



Effects of antioxidant activity of black maize in corn borer larval survival and growth

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Abstract

Antioxidant activity (AA) of black maize kernels attributed mainly to polyphenols has potential effects on health and possible defense functions against pests. Our objective was to evaluate the effects of maize polyphenols and AA in survival and growth of larvae of the corn borer *Sesamia nonagrioides*. We carried out two bioassays with *S. nonagrioides* larvae grown in artificial diet with white and black maize flour and control. AA was tested spectrophotometrically on each of the diets using four methods. The different measurements of AA were strongly correlated, indicating that these measurements were highly reliable. The control diet, the white-maize-diet and black-maize-diet with vitamin C and without H₂O₂ had the highest antioxidant activity. The processing of the maize flour altered the AA of the polyphenols. The control treatment had the highest AA, and vitamin C had stronger AA than polyphenols. AA of vitamin C hides that of polyphenols probably due to environmental effects, dilution of polyphenols, or interactions with other substances. Larvae grew more in the control diet and the addition of H₂O₂ had not significant effects on weight. There was a weak rank correlation between AA and larval weight. Mortality was lowest for the control diet with or without H₂O₂ followed by white maize with or without H₂O₂ and black maize without H₂O₂. Effects of polyphenols depend on other substances that might interact with them. The results indicate that antioxidant activity has insecticide effects on young larvae and, as the larvae grow, antioxidants have positive effects on larvae.

Additional key words: *Zea mays* L; polyphenols; anthocyanins; *Sesamia nonagrioides*.

Abbreviations used: AA (antioxidant activity); ABTS (2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid)); DPPH (2,2-diphenyl-1-picrylhydrazyl); FOLIN (Folin-Ciocalteu assay); FRAP (ferric reducing antioxidant power).

Authors' contribution: PR proposed the idea, carried out the bioassays and wrote the first draft. PS made the analyses of antioxidant activity. RAM made the statistical analyses. All authors have designed the experiment and reviewed the text.

Citation: Revilla, P.; Soengas, P.; Malvar, R. A. (2018). Effects of antioxidant activity of black maize in corn borer larval survival and growth. Spanish Journal of Agricultural Research, Volume 16, Issue 1, e1004. <https://doi.org/10.5424/sjar/2018161-11939>

Received: 23 Jun 2017. **Accepted:** 21 Mar 2018

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Funding: Cofounded by the Spanish National Plan for Research and Development, and European Regional Development Fund (FEDER) (grants AGL2013-48852-C3-1-R and AGL2016-77628-R).

Competing interests: The authors have declared that no competing interests exist.

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Introduction

Antioxidant activity (AA) has been associated to insecticide effects; therefore, plants with high AA could be used for developing eco-friendly repellents for the post-harvest protection of grain crops (Bedini *et al.*, 2016). Nesci *et al.* (2012) reported insecticidal activity of synthetic antioxidants on insects that were vectors of fungal infections. Swiatek *et al.* (2014) found association between AA and plant defense against biotic and abiotic stresses in transgenic maize. Garcia-Lara & Bergvinson (2014) reported a significant association between insect resistance and AA in a maize population improved for insect resistance. Therefore, molecules with AA are potential natural insecticides.

Selection for intensity of red kernel color in maize yields high levels of anthocyanins, that vary from 5.38 $\mu\text{mol/g}$ fresh weight in black maize kernels to 0.28 $\mu\text{mol/g}$ in white grains of the same variety, and produce higher AA in the kernels with darker color (Rodríguez *et al.*, 2013). Stonecipher *et al.* (1993) hypothesized that anthocyanins caused resistance to plant pathogens, but later research has questioned the role of anthocyanins in plant resistance to insects (Simmonds, 2003). There are no definitive evidences of effects of polyphenols on insect growth or survival in leaves (Costa-Arbulú *et al.*, 2001; Simmonds, 2003; Lev-Yadun & Kevin, 2008), but phenolic compounds may be associated with elevated chemical defenses (Simmonds, 2003; Karageorgou *et al.*, 2008). A possible role of free phenols in *Sesamia*

nonagrioides resistance has been hypothesized by Santiago *et al.* (2005) based on the higher level of these compounds in the pith of genotypes resistant to corn borer. Some authors have published that free phenols are toxic when incorporated into artificial diets (Dreyer *et al.*, 1981; Serratos *et al.*, 1987; Arnason *et al.*, 1992). However, the effects of free phenols on maize resistance to corn borer are not clear, and several hypothesis are being tested (Santiago *et al.*, 2006).

The most important corn borer in the Mediterranean area is *Sesamia nonagrioides* (Lefèbvre) (Lepidoptera: Noctuidae) (Malvar *et al.*, 1993; Cartea *et al.*, 1994; Cordero *et al.*, 1998; Butrón *et al.*, 1999; Velasco *et al.*, 2007). Late generations of corn borers feed on the grain of maize adult plants, causing direct yield losses (Larue, 1984). Laboratory bioassays are very useful for investigating the basis of plant resistance to insects, and have been used for the identification of biologically active compounds in crops (Harris, 1979; Schoonhoven *et al.*, 1998). Our objective was to evaluate the effects of maize polyphenols and AA in survival and growth of larvae of the corn borer *Sesamia nonagrioides*.

Material and methods

Plant material

The base population was the multicolor maize synthetic population EPS4. This synthetic was made in 1981 by mixing 100 kernels from each of three open-pollinated populations (Salcedo, Taboado and Cambados) from northwestern Spain with diverse kernel colors, followed by random mating for more than 10 generations (Rodríguez *et al.*, 2013). Two random 300-kernel samples of white and black kernels were separated from the population EPS4. These two samples were multiplied independently by plant-to-plant pollinations, using each plant solely as either male or female. The separated subsamples of white and black kernels were cleaned and grinded in a laboratory grinding mill. The flour was screened with a sieve of one millimeter in diameter.

Black kernels had high anthocyanin content 5.38 ± 0.018 $\mu\text{mol/g}$ fresh weight while white kernels had only 0.28 ± 0.018 $\mu\text{mol/g}$, while the difference in carotenoid content was lower (1.76 ± 0.064 vs 0.75 ± 0.064 $\mu\text{mol/g}$ in black and white kernels, respectively) (Rodríguez *et al.*, 2013). Differences in pigment content among kernels with diverse colors explained most differences in AA, as the AA of the hydrophilic fraction was highly correlated to that of the pigments, while differences among kernel colors for the AA of the lipophilic fraction were not significantly

different. Concerning the nutritive composition, this maize variety had around 11% of total protein, 5.5% of total fat, 1% of brute fiber, and 58% starch, without significant differences among kernel colors.

Bioassays

Two laboratory bioassays were conducted to investigate the effect of AA of white and black maize flour on growth and development of *S. nonagrioides* larvae. We used the artificial diet published by Farinós *et al.* (2004) as control treatment. The artificial diet was made starting with 1 liter of hot water, and then we added 2.5 g of benzoic acid and, after reaching the boiling point, 26 g of agar while homogenizing the mixture with an electric mixer. We introduced the bowl in cold water and, while the mix was cooling, we added 43 g of yeast, 40 g of wheat germ and 160 g of maize flour while homogenizing with the electric mixer. The control treatment was made with a type of standard commercial maize flour called polenta. While the mix continued cooling, we added 6 g of ascorbic acid, 1 g of nipagin (from Sigma) and 1.55 g of Wesson salt mixture (from Sigma). When the mix reached 50 °C and was homogeneous, we poured it into plastic boxes.

In the first bioassay, for each maize flour treatment, the polenta of the artificial diet was replaced with the same amount of white or black maize flour, respectively. A second sample of each (white or black, respectively) maize-diets included vitamin C (10 g/L). Therefore, there were five different diets: control, white maize, white maize + vitamin C, black maize, and black maize + vitamin C. The experiment followed a randomized complete block design with four repetitions. Sixty neonate larvae per repetition and treatment were randomly assigned to each diet by placing a 1 cm-diameter piece of diet plus one larva in each individual 35 mm-diameter Petri dish. The neonate larvae were obtained by taking eggs from maize plants grown in a greenhouse with a large population of moths hatched from larvae collected from the field; eggs were placed on pieces of artificial diet made following the recipe shown above for the control diet; when the larvae were hatched and reached a 2-mm size, the alive larvae were taken for the bioassay. The piece of diet was changed by a fresh piece two times per week in order to allow the larvae to eat fresh diet *ad libitum*. After two weeks, half of the larvae (30) of each treatment continued to be fed on the same diet while the other larvae were fed with the same diet but supplemented with 5% H_2O_2 in order to produce oxidative stress; this concentration of H_2O_2 is enough for inducing oxidative stress (Rao *et al.*, 1997). Initial larval weight was recorded and then the weight of each larva was taken two times per week. At

the same time we took larvae weights, we changed the piece of diet during four weeks, renewing the piece of diet and recording the number of dead larvae or pupa; therefore we took up to 8 weights per larvae.

A second bioassay was carried out in order to double check the effects of white vs. black maize flour, avoiding the interference of any other factor. In this bioassay we followed a simplified version of the first bioassay, wherein only the white and the black-flour treatments were performed. The recipes and methods were as in the first bioassay. This second bioassay was analyzed separately because the experimental design was different and, therefore, they could not be combined in a single analysis. Furthermore, the second bioassay intended to simplify the conditions in order to allow a direct comparison between black and white flour without interactions from other compounds.

Evaluation of antioxidant activity

Antioxidant activity of the diets was tested on random samples of each of the five diets with and without H₂O₂. For doing so, 5 g of each fresh diet were lyophilized. As the bioassays ended, lyophilized diets were grinded in an analytical grinding mill (Model A10, IKA, Germany). Two subsamples were taken from each sample and antioxidant capacity analyses carried out per duplicate. Freeze-dried and ground samples (10 mg) were extracted with 1 mL of 80% aqueous methanol in dark maceration for 24 h. After centrifugation (3700 rpm, 5 min), methanolic extracts were employed in order to determine AA by using four methods: ferric reducing antioxidant power (FRAP) (Benzie & Strain, 1996); 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) (Miller & Rice-Evans, 1997); 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Brand-Williams *et al.*, 1995); and Folin-Ciocalteu (FOLIN) (Singleton & Rossi, 1965). We used the four methods of AA determination following Sotelo *et al.* (2014) because, although normally they are highly correlated, these four methods measure slightly different aspects of AA, for example, with DPPH method, the reaction takes place in methanol, where for the other three methods, reaction occurs in water. Nevertheless, FOLIN is the most common method for AA determination.

All AA assays were carried out in a microplate spectrophotometer (Spectra MR; Dynex Technologies, Chantilly, VA, USA). Standards prepared with different concentrations of Trolox (0, 0.008, 0.016, 0.024, 0.032 and 0.04 mM) were measured for FRAP, DPPH and ABTS analyses and AA values were normalized to Trolox equivalents per gram of dry weight. Standards prepared with different concentrations of gallic acid (0, 0.008, 0.016, 0.024, 0.032 and 0.04 mM) were also

measured. Results of FOLIN assay were expressed in terms of micromoles of gallic acid equivalents per gram of dry weight.

Statistical analysis

Repeated measures analysis was used to analyze the weekly weight measurements of larvae. A growth curve of weight on time was estimated for each treatment, and homogeneity of linear and quadratic coefficients was tested for each pair of treatments. The analysis was made using the MIXED procedure of SAS (2008). All factors were considered random except treatment which was considered fixed. The covariance was calculated following Littell *et al.* (1996). Additionally, analysis of variance were made for each bioassay by using the MIXED procedure of SAS (2008), considering repetitions as random effects and color grains as fixed effects. And comparisons of means among treatments were calculated for larval weight for each time with the Fisher's protected LSD.

Finally, to analyze larval survival, the Kaplan–Meier estimates of the survival function were calculated for each treatment, and curves were compared using the log rank test (Cantor, 1997; Ordás *et al.*, 2002). Survival functions were significantly different if they deviated from the expected values of the null hypothesis (that survival functions are equivalent in all treatments). The statistic determines whether differences between survival functions are significantly different at any probability level, thus indicating that larvae have significantly different survival in some treatments than in others (LIFETEST procedure of SAS). Missing larvae were censored for larval survival analysis, which means that the analysis considered that larvae lived at least until they disappeared. When larvae reached pupal stage, the larvae survival was scored as reaching 30 days.

Results and discussion

Antioxidant activity of diets

The four measurements of AA were strongly correlated, with highly significant correlations coefficients that varied from $r^2 = 0.832$ between FOLIN and DPPH, to $r^2 = 0.996$ between FRAP and ABTS. Correlations were above 0.9 for ABTS and DPPH ($r^2 = 0.979$, $p < 0.001$), DPPH and FRAP ($r^2 = 0.979$, $p < 0.001$), ABTS and FOLIN ($r^2 = 0.928$, $p < 0.001$), and FRAP and FOLIN ($r^2 = 0.920$, $p < 0.001$). These values are in agreement with previous results indicating that correlations between methods for measuring AA

were very high (Huang *et al.*, 2005; Kusznierevicz *et al.*, 2008; Soengas *et al.*, 2012). Our coefficient correlations were higher than those previously reported for other crops (Zhi-Xiang *et al.*, 2011; Sotelo *et al.*, 2014), indicating that these measurements were highly consistent and reliable. Since the four measures of AA were highly correlated, we will focus on FOLIN (Table 1, Fig. 1).

Our results show that polenta had the highest AA, and that vitamin C had stronger AA than polyphenols. The recipe for the control diet already includes vitamin C because it has been designed for optimum growth of larvae (Farinós *et al.*, 2004). Adding H₂O₂ to the diet resulted in a non-significant reduction of AA, even though we used higher concentration of H₂O₂ than previous reports (Rao *et al.*, 1997). Vitamin C

Table 1. Antioxidant activity (mean ± standard deviation) of diets containing different combinations of maize flour, vitamin C and H₂O₂ for feeding larvae for *Sesamia nonagrioides*.

Maize flour	Vitamin C	H ₂ O ₂	FOLIN (mg galic acid/g DW)
White	No	Yes	1.93±0.03
		No	2.92±1.35
	Yes	Yes	5.58±0.56
		No	6.84±0.25
Black	No	Yes	1.68±0.34
		No	3.85±1.46
	Yes	Yes	4.84±0.10
		No	5.63±0.54
Control	Yes	Yes	5.047±0.37
		No	5.69±0.15

DW: dry weight

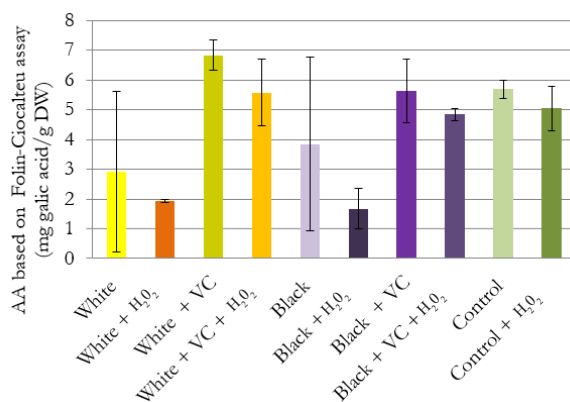


Figure 1. Antioxidant activity (AA) (error bars indicate standard deviation of the means) of diets containing different combinations of maize flour, vitamin C (VC) and H₂O₂ for feeding larva for *Sesamia nonagrioides* based on Folin method. DW: dry weight.

always increased AA, significantly for white flours. White flour had lower AA than black flour but, when an external antioxidant, such as vitamin C, or when an external oxidant, such as H₂O₂, is added, the order of AA is reversed in black and white flour because AA of vitamin C hides that of polyphenols. This could be due to an interaction between two antioxidants such as vitamin C and polyphenols in black flours or that the processing of the maize flour alters the AA of the polyphenols. Accordingly, several authors have shown that a cooking process, such as nixtamalization, reduces pigment content and antioxidant capacity (Del Pozo-Insfran *et al.*, 2006; De la Parra *et al.*, 2007; López-Martínez *et al.*, 2011; 2012). The causes of AA loss can be temperature and acidity, which have been reported as agent of antioxidant reduction by Li *et al.* (2011) who reported that citric acid significantly affects polyphenols content and AA. Furthermore, adding maize flour to the diet implies a dilution of AA that minimizes the presumable AA of polyphenols, as previously shown by Rodríguez *et al.* (2013), who reported a reduction in pigments' content and their respective antioxidant capacity due to dilution. Furthermore, as Petroni *et al.* (2014) stated, one major limitation in assigning a health property to polyphenols is the influence of other metabolites in the diet, acting as possible confounding factors. Indeed, there could be interactions between antioxidant substances, as indicated by Huang *et al.* (2005).

Effects of antioxidant activity on larval development and survival

In the first bioassay, the variation of larval weight as they feed on diets, indicate that the larvae always grow more in the control diet, designed for optimum nutrition of larvae, than in the maize-diets, and the addition of H₂O₂ has not significant effects on larval growth (Table 2). Besides, larvae fed on flour without oxidative stress (without H₂O₂) have grown more in diets with more antioxidants (polyphenols and / or vitamin C). The difference in growth coefficient between black flour + vitamin C and white flour was significant. In contrast, when larvae were fed on diets with oxidative stress (with H₂O₂), the larvae grew more in the diet with white flour + vitamin C. We would not expect that this diet have had the highest antioxidant concentration but it was the diet with H₂O₂ with the highest AA. This is probably due to vitamin C - polyphenols interaction discussed above.

In the second bioassay, differences were neither significant between coefficients of regression of larval weight nor between larval weight at any stage of development, corroborating the previous results

Table 2. Lineal regression coefficients (b) and standard errors (SE), and values of the log-rank statistic for homogeneity of survival of larval weight over time when fed with control diet, replacing maize polenta with white or black maize flour and with or without vitamin C, and adding hydrogen peroxide as oxidative stress.

Maize flour	Vitamin C	H ₂ O ₂	Intercept	b ± SE	Log-Rank ²
White	No	Yes	-32.8 a ¹	13.3±0.6 a	-7
		No	-23.8 a	6.9±0.6 c	-5
	Yes	Yes	-35.3 a	10.2±1.2 b	16
		No	-23.0 a	7.8±1.2 bc	15
Black	No	Yes	-28.8 a	7.8±0.8 c	2
		No	-26.0 a	7.6±0.6 bc	-6
	Yes	Yes	-26.8 a	7.6±1.1 c	14
		No	-31.1 a	9.3±0.9 b	10
Control	Yes	Yes	-32.8 a	13.3±0.6 a	-26
		No	-34.8 a	12.1±0.6 a	-13

¹Means followed by the same letter, within each column, were not significantly different according to Ls-means comparisons at $p < 0.15$ ²Log-rank statistic shows significant differences among survival curves at $p \leq 0.05$.

which showed that differences among black and white flour alone were not uncovered with this experimental design.

Larval weight was not related to survival as shown by the low correlation between larval weight and Log-rank ($r^2 = 0.30$, $p = 0.392$). Survival analyses indicated that the proportion of dead larvae was lowest for the control diet with or without H₂O₂ (Table 2). The values of Log-rank were also negative for white maize with or without H₂O₂ and for black maize without H₂O₂. Mortality was highest for both white and black maize irrespective of the addition of vitamin C or H₂O₂. These results show

that the relationship between AA and mortality was not clear; in fact, rank correlations between AA and Log-rank was not significantly different from zero ($r^2 = -0.14$, $p = 0.701$).

The weights recorded in the first bioassay showed that the larvae grown in the control diet had always the highest weights (Table 3). At first stages of development, higher concentration of antioxidants may be detrimental to larvae, *i.e.* larvae fed on white flour weighed significantly more than those fed on black flour with vitamin C. As larvae grew up, black flour with vitamin C increased larval weight to values that

Table 3. Larval weight along time when fed with control diet, replacing maize polenta with white or black maize flour and with or without vitamin C.

Maize flour	Vitamin C	H ₂ O ₂	Number of days of bioassay							
			4	7	11	14	18	21	25	28
White	No	Yes	-	-	-	-	113 b	164 b	234 b	239 b
		No	4 b	14 b	33 b	63 b	90 b	136 c	174 b	175 b
	Yes	Yes	-	-	-	-	147 b	207 b	217 b	193 b
		No	3 bc	12 ab	38 b	76 b	125 b	164 bc	181 ab	165 ab
Black	No	Yes	-	-	-	-	102 b	153 b	190 b	207 b
		No	3 bc	11 b	43 b	47 b	85 b	139 c	194 b	189 b
	Yes	Yes	-	-	-	-	110 b	162 b	188 b	180 b
		No	2 c	6 b	21 b	58 b	132 b	208 ab	241 ab	210 ab
Control	Yes	Yes	-	-	-	-	229 a	246 a	306 a	357 a
		No	6 a	35 a	82 a	161 a	193 a	258 a	263 a	279 a

¹Means followed by the same letter, within each column, were not significantly different according to Ls-means comparisons at $p < 0.15$

were not significantly different from the control from week sixth to eighth, and so did white flour with vitamin C for weeks seventh and eighth. Therefore, vitamin C has positive effects on larval growth. Besides, there was a weak relationship between AA and larval weight; actually, rank correlations between AA and larval weight were positive and moderate ($r^2 = 0.51$, $p=0.136$).

The effects of AA of polyphenols on growth of corn borers can be due to a possible insecticide effect as proposed by Bedini *et al.* (2016) or to the positive effect of antioxidant nutrients on health as shown by Petroni *et al.* (2014). As previous authors have shown, polyphenols could have both positive and negative effects on larval health and growth, depending on other substances that might interact with them (Costa-Arbulú *et al.*, 2001; Karageorgou *et al.*, 2008; Lev-Yadun & Kevin, 2008).

As conclusion, these results indicate that during the first stages of larval development, antioxidants have an insecticide effect, while, as the larvae grow up, antioxidants favors larvae growth. Furthermore, the antioxidant activity of maize polyphenols interacts with other antioxidant substances.

Acknowledgments

The authors thank Amando Ordás for providing the plant material (the maize synthetic EPS4) used for this experiment and Ana Alonso for technical support.

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