Plant Pathology

Occurrence of *Fusarium* species in maize kernels grown in Northwestern Spain

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Abstract

Efforts are required to understand the epidemiology of the *Fusarium* disease by focusing more precisely on the relationship between environmental variables and the disease presence. The objectives of the present study were to monitor the occurrence of *Fusarium* species in maize kernels in Northwestern Spain in order to determine the potential risk of mycotoxin contamination, and to identify environmental traits affecting the composition of the *Fusarium* species identified.

The environmental mean of *F. verticillioides* presence ranged from 33 to 99 %, supporting the idea that the fumonisin contamination is the main maize-based feed and food safety concern in this area, although emerging mycotoxins such as moniliformin, fusaproliferin and beauvericin should be also taken into account. Under the particular environmental conditions of this region we must point out temperature and humidity in relation to the *Fusarium* spp. occurrence. We determine that warmer temperatures at later stages of kernel development and during kernel drying increase the frequency of *F. verticillioides* in maize kernels; while the presence of *F. subglutinans* is impacted by higher relative humidity at the silking stage and cooler temperatures during the kernel drying period. The management of sowing and harvest dates can be effective in order to modulate the fungal presence and growth.

Key words: *Fusarium*, *Zea mays*, fumonisin; environment; presence; kernel, silk

Molds belonging to the genus *Fusarium* are widely found infecting maize kernels in temperate regions. The occurrence of *Fusarium* species is a food and feed safety problem because most of them produce mycotoxins (Logrieco et al., 2003,). Symptoms of mycotoxicosis depend on the type of mycotoxin, concentration, length of exposure and characteristic of the exposed individual (e.g. age and health), but mycotoxins could especially cause injuries in liver, kidneys, and immune, endocrine and/or nervous systems (Bennett & Klich, 2003). They can be mutagenic and carcinogenic; potential carcinogenic risk for some mycotoxins has been rated by the International Agency for Research on Cancer (IARC, 1993). Therefore, legislation to limit the amount of some mycotoxins has been implemented in many parts of the world (FAO, 2004) in order to minimize human health risk.

Climatic conditions determine the predominance of a particular species or group of species which cause different types of maize ear rot. In cooler temperate regions, Gibberella ear rot is predominant and is mainly caused by *F. graminearum* and related species such as *F. culmorum*, *F. cerealis* and *F. avenaceum* (Munkvold, 2003, Logrieco et al., 2002, Bottalico, 1998). In warmer regions, Fusarium ear rot is prevalent and is the result of kernel infection by *F. verticillioides* and other species of the *Gibberella fujikuroi* complex, such as *F. proliferatum* and *F. subglutinans.* All these species are mycotoxigenic and, depending on the particular species, can produce trichothecenes, fumonisins and/or zearalenone, and other mycotoxin comparatively less important such as moniliformin, beauvericin, fusaproliferin, fusaric acid or enniatins (Logrieco et al., 2002, Jestoi, 2008). In Spain, maize kernel seemed to be predominantly infected by *F. verticillioides* and in a lesser extent by *F. proliferatum*, both known as fumonisin producers (Butron et al., 2006, Jurado et al., 2006, Arino et al., 2007). Significant differences among years and locations for *Fusarium* spp. incidence in maize kernels has

been reported in many geographical areas (Bottalico, 1998, Goertz et al., 2010, Boutigny et al., 2012, Covarelli et al., 2011). Bakan et al. (2002), analyzing kernel infection by *Fusarium* ssp., found that *F. proliferatum* was more abundant in northeastern Spain. Our experimental plots are located in northwestern Spain, where climatic characteristics during kernel filling are very different from northeastern Spain conditions, and those climatic differences could be responsible for differences in the *Fusarium* species identified in the area (Marin et al. 1996; Butron et al., 2006). Attending to the fumonisin contamination, Sanchis et al. (1995) had already pointed out the potential fumonisin contamination in many Spanish corn-based products containing both *Fusarium* species, while a previous papers from our group noted fumonisin contamination of maize flours above the levels established in the European Regulation (Butrón et al., 2006).

Although yearly and geographical variation in the diversity of *Fusarium* in maize kernels has been noted, we have no information attending the environmental traits affecting biodiversity other than the wetter regions seemed to favor greater *Fusarium* contamination than the drier regions (Cantalejo et al. 1998). Therefore, the objectives of the present study were: (i) to monitor the occurrence of *Fusarium* species in maize kernels in Northwestern Spain in order to determine the potential risk of contamination by several mycotoxins; and (ii) to identify environmental traits associated with the variability in the *Fusarium* species composition in the area.

Materials and methods

Field experiments. Six maize hybrids derived from crosses among inbred lines EP39, CM151, EP42 and EP47 were used to monitor the prevalence of *Fusarium* spp. on maize kernels under natural infection. As corn borer attack has been associated to increased kernel infection by fungus (Smith & White, 1988), two inbred lines (EP39 and CM151) were selected as resistant to the Mediterranean corn borer (*Sesamia nonagrioides* Lef.) attack and the other two (EP42 and EP47) as susceptible (Santiago et al., 2003). Hybrids were evaluated at early (end of April) and late (middle of May) sowings in 2007 and 2008 in three locations in Northwestern Spain and were harvested in two dates. Locations were Pontevedra (42º 24' N, 8º 38' W, 50 m above sea level) and Barrantes (42º 30' N, 8º 46' W, 50 above sea level), both placed close to the coast, and Valongo (42º 26' N, 8º 27' W, 500 above sea level), situated in the inlands. Therefore, hybrids were evaluated in a total of 24 environments (combination of 2 years-3 locations-2 sowing dates-2 harvest dates). A split-plot design with three replications was used for each trial (year-location-sowing combination); hybrids were assigned to main plots and harvest times to sub-plots. Main plots consisted in two rows with 13 two-kernel hills per row, rows being 0.80 m apart from each other and hills 0.21 m apart. After thinning the final density was around 60 000 plants ha^{-1}. Within each plot, ears from one row (sub-plot) were harvested at the beginning of October (early harvest) and from the other row one month later (late harvest). Harvested ears were shelled and kernels were dried at 35 ºC for one week and maintained at 4 ºC and 50 % humidity until analyses were performed.

Environmental variables. A meteorological station was installed at each location for recording climatic data every 12 minutes. Next climatic variables were computed based on recorded climatic data: average of daily mean temperature (ºC), mean of daily

maximum temperatures (ºC), mean of daily minimum temperatures (ºC), mean of daily relative humidity (%), rainfall (mm), number of days with minimum temperature ≤ 15 ºC, number of days with maximum temperature ≥ 30 ºC, number of days with mean temperature ≥ 10 °C and < 15 °C, ≥ 15 and < 20 °C, ≥ 20 and < 25 °C, ≥ 25 and < 30 °C, and number of days with rainfall ≥ 2 mm. These climatic variables were selected according to previous reports on the influence of climatic factors on mold development in wheat and maize (Marin et al., 2004, de la Campa et al., 2005, Maiorano et al., 2009, Schaafsma & Hooker, 2007). These parameters were calculated for the next periods: the entire maize growing period, from sowing to harvest; the maize vegetative period, from sowing to silking; the maize reproductive period, from silking to harvest; the flowering period, from 15 days before silking to 15 days after silking; critical period 1 (C1), between 10 and 4 days before silking; critical period 2 (C2), between 4 days before silking and 2 days after silking, critical period 3 (C3), between 2 and 8 days after silking; critical period 4 (C4), between 8 and 14 days after silking; milk-dough kernel stage, between 16 and 30 days after silking; dent kernel stage, between 31 and 45 days after silking; kernel developing period, from silking to physiological maturity; kernel drying period, from physiological maturity to harvest.

Other environmental variables included and recorded at harvest were: maize husk coverage, evaluated by a visual scale from 0 (loose husks with visible cob) to 5 (tight husks) (Wiseman & Isenhour, 1992); kernel damage by corn borers on a visual rating from 1 (100% of ear totally damaged by borers) to 9 (no damage); tunnel length, maize stem damage by borers expressed in cm; kernel humidity (%); kernel damage by *Sitotroga cerealella*; percentage of kernels with damaged pericarp; and thickness of pericarp expressed in μm.

Identification of *Fusarium* species. Fifty kernels from each sub-plot were used for estimating the presence of each *Fusarium* species in maize kernels in 2007 and 2008. Maize kernels were grown on KOMADA medium which is selective for *Fusarium* spp. (Komada, 1975). Monosporic isolates were obtained and were grown on PDA (Potato Dextrose Agar), SNA (Spezieller Nährstoffarmer Agar) and CLA (CarnationLeaf Agar) media for determining specific characteristics of each isolate (Leslie & Summerell, 2006). In addition, a molecular identification of the species was also performed:

Fungal DNA was directly extracted from mycelia of monosporic cultures grown on plates, using the commercial kit E.Z.N.A.® Fungal DNA Mini (Omega bio-tek). All monosporic isolates were tested by PCR. PCR reactions were carried out with primers ITS1 and ITS4 (White et al., 1990) to amplify the ITS region of rDNA, and with primers EF1 and EF2 (O'Donnell et al., 2000) for the elongation factor 1α gene (EF-1α). ITS-PCR reactions were carried out in microcentrifuge tubes each containing one PuReTaq^{M} Ready-To-Go™ PCR Bead (GE Healthcare), 1 μL genomic DNA, 0.3 μL of each primer (10 μM), and sterile water up to a final volume of 25 μL. Elongation factor 1α gene PCR-reaction contained 1 μL of genomic DNA, 25 pmol of each primer, 200 μL of dNTPs, 1U of Green Taq DNA polymerase (GenScript, USA), 1X standard PCR buffer and sterile water up to a final volume of 25 μL.

Both DNA amplification reactions were carried out in a Thermocycler Biometra T3000 (Whatman) under the following conditions: one cycle at 94°C for 5 min; 35 cycles at 94°C for 30 s, 55ºC (for ITS1/ITS4) or 53ºC (for EF1/EF2) for 30 s, 72°C for 1 min; and a elongation final at 72°C for 10 min. Products from PCR reactions were electrophoresed on a 2% agarose gel, then stained with ethidium bromide, and visualized with a UV transilluminator. The size of PCR products was estimated by comparison with a 100 bp standard ladder (Marker XIV, Roche Diagnostics). Amplified

products were sequenced with the same primers used for PCR reactions in an ABIPrism 3130 Genetic Analyzer (Applied Biosystems). Sequences obtained were analyzed with the BLAST alignment program of the NCBI and comparing with those deposited in GenBank [National Center for Biotechnology Information (NCIB), 2012]. The molecular identification of a species was accepted when the percentage of sequence identity was above 98%.

Statistical analyses. The averaged percentage of presence of each *Fusarium* species at each of the 24 environments (combination of 2 years-3 locations-2 sowing dates-2 harvest dates) was computed as the mean of individual percentages in 18 sub-plots (six different maize hybrids replicated three times). Combined analyses of variance (ANOVA) for *Fusarium* spp. occurrence were computed with the GLM procedure of SAS following a split-plot design (SAS 2007). All sources of variation were considered as fixed factors. Comparisons of means among years, locations, sowing dates and harvest dates were made by Fisher's protected least significant difference (LSD). In addition, Pearson correlations analyses between *Fusarium* spp. were calculated.

In order to examine the relationships between the environmental variables and the *Fusarium* species in the kernels a redundancy analyses (RDA) was performed using CANOCO (Ter Braak & Smilauer, 1997). Previously, a detrented correspondence analysis (DCA) had been performed to determine if data could fit a linear ordination model as RDA or not, following recommendations by Lepš and Šmilauer (2003). Analyses were applied to the averaged percentage of presence of each *Fusarium* species in maize kernels at each environment. RDA computations were performed on centered and standardized data, and run with a forward selection of the environmental variables procedure and the associated Monte Carlo permutation test (499 unrestricted

189 permutations) to exclude environmental variables that did not contribute significantly

190 (p>0.05) to the variation of the *Fusarium* species.

Results

Nine different *Fusarium* species were isolated from maize kernel samples (Table 1). Five species were found in all locations: *F. verticillioides*, complex *F. subglutinans sensu lato*, *F. proliferatum*, *F. poae* and *F. oxysporum*. The prevalent species in the 24 environments was *F. verticillioides*; the environmental average of *F. verticillioides* presence ranged from 33 to 99 %. The second most abundant was the complex, *F. subglutinans sensu lato,* which was present in all environments at percentages varying from 1 to 27 %. The species identified and also included in this complex were *F. begoniae* and *F. sterilihyphosum*. The remaining *Fusarium* species (*F. proliferatum*, *F. poae*, *F. oxysporum, F. cerealis, F. equiseti, F. solani,* and *F. culmorum*) were present sporadically across environments and never surpassed a kernel presence of 4 % (data not shown).

There were no differences between years, locations, sowing dates or harvest dates for the diverse *Fusarium* species identified with the exception of *F. verticillioides*. *F. verticillioides* presence was higher in coastal locations (Pontevedra and Barrantes) compared to the inland location (Valongo). In addition, early sowing (86.19 % early sowing vs. 74.55 % late sowing) and late harvest (73.52 % early harvests vs. 80.94 % late harvests) showed the highest occurrence. No significant differences between years were observed for *F. verticillioides* presence.

There was simple positive correlation among abundances for *F. oxysporum* and *F. solani* ($r = 0.67$, $P \leq 0.001$), *F. cerealis* and *F. poae* ($r = 0.56$, $P \leq 0.01$), as well as *F. equiseti* and *F.culmorum* ($r = 0.77$, $P \leq 0.001$), *F. equiseti* and *F. subglutinans sensu lato* ($r = 0.59$, $P \leq 0.01$), and *F. culmorum* and *F. subglutinans sensu lato* ($r = 0.70$, $P \leq 0.001$). It is important to note that these correlations are based on very low percentages of presence for those species.

The redundancy analysis was performed using significant non-categorical environmental factors as explicative variables. The results of the Monte Carlo permutation tests revealed the statistical significance ($p \leq 0.05$) of the effects of three environmental variables on *Fusarium* species composition: number of days with mean temperature ≥ 15 and < 20 °C during drying kernel period, averaged relative humidity at C3 (between 2 and 8 days after silking), and number of days with minimum temperature ≤ 15 ºC at dent kernel stage (Table 2). The first two axes of the redundancy analysis using these three environmental variables as explicative variables explained the 71.2 % of the variability for *Fusarium* species ocurrence (Figure 1), the 75.0 % of the variability for *F. verticillioides* and 49.0 % of the variability for *F. subglutinans sensu lato* presence (Table 3). Days with mean temperature ≥ 15 and < 20 °C at drying kernel period and days with minimum temperature ≤ 15 ºC at dent kernel stage had an important contribution to the gradient for the first axis which explained the 75 % of variability for *F. verticillioides* (Table 3). The averaged relative humidity during C3 period (between 2 and 8 days after silking) and days with mean temperature ≥ 15 and < 20 °C at drying kernel period had an important effect on the second axis. Both the axes explained 49 % of variability for *F. subglutinans sensu lato* and between 6 and 21% of variability for *F. poae*, *F. proliferatum*, *F. oxysporum*, *F. cerealis*, *F. equiseti, F. solani* and *F. colmorum* (Table 3). Increased days with mean temperature 15 ºC ≤ and < 20 ºC at drying kernel period and fewer days with minimum temperature ≤ 15 ºC at dent kernel stage favored the occurrence of *F. verticillioides* in maize kernels (Figure 1); while the presence of *F. subglutinans* augmented with increased relative humidity at C3 period and fewer days with mean temperature 15 ºC ≤ and< 20 ºC during kernel drying (Figure 1).

240 **Discussion**

241 All species isolated from maize kernel samples were previously found in maize
242 grown in Europe (Dorn et al., 2009, Goertz et al., 2010, Logrieco et al., 2002). These
243 *Fusarium* species are, in general, mycotoxigenic, and produce fumonisins,
244 trichothecenes, zearalenone, moniliformin, beauvericin, enniatins and fusaric acid
245 (Leslie & Summerell, 2006, Logrieco et al., 2003, Jestoi, 2008). The results confirmed
246 that *F. verticillioides* is the prevalent species in Northwestern Spain (Munoz et al.,
247 1990, Butron et al., 2006).

248 *F. verticillioides* is the most frequently isolated species from maize pink ear rot
249 which is commonly observed from southern to central European areas; while the
250 predominant species causing maize red ear rot is *F. graminearum* which is increasingly
251 distributed from central to northern European regions (Logrieco et al., 2002). In warm
252 southern European areas, *F. verticillioides* is associated with *F. proliferatum*, while
253 displacement toward Central Europe increases the presence of *F. subglutinans* in
254 detriment of *F. proliferatum*. In this study, *F. proliferatum* was scarce and *F.*
255 *graminearum* was not present, while *F. verticillioides* was highly predominant and *F.*
256 *subglutinans sensu lato* was the most abundant group in agreement with the trend
257 observed in surveys performed in the last ten years in maize growing areas around the
258 world where *F. verticillioides* associated with *F. subglutinans* are becoming the
259 dominant species (Bottalico, 1998). Non-detected presence of *F. graminearum* could be
260 consequence of early establishment of *F. subglutinans* that may act as a biological
261 control mechanism against invasion by *F. graminearum* (Cooney et al., 2001) and/or the
262 possible competence between *F. verticilliodes* and *F. graminearum* (Marin et al., 2004,
263 Reid et al., 1999). Environmental conditions at Northwestern Spain, mild temperatures
264 along the year and moderate risk of ear damage by corn borers, can be related to the

265 species distribution. Corn borer damage is associated with increased infection by *F.*
266 *subglutinans* and *F. verticillioides* in detriment of infection by *F. graminearum* (Lew et
267 al., 1991). In addition, more extreme temperatures would favor *F. graminearum*
268 (colder) or *F. proliferatum* (warmer) presence (Logrieco et al., 2002).

269 *F. verticillioides* is a fumonisin producer , and *F. subglutinans* produces a range
270 of mycotoxins including moniliformin, fusaproliferin, beauvericin and fumonisin
271 (Jestoi, 2008).The fumonisin producing capacity of the *F.verticillioides* isolates in the
272 area has been noted (Cao, 2013). In addition, previous studies show the risk of
273 fumonisin occurrence in maize kernels in Northwestern Spain (Butrón et al. 2006; Cao
274 et al, 2013). The higher presence of *F. verticillioides* showed up by the results, obtained
275 in a wide range of environments in natural conditions, support the idea that the
276 fumonisin contamination is the main maize-based feed and food safety concern in this
277 area, although emerging mycotoxins such as moniliformin, fusaproliferin and
278 beauvericin should be also taken into account.

279 The influence of the geographical location on the variability of *F. verticillioides*
280 is important as long as climatic conditions vary across locations (Boutigny et al., 2012).
281 *F. verticillioides* presence was higher in coastal locations compared to the inland
282 location as expected because the coastal climate is more temperate. Variation due to
283 years was not significant; in southern European areas minor differences among years for
284 *Fusarium* variability have been reported (Covarelli et al., 2011, Dorn et al., 2009), while
285 important shift from one year to another for *Fusarium* spp. composition have been
286 found in northern European regions (Goertz et al., 2010, Dorn et al., 2009). About the
287 sowing and harvest dates, we corroborate the role of agronomic practices in order to
288 regulate the occurrence of *F. verticillioides* (Blandino et al, 2009), although slight
289 effects in the *Fusarium* presence has been noted in this particular study, probably with

290 no effect in the subsequent fumonisin contamination. The positive correlation among
291 abundances for *F. subglutinans sensu lato*, *F. equiseti* and *F. culmorum*, as well as
292 between *F. cerealis* and *F. poae,* corroborate that these species are adapted to similar
293 environmental conditions, those encountered in central and northern European areas
294 (Logrieco et al., 2002).

295 Efforts are required to understand the epidemiology of the *Fusarium* disease by
296 focusing more precisely on the relationship between environmental variables and the
297 disease-cycle. Temperature must be considered as an environmental factor that
298 influences spore production under field conditions, in addition to humidity (Indira and
299 Muthusubramanian 2004). In the same way, the mycotoxin contamination is affected by
300 climatic factors such as temperature and relative humidity available for pre and / or
301 post-harvest (Paterson & Lima, 2010). Attending to *F. verticillioides*, the two main
302 abiotic factors associated with the its life cycle are temperature and water activity
303 (Marin et al., 2004; Samapundo et al., 2005), they were considered the main factors in
304 modeling fungal development and fumonisin synthesis (Maiorano et al. 2009, De la
305 Campa et al., 2005). Likewise, under the particular environmental conditions of
306 Northwestern Spain we pointed out temperature and humidity in relation to the
307 *Fusarium* spp. occurrence. We conclude that warmer temperatures at later stages of
308 kernel development and kernel drying period favored the presence of *F. verticillioides*
309 in maize kernels; while the presence of *F. subglutinans sensu lato* augmented with
310 increased relative humidity at the stage of exposed fresh silks and cooler temperatures at
311 the kernel drying period. These results agree with the idea that *F. subglutinans* is
312 favored by cooler temperature and more humid conditions (Logrieco et al., 2002, Goertz
313 et al., 2010, , Boutigny et al., 2012) compared to *F. proliferatum* and *F. verticillioides*.

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435 **Table 1.** Averaged percentages of kernels with presence of *Fusarium* spp. isolates in
436 2007 and 2008 at three locations in Northwestern Spain. The numbers of positive
437 samples are within parenthesis.

***Fusarium* spp.**	**2007**	**2008**
F. verticillioides	75.75 (196)	78.69 (197)
F. subglutinans sensu lato	4.64 (45)	10.34 (85)
F. poae	1.01 (20)	0.07 (2)
F. proliferatum	0.78 (4)	0.05 (1)
F. oxysporum	0.07 (2)	0.96 (11)
F. cerealis	0.15 (1)	0.05 (2)
F. equiseti	0.00	0.17 (4)
F. solani	0.00	0.05 (2)
F. culmorum	0.00	0.10 (2)
Total % of positive kernels	82.40	90.49
Total % of negative kernels	17.60	9.51

438 **Table 2.** Statistics of the environmental variables retained after the Monte Carlo
439 permutation test and included in the RDA for *Fusarium* species composition in maize
440 kernels cultivated in 24 environments (two years, three locations, two sowing dates and
441 two harvest dates) in Northwestern Spain.

Variables[1]	*F*	*p*	Cumulative variance
Tm15-20S	15,87	0,002	0,42
HumC3	12,14	0,002	0,63
Tmin15D	5,65	0,016	0,71

442 [1]Tm15-25S: number of days with mean temperature ≥ 15 ºC and < 20 ºC at the kernel
443 drying period; HumC3: relative humidity at the critical period C3 (between 2 and 8 days
444 after maize silking); Tmin15D: number of days with minimum temperature ≤ 15 ºC at
445 the maize kernel dent stage.

446 **Table 3.** Accumulated variability for each *Fusarium* species abundance at 24
447 environments (two years, three locations, two sowing dates and two harvest dates) in
448 Northwestern Spain explained by three selected significant variables (days with mean
449 temperature ≥ 15 ºC and < 20 ºC at the kernel drying period, relative humidity at the
450 critical period C3 (between 2 and 8 days after maize silking), and days with minimum
451 temperature ≤ 15 ºC at the maize kernel dent stage).

Variability explained	**Axis 1**	**Axis 2**	**Axis 3**	**Axis 4**
F. verticillioides	0.75	0.75	0.75	0.99
F. subglutinans sensu lato	0.01	0.49	0.49	0.65
F. poae	0.01	0.15	0.32	0.32
F. proliferatum	0.06	0.06	0.06	0.09
F. oxysporum	0.05	0.14	0.16	0.16
F. cerealis	0.10	0.17	0.17	0.19
F. equiseti	0.01	0.10	0.12	0.28
F. solani	0.02	0.18	0.19	0.19
F. culmorum	0.08	0.21	0.21	0.31

Figure 1. Redundancy analysis (RDA) of variability for *Fusarium* species[1] presence restricted to the variability explained by three environmental variables[2].

[1]Each *Fusarium* species was designated using the initial of the genera (F) and the initial letters of the Latin specific name: *Fver* stands for *F. verticillioides*, *Fsub_sl* for *F. subglutinans sensu lato*, *Fpro* for *F. proliferatum*, *Fcul* for *F. culmorum*, *Fequ* for *F. equiseti*, *Fpoa* for *F. poae*, *Foxy* for *F. oxysporum*, *Fsol* for *F. solani*, and *Fcer* for *F. cerealis*.

[2]Tm15-20S = Mean temperature ≥ 15 ºC and < 20 ºC at the kernel drying period; HumC3 = relative humidity at the critical period 3 (between 2 and 8 days after maize silking); and Tmin15D = number of days with minimum temperature ≤ 15 ºC at the maize kernel dent stage.