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Are variations in kernel-related morphometric and chemical parameters correlated with differences in *Sitophilus oryzae* attack in maize?

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Abstract

Insect infestation during seed storage affects cereal productivity. *Sitophilus oryzae*, which is present in various climates, is one of the primary pests for maize (*Zea mays*) seeds. In this study, kernels of a set of eight inbred lines of importance for both maize breeding and research activities were characterised for morphological and biochemical parameters as well as for their susceptibility to *S. oryzae*. *S. oryzae* was reared in laboratory-controlled conditions and the susceptibility index (SI, range: 0-11), which depends on both number of emerging adults and their median developmental period, was measured. Maize kernels of each of the eight maize lines were put in contact with less than 3-day old males and females of *S. oryzae* for 13 days. Infested kernels were checked daily for new adult emergence. Four maize lines were susceptible to *S. oryzae* attack (SI = 7.5 to 9.7) while the others appeared to be moderately resistant (SI = 4.6-6.9). Our results highlighted the occurrence of variability for insect susceptibility among different maize genotypes, however, variations in the kernel traits analysed do not allow us to make predictions about the response to insect attack.

Keywords: coleoptera, maize inbred lines, pest susceptibility, rice weevil, seed traits, *Zea mays*

Introduction

The productivity of maize is at risk due to the incidence of different pests and pathogens (Balconi *et al.*, 2010, 2014; Torri *et al.*, 2015) attacking maize kernels both pre- and post-harvest. In particular, maize is one of the agricultural commodities susceptible to infestation by storage insects, which may cause a huge loss of quality and germination of the seeds (Derera *et al*., 2014). Insect attack damages both the endosperm and the embryo. Maize endosperm is the largest domain in the kernel and represents a good source of feeding material since it accumulates starch and protein, while the embryo, which comprises root and leaf primordia, represents the succeeding plant generation (Consonni *et al*., 2005).

Three species of the genus *Sitophilus* Schoenherr are pests of cereal seeds. *S. granarius* (L.) was the first species described in 1758 by Linnaeus. *S. oryzae* (L.) was subsequently detected on rice and *S. zeamais* Motschulsky was described on maize, but each of the species can thrive on all cereal seeds. *S. oryzae* and *S. zeamais* are more widespread than *S. granarius* since the latter is wingless. *S. oryzae* (L.) is one of the primary pests of stored maize, found not only in warm and tropical areas but also in temperate climates (CABI, 2018). The female chews a hole with the mandibles and lays one egg in each kernel. These species develop over a wide temperature range, the lower and upper temperatures are 15°C (Nakakita *et al*., 1997) and 35°C (Mansoor *et al*., 2017), respectively. The feeding damages the seeds, which also allows the development of fungi, thus causing a reduction in germination (Muzemu *et al.*, 2013; Zunjare *et al*., 2014). Crop yield losses due to these harmful organisms can be substantial and may be prevented, or reduced, by crop protection measures.

How and if nutritional properties, endosperm texture or tegument hardness may influence insect attacks has still to be elucidated. Several authors have considered how the physical characteristics of endosperm and embryo, such as hardness, thickness and seed size, influence resistance of genotypes to insect infestation (Ivbiljaro, 1981; Ashamo, 2001; Lale *et al*., 2013; Limonta *et al*., 2013; Akpodiete *et al*., 2015), while Zunjare *et al*. (2016) affirmed that "pericarp thickness and seed hardness did not impart resistance".

In this work, kernel-related traits taken into consideration included morphometric parameters, endosperm texture and nutritional properties. Beside pericarp thickness, seed hardness can be related to the ratio between the vitreous or semi-vitreous component of the endosperm, which is the hard portion located at the periphery, and the white floury component, which is the soft portion present in the central part of the kernel. On this basis, dent genotypes, characterised by the presence of little vitreous endosperm, and flint genotypes showing a larger proportion of vitreous endosperm (Gayral *et al*., 2016), have been analysed.

As to kernel nutritional properties, lines differ in starch content, and proportion of two types of glucose homopolymers that accumulate in the maize endosperm, *i.e.* the linear amylose molecule and the branched amylopectin, which depends on the activity of enzymes involved in starch chain elongation and branching (Zeeman *et al*., 2010). Total protein content, anthocyanin presence and total antioxidant capacity (TAC), were also taken into consideration.

The aim of this study is to verify whether variation in kernel traits including morphometric parameters, endosperm texture, pericarp thickness, and nutritional properties, such as starch content, total protein content and total antioxidant capacity (TAC), can be correlated with variation in susceptibility to *S. oryzae* attack.

Materials and methods

Plant materials

All genetic materials (inbred lines) included in this work belong to the germplasm collection of CREA Bergamo, (www.ecpgr.cgiar.org/working-groups/maize/maize-wg). Inbred lines have been maintained thorough sib-mating at the experimental station of the CREA Institute "Azienda La Salvagna" (249 m., 45°68'N, 9°64'E) for 10 years. Uniformity and stability of traits among plants of each line have been observed across generations through: i) field evaluations of agronomical traits, ii) ear and seed morphometric records.

For this work, ears were dried at 40°C for a week to about 12-13% moisture content and stored at room temperature. Kernels were removed from ten mature ears using an electric sheller, and kernels from each line were mixed thoroughly.

The eight inbred lines have different origin and seed features (table 1; figure 1). B73 is a historic American line selected by the Iowa State University that dominated the world scene for over 30 years (Russell, 1972). Even today, a considerable portion of modern germplasm has genetic traits that can be traced back to B73. Its first genome sequence was released in 2009 (Schnable *et al*., 2009). In the B73*Pl1* line, the introgression of a functional allele of the *Purple plant1* (*Pl1*; Cone *et al*., 1993) gene leads to the

Table 1. Main features of the maize inbred lines used in this study.

Maize line	Origin	Flowering time	Seed phenotype	Utilisation
B73	Selected from SSS (Stiff Stalk Synthetic)	Medium	Wild type	As female line to produce hybrids
B73*Pl1*	Selected from SSS (Stiff Stalk Synthetic)	Medium	Wild-type, red pericarp due to introgression of the Pl allele	-
Lo1411*wx*	Cross between the Pioneer waxy hybrid P3394E and the inbred line Lo1067	Medium	Waxy, due to homozygosity of the *waxy* (*wx*) allele	As female line to produce wx hybrids
Lo1096*wx*	Cross between Pioneer P1540 and a *waxy* inbred line	Medium	Waxy, due to homozygosity of the *waxy* (*wx*) allele	As male line to produce wx hybrids
Lo1488	Derived by PR33A46 Pioneer hybrid	Medium	Wild type	As male line to produce medium-late hybrids
Lo1496	Cross between BP42 945A and P3730 lines	Early	Wild type	As male line to produce hybrids
Lo1521*ae*	Cross between Lo1309ae and Lo1095ae inbred lines	Medium-late	Tarnished endosperm due to homozygosity of the *amylose extender*1 (*ae1*) allele	As female line to produce *ae* hybrids
Lo1530	Pioneer PR31G98 hybrid	Late	Wild type	As male line to produce hybrids

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Figure 1. Representative images of kernels of the eight maize inbred lines.

accumulation of anthocyanin pigment in different plant organs, such as seed pericarp, anthers and leaves. The Lo1411*wx* and Lo1096*wx* lines carry defective alleles of the *waxy* gene encoding a granule-bound starch synthase. Mutations at the *waxy* locus eliminate amylose synthesis, resulting in 100% amylopectin in the endosperm (Tsai, 1974; Wessler and Varagona, 1985).

Lo1521*ae* is homozygous for the *amylose extender*1 (*ae1*) mutant allele. The product of *ae1* is a starch branching enzyme (Kim *et al*., 1988); mutants in the *ae1* gene produce starch with a much higher amylose-content than amylopectin, showing significantly longer branch-chains than the normal maize starch (Liu *et al*., 2013).

Morphometric analysis

Maize kernels were imbibed for one hour in distilled water and longitudinally cut with a scalpel. Sectioned kernels were then observed with the Axio Zoom.V16 microscope; images were acquired as a series of Z-stack with the Axiocam 506 color camera and assembled with the ZEN 2 pro software (Zeiss Carl Zeiss Microscopy GmbH, Jena, Germany). To obtain fluorescence images, samples were exposed to UV light (100-400 nm) and the autofluorescence acquired through a GPF filter (wavelength from 395 to 475 nm). Images of whole kernels and pericarp were taken at 5× and 20× magnification, respectively. Measures of kernel length and wideness and pericarp thickness were elaborated by means of ImageJ software (https://imagej.nih.gov/ij/index.html).

Pericarp thickness measures were taken in the abaxial region of the kernel in the position indicated by arrows in figure 2 (B73 fluorescence image). For each parameter, 10 kernels were analysed for each genotype, with three technical replicates.

Chemical analyses of seeds

Subsamples were taken and seeds milled with a ZM 200 Retsch Ultra-Centrifugal mill equipped with a DR 100 vibratory feeder (Retsch GmbH, Haan, Germany) to a 1 mm sieve size and stored at 4°C.

Flour samples were scanned in duplicate in the visible and NIR regions of the electromagnetic spectrum in reflectance (400-2500 nm) at 2 nm intervals using a scanning monochromator NIRS 6500 (NIRSystems) (Foss Italia). Crude protein, crude lipid and starch (% on a dry matter basis) were calculated using NIRS prediction equations by means of the MATLAB program. MATLAB- Spectra were exported as csv files and processed using Matlab7.9 (The Math Works Inc., Natick, MA, USA) and PLS Toolbox7.9 (Eigenvector Research Inc., Manson, WA, USA).

Total antioxidant capacity (TAC) was expressed as mmol of Trolox equivalent (TE) for a kg of dry matter and determined by a specific calibration equation (Redaelli *et al.*, 2016).

Insect rearing

S. oryzae was laboratory-reared in the Department of Food, Environmental and Nutritional Sciences, Università degli Studi of Milan, on maize kernels at 27 ± 1°C and 70 ± 5% RH. To obtain newly emerged adults, 200 adults (mixed population) were collected from the mass rearing and put in a glass jar (1 L) with 400 g of maize; the adults were removed after 13 days. The jar with the maize kernels was kept in the thermostatic cell to allow the development of laid eggs. The new adults emerged in about 28 days and were selected for the tests.

Set up of screening for susceptibility to S. oryzae

The first step was to determine the minimum amount of kernels to use in the experiments. Three trials were set up in which insects were grown in 156 mL glass jars with 100, 200 and 300 kernels respectively, each replicated five times. Five male/female couples of *S. oryzae*, three days old, were put in each jar. Sex was determined by examining the thickness and length of the rostrum and the 6° abdominal sternite aspect (Dinuta *et al.*, 2009; Manivannan *et al.*, 2017). Couples were left for 13 days to allow mating and then removed. The number of F_1 adults in the different groups was daily recorded. In the trial with 100 kernels, 36.4 ± 8.95 (± s.e.) adults were recorded, a number that is significantly different from those recorded in the experiments with 200 and 300 kernels, 114.4 ± 9.94 and 124.2 ± 13.36, respectively. The number of adults recorded on 200 and 300 kernels was not statistically different (one-way ANOVA $F_{2, 12} = 19.427$, $P \leq 0.001$, LSD range test).

Taking account of the previous results, experiments with the different maize lines were carried out in 156 mL glass jars with 200 kernels. Tests with each of the eight lines were replicated three times. Five males and five females of *S. oryzae*, less than three days old, were put in each jar. Adults were held in the glass jar to allow mating and oviposition for 13 days and then removed. Infested kernels were kept into the glass jar and checked daily for new adult emergence. The Susceptibility Index (SI) was calculated according to Dobie (1974):

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$$SI = \frac{lnF1}{MDP} \times 100$$

Where F1 = first-generation emerging adult total number; MDP = median developmental period. SI ranged from 0 to 11; when the value was from 0 to 4 the line was considered resistant (R), from 4.1 to 7 moderately resistant (MR), from 7.1 to 10 susceptible (S) and more than 10.1 very susceptible (VS).

Statistical analyses

Data, normally distributed, were submitted to one-way ANOVA and least significance difference test (α = 0.05) and Pearson product-moment correlation (IBM SPSS Statistics 25).

Results

Kernel related parameters

The proportion of vitreous and floury endosperm varies among lines (figure 2). Vitreous endosperm was greater than floury endosperm in Lo1496, equal in B73, B73*Pl1*, Lo1411*wx* and Lo1521*ae*, and less in Lo1488 and Lo1530. Variation among lines was also observed for kernel length and width, as well as for pericarp thickness (table 2) as measured from fluorescence images. Pericarp thickness showed the highest and the lowest values, respectively, in Lo1488 and B73*Pl1*. Lo1411*wx* showed the highest 1000-kernel weight (306.5 g). The genotype with the lowest weight was Lo1530 (175.0 g).

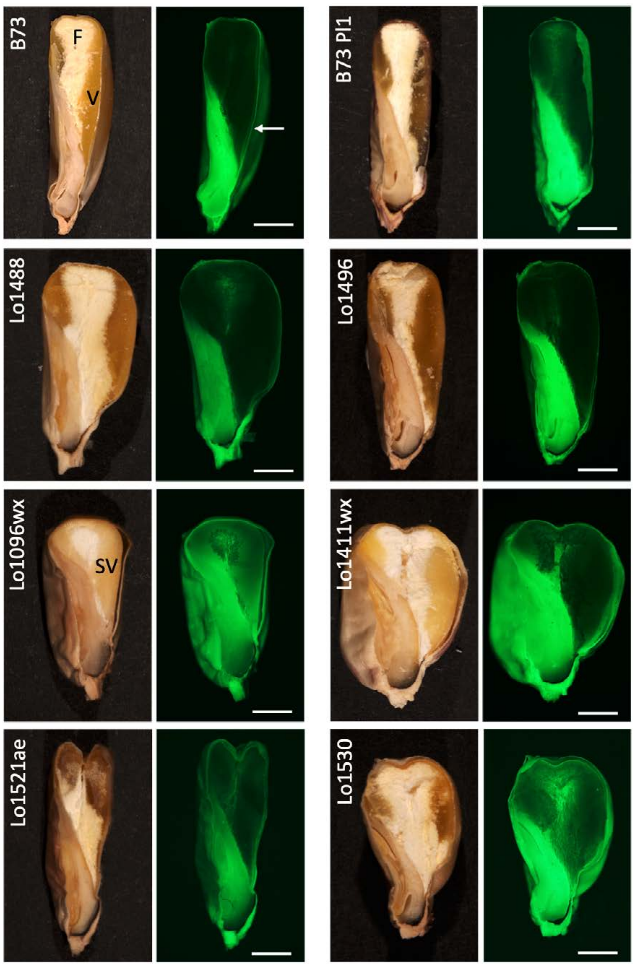

Figure 2. Representative images and fluorescence (green) images of sectioned kernels of the eight maize inbred lines. Floury (F) and vitreous (V) portions of the endosperm are indicated in the B73, while semi-vitreous (SV) is indicated in the Lo1096*wx* seed sections. The arrow indicates the position in which pericarp thickness was measured.

Table 2. Morphometric parameters in eight maize inbred lines.

Maize line	1000 kernel weight (g)	Proportion of vitreous/ semi-vitreous endosperm	Kernel length (mm)	Kernel width (mm)	Pericarp thickness (µm)
	mean ± SE		mean ± SE	mean ± SE	mean ± SE
B73	252.50 ± 3.09 b	Intermediate	10.42 ± 0.25 a	3.96 ± 0.47 b	68.62 ± 1.11 d
B73*Pl1*	240.50 ± 2.75 c	Intermediate	10.13 ± 0.16 a	3.99 ± 0.15 b	49.93 ± 0.68 f
Lo1096*wx*	227.50 ± 2.22 d	Large (s)	8.61 ± 0.11 c	3.55 ± 0.08 bc	91.45 ± 1.16 b
Lo1411*wx*	306.50 ± 2.63 a	Intermediate (s)	9.43 ± 0.20 b	5.26 ± 0.10 a	88.05 ± 1.81 bc
Lo1488	238.00 ± 3.65 c	Small	10.13 ± 0.16 a	4.84 ± 0.21 a	104.56 ± 1.98 a
Lo1496	252.50 ± 1.71 b	Large	10.42 ± 0.20 ab	4.28 ± 0.13 ab	84.36 ± 2.21 c
Lo1521*ae*	249.50 ± 2.63 b	Intermediate	9.18 ± 0.17 b	3.03 ± 0.19 c	63.97 ± 1.65 e
Lo1530	175.00 ± 1.29 e	Very small	9.07 ± 0.25 bc	4.71 ± 0.28 a	53.18 ± 1.61 f

One-way ANOVA for 1000-kernel weight (g) $F_{7,\,24} = 194.25$, $P < 0.00$; length $F_{7,\,72} = 11.739$, $P < 0.00$; width $F_{7,\,72} = 8.636$, $P < 0.00$; pericarp $F_{7,\,72} = 149.152$, $P < 0.00$. Means followed by a different letter in a column are significantly different ($\alpha = 0.05$, LSD multiple range test). s: semi-vitreous.

The starch content ranged from 64.9 to 72.2% dry weight in Lo1096*wx* and Lo1496, respectively, with an average of 69.35% and the lipid content ranged from 2.9 to 4.9% dry weight in Lo1488 and Lo 1096*wx*, respectively with an average of 3.71% (table 3). The level of proteins observed in different inbred lines was the qualitative parameter that showed the lowest variation; it varied from 11.7 to 13.3% dry weight with an average of 12.38%. The highest protein content was measured in Lo1096*wx* and the lowest in Lo1530.

Table 3. Seed chemical composition and total antioxidant capacity (TAC) in the eight maize inbred lines.

Maize line	Starch (% dry weight)	Lipid (% dry weight)	Protein (% dry weight)	TAC (mmol TE kg^{-1} dry weight)
	mean ± SE	mean ± SE	mean ± SE	mean ± SE
B73	70.49 ± 0.99 ab	4.41 ± 0.04 b	12.29 ± 0.11 cd	14.18 ± 0.22 cd
B73*Pl1*	66.85 ± 1.03 bc	3.08 ± 0.06 e	12.25 ± 0.09 cd	13.78 ± 0.16 d
Lo1096*wx*	64.86 ± 0.77 bc	4.88 ± 0.12 a	13.29 ± 0.03 a	15.96 ± 0.33 a
Lo1411*wx*	70.01 ± 0.57 ab	3.54 ± 0.05 d	12.03 ± 0.09 cd	15.10 ± 0.00 bc
Lo1521*ae*	68.11 ± 0.97 b	4.03 ± 0.17 c	12.33 ± 0.10 cd	15.31 ± 0.05 ab
Lo1488	71.83 ± 0.54 a	2.88 ± 0.00 e	12.62 ± 0.01 bc	13.15 ± 0.14 de
Lo1496	72.25 ± 0.73 a	3.47 ± 0.01 d	12.70 ± 0.20 b	13.05 ± 0.06 e
Lo1530	71.71 ± 0.58 a	3.43 ± 0.00 d	11.68 ± 0.06 e	14.47 ± 0.33 c

Means followed by a different letter in a column are significantly different ($\alpha = 0.05$, LSD multiple range tests).

The antioxidant capacity varied from 13.0 to 16.0 mmol TE kg^{-1} dry weight with an average of 14.37 mmol TE kg^{-1} (table 3). The highest antioxidant capacity was measured in Lo1096*wx* while the lowest was detected in Lo1496.

From this qualitative analysis, it emerged that Lo1096*wx* showed a low starch content (64.9% dm) accompanied by the highest level of protein (13.3% dry weight), lipid content (4.9% dry weight) and antioxidant level (16 mmol TE kg^{-1} dry weight).

Insect susceptibility

The highest numbers of adults were recorded on B73*Pl1* and Lo1096*wx*, the lowest on Lo1411*wx* (table 4.) The shortest development period was on B73*Pl1* and the longest on Lo1496. Pearson product-moment correlation indicates a strong significant negative association between the number of emerged adults and the length of the development period ($r = -0.61$, $N = 24$, $P < 0.001$). The lines B73, B73*Pl1*, Lo1096 and Lo1530 were susceptible, since they showed a SI value greater than 9, while the others appeared to be moderately resistant (table 4).

Table 4. Mean number (± S.E.) of adults (F1) emerged, mean development period (MDP days) (± S.E.) of *Sitophilus oryzae*, susceptibility index (SI) and SI value recorded on the tested maize lines.

Maize line	F1 Progeny emergence	MDP (days)	SI (%)	SI value
	mean ± SE	mean ± SE		
B73	31.66 ± 3.71 ab	49.15 ± 1.01 bc	9.1	S
B73*Pl1*	43.00 ± 1.00 a	46.27 ± 0.62 d	9.7	S
Lo1096*wx*	37.66 ± 8.57 a	47.96 ± 0.99 bcd	9.2	S
Lo1411*wx*	7.33 ± 2.85 d	51.77 ± 1.09 ab	4.6	MR
Lo1488	15.00 ± 1.15 cd	50.91 ± 1.02 ab	6.4	MR
Lo1496	14.00 ± 4.16 cd	52.26 ± 1.13 a	5.9	MR
Lo1521*ae*	16.00 ± 2.52 cd	48.71 ± 1.15 bcd	6.9	MR
Lo1530	23.66 ± 0.88 bc	51.53 ± 0.95 ab	7.5	S

One-way ANOVA: F1 progeny emergence $F_{7, 16} = 10.555$, $P < 0.00$; MDP $F_{7, 558} = 4.6$, $P < 0.00$. Means followed by a different letter in a column are significantly different ($\alpha = 0.05$, LSD multiple range test). Susceptibility index (SI): 0 to 4 = resistant (R); 4.1 to 7 = moderately resistant (MR); 7.1 to 10 susceptible (S).

To explore the correlation between the different values observed for the kernel morphogenetic parameters and susceptibility to *S. oryzae*, radar charts were constructed (figure 3) that report the number of adults together with kernel length and width measures (A) and pericarp thickness, (B) in the different inbred lines. The number of adults did not correlate with the length and the width of the kernels.

Although it appeared that B73*Pl1* had the highest number of adults and the thinnest pericarp, while Lo1411 had the lowest number of adults and the thickest pericarp, these correlations were not supported by the statistical analysis. Pearson product-moment correlation only indicated a moderate negative correlation between the number of emerged

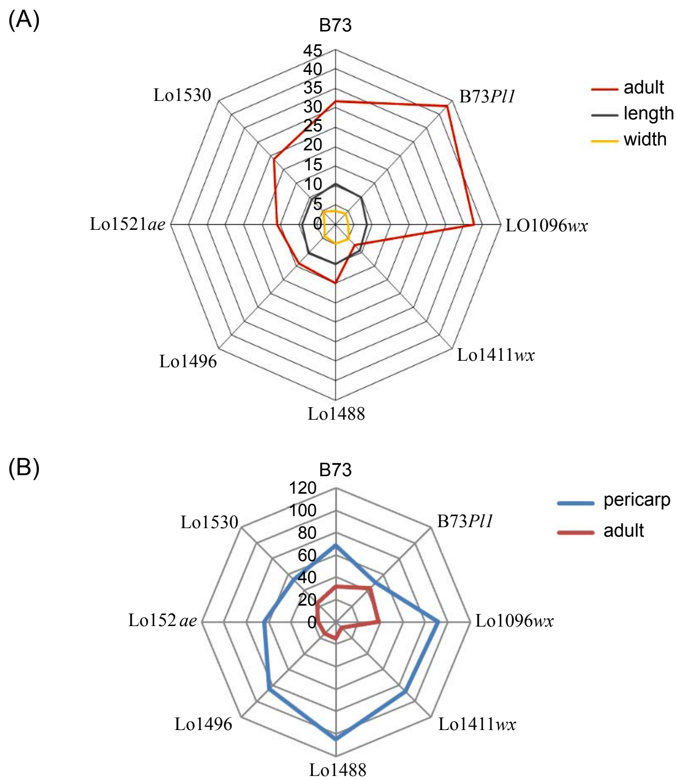

Figure 3. (**A**) Correlation between the number of F_1 progeny of *Sitophilus oryzae*, length and width (mm) of kernels in the different maize lines; (**B**) correlation between the number of F_1 progeny of *Sitophilus oryzae* (L.) and pericarp thickness (μm).

adults and the pericarp thickness ($r = -0.45$, N = 8, n.s.) (figure 3). Moreover, the number of emerged adults varied among different lines that showed similar chemical composition. The number of adults on the different lines was not correlated to starch content ($r = 0.21$, N = 8, n.s.), lipid content ($r = -0.16$, N = 8, n.s.), protein content ($r = 0.60$, N08, n.s.), and TAC ($r = 0.06$, N = 8, n.s.). TAC and SI were also not correlated ($r = -0.1$, N = 8, n.s.).

Discussion

Various authors have argued that the morphometric characteristics of kernels are not related to the susceptibility to the insect (Gomez *et al.*, 1982; Zunjare *et al.*, 2014, 2016; Rahardjo *et al.*, 2017), while others assert that colour, hardness, thickness and kernel size may influence the resistance of cereals to insect storage pests (Ivbiljaro, 1981; Ashamo, 2001; Lale *et al.*, 2013; Akpodiete *et al.*, 2015). In our study, differences in kernel length and width, and in pericarp thickness, were not related to the number of

S. oryzae offspring (figure 3). For example, in the Lo1411*wx* and Lo1530 lines, which are characterised by the greatest width of the caryopsis, the least and the greatest number of adults respectively were observed. Moreover, the two most susceptible lines, B73*Pl1* and Lo1096*wx*, showed both the lowest (49.9 µm) and one of the highest values of pericarp thickness (91.4 µm), respectively. Our results also suggest that there is no correlation between the protein and starch content (table 3) and the numbers of the progeny of *S. oryzae* (table 4), as stated by Tongjura *et al.* (2010) regarding *S. zeamais*. Nwosu (2016), instead, observed the influence of protein in maize lines resistant to the same species. The lines considered by Nwosu presented a protein content ranging from 4 to 14%, while the protein content of the lines observed in this research ranged from 11.68 to 13.29, too small a range to determine differences in the biology of this insect. Moreover, this pest species feeds on the seed starch, which is not a limiting factor when the insect is fed on corn seeds, while amino acids are supplied to the insect by endosymbionts (Heddi *et al*., 2003). Seed lipid content, that ranged from 2.88 to 4.88 (table 3), does not affect insect behaviour; finally, no evident preference for the type of endosperm was highlighted by storage pest development. Similarly, no correlation was detected between the number of adults (table 4) and the TAC values (table 3) of the eight lines. Total antioxidant capacity (TAC) refers to the overall activity of different compounds, generally present in the maize seed, that contribute to its antioxidant properties, such as carotenoids, polyphenols, flavonoids and anthocyanins (Serpen *et al*., 2007; Brewer, 2011). This parameter, which is generally understood to be related to the *in vivo* defence mechanisms of the plant, which produces more antioxidant molecules to combat the pathogen attack, seemed not to affect the responses to S. *oryzae* attack in stored materials in this study.

The results obtained in the present work indicate the existence of variability for the susceptibility to *S. oryzae* in the sample of eight maize inbred lines analysed. Lo1411*wx*, Lo1488, Lo1496 and Lo1521*ae* were identified as moderately resistant genotypes, while B73, B73*Pl1* and Lo1530 were shown to be more susceptible. However, data obtained from the morphometric and chemical analysis of the kernels of the tested lines are not associated with susceptibility to *S. oryzae* attack. In other words, variations in the parameters taken into consideration are not predictive of the type of response to *S. oryzae* attack.

To our knowledge, this type of correlations up to now is not clearly understood, as there are multiple parameters involved and it is difficult to find a key parameter. We may speculate, in agreement with a previous study, that susceptibility to storage insect attack could be attributed to quantitative more than to single gene traits (Locatelli *et al*., 2019). A wider collection of genotypes will allow to investigate the molecular mechanisms at the basis of the kernel-insect interaction and eventually to analyse the genetic variability at the basis of insect susceptibility in different maize lines (Gafishi *et al*., 2012; Kasozi *et al*., 2016; Sodedji *et al*., 2018).

Considering that cereals could be stored for long periods before being distributed for sowing, and that *S. oryzae* is among the main pests that cause economically significant damage to seeds, when selecting new corn lines in breeding programmes, in addition to the agronomic characteristics, assessing the susceptibility to the attack of this species by biological tests must also be considered (Abebe *et al*., 2009).

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