrimination between naturally-aborted seed and seed emptied by seed bugs (Bates et al. 2002). The detection of cross-reacting antigens in seeds damaged by *L. corculus* using antibodies raised against *L. occidentalis* salivary gland extracts suggests that the same assay devel-

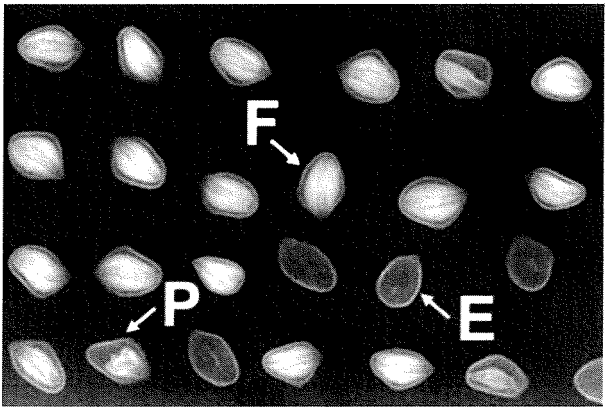

Fig. 4. Radiograph depicting full (F), empty (E), and partially-depleted (P) seeds of loblolly pine after exposure to feeding by *Leptoglossus corculus*.

Table 1. Comparison of percentages of loblolly pine seed fed on by *L. corculus* over a four-week period as estimated by radiography and determined by antibody assay

	Percentage damaged			
	Radiographic assessment (n = 50)			
Exposure duration (days)	Partially-filled seeds	Empty seeds	Total seeds	Antibody assay positive (n = 10)
0	0	0	0	0
7	50	0	50	70
14	40	14	54	80
21	49	29	78	100
28	37	33	69	90

oped to assess the impact of *L. occidentalis* could be used to help determine the role *L. corculus* feeding plays in current seed losses observed in commercial seed orchards of the southern USA. Adaptation of the antibody-based assay to suit the needs of seed testing laboratories might be desirable. Transfer of extracted proteins to commercially available solid supports such as micro-well plates for ELISA, nitrocellulose paper for dot/slot blots or development of dipstick (lateral flow) test strips (Bangs et al. 2002) might improve the efficiency of the antibody-based assay by eliminating the need for SDS-PAGE or Western blots. Sensitivity of the assay might be enhanced if antibodies derived from *L. corculus* salivary antigens were used. Ideally, a similar assay could also be devised for the shieldbacked pine seed bug, *Tetyra bipunctata* (Herrich-Schäffer) (Pentatomidae), the other major seed bug in

southern pine seed orchards (Hedlin et al. 1981). The development of species-specific antibody assays could allow seeds to be assessed at harvest for damage from multiple seed-feeding insect species.

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