Lipid, Polyamide, and Flavonol Phagostimulants for Adult Western Corn Rootworm from Sunflower (*Helianthus annuus* L.) Pollen

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Adult Diabroticites including western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte, consume pollen of corn, squash, sunflower, and other species. Short-chain neutral amino acids in methanol−water extracts of pollen have been previously identified in our laboratory as strong phagostimulants for *Diabrotica*. Bioassay-driven fractionation was used to characterize the interacting lipid and midpolarity phagostimulants for adult WCR in Giant Gray Stripe sunflower, *Helianthus annuus* L., pollen. Lipids rich in ω3-linolenic acid including triglycerides, free fatty acids, phosphatidylethanolamines, phosphatidic acids, and phosphatidylcholines were highly phagostimulatory. Other important phagostimulatory components included a hydroxycinnamic acid-polyamine amide, N^1,N^5,N^{10}-tri[(*E*)-*p*-coumaroyl]spermidine, and a flavonol, quercetin β-3-*O*-glucoside. The structural characteristics of these phagoactive compounds and their role in the pollinivory specialization of rootworm beetles are discussed.

Keywords: *Phagostimulant; α-linolenic acid; lipids; hydroxycinnamic acid-polyamine amide; flavonol; western corn rootworm; sunflower; pollen; feeding specialization*

INTRODUCTION

The pollinivory (pollen-feeding) trait is common in insects. The role of visual guides and olfactory cues have dominated investigations on pollinator orientation to floral and extrafloral rewards including pollens and nectars (Harborne, 1993). The role of taste perception in insect pollen feeding is essentially unknown. Although visual, olfactory, and physical aspects can be important in insect host finding and acceptance (Schoonhoven et al., 1998), contact chemoreception of nonvolatile nutrient and secondary chemicals usually directs the ultimate choice of food. Cucurbitacins, oxygenated tetracyclic triterpenes, responsible for the characteristic bitter taste of most wild Cucurbitaceae, are well-known as specific arrestants and feeding stimulants for adult Diabroticite beetles (Coleoptera, Chrysomelidae, Galerucinae) (Metcalf et al., 1980; Metcalf and Lampman, 1989). In fact, the strong phagostimulatory actions of cucurbitacins on adult Diabroticite beetles has led to their use in baits for adult rootworm pest control (Metcalf and Metcalf, 1992), but dependence on cucurbitacin baits has some limitations, including the cytotoxic effect of cucurbitacins (Miró, 1995).

The phagostimulatory sensitivity of Diabroticite species to cucurbitacins is not correlated with Cucurbitaceae specialization (Metcalf and Metcalf, 1992), indicating that other factors, including other phagostimulants or lack of feeding deterrents, may influence host-plant affinities among these beetles. Adults of major Diabroticites pests such as the western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte, include pollen in their natural diets (Ludwig and Hill, 1975; Samuelson, 1994) and consume pollen as a laboratory diet (Mullin et al., 1994; Hollister and Mullin, 1999). Although these beetles can feed on other tissues of corn and squash, their natural prevalence in flowers and high acceptance of pollen suggest a special affinity for pollens. Pollen, as a highly preferred *Diabrotica* food, may provide alternative phytochemical stimulants for selective rootworm control.

The chemistry of pollen has been widely investigated, particularly at an early stage (Barbier, 1971; Stanley and Linskens, 1974). Pollen is a rich food source with protein, free amino acids, sugars, starch, fats, various vitamins and inorganic salts, and varying amounts of secondary substances, but little is known about the gustatory cues that determine pollen consumption. *Diabrotica* beetles, major pests of corn (*Zea mays* L.) throughout Pan-America, of which WCR is the most pestiferous, have varied feeding preferences among tested pollen species (Hollister and Mullin, 1999). Knowledge of pollen chemistry and its involvement in Diabroticite gustation is an initial step in understanding the phytochemosensory basis for pollinivory.

Adult WCR readily consume pollens including corn, winter squash (*Cucurbita maxima* Duchesne), and common sunflower (*Helianthus annuus* L.). Extraction of pollens showed that free amino acids including alanine, serine, β-alanine, proline, and γ-aminobutyric acid are strong beetle feeding stimulants (Mullin et al., 1994; Hollister and Mullin, 1999). However, particularly for sunflower, additional phagostimulatory activity was found in the more nonpolar and amino acid-lacking extracts of the pollen. Sunflower is an important crop, particularly in the northern United States and in Canada. The *Helianthus* genus is remarkable for the breadth of chemicals synthesized. Antifeedants and neurotoxicants for adult rootworm have been isolated and identified (Mullin et al., 1991) from floral tissues of the cultivated sunflower, but not from the pollen

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10.1021/jf980858w CCC: $18.00
Published on Web 02/10/1999

[reviewed in Mullin et al. (1994)]. Using bioassay-driven fractionation, we present here the isolation and identification of additional strong phagostimulants for adult WCR from sunflower pollen that reside in more lipophilic extracts than for free amino acids.

MATERIALS AND METHODS

Plant and Insect Materials. Giant Gray Stripe sunflower was grown on a silt loam soil in a multicrop field plot at The Russell E. Larson Field Research Center at Rock Springs in Centre Co., Pennsylvania, in 1997. Inflorescences at or just before anthesis were cut with ~1 ft of stem using a sharp knife and immediately brought on ice from the field to the laboratory, where they were recut, placed in a reservoir of water, and allowed to shed pollen at room temperature into aluminum trays. Daily collections of fresh pollen were then sieved through 153 and 80 μm Nitex screens (Tetko Inc., Briarcliff Manor, NY) using a Vortex. Pollen was stored at −80 °C until use.

Pupae of a nondiapause strain of WCR were obtained from French Agricultural Research, Inc. (Lamberton, MN). Adults after eclosion were reared in plastic boxes on store-bought acorn squash fruit at 25 °C under a 16:8 light/dark photoperiod in an EGC growth chamber. Adult beetles ranging in age from 5 to 18 days were used in feeding bioassays.

Feeding Bioassay. A no-choice feeding test adapted from that of Kim and Mullin (1998) was used to measure phagostimulatory activity. An aliquot (4−5 μL) of pollen extract or fraction (5 mg of fresh pollen equivalent), authentic standard, or combinations of fractions or purified compounds was applied to one 6 mm diameter disk of regenerated cellulose (0.45 μm pore, Schleicher & Schuell, Keene, NH), air-dried, and placed in a 9 cm Petri dish arena with two randomly selected beetles, as described (Kim and Mullin, 1998). The beetles were allowed to feed for 24 h under the rearing chamber conditions as before except filter paper was used to shade dishes from the top. Disks were then air-dried; surface areas were determined to the nearest 0.01 mm^2 on an Optomax V image analyzer (Analytical Measuring Systems, Ltd., Saffron, Walden, U.K.) and then subtracted from the solvent control disk area to give the area consumed. A percentage consumption ratio for each disk was calculated by dividing the average solvent control disk area minus the treatment disk area by the control disk area and multiplying the result by 100. At least five replicates were used for each treatment. In preliminary binary choice tests between a treated and a solvent control disk, no consumption of the controls occurred. Because there were no significant differences between choice and no-choice tests, the latter test format was used for all behavioral measurements of phagostimulatory activity.

General Identification Methods. UV and IR spectra were obtained with Perkin-Elmer Lambda 3B and 1600 FTIR spectrophotometers, respectively. NMR experiments were performed at 25 °C on a Bruker AMX-2-500 operating at 500.13 MHz for ^{1}H and at 125.77 MHz for ^{13}C, and fast atom bombardment mass spectroscopy (FABMS) was measured on a Kratos MS-50 within the Pennsylvania State University, Department of Chemistry NMR and MS Facilities.

Reference Compounds. Trilinolenin (1,2,3-tri[(*cis,cis,cis*)-9,12,15-octadecatrienoyl]glycerol, 98%), trilinolein (1,2,3-tri-[(*cis,cis*)-9,12-octadecadienoyl]glycerol, 99%), α-linolenic acid (9,12,15-octadecatrienoic acid, 98%), L-α-phosphatidylcholine dicaproyl, and tripalmitin (1,2,3-trihexadecanoylglycerol, 99%), were obtained from Sigma. All other reagents and solvents were of reagent grade or better.

Phytochemical Isolation. Fresh pollen (20 g) collected in August 1997 was sequentially extracted with 5 × 100 mL of CH_2Cl_2/MeOH (1:1), 2 × 100 mL of MeOH, 5 × 100 mL of 80% aqueous MeOH, and 1 × 100 mL of H_2O at room temperature. For each extraction, the pollen suspension was magnetically stirred for 5 min, and the combined supernatants for all organic solvent extracts after filtration were then concentrated with a Büchi rotovapor at 35 °C down to an aqueous phase which was sequentially partitioned with CH_2Cl_2 (3 × 250 mL) followed by ethyl acetate (5 × 300 mL). The remaining aqueous phase and original water extract were combined, concentrated to 20 mL, and applied to Dowex 50 × 8-200 cation-exchange resin in the H^+ form (10 × 100 mm of resin) for fractionation into neutral and acidic (unbound water wash) and basic and amphoteric components (2 N NH_4OH eluate). The combined organic phases were concentrated at 35 °C and applied to two 10 × 500 mm open columns of silica gel 60 (70−230 mesh, E. Merck, Milwaukee, WI).

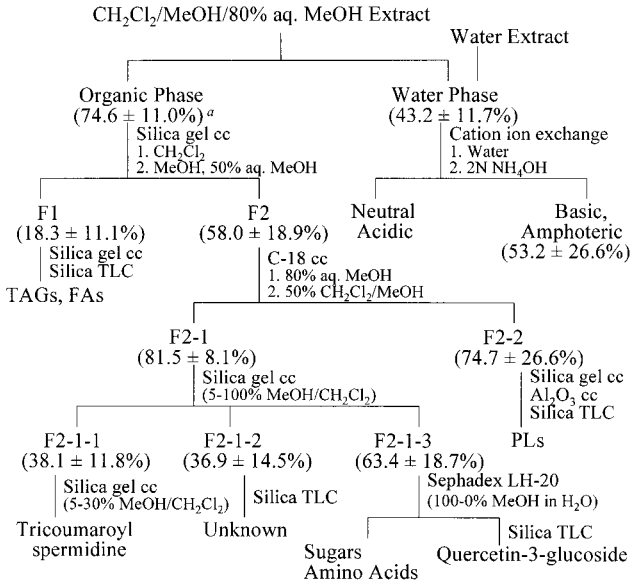

Figure 1. Isolation scheme for adult WCR phagostimulants from sunflower pollen. [a]Mean ± SE (n = 5) percentage disk consumption at 5 mg of fresh pollen equivalent.

The scheme used to chromatographically isolate from sunflower pollen some novel phagostimulants for adult WCR is outlined in Figure 1 (each fraction was adjusted to 1 g of fresh pollen equivalent/mL for bioassay). CH_2Cl_2 elution of the bound organics on the silica gel column gave fraction F1, whereas F2 was eluted with MeOH and 50% aqueous MeOH. Silica gel column chromatography of F1 with increasing CH_2Cl_2 in petroleum ether followed by MeOH in CH_2Cl_2 or with increasing diethyl ether (containing 1 ppm of BHT) in petroleum ether afforded feeding stimulatory triacylglycerides (TAGs) and fatty acids (FAs), which were further resolved by thin-layer chromatography (TLC) (silica gel 60 $F_{254,}$ 250 μm, E. Merck) using the appropriate CH_2Cl_2, petroleum ether/diethyl ether (90:10 or 80:2), or toluene/CH_2Cl_2/MeOH (98:2:0.2) developing system.

Fraction F2 was applied to an open column (25 cm × 100 mm) of C_{18} reversed-phase silica gel (Aldrich, Milwaukee, WI) and eluted sequentially with 0−80% MeOH in H_2O (all fractions combined as fraction F2-1) and with 0−50% CH_2Cl_2 in MeOH (combined as fraction F2-2); both eluates were stimulatory to WCR feeding. F2-1 was further fractionated on an open column of silica gel stepwise with 5−100% MeOH in CH_2Cl_2 to give three phagostimulatory fractions (F2-1-1 to F2-1-3). N^1,N^5,N^{10}-Tri-[(*E*)-*p*-coumaroyl]spermidine (Figure 2) was purified from F2-1-1 by repeated silica gel column chromatography with 5−30% MeOH in CH_2Cl_2 as the elution solvent. The undetermined phagoactive compounds were purified from fraction F2-1-2 by TLC developed with ethyl acetate/2-pentanone/formic acid/water, 5:3:1:1, and CH_2Cl_2/MeOH, 3:1. This same solvent system was used to resolve by TLC the flavonols in F2-1-3 after processing on a column (25 × 500 mm) of Sephadex LH-20 (Pharmacia Biotech, Piscataway, NJ) with increasing MeOH in H_2O to remove sugar and amino acid impurities. Open column chromatography of F2-2 on silica gel using increasing MeOH in CH_2Cl_2 and finally 50% H_2O in MeOH gave phagostimulatory phospholipid fractions. Phosphatidylcholines (PCs) and lysophosphatidylcholines (LPCs) were separated from other phospholipids (PLs) on an aluminum oxide (neutral, Alupharm Chemicals, New Orleans, LA)

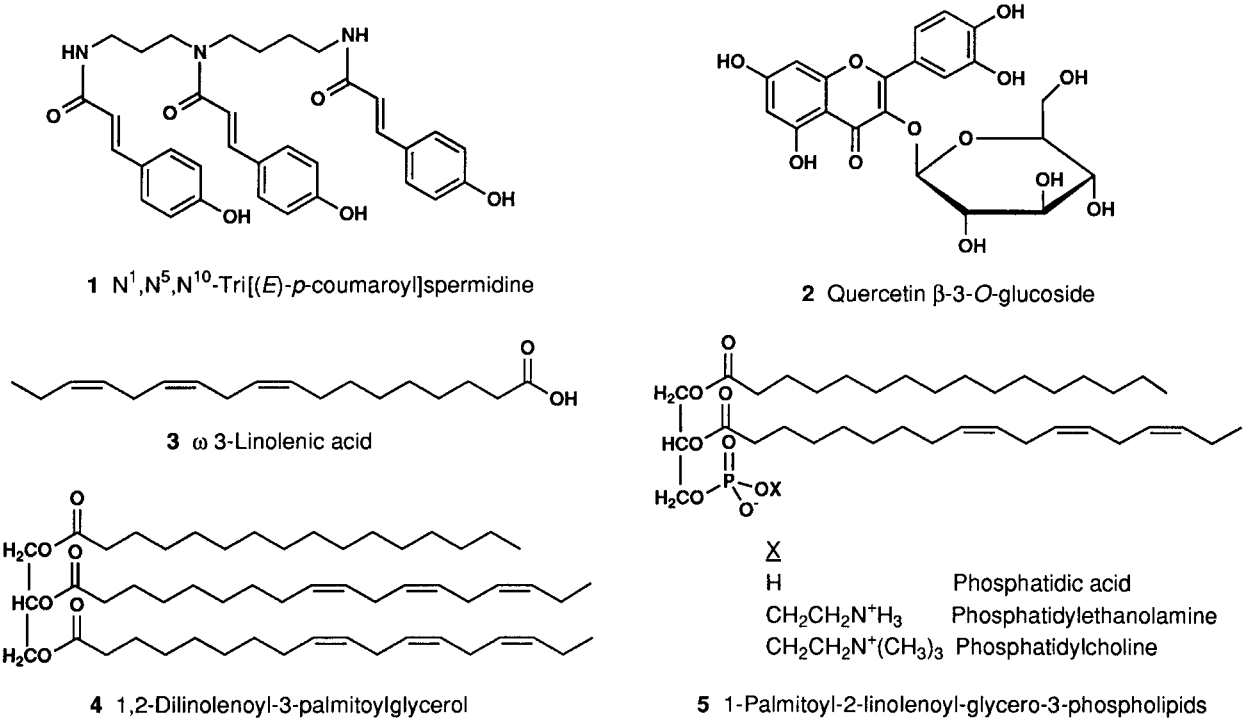

Figure 2. Structures of phagostimulants for adult WCR from sunflower pollen.

open column (10 × 500 mm) with MeOH/$CHCl_3$ (1:1) as the elution solvent. Phosphatidylethanolamines (PEs), phosphatidic acids (PAs), phosphatidylinositols (PIs), lysophosphatidylethanolamines (LPEs), PCs, and LPCs were purified by analytical TLC developed in $CHCl_3$/MeOH/H_2O (71:26:3) alone or consecutively with $CHCl_3$/MeOH/NH_4OH (65:25:5) and were identified by cochromatography with authentic standards (Sigma) and by their color reactions toward Phospray (Supelco, Bellefonte, PA), ninhydrin (Sigma), Dragendorff's reagent (Sigma), I_2 vapor, and H_2SO_4.

Pollen Content of Phagoactive Components. UV−visible reflectance densitometry (Schimadzu CS-9000U dual-wavelength flying-spot scanner, Columbia, MD) of HPTLC (Kieselgel 60, 10 × 10, E. Merck) resolved compounds was used to determine amounts of phagoactive components on a fresh pollen weight basis (Table 1). Analysis of fractions F1, F2-1, and F2-2 was used to approximate pollen contents. The purified hydroxycinnamic acid polyamide, flavonol, and the unknown(s) were resorted to for standard curve construction and HPTLC analysis of the three bioactive components of F2-1. Petroleum ether/acetone/2-propanol (5:3:2, **A**) and 224 nm, ethyl acetate/2-pentanone/acetic acid/water (5:3:1:1, **B**) and 365 nm, or system **B** and 267 nm were the solvent systems and absorbance maxima utilized, respectively, for HPTLC development and reflectance densitometry of these three phagostimulants. For TAGs and FAs in F1, two-dimensional HPTLC with petroleum ether/ethyl ether/formic acid (90:10:2, **C**) followed by CH_2Cl_2 (**D**) development, visualization with 50% aqueous sulfuric acid at 110 °C, and analysis at 485 nm was used for quantification, with purified TAGs and FAs as the standards. For PLs in F2-2, L-α-phosphatidylcholine dicaproyl was used as a calibration standard (R_f = 0.43) after development in $CHCl_3$/MeOH/H_2O (71:26:3, **E**), visualization with Phospray, and densitometry at 565 nm.

Fatty Acid Analyses. Free and released FAs after 1 N potassium hydroxide base hydrolysis of TAGs and PLs or phospholipase A2 (bee venom, Sigma) treatment of PEs were converted into methyl esters (Robyt and White, 1990) using a 14% boron trifluoride (BF_3)/methanol reagent (Sigma). Methyl esters dissolved in hexane were then analyzed on a Hewlett-Packard 5890 gas chromatograph equipped with a 30 m × 0.25 mm i.d. × 0.2 μm film thickness Rtx-2330 (10% cyanopropylphenyl−90% bis-cyanopropyl polysiloxane) capillary column (Restek Corp., Bellefonte, PA) using a flame ionization detector. Helium at 30 cm s^{-1} and a temperature program of 110 °C for 2 min increased at 2 °C/min to 200 °C was used to resolve unknown fatty acid methyl ester (FAMEs) mixtures for identification by cochromatography with standards (Sigma). FA compositions were calculated as percentages of the total peak area for significant (>0.2%) fatty acids in a lipid fraction.

Table 1. Feeding Stimulants for Adult WCR in Organic Solvent Extracts from Sunflower Pollen

pollen chemical			feeding activity	
compound	R_f[a]	content (μmol/g of pollen)	amount (nmol/disk)	% consumption[b] (24 h)
tricoumaroylspermidine	0.92, **A**	49.9	103	31.5 ± 11.5[d]
unknown(s)	0.45, **B**	2.8 (mg)	6 (μg)	37.5 ± 7.5[d]
quercetin 3-glucoside	0.53, **B**	9.7	17.2	31.3 ± 10.7[d]
TAGs	0.31, **C**; 0.86, **D**	12.6[c]	23.5	33.2 ± 14.3[d]
FAs	0.13, **C**; 0.05, **D**	2.9[c]	86.2	52.4 ± 18.3
PEs	0.79, **E**	4.3	20	40.6 ± 6.6
PAs	0.64, **E**	1.3	20	37.6 ± 10.4
PCs	0.43, **E**	9.1	20	29.8 ± 7.5[d]
PIs	0.30, **E**	0.5	2.1	ns[e]
LPEs	0.33, **E**	0.5	20	ns
LPCs, other PLs	0.12, **E**	0.4		

[a] By HPTLC, solvent systems in bold letters as defined under Materials and Methods. [b] Mean ± SE percentage disk consumption. [c] TAGs and FAs contents were calculated on the basis of their GLC analysis (Table 2). [d] Activities were measured at ∼2 mg of fresh pollen equivalent per disk. [e] Nonsignificant consumption compared to control.

***N*^{1},*N*^{5},*N*^{10}-Tri[(*E*)-*p*-coumaroyl]spermidine (1).** The UV (MeOH) λ_{max} 308, 297, 224, 212 in MeOH of this major hydroxycinnamic acid-polyamine amide shifted to λ_{max} 350, 309, 240, 213 with NaOH, demonstrating the *p*-coumaroyl moeity in the *E* form (Werner et al., 1995). Strong alkaline hydrolysis (Meurer et al., 1986, 1988) gave only *p*-coumaric acid based on cochromatography by TLC and on its UV absorbance with and without NH_3 vapor. Confirmation of **1** was obtained by FABMS and by ^1H and ^{13}C NMR in CD_3OD. The MS gave $[M + 1]^+$ 584.2, and both ^1H and ^{13}C NMR data

were consistent with those found in previous literature (Meurer et al., 1988; Werner et al., 1995).

Quercetin β-3-*O*-Glucoside (2). Confirmation of this flavonol glucoside occurred through UV spectroscopy using the shift reagents NaOH, NaOAC, NaOAC·H_3BO_4, $AlCl_3$, $AlCl_3$·HCl and ^{1}H NMR in DMSO-d_6, TLC cochromatography with authentic isoquercitrin (Extrasynthése, Genay, France), and release of quercetin (Sigma) and glucose after its hydrolysis in 1 M trifluoroacetic acid at 100 °C (Mabry et al., 1970; Markham, 1982). The glucose H-1 doublet at δ 5.38 with a large $J = 7.44$ Hz is evidence for a glucose β-linked to the 3-OH of quercetin; all other resonances were identical with those of isoquercitrin.

Linolenoyl (3)-Dominant TAGs (4) and PLs (5). TAGs was analyzed by FT-IR in $CHCl_3$, by ^{1}H and ^{13}C NMR in $CDCl_3$ and by GLC (above). An aliphatic ester with nonconjugated double-bond absorptions in the FT-IR was consistent with dominance of ω-3 olefin methyl resonance at δ 0.93 (*t*) in the ^{1}H NMR and the 1,4-all cis double bond δ 5.34 (*m*) and 127.2−130.2 resonances in the ^{1}H and ^{13}C NMR spectra, respectively. A dominance of α-linolenoyl in the TAG and the PL fractions was established and confirmed by FAMEs analysis as above.

RESULTS

Phagostimulant Isolation and Identification. WCR can be induced to feed on regenerated cellulose membranes coated with either organic solvent soluble or water soluble components of sunflower pollen extracts with percentage disk consumptions of 74.6 ± 11.0 and 43.2 ± 11.7, respectively, at 5 mg of pollen equivalent. Water soluble components responsible for phagostimulatory activity largely bind to strongly acidic Dowex and have been identified as free amino acids (Mullin et al., 1994; Hollister and Mullin, 1999), particularly the short-chain neutral amino acids (Kim and Mullin, 1998). Here we purified and identified the more lipophilic components of sunflower pollen responsible for stimulating feeding by WCR.

Silica gel chromatography of the organosolubles from the original combined methylene chloride/methanol extract of sunflower pollen resulted in a weakly active fraction F1 (18.3 ± 11.1% consumption at 5 mg of pollen equivalent) and a more polar and stronger phagostimulatory F2 (58.0 ± 18.9%) fraction. The latter after C_{18} reversed-phase chromatography gave two strongly stimulatory polar F2-1 (81.5 ± 8.1%) and midpolar F2-2 (74.7 ± 9.0%) fractions. Repeated silica gel chromatography of the most lipophilic components of F2-1 (Figure 1) gave the abundant N^1,N^5,N^{10}-tri[(*E*)-*p*-coumaroyl]spermidine (49.9 μmo/g of fresh pollen, Table 1) and other minor and much less active hydroxycinnamic acid polyamides (1.5 mg/g of pollen) that remain unidentified. Tricoumaroyl spermidine at 2 mg of fresh pollen equivalent per disk evoked 31.5 ± 11.5% consumption (Table 1). A more polar bioactive component from F2-1 was similarly purified (Figure 1) that elicited 37.5 ± 7.5% disk consumption at 2 mg of pollen equivalent (Table 1), but its identity remains unknown. The third and most polar phagostimulant in F2-1 was freed of sugars and amino acids using Sephadex LH-20 chromatography and then isolated on silica TLC to give mostly quercetin 3-glucoside (4.5 mg/g of pollen) with 31.3 ± 10.7% consumption (2 mg of pollen equivalent, Table 1) and some minor and less phagoactive flavonols.

Bioassay-driven fractionation of the constituents of F2-2 led to isolation of phospholipids of which PEs, PAs, and PCs were similarly active (Table 1). Significant disk consumption with these PLs occurred at 2−5 nmol/disk, which increased in a dose-dependent manner to 20 nmol and then did not increase further (Figure 3). Conversely, PIs and LPEs were not significantly active in the range 2−40 nmol/disk (Table 1; data not shown), and only low or trace amounts of other PLs were obtained.

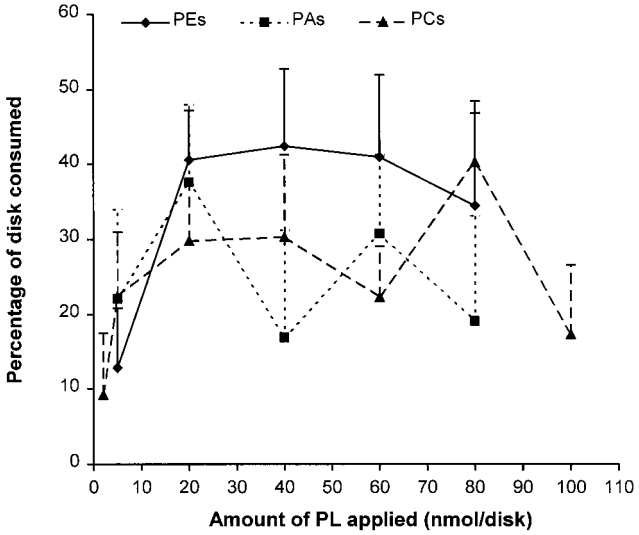

Figure 3. Dose−phagostimulation relationships for PLs of different polarity from sunflower pollen on adult WCR.

Table 2. FA Compositions of Lipid Fractions in Sunflower Pollen[a]

FA	t_r[b] (min)	TAGs	FAs	PEs	PAs	PIs	PCs	LPEs	LPCs
C16:0	14.28	21.1	43.8	51.2	54.8	57.9	56.0	94.6	65.1
C18:0	20.15	0.4	5.7	0.2	0.5	1.0	0.7	1.4	19.6
C18:1	21.38	1.4	4.1	0.2	0.2		0.2		
C18:2	23.69	9.0	11.9	13.9	9.7	4.5	11.7	4.0	9.8
C18:3	26.53	68.1	34.5	34.4	34.8	36.7	31.4		5.5

[a] Percentage of total peak areas. [b] Retention time after injection.

Removal of interfering carotenoids and other UV-quenching components from F1 through silica chromatography (Figure 1) led to an increase in its relative phagostimulatory activity and isolation of high amounts of TAGs (12.6 μmol/g of pollen) and lower amounts of FAs (2.9 μmol/g of pollen) (Table 1). Although the FA fraction was more phagostimulatory than TAGs on a weight basis (Table 1), the TAGs were much more abundant and accounted for most of the F1 bioactivity. Surprisingly, FA analysis revealed that ω3-linolenic acid (68.1%) dominated in TAGs followed by C16:0 (21.1%), C18:2 (9.0%), and then much lower or trace amounts of 18:1 and 18:0 (Table 2). A similar trend was found for FAs, and palmitic, linolenic, and linoleic essentially comprised the FAs of all phagostimulatory PLs (Table 2). Silver nitrate-impregnated silica TLC (Hamilton, 1986) of TAGs in sunflower pollen resolved them into five spots using 6% MeOH in CH_2Cl_2 with only one spot of R_f higher than trilinolein. This indicates that most TAGs in this pollen have two C18:3 moieties. Phospholipase A2 hydrolysis of PEs released 16.9% C16:0, 0.2% C18:0, 0.7% C18:1, 22.4% C18:2, and 59.9% C18:3, whereas base hydrolysis of the remaining LPEs gave 77.6% C16:0, 0.6% C18:0, 0.3% C18:1, 6.1% C18:2, and 15.4% C18:3. This is consistent with C16:0 dominating at position 1 and C18:3 at position 2, which is typical of unsaturated PLs (Hanahann, 1997).

Fatty Acid Structure−Phagoactivity Relationships. Lipids from lipophilic TAGs and FAs to polar PLs all induced WCR feeding, indicating that bioactivity may reside in the FA moiety. That the unusually high

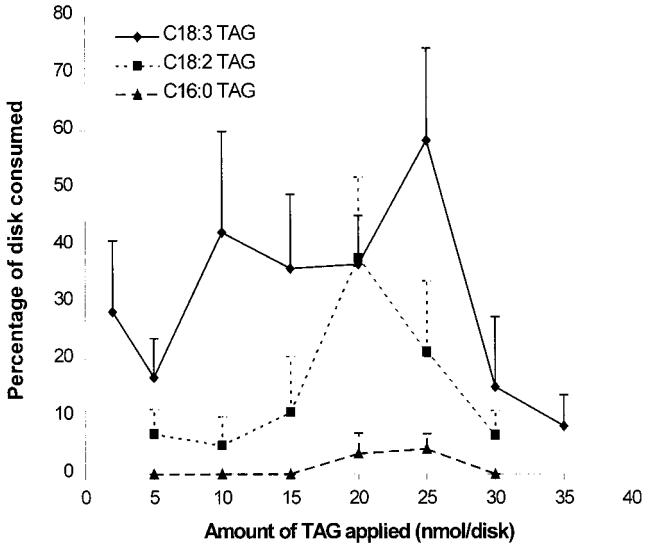

Figure 4. Dose−phagostimulation relationships for authentic TAGs with different degrees of unsaturation on WCR.

ω3-C18:3 content of pollen lipids is likely responsible for bioactivity was demonstrated by feeding experiments with authentic triglycerides in which ω3-trilinolenin > trilinolein ≫ tripalmitin in stimulating feeding (Figure 4). With the former TAG, beetle feeding was significantly induced at 2 nmol/disk and continued to increase with dose to 58.2 ± 16.1% consumption at 25 nmol/disk, after which the activity decreased. By contrast, the 18:2 TAG was only significantly phagostimulatory at 20 nmol/disk and then reduced activity with increasing dose (Figure 4). Free α-linolenic acid increased beetle feeding from 17.6 ± 7.7% at 20 nmol/disk to 42.3 ± 10.6% at 100 nmol/disk and then decreased consumption at higher doses (data not shown). Moreover, TAGs present in sweet corn (*Zea mays* L.) var. Bodacious pollen (Lin, unpublished data) are similarly phagostimulatory to WCR and rich in C18:3 (43.1%), as well as C16:0 (49.4%) and C18:2 (3.8%). Cationic (PEs, PCs) and anionic (PAs) phospholipids rich in ω3-linolenic acid were uniformly stimulatory to WCR feeding (Table 1), although the anomaly with PIs may be due to their neutral polyol headgroup.

DISCUSSION

Conjugates between hydroxycinnamic acids, particularly caffeic, ferulic, and *p*-coumaric acids, and the aliphatic polyamines putrescine, spermidine, and spermine are widely distributed in the plant kingdom, preferentially occurring in reproductive organs and seeds and involved in the flowering process and sexual organogenesis (Martin-Tanguy et al., 1978; Smith et al., 1983; Martin-Tanguy, 1985; Flores et al., 1989; Harborne, 1994). Hydroxycinnamic acid amides have also been linked to disease resistance, especially toward fungi and viruses, and to the protection of pollen grains from UV light damage (Ellestad et al., 1978; Smith et al., 1983; Martin-Tanguy, 1985). These phenolic amides are primarily distributed in the outer exine of the pollen grain (Gubatz et al., 1986). Here we show for the first time for any animal that a member of this class of secondary compounds, tricoumaryl spermidine, is a phagostimulant for a pollen feeding specialist, the western corn rootworm.

Plant pollens also accumulate the ubiquitous flavonoids (Ceska and Styles, 1984), whereas mostly flavonol glycosides have been shown to have important roles in pollen germination (Taylor et al., 1994). These phenolics also can reside at high concentrations in the exine, where they impart a distinctive yellow color (Wiermann and Uieth, 1983), and can function as pollen signals for insect herbivores (Harborne, 1993). We demonstrated that the prevalent sunflower pollen flavonol, quercetin 3β-glucoside (2 mg of pollen equivalent), is a significant phagostimulant for WCR (Table 1) and joins an increasing representation of both stimulants and deterrents for insect feeding among the flavonoids (Hedin et al., 1968; Mullin et al., 1991; Schoonhoven et al., 1998).

Primary nutritive lipids have been shown to stimulate feeding in other insect species (Hsiao, 1985). For larvae of WCR, linoleic and oleic acids but not stearic acid, when combined with carbon dioxide, are attractive (Hibbard et al., 1994). Octadeca-*trans*-2,*cis*-9,*cis*-12-trienoic acid found in mixed pollen was stimulatory to honey bee (Hopkins et al., 1969). Phospholipids of unknown FA composition, such as lecithin and PI from wheat germ oil, are phagostimulatory for two grasshoppers, *Camnula pellucida* and *Melanoplus bivittatus* (Thorsteinson and Nayar, 1963), whereas PIs, PSs, or PCs elicit Colorado potato beetle, *Leptinotarsa decemlineata*, larvae to feed (Hsiao and Fraenkel, 1968). In insect-pollinated plant species, lipids are major metabolic products during pollen development (Piffanelli et al., 1997) that accumulate primarily in the pollen coat or pollenkitt (Wiermann and Gubatz, 1992). Pollens often (Evans et al., 1990) but not always (Bianchi et al., 1990) have high contents of ω3-C18:3 FA, which are essential nutrients in *Diabrotica* (Ogg et al., 1993) and most insect diets. Thus, linolenoyl-rich lipids could serve as appropriate pollen-selective taste cues for pollen-feeding specialists.

Individually, any lipid or midpolarity phagostimulant isolated from organic extracts of sunflower pollen could not fully explain WCR pollen feeding. This has led us to pursue synergistic and additive interactions between pollen classes of phagostimulants. For example, a combination of the α-linolenoyl-rich lipids such as 15 nmol of trilinolenin with 20 nmol of L-alanine induces high disk consumption (87.7 ± 5.7%) for the mixture compared with 20.1 ± 7.4% for L-alanine or 35.9 ± 12.9% for trilinolenin alone. That synergistic interaction occurs between these more lipophilic components and the previously identified stimulatory amino acids of sunflower pollen will be detailed in a subsequent manuscript (Lin and Mullin, unpublished data). By this work, we aim to define the chemical basis for pollinivory and more generally the gustatory code that determines organ-specific plant feeding in insects.

CONCLUSIONS

Adult WCR use common primary nutrients, such as α-linolenoyl-rich lipids and amino acids, and secondary chemicals, such phenolic polyamides and flavonols, in pollen as feeding cues. The identification and elucidation of the interactions between phagostimulants and presumed antifeedants in these highly preferred pollen foods will assist in our understanding of the phytochemosensory basis for WCR and other Diabroticite beetle−plant interactions. Optimizing the combination of *all-cis*-ω3-linolenic acid lipids, tricoumaroylspermidine, and quercetin 3-glucoside with known phagostimulatory amino acids will aid in the development of

alternative bait formulations for adult Diabroticite control and ultimately the commercialization of environmentally safe biopesticides for these primary pests of corn and other crops.

ABBREVIATIONS USED

BHT, 2,6-di-*tert*-butyl-4-methylphenol; FAs, fatty acids; FAMEs, fatty acid methyl esters; LPCs, lysophosphatidylcholines; LPEs, lysophosphatidylethanolamines; PAs, phosphatidic acids; PCs, phosphatidylcholines; PEs, phosphatidylethanolamines; PIs, phosphatidylinositols; PLs, phospholipids; TAGs, triacylglycerides; WCR, western corn rootworm.

ACKNOWLEDGMENT

We thank A. J. Benesi and A. D. Jones for their help in NMR and MS analysis, and D. Conway for her technical assistance.

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Received for review August 3, 1998. Revised manuscript received December 24, 1998. Accepted January 6, 1999. We thank the USDA (NRICGP 95-37302-1807) for financial support.

JF980858W