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Fusarium verticillioides and *Fusarium graminearum* Infection and Fumonisin B_1 and Zearalenone Accumulation in Resveratrol-treated Corn

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Resveratrol antimycotoxigenic activity was tested against *Fusarium verticillioides* and *Fusarium graminearum* in corn. Both trans-resveratrol and RES VIN® (a commercial lyophilised polyphenolic product obtained from the skins of red wine grapes, which contains a 10.015% resveratrol) were tested for their efficacy to control mycotoxins accumulation in corn. In particular, their effects were tested against fumonisin B_1 (FB_1) and zearalenone (ZEA) accumulation in naturally contaminated corn with additional inocula of toxinogenic isolates of either *F. verticillioides* or *F. graminearum*. ZEA accumulation was reduced by 80% in *F. graminearum* inoculated samples, while no inhibition of FB_1 accumulation was observed in any of the treatments tested. Resveratrol has previously shown to have antifungal properties against certain fungal species. Thus, studies are needed using higher resveratrol concentrations in order to achieve wider antimycotoxigenic effect as reported in *in vitro* studies. Trans-resveratrol and RES VIN® had similar effects in the experiment carried out, suggesting that the use of the sub product of the wine industry could be a good alternative to synthetic resveratrol.

Key Words: resveratrol, fumonisin, zearalenone, *Fusarium verticillioides, Fusarium graminearum*

INTRODUCTION

Zearalenone (ZEA) and fumonisin B_1 (FB_1) are among the most common mycotoxins found in corn over the world. ZEA is mainly produced by *F. graminearum* Schwabe that causes ear rot of corn in many corn-producing areas of the world with cool, wet growing seasons (Sutton, 1982). The major effects of ZEA are estrogenic and mostly affect the urogenital system. It produces a condition known as hyperestrogenism in pigs and has also been implicated in some incidents of precocious puberty changes in children (Kuiper-Goodman et al., 1987). Fumonisins are a group of foodborne carcinogenic metabolites originally isolated from *F. moniliforme* (or *F. verticillioides*; Gelderblom et al., 1988). The structure of FB_1 was elucidated by Bezuidenhout et al. (1988) and it was shown to cause leukoencephalomalacia in horses (Marasas et al., 1988b). Both *F. verticillioides* and *F. proliferatum* have been related to the massive contamination of agricultural products, mainly corn, with fumonisins. The presence of fumonisins in corn in the Transkei region (South Africa) and in China has been correlated with a high incidence of esophageal cancer in humans (Marasas et al., 1988a; Yoshizawa et al., 1994). Both mycotoxin accumulation occurs in the field or during wet storage.

In the last few years some alternatives to synthetic fungicides in the prevention of *Fusarium* mycotoxin accumulation have been sought. Recently, essential oils from different plants have attracted much attention because of their antifungal properties. Essential oils of cinnamon, clove, lemongrass, oregano and palmarosa have been shown to inhibit growth of *F. verticillioides*, *F. proliferatum* and *F. graminearum* on a corn extract agar medium (Velluti et al., 2004a). When applied to sterilised corn grain at 0.95–0.995 a_w and 20–30 °C, these essentials oils only inhibited ZEA and FB_1 production at 0.995 a_w, and mainly at 30 °C (Velluti et al., 2003, 2004b, 2004c). However, when applied to naturally contaminated corn the effect of the essential oils was poor compared to the impact of natural mycoflora on ZEA and FB_1 accumulation (Marín et al., 2003, 2004).

Resveratrol, a phytoalexin, has shown potential value in the prevention and treatment of cardiovascular

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disease and certain cancer processes. Resveratrol is present in grapes, its concentration in seeds is lower than that in skins, whilst accumulation of resveratrol in juice/pulp was much lower than in skins and seeds (Magee et al., 2002). Resveratrol is produced by grape berries in response to grey mould as a resistance agent. Conditions leading to *Botrytis cinerea* infection enhance resveratrol production, but extensive fungal development may destroy the induced phytoalexin (Jeandet et al., 1995). It has been shown to inhibit oxidative enzymes in plants and animal systems (Fan and Mattheis, 2001). Its antioxidant and prooxidant activities have been compared to other antioxidants (BHT, BHA, phenol, propyl gallate, sodium tripolyphosphate, alpha-tocopherol and vainillin) widely used in foods, and found to be similar or higher (Murcia and Martínez-Tome, 2001).

Some authors have found a positive correlation between the ability to synthesise resveratrol and the resistance of the plant to fungal infection (Bavaresco et al., 1997). Grapes inoculated with *Aspergillus carbonarius*, *Aspergillus japonicus* and *Aspergillus ochraceus* showed an increase in their trans-resveratrol content compared to blanks. In addition, when trans-resveratrol was added to a grape medium, growth of *A. carbonarius* was retarded while ochratoxin A (OTA) accumulation increased significantly, compared to that produced in the same medium with no trans-resveratrol (Bavaresco et al., 2003).

The use of resveratrol to prevent ochratoxin A and ZEA accumulation in wheat and corn seeds, respectively, has been evaluated by Fanelli et al. (2003) and Ricelli et al. (2004). They found that a 230 mg/g concentration in seeds could lead to a sharp reduction of mycotoxin content in those samples.

The aim of this work was to test the efficacy of resveratrol in naturally contaminated corn *in situ*, in order to control FB_1 and ZEA production.

MATERIALS AND METHODS

Material

Culture Material

F. verticillioides (Sacc.) Nirenberg (25N) and *F. graminearum* Schwabe (CECT 2150) were used. The first one is a known FB_1 producer, and the second one is a known ZEA producer. The former strain belongs to Food Technology Department fungi collection of the University of Lleida, Spain, and the latter to the Culture Type Spanish Collection.

Resveratrol

Trans-resveratrol was purchased from Sigma, St Louis, MO, USA. RES VIN® is the commercial name for a lyophilised polyphenolic product obtained from the skins of red wine grapes, which contains a 10.015% resveratrol.

Both trans-resveratrol and RES VIN® were dissolved in ethanol at the concentration of 25 mg/mL resveratrol.

Methods

Moisture Equilibration and Inoculation

Spanish dent corn grain was used. The initial water activity (a_w) of the grain was 0.71, equivalent to a moisture content of 139 g/kg. No ZEA was detected, while FB_1 was present in a concentration of 0.7 mg/kg. The corn grain was weighed into sterile flasks (500 g per flask) and rehydrated to the 0.95 a_w level by addition of sterile distilled water. The amount of water was calculated from the moisture adsorption curve of the grain. Flasks were vigorously shaken and allowed to equilibrate at 4°C for 96 h, with periodic shaking. Four millilitres of resveratrol solutions were added to each flask and repeatedly mixed; 4 mL ethanol was added to control treatments. To allow the evapouration of ethanol, the flasks, plugged with cotton wool, were put at 25 °C for 2 h and periodically mixed. Each flask was inoculated with 5 mL of a spore suspension of either *F. verticillioides* or *F. graminearum* (500 CFU/g); 5 mL of tween-water was added to control flasks. Final a_w values were confirmed by using a water activity meter (AquaLab, Pullman, Washington, DC, USA).

The total number of treatments was nine as detailed in Table 1, and all of them were performed in triplicate.

Incubation and Direct Plating

Flasks were incubated at 20°C for 28 days. After this time, 100 grains from each treatment were surface disinfected and directly plated in MEA and DG18, while the remaining grains were frozen at –20°C for later FB_1 and ZEA analyses. Plates were examined and infection by *Fusarium* Liseola section, *F. graminearum*, and other common genera observed were recorded.

Table 1. Experimental design (list of treatments).

Treatment no.	Antifungal	Inoculum
1	None	None
2	None	*F. verticillioides*
3	None	*F. graminearum*
4	Resveratrol	None
5	RES VIN®	None
6	Resveratrol	*F. verticillioides*
7	RES VIN®	*F. verticillioides*
8	Resveratrol	*F. graminearum*
9	RES VIN®	*F. graminearum*

Mycotoxin Analyses

The European Committee for Standardisation (CEN 2000) method for determination of FB_1 by HPLC was followed. Subsamples (25 g) were ground and extracted by blending in 50 mL methanol-water (3 + 1). Extracts were filtered. Filtrate (10 mL) was loaded on a preconditioned strong anion exchange (SAX) column and eluted with 0.5% acetic acid in methanol. The eluate was evaporated to dryness in a rotavapour, redissolved in methanol, and finally evaporated under a gentle stream of nitrogen and dissolved in methanol for HPLC. A sample was coupled to an OPA (*o*-phthaldialdehyde) reagent and assayed by HPLC, by comparison with external standards, using methanol plus 0.1 M dihydrogen sodium phosphate (3 + 1) (pH 3.35) as mobile phase at a 1 mL/min flow rate.

For ZEA quantification the AOAC method for α-zearalenol and ZEA in corn by HPLC was followed (AOAC 1997).

The reference standards of both FB_1 and ZEA were purchased from Sigma, St Louis, MO, USA.

Dry Matter Determination

The dry matter content of each sample was determined by drying subsamples of approximately 10 g at 105 °C for 17 h (ISTA 1976). Thus, all results are presented on a dry weight basis.

Statistical Analyses of the Data

A full factorial design with three replicates per treatment was carried out. Responses were fungal infection by the *Fusarium* species and other natural main contaminants, concentration of FB_1 and concentration of ZEA. Analysis of variance was performed using SAS version 8.02 (SAS Institute, Inc., Cary, NC, USA). Statistical significance was judged at the 5% level. Additionally, principal component analysis (PCA) was used in order to view interrelationships between the different variables assayed; The Unscrambler® version 7.6 was used.

RESULTS

Impact of Resveratrol Treatments on Natural and Inoculated Mycoflora

The main fungal genera found in corn were *Fusarium*, *Hyphopichia*, *Penicillium*, *Eurotium* as well as some *Zygomycetes*. The main point that clearly affected the results of the treatments was that naturally contaminated corn contained itself more than 90% of both *Fusarium* Liseola section and *Hyphopichia burtonii* contamination (Figure 1).

Two different media were used for quantification of fungal infection, the main difference between both media being that DG18 did not allow growth of

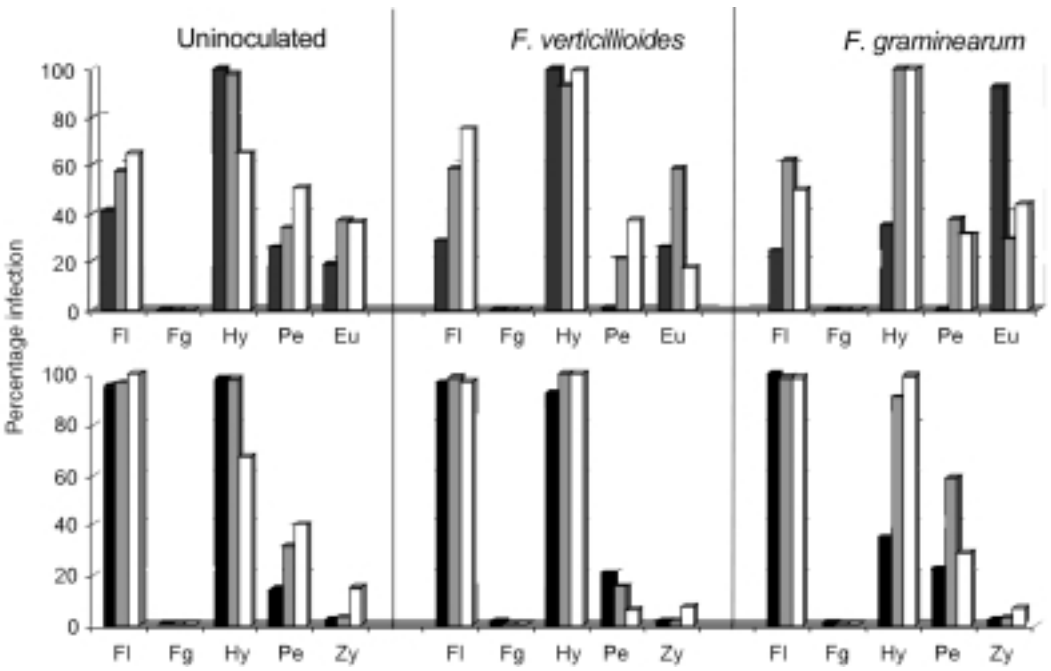

Figure 1. Percentages of infection of corn grains by the main groups of fungi found (Fl, *Fusarium* Liseola section; Fg, *Fusarium graminearum*; Hy, *Hyphopichia*; Pe, *Penicillium*; Eu, *Eurotium*; Zy, *Zygomycetes*) in DG18 (upper) and MEA (lower) media (■, control; ▒, resveratrol; □, RES VIN®).

Zygomycetes while no *Eurotium* isolates were detected in MEA. On the other hand, direct plating showed not to be suitable for *F. graminearum* detection, because in a heavy-contaminated corn by *Fusarium* Liseola section, the later probably grew better and prevented *F. graminearum* detection. Percentages of infection by *Fusarium* Liseola section were significantly higher in MEA than in DG18 medium.

The analysis of variance showed that neither inoculation nor resveratrol treatments had a significant effect on infection by *Fusarium* species; also no difference in *Fusarium* infection was found neither with additional *Fusarium* inoculum nor with resveratrol treatments (data not shown). No significant difference was either found for the remaining genera except for *Hyphopichia*, whose incidence increased with both trans-resveratrol and RES VIN® when additional *F. graminearum* was inoculated.

The PCA displayed in Figure 2 summarises the possible relationship between the different fungal groups for the whole set of samples. PC1 explained 34% of variability of data, while PC2 explained the 21%. PC1 separated those samples which presented high infection by *Hyphopichia.* There was a remarkable negative relationship between the presence of *Hyphopichia* and that of the remaining groups, mainly *Penicillium*, *Eurotium* and *Zygomycetes*, while on the other hand, presence of *Zygomycetes* and *Penicillium* is slightly negatively related to *Eurotium*, as explained by PC2.

Impact of Resveratrol Treatments on FB_1 Accumulation by Natural and Inoculated Mycoflora

Treatments without resveratrol reaching mycotoxin levels of 2.5–3.5 mg/kg were found after 4 weeks incubation period at 20 °C in either inoculated or uninoculated treatments, meaning that naturally existing *Fusarium* Liseola isolates were probably responsible for this mycotoxin accumulation, while additional

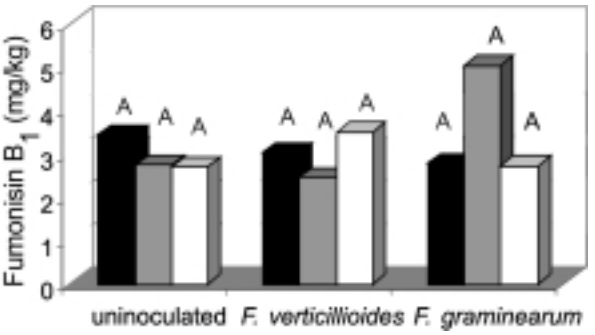

Figure 3. Fumonisin B_1 accumulation in corn at 20 °C for 28 days as affected by the different inoculation and resveratrol treatments (■, control; ▒, resveratrol; □, RES VIN®). Bars with different letters show significantly different treatments ($p < 0.05$).

F. verticillioides and *F. graminearum* inocula were not high enough to affect the natural FB_1 formation in either a positive or a negative side – no significant differences were found (Figure 3). Moreover 2–5 mg/kg concentrations were found when either trans-resveratrol or RES VIN® were added to corn, no significant reduction in FB_1 occurred.

Impact of Resveratrol Treatments on ZEA Accumulation by Natural and Inoculated Mycoflora

A mean concentration of 68 μg/kg was found in untreated uninoculated controls, while no ZEA was detected in *F. verticillioides* inoculated controls, and a significantly higher amount was found in *F. graminearum* inoculated control (>500 μg/kg; Figure 4). Referring to resveratrol treatments, the only significant effect was found for *F. graminearum* inoculated samples where an 80% reduction in ZEA accumulation was found regardless of the use of either trans-resveratrol or RES VIN®.

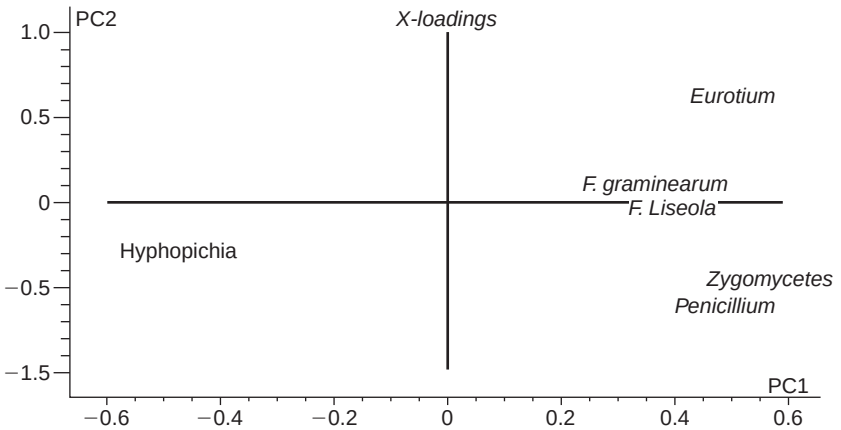

Figure 2. PCA showing relations among the main groups of fungi found.

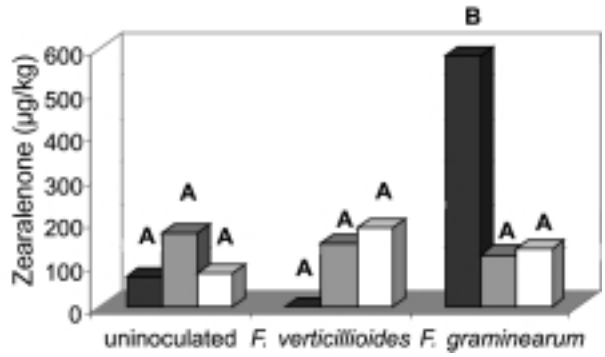

Figure 4. Zearalenone accumulation in corn at 20°C for 28 days as affected by the different inoculation and resveratrol treatments (■, control; □, resveratrol; □, RES VIN®). Bars with different letters show significantly different treatments ($p < 0.05$).

Both FB_1 and ZEA concentrations in samples were lower in those samples with high incidence of *Hyphopichia* and when the infection by *Penicillium* and *Zygomycetes* was low (Figure 5; explained variability: PC1 39%, PC2 18%).

DISCUSSION

Most of the investigations of the fungitoxic character of resveratrol have been carried out on its role against *B. cinerea*, which is the most destructive of the diseases of grapes (Adrian et al., 1997). *In vitro*, resveratrol behaved as an antimicrobial against filamentous fungi such as *Penicillium expansum* and *Aspergillus niger* (Filip et al., 2003). Resveratrol has also been shown to delay fungal spoilage of stored apples (Gonzalez Ureña et al., 2003).

The present study was carried out directly in naturally contaminated corn to overcome the drawbacks of previous experiments where the successful results obtained in *in vitro* media or sterilised corn could not be confirmed in naturally contaminated corn. In addition, the efficacy of resveratrol in preventing *Fusarium* species growth and their mycotoxins accumulation in sterile media or corn has been shown before (Fanelli et al., 2003; Ricelli et al., 2004). Moreover, they found a decreased effectiveness of resveratrol when using unsterilised seeds compared to irradiation-sterilized ones (Fanelli et al., 2003). The effect of accompanying mycoflora has been crucial for the interpretation of results, e.g. the high initial contamination by *Fusarium* Liseola section probably acted as a natural control factor which masked the artificial inoculation of *F. verticillioides* and probably interfered in the possible action of resveratrol. The additional inoculum (500 cfu/g) was probably too low compared to the overall initial microbial load, expected to be about 10^4 cfu/g. Thus, regarding fungal infection, no decrease was observed for *Fusarium* Liseola infection in resveratrol-treated corn. On the other hand, *F. graminearum*, although present, could not be quantified in terms of fungal infection due to the invasion of test plates by *Fusarium* Liseola section, thus, the effects of resveratrol treatment on *F. graminearum* infection could not be established.

The resveratrol concentration (200 mg/kg) was chosen according to previous results obtained in cereals (Fanelli et al., 2003; Ricelli et al., 2004), with no concluding results with concentrations of 12 and 23 mg/kg, but promising ones at 230 mg/kg.

No effect of resveratrol against FB_1 accumulation was observed. Moreover, no differences were found depending on either additional inoculum or competence by *F. graminearum*, suggesting that the massive initial inoculum (both *F. verticillioides* and *F. proliferatum*, the major FB_1 producers are included in the Liseola section) made all those treatments useless.

Interestingly, ZEA accumulation increased with additional inoculum, while decreased (almost significantly) by inoculating additional *F. verticillioides*,

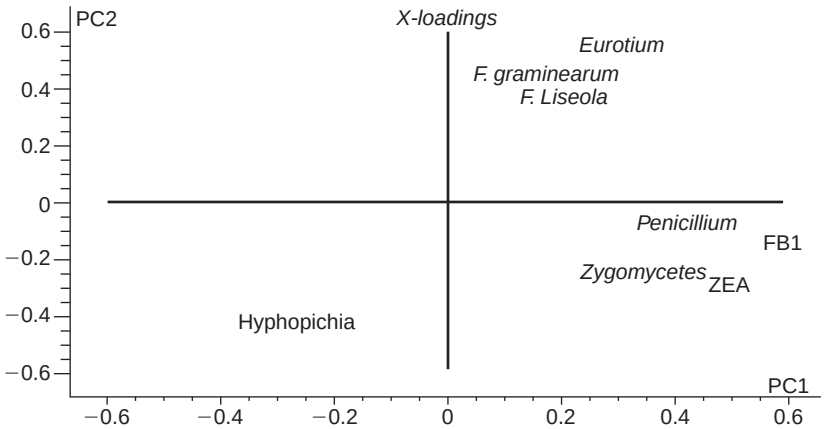

Figure 5. PCA showing relations among the main groups of fungi found and mycotoxins accumulation.

suggesting a low initial inoculum (the existence of an initial *F. graminearum* or other ZEA-producing species load, although not detected in the microbiological assay, was corroborated by the production of a mean ZEA level of 68 μg/kg). Resveratrol treatments did not control this production, although an 80% ZEA reduction was observed when compared to controls when an additional inoculum was used, due to either inhibition of *F. graminearum* growth, ZEA biosynthesis or both. Both total ergosterol concentration and ZEA significantly decreased in unsterilised corn with 230 mg/kg resveratrol compared to controls after 30 days at 25 °C, while no significant differences were found when using 12–23 mg/kg resveratrol concentrations (Fanelli et al., 2003). They concluded that the lowering in ergosterol content means that resveratrol might have an antimicrobial effect over the mycoflora present in corn, and not only on *F. graminearum*.

One of the main stages in the development of new natural pesticides is the study of the toxicological and environmental properties of the compound to be used; the lack of toxicity of resveratrol has already been demonstrated. In addition, in the case of resveratrol, a considerable number of investigations are currently focused on the health benefits of resveratrol consumption (German and Walzem, 2000).

CONCLUSIONS

The inhibiting effect of resveratrol on ZEA production by *F. graminearum* has been confirmed; no effect was found, however, on FB_1 accumulation. Finally, despite the low effectiveness of the resveratrol treatments, no differences were found in any case when using either synthetic trans-resveratrol or RES VIN®, suggesting that further studies on optimisation of resveratrol concentration of treatments could be done by using the sub product of the wineries, being a cheaper source of resveratrol than the synthetic one.

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