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Influence of Foliar Fungicide Treatment on Lipolytic Enzyme Activity of Whole Wheat

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Abstract
Lipolytic enzymes play a key role in the deterioration of whole wheat flour upon storage but may also be involved in plant disease and stress tolerance while the crop is in the field. Therefore, the purpose of this study was to determine the effect of foliar fungicide treatment on lipolytic activity in wheat. A significant cultivar × fungicide × year interaction for esterase (*p*-nitrophenyl butyrate as substrate [EA-B]) and lipoxygenase (LOX) activities was observed; however, a large portion of the variability was owing to year (environment). Fungicide influenced lipase (olive oil as substrate [LA-O]), EA-B, and LOX activities. Lipase (*p*-nitrophenyl palmitate as substrate [LA-P]) showed variation in terms of cultivar and year rather than the application of fungicide. Partial correlation (year as a partial variable) between LA-P and EA-B activities was observed ($r = 0.78$, $P < 0.001$), although neither was correlated with LA-O. The influence of foliar fungicide on lipolytic enzyme activities depends mostly on growing conditions but is also affected by disease stress, disease resistance of the varieties tested, and the substrate being used in the assay.

The 2015 Dietary Guidelines for Americans (USDA and USDHHS 2015) recommended the consumption of at least half of grain-based foods as whole grains. In a 2015 survey of 1,510 U.S. adults, 63% of respondents indicated that they consume whole grains at least half of the time, and

31% indicated that they nearly always choose whole grains (Whole Grains Council 2015). This was in contrast to only 4% in 2010. Additionally, the market for whole grain and high fiber foods is expected to grow at a compound annual growth rate of 6.6% through 2022 (Stratistics 2016). Thus, although whole wheat flour production is only about 6% of total wheat flour production today (USDA 2016), it can be expected to grow to meet consumer demands.

Whole wheat flour includes the bran and germ fractions of the kernel together with the endosperm. A majority of the enzymatic activities are present in the bran and germ fractions of wheat (O'Connor and Harwood 1992; Every et al. 2006) and may contribute to the functionality of whole wheat flours (Tait and Galliard 1988; Hansen and Rose 1996). Of particular relevance is the activity of lipolytic enzymes in the bran and germ, which have been the subject of study for many years and which can contribute to whole wheat flour deterioration (Tait and Galliard 1988).

Lipolytic enzymes play a vital role in the functional changes in whole wheat (*Triticum aestivum* L.) flour during storage (Doblado-Maldonado et al. 2013). Lipase (triacylglycerol hydrolase, EC 3.1.1.3) contributes hydrolytic rancidity to whole wheat flour by producing free fatty acids through the hydrolysis of triacylglycerols (O'Connor and Harwood 1992). Subsequently, linoleic and linolenic acids generated by lipase can be oxidized by lipoxygenase (LOX, EC 1.13.11.12) in hydrated flour (Gardner 1987). Ultimately, hydrolytic and oxidative mediated degradation (collectively lipolytic degradation) causes the development of rancidity, resulting in poor bread quality (Tait and Galliard 1988) and unacceptable sensory properties (Hansen and Rose 1996; Bin and Peterson 2016).

Lipase activity is concentrated in the pericarp and germ portions of the kernel, with the majority located in the pericarp (O'Connor and Harwood 1992). The total lipase activity of wheat may originate from a combination of endogenous lipases as well as microbial lipases on the surface of the grain (O'Connor and Harwood 1992). Fungal lipases on the surface of the grain may be involved in virulence of plant pathogens (Gaillardin 2010). During germination, endogenous lipase activity increases, and it was found to be a function of coleoptile length and germination conditions (Brijs et al. 2009). High lipase activity was found when wheat was germinated in the dark and at higher temperatures (Brijs et al. 2009).

In contrast to lipase, the germ of wheat shows maximum LOX activity followed by the pericarp (Every et al. 2006). LOX activity in plants is associated with providing hydroperoxide substrates and volatile aldehydes for the synthesis and activation of plant defense mechanisms against pathogens (Prost et al. 2005; Matsui 2006). The involvement of LOX was found in the mechanisms for disease resistance in tobacco leaves (Shah 2005) and

against fungal pathogens. During germination of maize, activity of LOX was increased in parallel to lipase activity (Lin et al. 1983).

One important agronomic practice of interest during wheat production is in-season application of fungicide to prevent or minimize yield loss owing to fungal disease in wheat (Dimmock and Gooding 2002; Ruske et al. 2003). As stated in the preceding discussion, it is likely that lipase activity in grain is related to fungal contamination, and LOX activity could be related to disease resistance (stress tolerance). Therefore, quantification of the lipolytic activities in wheat in the context of in-season fungicide application needs to be addressed.

True lipases are active on ester-linkages on water-insoluble substrates at the oil–water interface. Related enzymes, esterases (carboxyl ester hydrolases, EC 3.1.1.1), are active on the same chemical bond but show a propensity toward water-soluble substrates. Several water-soluble and water-insoluble substrates are commonly used to assay lipase. Three of the most common substrates that have been used to assay cereal grain lipases are triacylglycerol (typically triolein or olive oil), *p*-nitrophenyl palmitate, and *p*-nitrophenyl butyrate (Prabhu et al. 1999; Suzuki et al. 2004; Wrolstad et al. 2005; Palacios et al. 2014). *p*-Nitrophenyl butyrate is a water-soluble substrate and would therefore be expected to capture general esterase activity. Triacylglycerols and *p*-nitrophenyl palmitate are not water-soluble and thus should capture true lipase activity, although *p*-nitrophenyl palmitate would be more convenient to assay than triacylglycerol owing to its color generation upon hydrolysis. Because loss of flour quality seems to be a function mostly of liberation and subsequent oxidation of unsaturated long-chain fatty acids, it would seem that true lipase activity would be more relevant to wheat quality than general esterase activity. Previous studies have based conclusions on the lipase activity measured by one substrate (Doblado-Maldonado et al. 2013; De Almeida et al. 2014). However, because the activity could differ among substrates it is relevant to compare lipase activity measurements among substrates.

Therefore, this study had both a primary and a secondary objective. The primary objective was to evaluate the consequences of in-season foliar fungicide treatment on the activities of lipolytic enzymes in wheat. The secondary objective was to determine the relationships among lipase and esterase activities in wheat assayed using different substrates.

Materials and Methods

Experimental Design. Hard red winter wheat samples were produced at the agronomy research farm located at Havelock, Nebraska, U.S.A.

(40°51′15.077″ N and 96°36′46.828″ W) under rain-fed conditions during two growing seasons (2014 and 2015). Six hard red winter wheat cultivars that are commonly produced in this region were used in this study: Freeman (registration number CV-1098, PI 667038) (Baenziger et al. 2014), Millennium (registration number CV-908, PI 613099) (Baenziger et al. 2001), Overland (registration number CV-1020, PI 647959) (Baenziger et al. 2008), Pronghorn (registration number CV-848, PI 593047) (Baenziger et al. 1997), Robidoux (registration number CV-1064, PI 659690) (Baenziger et al. 2012), and Settler CL (registration number CV-1051, PI 653833) (Baenziger et al. 2011). Wheat grains used in this study were randomly sampled from four field replications that received two fungicide treatments: with (F1) and without (F0) foliar fungicide (Prosaro 421 SC, [prothioconazole + tebuconazole]) application at the rate of 455 mL/ha with the addition of a nonionic surfactant at 0.125% v/v when all plants reached flag leaf stage (Zadoks growth stage 39).

Weather and Agronomic Data. Weather data for the field experiment site were obtained from the Automated Weather Data Network of the High Plains Regional Climate Center (www.hprcc.unl.edu). Only weather conditions from flowering to harvest were reported.

Wheat cultivars were planted on October 2, 2013, for 2014 trials and September 17, 2014, for 2015 trials. Plants were harvested on July 9, 2014, and July 13, 2015. Days to flowering (FD) was the number of days after April 30 when 50%of the plants had protruded anthers (Zadoks growth stage 65). Visual assessment of disease severity (DS) evaluation was made for all foliar diseases combined from the top two leaves in each plot as percent diseased leaf area on a scale of 0–100% (Bhatta 2015).

Kernel Characteristics. Total grain protein (TGP) concentration was determined with a near-infrared analyzer (DA 7250, Perten Instruments, Springfield, IL, U.S.A.), calibrated to combustion analysis using a LECO FP528 (AACC International Approved Method 46-30.01) as described (Bhatta 2015).

Thousand kernel weight (TKW) was measured with a model ESC-1 seed counter (Agriculex, Guelph, ON, Canada) by weighing 1,000 kernels from each genotype on each plot.

Falling number (FN) was analyzed with AACCI Approved Method 56-81.03. Values of >300 s were considered to indicate no sprout damage.

Lipase Activity Using Olive Oil as the Substrate. Lipase activity using olive oil as the substrate was determined as previously described (Dobladó-Maldonado et al. 2013). Briefly, wheat grains were milled with a cyclone mill

(UDY, Fort Collins, CO, U.S.A.) equipped with a 1 mm screen. Whole wheat flour (200 mg) was defatted with 1 mL of hexane (1:5 wt/vol). The supernatant was discarded after centrifuging (13,793 × *g*, 5 min). Lipid extraction with hexane was repeated again. The defatted pellet was dried until there was no hexane odor, and then 0.12 mL of refined commercial olive oil (Vigo Importing, Tampa, FL, U.S.A.) and 0.03 mL of water were added and mixed with a toothpick until the mass appeared homogenous. The samples were then incubated at 40°C for 16 h. After incubation, 1.2 mL of 2,2,4-trimethylpentane was added, and the mixture was shaken vigorously for 1 min and then centrifuged. Supernatant (1 mL) was mixed with 0.2 mL of cupric-acetate pyridine reagent. The absorbance of the 2,2,4-trimethylpentane layer was then measured at 715 nm. A standard curve was created by using oleic acid (Alfa Aesar, Thermo Fisher Scientific, Waltham, MA, U.S.A.) (0–10mM in 2,2,4-trimethylpentane). Lipase activity measured using olive oil as the substrate was abbreviated as LA-O. The units for LA-O were U/g, where U was defined as the micromoles of oleic acid equivalents liberated per hour during the reaction.

Lipase Activity Using *p*-Nitrophenyl Palmitate as the Substrate. Lipase activity using *p*-nitrophenyl palmitate as the substrate was determined as described by Wrolstad et al. (2005) with a few modifications. To 1 g of milled sample, 5 mL of deionized water was added. The samples were vortexed and then shaken horizontally for 30 min at room temperature and then centrifuged (4,500 × *g*, 15 min). The supernatant was separated and used as crude enzyme extract. To 0.5 mL of crude enzyme extract, 1.25 mL of Tris-Cl buffer (0.1M, pH 8.2) and 1.25 mL of substrate solution were added. The substrate solution was prepared by adding 15.9 mg of *p*-nitrophenyl palmitate (1492-30-4, Sigma-Aldrich, St. Louis, MO, U.S.A.), 17 mg of sodium dodecyl sulfate, and 1 g of Triton X-100 to a total volume of 100 mL of deionized water. This solution was heated in a water bath at 65°C for 15 min to remove turbidity and then cooled to ambient temperature prior to use. The assay mixture was incubated at 37°C for 15 min, and then the absorbance was analyzed at 400 nm. A standard curve was prepared using *p*-nitrophenol (100-02-7, ACROS, Thermo Fisher Scientific) (0–0.1mM in Tris-Cl buffer). Lipase activity measured using *p*-nitrophenyl palmitate as the substrate was abbreviated as LA-P. The units for LA-P were U/g, where U was defined as the micromoles of *p*-nitrophenol released per hour.

Esterase Activity Using *p*-Nitrophenyl Butyrate as the Substrate. Esterase activity was analyzed using *p*-nitrophenyl butyrate (2635-84-9, Sigma-Aldrich) as the substrate. The crude enzyme extract and substrate solution

were prepared analogous to the LA-P assay, except the SDS and Triton X-100 were not added to the substrate solution. Esterase activity measured using *p*-nitrophenyl butyrate as the substrate was abbreviated as EA-B. The units for EA-B were U/g, where U was defined as the micromoles of *p*-nitrophenol released per hour.

LOX Activity. LOX was analyzed following Gökmen et al. (2007) with a few modifications. To 1 g of flour, 5 mL of phosphate buffer (pH 6.5) was added, and the tubes were horizontally shaken. The mixture was centrifuged at 5,000 × *g* for 15 min at 4°C. The supernatant was used as the crude enzyme extract for further analysis. For the substrate solution, linoleic acid (L-1376, Sigma-Aldrich) (157 µL) and Tween 20 (157 µL) were mixed and then emulsified into 5 mL of distilled water, after which the volume was then brought to 200 mL with phosphate buffer (pH 6.5). The substrate solution was flushed with nitrogen to prevent any oxidation. Crude enzyme extract (100 µL) was added to 2.9 mL of substrate solution in a water bath at 30°C. After 2.5 min, 1 mL was transferred to 4 mL of 0.1M sodium hydroxide to stop the enzymatic reaction and improve the clarity of the solution by the formation of Na-salt with linoleic acid (Wrolstad et al. 2005). The units of LOX activity were U/g, where U was defined as the numeric increase in absorbance at 234 nm per minute of the reaction.

Data Analysis. Data were analyzed with SAS software (version 9.4, SAS Institute, Cary, NC, U.S.A.). Data were initially analyzed using a three factor factorial ANOVA. Cultivar, treatment, year, and their interactions were fixed effects, and replication nested within year was a random effect. Owing to large differences in environmental conditions between 2014 and 2015, data were also analyzed by year to estimate the factorial effects of cultivar and foliar fungicide treatments and associated interaction in the two environments (years). Differences among least squares means were gauged using Fisher's protected least significant difference test at $P \leq 0.05$. Pearson's partial correlation analysis was calculated on least squares means to examine the relationship among measured variables; DS, FD, TGP, TKW, FN, LA-O, LA-P, EA-B, and LOX.

Results

Weather and Agronomic Data. The average air temperature was similar for both growing seasons (Table I), although a considerable difference in rainfall was observed. As expected, the differences between these

Table I. Mean Temperature and Total Precipitation in Two Growing Seasons (2014 and 2015) at Havelock, Lincoln, Nebraska, U.S.A.[a]

	Year 2014		*Year 2015*	
Month	*Temperature (°C)*	*Precipitation (mm)*	*Temperature (°C)*	*Precipitation (mm)*
April	11.5	88.9	12.2	50.5
May	18.4	134	16.4	277
June	23.3	150	23.0	195
July	23.9	13.0	25.1	60.7
Mean or total	19.3	385	19.2	583

a. High Plains Climate Center, University of Nebraska–Lincoln.

Table II. Analysis of Variance (Mean Squares) for All Measured Variables[a]

Source of Variance	*df*	*DS*	*FD*	*TGP*	*TKW*	*FN*	*LA-O*	*LA-P*	*EA-B*	*LOX*
Full model										
Year	1	4,597***	8.16	46.48***	993.11***	232,610***	86.7***	9,135***	80,256***	132***
Error a = rep(year)	6	91.4	5.1	0.2	4.7	326.7	0.3	21	167.2	0.2
Cultivar	5	1,803***	39.14***	2.52***	27.3***	9,022*	0.91***	156***	1,936***	25.7***
Treatment	1	30,099***	1.52	0	189.8***	28,998**	0.76*	47.97	1,621**	1.26***
Cultivar × treatment	5	889***	0.19	0.4	1.82	1,074	0.07	9.84	108.9	10.6***
Cultivar × year	5	343*	7.79**	1.92***	18.2***	6,014*	0.63***	156***	1,815***	7.42***
Treatment × year	1	12,027***	1.52	0.04	56.16***	4,081.2	0.47*	28.29	1,295*	0.66***
Cultivar × treatment × year	5	101.6	0.95	0.12	3.9	6,229*	0.09	7.38	375*	7.62***
Error	66	127	1.9	0.4	2.6	2,148	0.1	12.3	121	0.1
Year 2014										
Cultivar	5	1,407**	8.28***	0.7	24.15***	10,799*	0.044*	3.528	77.16	5.04***
Treatment	1	2,050*	0	0.07	19.5**	5,814.9	0.02	1.4**	12.86	2.29***
Cultivar × treatment	5	503*	0.27	0.42	1.15	1,107.6	0.003	0.56	83.59	0.414*
Error	33	201	0.9	0.7	2.3	2,769	0.01	0.56	64.3	0.09
Year 2015										
Cultivar	5	811***	39.9***	3.62***	20.44***	4,428*	1.70***	312***	3,540***	46***
Treatment	1	40,221***	3	0.02	220.9***	27,333**	1.47*	75.02	2,903**	0.08
Cultivar × treatment	5	478***	0.9	0.09	4.48	6,108*	0.184	16.94	407.1	28.2***
Error	33	53.7	3	0.1	2.8	1,527	0.23	24.2	177	0.2

a. df = degrees of freedom; DS = disease severity; FD = days to flowering; TGP = total grain protein; TKW = thousand kernel weight; FN = falling number; LA-O = lipase activity using olive oil as the substrate; LA-P = lipase activity using *p*-nitrophenyl palmitate as the substrate; EA-B = esterase activity using *p*-nitrophenyl butyrate as the substrate; and LOX = lipoxygenase activity. Asterisks *, **, and *** indicate $P < 0.05$, 0.01, and 0.001, respectively.

environments provided a significant year effect on DS that varied with cultivar and foliar fungicide treatment (Table II). Major diseases observed in 2014 were *Septoria tritici* blotch and tan spot, whereas in the wetter season (2015), *Fusarium* head blight and stripe rust predominated. Freeman

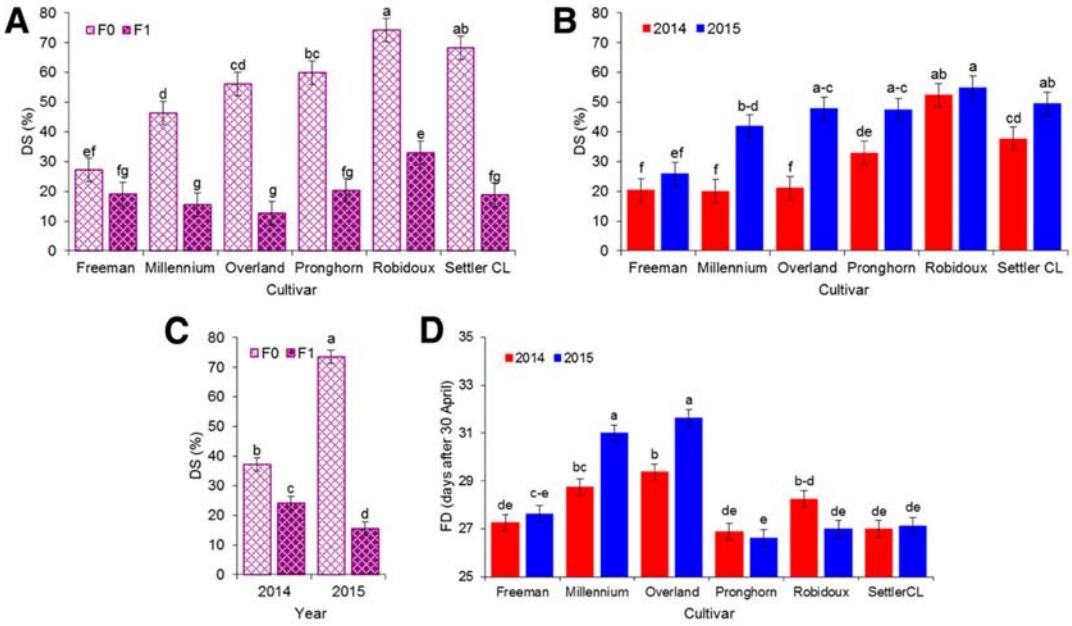

Fig. 1. Plots of significant cultivar, treatment, and year effects on measured agronomic characteristics: **A–C)** disease severity (DS); and **D)** flowering date (FD). F0 = no fungicide application; F1 = fungicide application; and means with different letters are significantly different within each subplot.

showed similar DS regardless of the fungicide treatment (Fig. 1A). When fungicide was applied, Robidoux showed the highest DS, whereas the rest of the cultivars showed similar DS. Millennium, Overland, Pronghorn, and Settler CL had higher DS in 2015 than in 2014 (Fig. 1B), indicating that these cultivars were more susceptible to stripe rust and *Fusarium* head blight than to *Septoria tritici* blotch and tan spot. Application of fungicide against pathogens was found to be more effective when the environment for disease development was favorable: in 2015 DS was reduced from 74 to 16% when fungicide was applied (Fig. 1C).

The average FDs were 27.9 days in 2014 and 28.3 days in 2015. These days refer to the number of days after April 30. A significant cultivar × year interaction was observed for FD (Table II). Except for Millennium and Overland, other cultivars had similar FD in both years (Fig. 1D). In 2015, Millennium and Overland had higher FD than in 2014.

Kernel Characteristics. TGP of cultivars varied over the years (Table II): TGP of all the cultivars was higher in 2015 than in 2014 (Fig. 2A). Robidoux (in 2015) had the highest TGP.

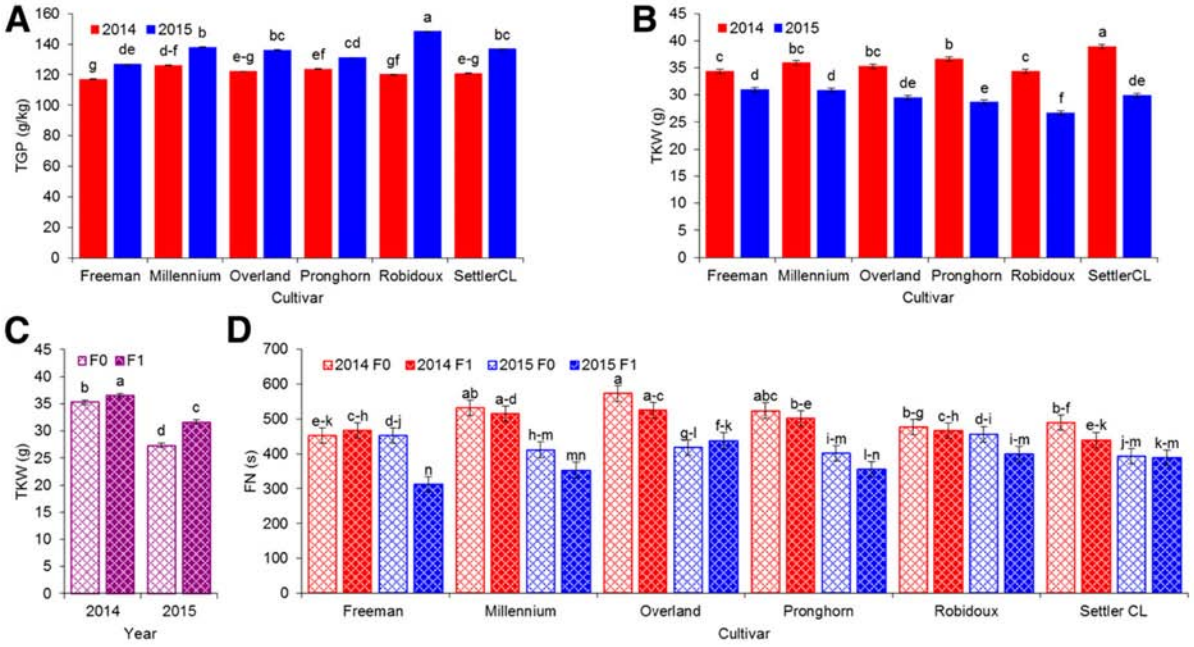

Fig. 2. Plots of significant cultivar, treatment, and year effects on measured kernel characteristics: **A)** total grain protein (TGP); **B and C)** thousand kernel weight (TKW); and **D,** falling number (FN). F0 = no fungicide application; F1 = fungicide application; and means with different letters are significantly different within each subplot.

The cultivar × year and treatment × year interactions were significant for TKW(Table II). TKW of all cultivars was higher in 2014 than in 2015 (Fig. 2B). In 2015, except Robidoux all cultivar had similar TKW. TKW of cultivars treated with fungicide was higher in both years (Fig. 2C).

A significant cultivar × treatment × year interaction was observed for FN (Table II); however, true sprouting damage was not observed in either year (FN > 300 s) for all cultivars. The variation in FN was mainly contributed by year (81%). Millennium, Overland, and Pronghorn had higher FN in 2014 than in 2015 (Fig. 2D). The range of FN was 311–457 s in 2015 and 459–549 s in 2014. Application of fungicide was not found to affect FN except for Freeman in 2015.

Lipase Activity Using Olive Oil as the Substrate. The cultivar × year and treatment × year interactions were significant for LA-O (Table II). The application of fungicide did not affect LA-O in 2014 but reduced LA-O in 2015 (Fig. 3A). For all cultivars, LA-O was an order of magnitude higher in 2015 compared with 2014 (Fig. 3B). There were no differences among cultivars for their LA-O in 2014. In 2015 the highest and lowest LA-O values were found in Millennium and Pronghorn, respectively. Interestingly, Freeman showed an increase in LA-O despite the low DS in 2015.

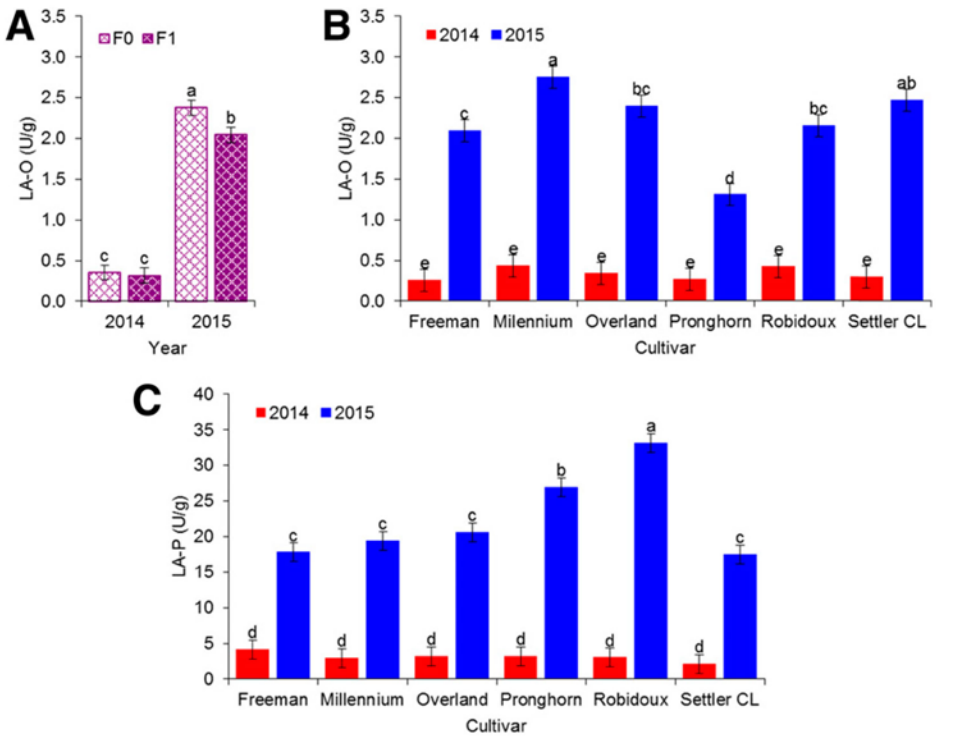

Fig. 3. Plots of significant cultivar, treatment, and year effects on lipase activity: **A–B)** as measured with olive oil as the substrate (LA-O); and **C)** as measured with *p*-nitrophenyl palmitate as the substrate (LA-P). F0 = no fungicide application; F1 = fungicide application; and means with different letters are significantly different within each subplot.

Lipase Activity Using *p*-Nitrophenyl Palmitate as the Substrate. A significant cultivar × year interaction was observed for LA-P mainly owing to the effect of year (Table II). As seen with LA-O, a similar dramatic increase was seen in LA-P when comparing 2014 to 2015 (Fig. 3C). Additionally, there were no differences among cultivars for LA-P in 2014, but in 2015 significant differences were observed. In contrast to results for LA-O, fungicide treatment did not affect LA-P activity. The cultivars also had different rankings for LA-P: Robidoux showed the highest LA-P in 2015 followed by Pronghorn, and rest of the cultivars had similar LA-P.

Esterase Activity Using *p*-Nitrophenyl Butyrate as the Substrate. A significant three-way interaction (cultivar × treatment × year) was observed for EA-B (Table II), with a large portion of the variability contributed by year (89%). EA-B of all the cultivars was higher in 2015 compared with 2014, with Robidoux having the highest EA-B (Fig. 4). Application of fungicide was found to influence EA-B in 2015 for Freeman and Overland, with EA-B

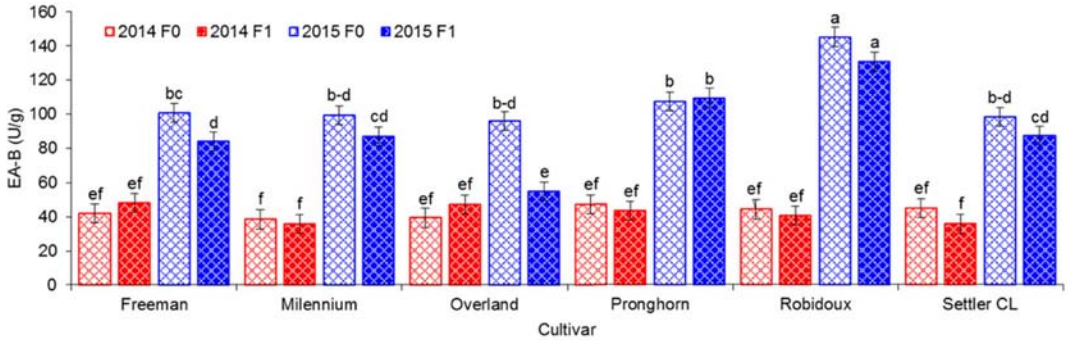

Fig. 4. Plot of significant cultivar, treatment, and year effects on esterase activity measured with *p*-nitrophenyl butyrate as the substrate (EA-B). F0 = no fungicide application; F1 = fungicide application; and means with different letters are significantly different.

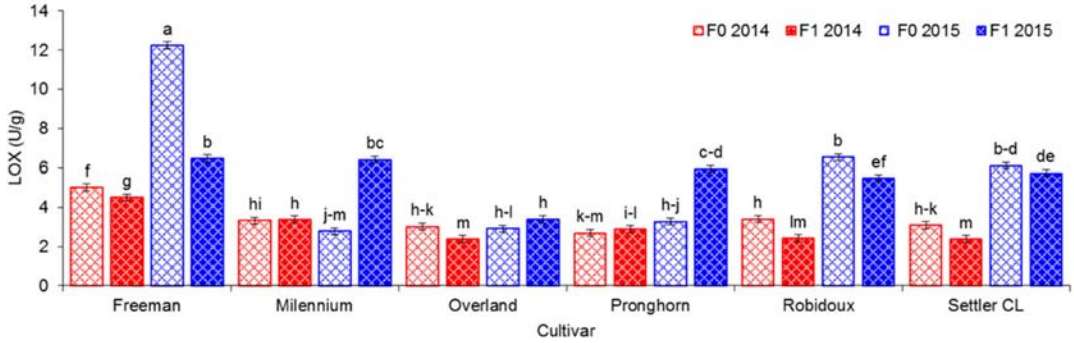

Fig. 5. Plot of significant cultivar, treatment, and year effects on lipoxygenase (LOX) activity. F0 = no fungicide application; F1 = fungicide application; and means with different letters are significantly different.

being higher when fungicide was not applied. In 2014, there were no differences among cultivars for EA-B, and the application of foliar fungicide did not influence EA-B. Overall, it appeared that fungicide treatment had minimal impact on EA-B, which was similar to findings with LA-P.

LOX Activity. The cultivar × treatment × year interaction was significant for LOX (Table II). As with EA-B, most of the variation was attributed to the difference between the years, although in contrast to EA-B, both the three-way interaction and the two-way interaction of cultivar × treatment contributed substantially to the source variance (5.8 and 11%, respectively), indicating different responses among the cultivars to the fungicide application depending on year. LOX activity decreased with the application of fungicide in both years for Freeman and Robidoux (Fig. 5). This suggested the consistent response of these cultivars to the application of fungicide

Table III. Pearson Partial Correlation Coefficients (Year as a Partial Variable) Among Response Variables[a]

Variables	*FD*	*TGP*	*TKW*	*FN*	*LA-O*	*LA-P*	*EA-B*	*LOX*
DS	–0.16	0.13	–0.68***	–0.05	0.33	0.35	0.41*	0.44*
FD	...	0.09	0.15	0.22	0.48*	–0.35	–0.57**	0.08
TGP	...	...	–0.21	0.25	0.28	0.49	0.42**	0.28
TKW	...	...	...	–0.05	–0.12	–0.62**	–0.53**	–0.51*
FN	...	...	...	...	0.03	–0.002	0.01	
–0.0003								
LA-O	...	...	...	...	...	–0.32	–0.13	0.20
LA-P	...	...	...	...	...	...	0.78***	0.26
EA-B	...	...	...	...	...	...	...	0.24

a. DS = disease severity; FD = days to flowering; TGP = total grain protein; TKW = thousand kernel weight; FN = falling number; LA-O = lipase activity using olive oil as the substrate; LA-P = lipase activity using *p*-nitrophenyl palmitate as the substrate; EA-B = esterase activity using *p*-nitrophenyl butyrate as the substrate; and LOX = lipoxygenase activity. Asterisks *, **, and *** indicate $P < 0.05$, 0.01, and 0.001, respectively.

across years. In 2014, LOX activities of Overland and Settler CL were reduced when fungicide was sprayed, but no such effect was observed in 2015. Interestingly, in 2015, Millennium and Pronghorn showed higher LOX activity in the case of fungicide treatment. Fungicide application had no effect on these cultivars in 2014. The growing conditions and interaction of the cultivar × treatment × year were the major factor for such differences in LOX activity.

Correlations Among Response Variables. DS was negatively correlated with TKW (Table III). DS was correlated with EA-B and LOX. A significant positive correlation of FD was observed with LA-O and a significant negative correlation with EA-B. TGP and EA-B were correlated. TKW was negatively correlated with LA-P, EA-B, and LOX. LA-P and EA-B were highly correlated.

Discussion

The objective of this study was to determine the influence of fungicide application on lipolytic activities of wheat and to determine the relationship among measured enzymatic activities. Our results suggested that the influence of foliar fungicide on lipolytic enzyme activity in wheat is dependent mainly on the growing conditions of the plants. The presence of disease in

the field, disease resistance of the cultivars, and the enzyme being studied are crucial in determining the influence of fungicide on enzymatic activities. Environmental conditions in 2014 were characterized by typical rainfall and low DS in the field providing a normal growing season for plants. However, high rainfall in 2015 favored disease outbreak including *Fusarium* head blight and stripe rust. The activation of endogenous enzymes expressed during stressed conditions together with the presence of exogenous enzymes (fungal/microbial enzymes) may have resulted in higher enzymatic activities in many cultivars in 2015.

Significant cultivar × year × treatment interactions were observed among cultivars for EA-B, LOX, and FN. However, the major portion of variation was attributed to different growing conditions across years. In addition to the difference in growing conditions between the two years, it is expected that the efficacy of foliar fungicide is dependent on DS and disease resistance for each cultivar (Wegulo et al. 2011). This may be the reason for differences in enzymatic activities in 2015 caused by the application of foliar fungicide. The influence of foliar fungicide on enzyme activity is also dependent on the enzyme being measured and the substrate being used in the assay. For instance, LA-P of the cultivars was not influenced by the application of foliar fungicide, whereas LA-O, which is also a measure of lipase activity, was influenced by fungicide.

Lipase activities using different substrates, LA-O and LA-P, were not correlated. This may be owing to the presence of different enzymes or other compounds that inhibit or activate the enzymes, because LA-O was measured on whole milled kernels and LA-P was measured on an extract. The affinity of lipase enzymes for different substrates may be another reason for the lack of correlation between LA-O and LA-P (O'Connor and Harwood 1992). Further research is needed to determine which substrate gives a better indication of flour stability during storage.

The higher activity of most enzymes in 2015 compared with 2014 suggests that disease pressure influences the activity of lipolytic enzymes. However, significant correlations with DS were only found for EA-B and LOX. This suggested that DS is not the only factor to change lipolytic activity in wheat. The presence of mold or injury to the plant may have elevated lipase activity despite low DS in those cultivars. Another explanation may be that the disease pressure was enough to increase enzymatic activities, but visual signs and symptoms of disease were yet to be expressed in the plants.

Because enzyme activities generally increase during sprouting, FN was assayed to determine if lipolytic activities were related to preharvest sprouting. No sprouting damage was observed in either year of this study, and no enzyme activities were correlated with FN. Therefore, differences

in lipolytic activity among cultivars in this study were not owing to preharvest sprouting. However, high rainfall and disease pressure in 2015 resulted in lower FN than in 2014.

Different agronomic characteristics, including FD, TGP, and TKW, were accounted for in this study because previous studies have shown relationships between these variables and (exogenous) enzymatic activity (Dornez et al. 2006). Owing to higher rainfall in 2015, FD was delayed, whereas TKW was lower owing to higher DS. TKW and enzyme activity (LA-P, EA-B, and LOX) were negatively correlated in our study. This may be because the majority of enzymes in our study are located in the outer portions (bran and germ) of the kernel (O'Connor and Harwood 1992; Every et al. 2006) and smaller kernels have more surface area per unit weight of wheat.

Conclusions

Enzymatic activities were mostly dependent on growing conditions, with a much lower contribution of cultivar and fungicide treatment. When the environment was conducive to disease development, fungicide application influenced DS, TKW, LA-O, EA-B, LOX, and FN. In the future, it would be interesting to determine how specific diseases influence lipolytic activities in wheat. EA-B, LOX, and FN were dependent on the interaction of cultivar, treatment, and growing conditions. The variation in lipolytic activities in terms of cultivars widens the scope in crop management and variety selection in order to develop and grow wheat varieties with low lipolytic activities for the whole grain flour market. However, further studies on relationship of lipolytic activities and stability of flour under different storage conditions, levels of anticipated field stress conditions, and different agronomic practices should be explored to predict if low lipolytic activities in flour are predictive of improved flour shelf life and quality.

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