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Assessing pigmented pericarp of maize kernels as possible source of resistance to fusarium ear rot, *Fusarium* spp. infection and fumonisin accumulation

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ABSTRACT

One of the purposes of maize genetic improvement is the research of genotypes resistant to fusarium ear rot (FER) and fumonisin accumulation. Flavonoids in the pericarp of the kernels are considered particularly able to reduce the fumonisin accumulation (FUM). The aim of this field study was to assess the effect of flavonoids, associated with anti-insect protection and *Fusarium verticillioides* inoculation, on FER symptoms and fumonisin contamination in maize kernels. Two isogenic hybrids, one having pigmentation in the pericarp (*P1-rr*) and the other without it (*P1-wr*), were compared. *P1-rr* showed lower values of FER symptoms and FUM contamination than *P1-wr* only if the anti-insect protection and the *F. verticillioides* inoculations were applied in combination. *Fusarium* spp. kernel infection was not influenced by the presence of flavonoids in the pericarp. Artificial *F. verticillioides* inoculation was more effective than anti-insect protection in enhancing the inhibition activity of flavonoids toward FUM contamination. The interactions between FUM contamination levels and FER ratings were better modeled in the pigmented hybrid than in the unpigmented one. The variable role that the pigment played in kernel defense against FER and FUM indicates that flavonoids alone may not be completely effective in the resistance of fumonisin contamination in maize.

1. Introduction

Fusarium ear rot (FER) is one of the most widespread diseases affecting maize (*Zea mays* L.) ears in temperate regions and it can result in high yield losses due to a decrease of grain matter content, kernel density and total grain yield (Presello et al., 2008). Climatic conditions during the growing season are determinant factors for FER incidence and severity (Venturini et al., 2015). FER epidemics are particularly favored at flowering and kernel drying in presence of warm and dry conditions (Cao et al., 2014; Miller, 2001). Beside climatic conditions, many other factors, such as cultural practices, host susceptibility, kernel damage by insects and pest management strategy, contribute to FER (De Curtis et al., 2011; Parson and Munkvold, 2012). In most maize-growing areas of southern Europe, such as northern Italy, FER is caused by several *Fusarium* species belonging to the *Fusarium fujikuroi* species complex (FFSC) (Aguín et al., 2014; Shala-Mayrhofer et al., 2013; Venturini et al., 2011). The most important FFSC species responsible of FER are the conidial anamorphs of the biological species *F. verticillioides* (Sacc.) Nirenberg followed by *F. proliferatum* (Matsush.) Nirenberg ex Gerlach & Nirenberg and *F. subglutinans* (Wollenw. & Reinking) P.E. Nelson, Toussoun & Marasas (White, 1999). FFSC strains may occur on maize as seedborne endophytes or infect the plant at various developmental stages without inducing visible disease symptoms (Munkvold et al., 1997; Venturini et al., 2011). Insect wounds and exposed silks constitute the most important pathways for FFSC conidia to colonize kernels (Alma et al., 2005; Duncan and Howard, 2010). In addition, systemic movement of FFSC strains from infected seed and stalk to developing maize kernels has also been reported as an infection pathway (Munkvold, 2009). In northern Italy one of the most relevant pests of maize implicated with FER incidence and severity are corn borers, in particular the European corn borer (ECB) *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) (Mazzoni et al., 2011), although other insects are likely to facilitate FER as well (Cao et al., 2014; Parson and Munkvold, 2012). Strains belonging to FFSC, mainly *F. verticillioides* and *F. proliferatum*, are mycotoxin producing fungi. Thus, FFSC infections of maize can result in accumulation of mycotoxins such as fumonisins (FUM) in maize kernels. Fumonisin B_1 (FB1) contamination in maize grain is of concern because of its causal role in equine leukoencephalomalacia, porcine pulmonary oedema, liver and renal carcinogenicity in laboratory rodents and possibly even human carcinogenicity (Shephard et al., 2013).

Abbreviations: (AIP), anti-insect protection; (FER), fusarium ear rot; (FERi), fusarium ear rot incidence; (FERs), fusarium ear rot severity; (FFC), *Fusarium fujikuroi* species complex incidence; (FUM), fumonisin contamination; (FVI), *Fusarium verticillioides* inoculation; (IDi), insect damage incidence; (IDs), insect damage severity.

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Northern Italian grown maize undergoes variable levels of fumonisin contamination depending on several factors such as climatic variables, maize maturity class, and growing weeks (Maiorano et al., 2009; Torelli et al., 2012; Venturini et al., 2015). The application of cultural practices to reduce fumonisin levels is not always effective and environmentally safe methods to control FER and FUM levels in maize (De Curtis et al., 2011; Folcher et al., 2009). Maize genetic improvement seems to be the most sustainable approach to control FER and minimize fumonisin accumulation. However, difficulties might be encountered in the search for resistant maize genotype, because maize resistance to both FER and FUM is under polygenic control and it is quantitative in nature. No source of complete resistance has been identified for either FER or FUM (Mesterházy et al., 2012). Focus of many researches, FER and FUM resistance has brought maize breeders to consider diverse approaches that are currently under investigation (Atanasova-Penichon et al., 2014; Picot et al., 2013). Among them, the development of maize genotypes with kernel pericarp enriched with phenolic compounds seems particularly promising in the reduction of mycotoxin accumulation (Pilu et al., 2011; Sampietro et al., 2013). In addition, phenolic pigments accumulated in the maize seeds are associated with antioxidant power and thought to be highly beneficial for human health (Rodrıguez et al., 2013). Phenolic compounds in kernel pericarp such as flavonoids, especially those regulated by the *p1* gene (Grotewold et al., 1994), reduced fumonisin accumulation (Pilu et al., 2011). Flavonoids are thought to act as a physical barrier against fungal infection by hardening maize kernel pericarp and therefore reducing the mycelial progress from infected to intact kernels. Moreover, the antifungal activity of flavonoids is probably exerted by complexing irreversibly with nucleophilic amino acids in fungal proteins leading to inactivation of the proteins and loss of their functions (Treutter, 2006). Inhibitory effect of flavonoids on fumonisin biosynthesis has not yet been fully elucidated, but it can be hypothesized that flavonoids may inhibit redox enzymes encoded by the FUM6 gene blocking fumonisin production (Kim et al., 2006).

In a previous field study FER and fumonisin levels of two isogenic maize hybrids, one able to accumulate flavonoids and one colourless, were compared. That research showed that the effects of flavonoids in maize pericarp varied depending on the experimental site and was not stable across the years (Venturini et al., 2015). The objective of the current study was to investigate the stability in the field of pigmented maize resistance to FER and FUM contamination in response to artificial inoculation of the ears with *F. verticillioides* strains and/or to the apposition of physical barrier against insects. The effect of flavonoids on frequency and severity of insect damage and FER, FFSC kernel contamination and FUM accumulation was evaluated.

2. Materials and methods

2.1. Fungal inoculum

Eight *F. verticillioides* strains (Fv2003, Fv2010, Fv2170, Fv2198, Fv2221, Fv2232, Fv2233 and Fv2306) were used in this study. These strains were isolated from naturally infected maize kernels in 2011, identified following morphological and biological species concepts (Leslie and Summerell, 2006), and belong to the fungal culture collection of the Mycology Laboratory at the Department of Agricultural and Environmental Sciences — Production, Land, Agrienergy (DiSAA-PTA, University of Milan, Milan, Italy). The isolates were maintained on potato dextrose agar (PDA, Difco®, Becton & Dickinson Co., Sparks) at 4 °C and stored as conidial suspensions in 20% glycerol at −80 °C. Preliminary results performed in the laboratory indicated that all the *F. verticillioides* isolates showed high levels of pathogenicity on maize seeds following the method described by Venturini et al. (2013). The *F. verticillioides* isolates were also tested for the fumonisin production *in vitro* as described by Glenn et al. (2008) (data not shown). They produced from 174.4 to 373.4 μg/g of fumonisin *in vitro*. When inoculum was required, *F. verticillioides* strains were grown on PDA slants at 25 °C under combined visible (white) and ultraviolet (black) light (12 h per day) for 7 days. Conidial suspensions were prepared the day before the field inoculation assays by adding 6 mL of sterile distilled water to the each PDA slant and gentle shaking. The conidia were harvested by filtering the suspensions through two layers of cheesecloth and then enumerated and adjusted to 1.0×10^6 conidia/mL with a haemocytometer (Kova®, Hycor Biomedical Co., Indianapolis). To obtain a mixed inoculum, the conidial suspensions of each *F. verticillioides* strain were mixed together and stocked overnight at 4 °C.

2.2. Experimental design and inoculation procedure

Field experiments were carried out in 2013 in Landriano (PV) at 45°190N, 9°160E, 88 m a.s.l. The field was in a maize–maize rotation and standard soil fertilization, irrigation and weed management practices were applied.

The experimental design was a randomized complete block arranged as a split plot with three replicates. Two isogenic maize hybrids, one with *P1-rr* allele providing pigmentation in pericarp, due to accumulation of phlobaphene pigments, and the other carrying *P1-wr* allele without phlobaphene accumulation in pericarp (Pilu et al., 2011), were sown in late April. Hybrids were applied to main plots representing *P1-wr* and *P1-rr* genotypes, respectively. Each plot consisted in four 70-m rows, 0.7 m apart, and sown at a density of 7 seeds m^{-1}. The growth stages were monitored weekly following the BBCH (Biologische Bundesanstalt, Bundessortenamt and Chemical industry) scale (Lancashire et al., 1991). Treatments were used as sub-plots and were randomized within the two main plots. Treatments included: anti-insect protection (AIP) and *F. verticillioides* inoculation (FVI). Experimental units were single rows consisting of 60 plants (20 plants per replicate). Anti-insect protection was realized by covering all primary ears in the AIP experimental units, at silking (BBCH 61, silking was assigned when silk emergence could be observed in 50% of maize ears), with a bag (400 × 200 mm) made by transparent insect-proof polyethylene net, mesh size 0.22 mm (Retificio Padano s.r.l., Ospitaletto, BS, Italy), sealed at the basal part of the ear (Castellano et al., 2008). The primary ears of plants within the FVI units were inoculated 7 days after flowering when silks were still green but started to dry out from the tips (Miedaner et al., 2010). Silk channel inoculation was performed by injection of 1 ml inoculum into the silk channel of the primary ear of each plant. Negative controls consisted of experimental units of not covered ears (nAIP) or sterile water-inoculated ears (nFVI). Meteorological data were recorded by an automated weather station placed next to the field, with an hourly step.

2.3. Insect damage and FER incidence and severity

Insect damage (ID) and FER incidence and severity were assessed at physiological maturity on 20 ears per replicate. Incidence of ID (IDi) was calculated as the percentage of ears with visible damage due to larval activity. Incidence of FER (FERi) was calculated as the percentage of ears with symptoms per replicate. ID severity (IDs) was calculated as the percentage of kernels per ear with visible damage due to insect activity. A scale of 0–6 was used in which each numerical value corresponds to a percentage interval of surfaces exhibiting visible kernel injuries due to larval activity according to the modified version of a 1–7 scale suggested by Blandino et al. (2008): 0 no injuries, 1 = 1–5%, 2 = 6–10%; 3 = 11–20%, 4 = 21–35%, 5 = 35–60% and 6 > 60%.

FER severity (FERs) was calculated as the percentage of surface with symptoms per ear. A modified version of a 1–7 scale proposed by Reid and Zhu (2005) was adopted, in which each numerical value corresponds to a percentage interval of surfaces exhibiting visible symptoms of the disease such as rot, mycelial growth and kernels with 'starburst' streaks within the pericarp, according to the following scale: 0 = no symptoms, 1 = 1–3%, 2 = 4–10%; 3 = 11–25%, 4 = 26–50%, 5 = 51–75% and 6 = 76–100%. ID and FER severity indexes expressed as