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Is it possible to control fumonisin contamination in maize kernels by using genotypes resistant to the Mediterranean corn borer (*Sesamia nonagrioides* Lef.)?

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Abstract

Insect activity has long been associated with *Fusarium* infection. The objectives of the current study were (i) to estimate the impact of Mediterranean corn borer, *Sesamia nonagrioides* Lef. (MCB), damage on fumonisin contamination in the maize kernel by comparing fumonisin contamination under infestation and protected conditions and (ii) to evaluate the potential use of genotypes resistant to this borer as controlling factors of fumonisin contamination. Genotypes with increased kernel damage by borers tended to increase fumonisin accumulation under infestation conditions. In particular environments, other factors influenced fumonisin contamination more than damage by borers. When ear damage by borers is significant, maize resistance to ear damage could contribute to the reduction of fumonisin contamination in the kernels. Genotype such as EP42 x EP77 that combines low ear damage by borers and low fumonisin level across environments is a good choice in order to control fumonisin contamination. The use of an applicable methodology in order to identify MCB resistant genotypes to ear attack under artificial infestations might be a promising approach.

KEY WORKS: *Sesamia nonagrioides*, *Fusarium verticillioides*, fumonisin, borer damage, *Zea mays*, maize.

Fumonisins, which are mycotoxins produced mainly by *Fusarium verticillioides* (Sacc.) Nirenberg (syn. *F. moniliforme* J. Sheld.) and *Fusarium proliferatum* (T. Natsushima) Nirenberg, in maize cultivated in temperate areas (Logrieco et al. 2003) have been receiving a great deal of attention because fumonisins B1(FB_1) and B2 (FB_2) are the most common mycotoxins found in maize kernels throughout the world (Shephard et al. 1996; Placinta et al. 1999; Soriano and Dragaci 2004). These *Fusarium* species are the most prolific fumonisin producers, and 17900μg/g of FB_1 have been recorded in cultures of *F. verticillioides*, and 31000 μg/g of FB_1 in cultures of *F. proliferatum* (Shephard et al. 1996). In Spain, 100% of *F. proliferatum* and more than 70% of *F. verticillioides* isolates are toxigenic (Sanchís et al. 1995; Abarca et al. 2000). Wild and Hall (1996) review reports that fumonisins cause disorders in horses, swine, rats, and mice among others. High contamination levels of fumonisins in food may cause oesophageal cancer in humans (Avantaggiato et al. 2003).

The levels of fumonisin contamination permitted by the European Union since November 2007 are 1000 μg/kg in maize dedicated to direct human consumption (European Regulation 2006). There is previous information about the occurrence of fumonisins in maize in Spain (Arino et al. 2007; Castella et al. 1999; Cantalejo et al. 1998; Sanchís et al. 1994). Particularly, in northwestern Spain, among ten hybrids evaluated in natural conditions at different locations only one of them showed an average fumonisin contamination below 1000 μg/kg (Butrón et al. 2006a). The most abundant species was *F. verticillioides*.

Insect activity has long been associated with *Fusarium* infection in maize kernels. Stem borers such as the pyralid *Ostrinia nubilalis* (Hübner) (ECB) and the noctuid *Sesamia nonagrioides* Lefèbvre (MCB) have been reported to favor kernel infection by *F. verticillioides* due to pericarp damage made by borers and their action as vectors (Smith and White 1988; Bakan et al. 2002; Avantaggiato et al. 2003; Blandino et al. 2010a; Blandino et al. 2010b). These borers are the main insect pests of maize in Mediterranean countries (Cordero et al. 1998; Velasco et al. 2007; Blandino et al. 2010a). In northwestern Spain, the first generation MCB attacks plants during the

mid-vegetative stage, and the second generation attacks during the reproductive stages (from early milk stage to maturity) causing a high percentage of broken plants, a heavy yield reduction and decline in grain quality. The second-generation larvae, above all, play an important role in the epidemiology of *Fusarium* ear rot in maize, and the ear damage by borers can increase mycotoxin contamination of kernels (Avantaggiato et al. 2003; Blandino et al. 2010a). According to Sobek and Munkvold (1997), borer damage promoted ear rot by spore transport and by alteration of the kernels epidermis. Consequently, control measures against the larvae are necessary to prevent yield losses and to guarantee low contamination.

A reduction in borer injury and, as a result, in mycotoxin contamination, is observed in maize genetically engineered with *Bacillus thuringiensis* crystal toxin which has lethal effects against lepidopteran species, compared to non-transgenic maize (Munkvold et al. 1997; Bakan et al. 2002; Ostry et al. 2010; Folcher et al. 2010). Although the use of some transgenic crops has been approved by the European Union, they will be probably subjected to restrictions in the food chain in the near future. In addition, specialized agriculture strategies such as the organic agriculture that, in general, avoid transgenic crop cultivation or the strong opposition to the cultivation of genetic modified organisms by the European consumers must be considered.

Agrochemicals can also be used for reducing damage by stem borers (Mazzoni et al. 2011). Folcher and coauthors (2009) showed that the insecticide deltamethrin, which controlled the two main maize borers ECB and MCB, was efficient in reducing mycotoxin levels in maize fields, whereas several other authors showed the efficiency of pyrethroids and organophosphate insecticides as well as the control of timing applications (Saladini et al. 2008; Blandino et al. 2010a; Blandino et al. 2010b). The poor efficacy of chemical control, due to the entirely endophytic life of MCB, has been found in some experimental work (Spanu et al. 1993). A third alternative for controlling damage by corn borers would be the use of conventional breeding for obtaining maize with low susceptibility to MCB attacks. This could lead to the development of promising varieties showing low levels of damage by borers and fumonisin contamination, these

105 varieties would be compatible with any kind of agriculture including organic cultivation
106 (Avantaggiato et al. 2003).

107 In spite of the vast amount of literature available on the control of Lepidoptera and/or
108 fumonisin contamination, the evaluation of genotypes comprising good resistance to MCB in
109 relation to fumonisin contamination has not been evaluated previously. The objectives of the
110 current study were (i) to estimate the impact of borer damage on fumonisin contamination in
111 maize kernel by comparing fumonisin contamination under infestation and protected conditions
112 and (ii) to evaluate the potential usage of genotypes resistant to borers as controlling factors of
113 fumonisin contamination.

Materials and Methods

Field experimental design. Six maize inbred lines previously classified attending to their degree of resistance to stem and ear attack by MCB (Butrón et al. 1998; Butrón et al. 1999; Butrón et al. 2006b) were used as parents of a set of 15 hybrids (Table 1). In 2008 and 2009, the hybrids obtained were evaluated in a split-plot design with three replicates at Pontevedra, a location in north-western Spain (42º 25´N, 4º 57´W, and 20 m above sea level). Treatments (artificially infested with MCB or treated with insecticide) were assigned to main plots and genotypes to subplot units. Each subplot consisted in a single row of 15 plants, the distance between consecutive rows was 0.8 m and 0.18 m between hills within rows, and the final density was approximately 70.000 plants ha^{-1}. The soil type was acid sandy loam. Trials were irrigated once, and cultural operations, fertilization, and weed control were carried out according to local practices. At flowering, 5 competitive plants were artificially infested with one egg mass of about 40-50 eggs between the main ear and the stem as described by Butrón and coauthors (1998). The MCB rearing method used was described by Eizaguirre and Albajes (1992). The insecticide applied was the granular formulation Clorifos 5-G (5% Chlorpyrifos) at 40 kg ha^{-1}; chlorpyrifos is an organophosphate insecticide Category II, showing stable toxicity at all temperatures (Musser and Shelton 2005). The insecticide was applied locally in the leaf axil of the maize plants. Four insecticide applications were applied, first was done at the early flowering stage and, then, every 20 days. The efficacy of this insecticide reducing MCB damage has been noted previously (Ordás et al. 2012).

Corn borer damage. At harvest, traits recorded were as follows: tunnel length (stems of 5 plants per plot were longitudinally split to measure the total length in centimeters per plant of tunnels made by borers) and visual ratings for ear and shank appearance (on a 1–9 point subjective scale determined as follows: 1 = >90% damaged, 2 = 81–90% damaged, 3 = 71–80% damaged, 4 = 61–70% damaged, 5 = 41–60% damaged, 6 = 31–40% damaged, 7 = 21–30% damaged, 8 = 20–1% damaged and 9 = 0%) (Sandoya et al. 2008).

Fumonisin B_1 and B_2 quantifications. Five main ears from the each subplot were collected at harvesting time and evaluated for fumonisin concentrations. Fumonisin quantification was undertaken by the technical service of the Food Technology Departament of the University of Lleida according to the European Regulation No. 2006/401/EC. A 200g representative sample of grains from each subplot was dried and milled. A 10 g representative sub-sample of the milled material was analysed for fumonisin B_1 and B_2. Samples were extracted by shaking for at least 20 min with a 50 mL of water + methanol + acetonitrile (50:25:25) containing 1g sodium chloride. The supernatant was filtered through Whatman filter nº4 and 10 mL were diluted with 40 mL phosphate buffered saline (PBS) and purified through an immunoaffinity column (Fumoniprep ® R-Biopharm Rhône LTD, Glasgow, UK). After washing the column with 20 mL PBS, fumonisins were slowly eluted with 1.5 mL methanol into graduated glass vial. 1.5 mL MiliQ water was subsequently eluted obtaining a final volume of 3 mL methanol + water (50:50). Fluorescent derivates of the fumonisins were formed using o-phthaldialdehyde (OPA) and analysed by HPLC (Waters Separation Module 2695, Milford, MA, USA) with a fluorescence detection system (Waters Multi λ Fluorescence Detector 2475; λ_{ex} = 335nm, λ_{em} = 440nm). Analysis by injection of 100 μL was carried out using a Spherisorb ® OSD2 column (150 mm x 4.6 mm i.d.; 5 μM particle size) and precolumn (Spherisorb ® S5OSD2 (10 mm x 4.6 mm i.d.). The mobile phase was methanol + sodium phosphate 0.1M (77:23) at pH 3.35, with a linear gradient over 12 min at a flow rate of 1mL min^{-1}. Toxin quantification was performed using external standards purchased from Sigma (Sigma, St. Louis, MO, USA).

Statistical analyses.

Individual and combined analyses of variance (ANOVA) for corn borer damage and fumonisin contamination were computed with the GLM procedure of SAS following a split-plot design (SAS 2007). Years, replications and their interactions were considered random factors, and genotype and treatment were considered as fixed factors. Comparisons of means among treatments and genotypes were made by Fisher's protected least significant difference (LSD).

166 Two types of correlations were carried out: simple correlation coefficients between fumonisin
167 concentration and ear appearance under artificially infested conditions were calculated for each
168 year (N = 15). In addition, in order to minimize genotypic differences attending to the borer
169 resistance, correlations between the differences for borer damage traits, computed as the
170 difference between infested and protected plants, and fumonisin contamination were evaluated in
171 for each year (N = 15). With the aim of obtain positive differences between treatments,
172 differences for tunnel length and fumonisin content were computed as the difference between
173 infested and protected plants; and for ear and shank appearance as the difference between
174 protected and infested plants. We use the CORR procedure of SAS (SAS 2007).
175

176 **Results and Discussion**

177 In the combined analysis of variance there were significant differences ($P < 0.05$) among years for
178 all traits measured (Table 2). Differences among treatments were not significant for any trait, but
179 year × treatment interaction was significant for tunnel length and shank appearance. Differences
180 among genotypes were significant for all traits with the exception of the ear appearance. For this
181 last trait the triple interaction year × treatment × genotype was significant (Table 2). In the
182 individual analyses of variance, there were significant differences between treatments for borer
183 damage traits in 2008 and 2009, while significant differences among genotypes were only
184 significant in 2009 (data not shown). The insecticide treatment was efficient in reducing damage
185 by borers, especially in the stem, but showed no effect on kernel contamination with FB_1, FB_2
186 and $FB_1 + FB_2$ (Table 3), in disagreement with results reported by several authors (Munkvold and
187 Desjardins 1997; Saladini et al. 2008; Blandino et al. 2010b; Folcher et al. 2009), although some
188 other studies mentioned its limited efficacy depending on the year (Torelli et al. 2012).
189 Nevertheless, the correlation coefficients between the difference between protected and infested
190 plants for ear appearance and fumonisin content were significant or very close to the significant
191 level in 2008 ($r = 0.49$, $P \leq 0.06$) and 2009 ($r = 0.51$, $P \leq 0.05$). Differences for tunnel length or
192 shank damage were not correlated to the fumonisin content. This was expected because *Fusarium*
193 *verticillioides* and *F. proliferatum* penetrate mainly into the maize kernel through silks or broken
194 pericarp, and the systemic infection through the stem is less important. Therefore, genotypes with
195 increased kernel damage by borers tended to increased fumonisin accumulation under infestation
196 conditions. In previous studies, ear damage by borers was also the damage trait most related with
197 fumonisin contamination (Avantaggiato et al. 2003; Blandino et al. 2010a; Alma et al. 2005).
198 Furthermore, expression of CryIA(b) in plant tissues other than kernels did not consistently
199 affect *Fusarium* symptoms or infection (Munkvold et al. 1997).

200 A previous study reported a high correlation between fumonisin content and percentage
201 of insect-damaged ears (Avantaggiato et al. 2003). Meanwhile, in the current study, there is a weak

202 association between both traits. It is interesting to note that in the previous study the percentage
203 of damaged ears was between 20 and 45 %, and the level of fumonisin contamination varied from
204 3 to 26 μg/g (Avantaggiato et al. 2003). However, in the current study the reduced variability for
205 kernel attack by borers (less than 20 % of kernels were damaged by borers) could make it difficult
206 to establish a clear relationship between ear appearance and fumonisin contamination. In our
207 conditions, MCB larvae usually attack stems, but can also damage ears in a lesser degree (Cordero
208 et al. 1998). Velasco and coauthors (2007) described the average behavior of MCB across four
209 locations and seven years, and they concluded that 67% of the larvae of MCB were found in the
210 stem below the main ear, while 18% of MCB larvae were found properly in the ear. This is
211 indicative of the low importance of ear damage by MCB larvae compared to stem damage. In
212 spite of the high efficiency of the infestation methodology carried out, and in view of the larvae
213 feeding behavior, we suggest an improvement of the infestation technique in order to increase
214 kernel attack by MCB larvae. In the present study, eggs were always placed between the shank of
215 the primary ear and the stem trying to mimic moth behavior. However, if we are interested in ear
216 resistance to borers, several studies mentioned a variation in the infestation methodology
217 (Velasco et al. 1999; Cartea et al 2001): the egg mass is placed among the silks, allowing the
218 detection of more differences for ear resistance among genotypes. In fact, ear tip infestation
219 resulted in more damaged grains, because the barriers that protect the grain were bypassed
220 (Velasco et al. 1999). Thereby, direct selection of kernel damage throughout the appropriate
221 infestation methodology will be even more effective than the current infestation methodology
222 used to date to get resistant genotypes. We have to take into consideration again that the main
223 damage by this borer is focus in the stem, so the use of genotypes resistant in the stem is a good
224 starting point to evaluate thereafter the ear damage resistance with the proper infestation in the
225 ear, as recommended in the current study.
226 As ear appearance under infestation is a measure of resistance level of genotypes to kernel
227 attack, simple correlation coefficients between ear appearance and fumonisin content in the

228 artificially infested treatment were calculated to establish if it is possible to control fumonisin
229 contamination in maize kernels by using genotypes resistant to borers. The correlation
230 coefficients between both traits were r = -0.34 (p = 0.22) and r = -0.87 (p < 0.001) in 2008 and
231 2009, respectively. The lack of relationship between kernel damage by borers and fumonisin
232 content in 2008 was expected because kernel damage was not significant in that year. Artificial
233 infestation was performed in 2008 as usual, but environmental conditions that disturb the
234 development of the pest can drastically limit insect population. Unfavorable conditions for MCB
235 development were observed in 2008 because records of natural infestation by capture in
236 pheromone cone traps were lower in 2008 than in previous or following years. In addition, no
237 differences for ear appearance were detected among genotypes under infestation conditions in
238 2008; meanwhile differences for ear appearance were significant in 2009 (Table 4). Several studies
239 pointed out that correlations between insect damage and fumonisins measured in the same
240 genotypes could be more significant in some environments (i.e. years), suggesting that
241 environmental factors are influencing the plant's ability to defend itself in the absence of insects
242 (i.e. the fungus is able to invade on its own) and/or the ability of the fungus to spread at different
243 rates after insect attack (Dowd 2003). In our conditions, kernel damage by MCB larvae was a
244 secondary risk factor for fumonisin contamination [damage by borers was significantly lower in
245 2008 compared to 2009, but fumonisin content was significantly higher (Tables 2 and
246 3)].Therefore it would not be possible to control fumonisin contamination across years by using
247 resistant genotypes to ear attack by MCB larvae such as EP39 × EP77. However, genotypes such
248 as EP42 × EP77 that combine low ear damage by borers and low fumonisin level across
249 environments could be a good election (Table 4).
250 In summary, we did not determine a relationship between borer attack and fumonisin
251 contamination, nevertheless, in particular environments, genotype differences in ear resistance to
252 borer attack could influence genotype differences to fumonisin contamination of the kernels.
253 More experimental work needs to be done to prove that using genotypes resistant to borers are a

viable option to control fumonisin contamination. The use of an applicable methodology in order to identify MCB resistant genotypes to ear attack under artificial infestations might be the most promising approach.

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375 Table 1. Pedigree, and ear and stem resistance to MCB for the six parental inbred lines used in
376 this study.

Inbred line	Pedigree	Stem resistance[a]	Ear resistance[b]
CM151	Mt42 × WF9[2]	resistant	susceptible
EP39	Fino	resistant	resistant
EP42	Tomiño	susceptible	susceptible
EP47	(EP4 × A239) EP4[2]	susceptible	susceptible
EP73	EPS5	susceptible	susceptible
EP77	EP31 × CM109	resistant	resistant

377 [a] Stem resistance classification based on tunnels length or percentage of damage on the stem after
378 artificial infestation in the field (Butrón et al. 1998; Butrón et al. 2006a).
379 [b] Ear resistance classification based on ear general appearance after artificial infestation in the
380 field (Eizaguirre and Albajes, 1992; Butrón et al. 1998; Butrón et al. 2006a).

381 Table 2. Mean squares from the combined analysis of variance for the borer damage traits and fumonisin contamination evaluated in fifteen hybrids

382 under two treatment conditions (infested with *S. nonagrioides vs* protected with insecticide) in 2008 and 2009.

		Borer Damage Traits			Fumonisin Contamination		
Source of Variation[a]	**df**	**Stem tunnel length (cm)**	**Ear appearance (1-9)**[c]	**Shank appearance (1-9)**[c]	**Fumonisin B_1 (μg/g)**	**Fumonisin B_2 (μg/g)**	**Fumonisins $FB_1 + FB_2$ (μg/g)**
Year	1	18395.819 *	4.355 **	253.551 **	406.176 **	37.723 **	693.072 **
Replication (Y)	4	43.148	0.483 *	3.978 *	24.584 **	1.885 *	39.073 **
Treatment	1	22995.124	0.782	138.513	0.427	0.204	0.038
Y x T	1	6122.861 *	0.404	36.360 **	0.883	0.004	1.026
R x T(Y)	4	51.288	0.197	0.683	3.949	0.226	5.868
Genotype	14	393.086*	0.254	3.053 *	7.303 *	0.901 *	12.741*
T x G	14	231.271	0.119	1.856	2.788	0.532	5.174
Y x G	14	72.750	0.264	0.717	4.597	0.620	8.322
Y x T x G	14	95.319	0.274 *	1.098	3.529	0.511	6.213
Error[b]	112	137.635	0.157	1.377	3.617	0.517	6.260

383 *, **, Probability level of 0.05 and 0.01, respectively.

384 [a] Year: Y; Replication: R; Treatment: T; Genotype: G.

385 [b] Degrees of freedom for Fumonisin B_2 were 111.

386 [c] Ear and shank appearance (on a 1–9 point subjective scale determined as follows: 1 = >90% damaged, 2 = 81–90% damaged, 3 = 71–80% damaged, 4

387 = 61–70% damaged, 5 = 41–60% damaged, 6 = 31–40% damaged, 7 = 21–30% damaged, 8 = 20–1% damaged and 9 = 0%).

388 Table 3. Means of borer damage traits and fumonisin contamination of two treatments (infested with *S. nonagrioides vs* protected with insecticide)

389 evaluated in fifteen hybrids in 2008 and 2009.

		Borer Damage Traits			Fumonisin Contamination		
Year	Treatment	Stem tunnel length (cm)	Ear appearance (1-9)[a]	Shank appearance (1-9)[a]	Fumonisin B_1 (μg/g)	Fumonisin B_2 (μg/g)	Fumonisins $FB_1 + FB_2$ (μg/g)
2008	Insecticide	3.99 b	8.49 a	8.75 a	3.85 a	1.08 a	4.93 a
	Sesamia	14.93 a	8.46 a	7.90 b	3.61 a	1.14 a	4.75 a
LSD (P≤0.05)		7.69	---	0.66	---	---	---
2009	Insecticide	12.54 b	8.28 a	7.27 a	0.71 a	0.15 a	0.86 a
	Sesamia	46.81 a	8.05 b	4.62 b	0.75 a	0.23 a	0.98 a
LSD (P≤0.05)*		5.02	0.46	0.82	---	---	---

390 *For each year, means within a column followed by the same letter are not significantly different (P≤0.05).

391 [a] Ear and shank appearance (on a 1–9 point subjective scale determined as follows: 1 = >90% damaged, 2 = 81–90% damaged, 3 = 71–80% damaged, 4

392 = 61–70% damaged, 5 = 41–60% damaged, 6 = 31–40% damaged, 7 = 21–30% damaged, 8 = 20–1% damaged and 9 = 0%).

393 Table 4. Means of fifteen hybrids evaluated for fumonisin contamination and ear appearance in individual (under infestation with *Sesamia nonagrioides* in

394 2008 and 2009) and combined analysis of variance (under infestation with *S. nonagrioides* and protection with insecticide in both years).

	Infested with *Sesamia nonagrioides*				**Combined Treatments**	
	Year 2008		**Year 2009**		**Combined Years**	
Genotype	**Fumonisins $FB_1 + FB_2$ (μg/g)**	**Ear appearance (1-9)[a]**	**Fumonisins $FB_1 + FB_2$ (μg/g)**	**Ear appearance (1-9)[a]**	**Fumonisins $FB_1 + FB_2$ (μg/g)**	**Ear appearance (1-9)[a]**
EP39 × CM151	1.10 a	8.66 a	1.57 abc	7.86 bcd	1.79 cd	8.53
EP39 × EP42	5.44 a	8.33 a	0.00 c	8.46 a	3.65 abc	8.45
EP39 × EP47	6.95 a	8.88 a	1.95 abc	7.73 cd	4.66 ab	8.27
EP39 × EP73	6.51 a	8.66 a	2.73 a	7.73 cd	4.76 a	8.26
EP39 × EP77	5.33 a	8.66 a	0.03 c	8.46 a	2.68 bcd	8.53
EP42 × CM151	3.55 a	8.66 a	0.07 c	8.46 a	2.33 cd	8.47
EP42 × EP47	5.17 a	8.44 a	0.26 bc	7.93 abcd	2.09 cd	8.24
EP42 × EP73	7.48 a	8.11 a	0.87 abc	7.93 abcd	2.88 abcd	8.22
EP42 × EP77	1.76 a	8.55 a	0.03 c	8.20 abc	1.79 cd	8.42
EP47 × CM151	3.12 a	8.11 a	0.80 abc	8.06 abcd	2.55 cd	8.20

EP47 × EP73	6.76 a	8.00 a	0.75 abc	7.93 abcd	2.92 abcd	8.05
EP47 × EP77	8.15 a	8.11 a	0.02 c	8.20 abc	3.60 abc	8.35
EP73 × CM151	1.89 a	8.66 a	2.46 ab	7.80 cd	2.57 cd	8.32
EP73 × EP77	4.72 a	8.66 a	3.02 a	7.6 d	3.79 abc	8.11
EP77 × CM151	3.40 a	8.33 a	0.20 bc	8.40 ab	1.24 d	8.37
LSD (P≤0.05)*	----	----	2.38**	0.56	2.02	----

*Means within a column followed by the same letter are not significantly different (P≤0.05).

** Means within this column are significantly different at P≤0.1.

[a] Ear and shank appearance (on a 1–9 point subjective scale determined as follows: 1 = >90% damaged, 2 = 81–90% damaged, 3 = 71–80% damaged, 4 = 61–70% damaged, 5 = 41–60% damaged, 6 = 31–40% damaged, 7 = 21–30% damaged, 8 = 20–1% damaged and 9 = 0%).