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THE NEEM [*AZADIRACHTA INDICA* A. JUSS (*MELIACEAE*)] OIL REDUCTION IN THE *IN VITRO* PRODUCTION OF ZEARALENONE BY *FUSARIUM GRAMINEARUM*

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ABSTRACT

Zearalenone, a mycotoxin produced by fungi of the genus *Fusarium*, including *F. graminearum*, triggers reproduction disorders in certain animals and hyperestrogen syndromes in humans. Current research investigates three concentrations of neem oil extract (0.1, 0.25 and 0.5%) in reducing the production of zearalenone. Neem oil extract decreased zearalenone amount in the three concentrations but highest inhibition (59.05%) occurred at 0.1%.

Key words: Zearalenone, *Fusarium graminearum*, neem oil extract.

Zearalenone (ZEA) is a non-steroid estrogen mycotoxin produced, by several fungi of the genus *Fusarium*, including *F. graminearum*, mainly in countries with a temperate and humid climate (18). The fungus causes reproduction disorders in animals (mainly in pigs) and hyperestrogen syndromes in humans. ZEA has also revealed to be hepatotoxic, hematotoxic, immunotoxic and genotoxic (22). *F. graminearum* Schwabe [sexual state: *Gibberella zeae* (Schwein.) Petch] infects cereals and causes several undesirable effects, including mycotoxins production in food with toxic potential in animals and human beings (6, 7, 8, 18). Synthetic chemical have been employed to control fungi in grains (4). However, the compounds revealed fungus resistance problems, besides the emergence of secondary pests (3). Plant natural extracts may be an alternative to substitute the synthetic chemical agents. Vegetal oils have been used as inhibitors of toxicogenic fungi and may be safer for consumption. In fact, an increasing demand for mycotoxin-free food and commodities may be perceived (2,19). Extracts from neem (*Azadirachta indica* A. Juss), employed in pest control in plant cultures, (15, 20) reveal bioactive substances in all parts of the tree, especially in its seeds (12). It has been shown that neem seeds oil has fungitoxic effects (10), whereas *in vitro* investigations revealed the inhibitory effects of neem extracts in the production of several mycotoxins (1, 5, 13, 14). Owing to the toxic effects and financial losses caused by ZEA and to the fungitoxic potential of neem extract, current research verifies, through the *in vitro* method, whether neem oil extract reduces ZEA production by *F. graminearum*. ZEA was produced by the isolate of *F. graminearum* UEL 2118, producer of ZEA, and other mycotoxins (21), stored in Potato Dextrose Agar (PDA) in the fungus collection at the Laboratory of Chemistry and Physiology of Microorganisms of the Biochemistry Department of the State University of Maringá, Maringá PR Brazil. The isolate was first grown in a

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potato dextrose broth, at 25ºC, during seven days, and then cultivated in a rice medium to produce ZEA. Neem oil extract (NO) came from the commercial product BioNeem® produced from neem seeds. The method for the production, extraction and analysis followed Richardson et al (16). Medium for ZEA production was prepared by adding 5 g of rice to 3 ml of deionized water in 100 ml Erlenmeyer flasks, or rather, the control medium for the production of ZEA. Media for tests were prepared likewise, albeit with the addition of NO (BioNeem®). Media with final extract concentrations 0.1, 0.25 and 0.5 % were obtained and were autoclaved at 121°C for 21 minutes in two consecutive days. After pre-culture of isolate 2118 in PDA broth at 25ºC for seven days, it was ground in a homogenizer during 30 seconds. Further, 500 µl of the culture were inoculated with a micropipette in rice media: control (without NO) and tests (with 0.1, 0.25 and 0.5 % NO). They were incubated for 28 days at 25ºC for the production of ZEA. For extraction, detection and quantification of ZEA, 10 ml of ethyl acetate were added to cultures after incubation. Cultures were fragmented with a spatula, stirred and, after a 24-hour decantation, transferred to another flask and filtered with filter paper. Above procedure was repeated twice and then the two extracts were mixed. The extracts obtained were partitioned twice with 10 ml hexane to remove interfering lipids. Final extract was concentrated in a rotary evaporator until completely dried. High Performance Liquid Chromatography (HPLC) to quantify ZEA was according Machado and Kemmelmeier (9). Extracts for HPLC were concentrated and diluted in methanol (chromatographic degree) and previously filtered through a 0.45 µm disposable syringe filter membrane (MFS-13 Micro Filtration Systems, California U.S.A) and analyzed (20 µl) with a Shimadzu® liquid Chromatograph (Tokyo, Japan) equipped with a LC-10AD pump, a Rheodyne® injector, a SPD-10A UV detector, a CBM-101 Communications Bus Module and a Class-CR10 workstation system. A reversed-phase Shimpack® CLC-ODS (M0 column (150 x 4.6 mm, 5 µm) was used at 30°C, with an equivalent pre-column (10 x 4.5 mm). The mobile phase was methanol : water (65:35 v/v) with 0.5 mM of sodium hydroxide with a flow of 0.5 ml/minute. Absorption was measured at 275 nm. Data collection and integration were performed with Class-CR10 software (Shimadzu®, Tokyo, Japan). ZEA standard (Sigma®) (100 µM) was used and amount of ZEA was calculated and expressed in µg of ZEA per gram of rice. All experiments were repeated four times and treatment results were statistically evaluated by two-way variance analysis (ANOVA), with 1% probability ($p<0.01$). When significant differences existed, means were analyzed by Tukey's test ($p\leq0.05$), with statistical program SAS (SAS. Statistical Analysis System. Sas Institute Inc., Cary, NC, USA, 2001).

Results show that the production of ZEA in neem-less medium (control) was higher than that in extracts with NO (tests) (Figure 1 and Table 1).

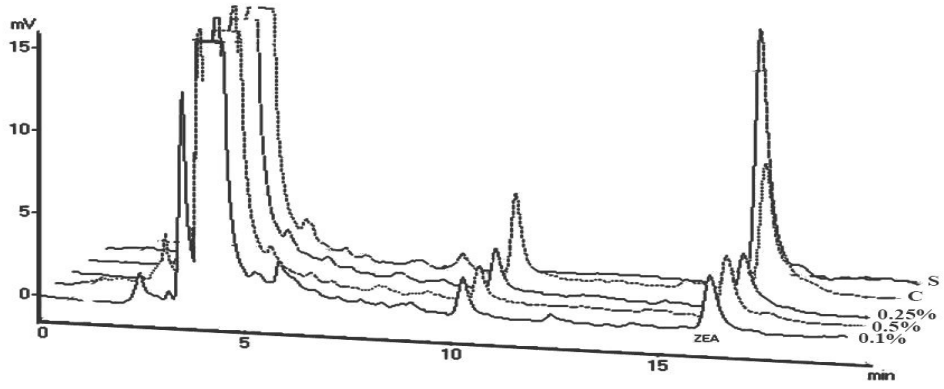

Figure 1. Profile of ZEA elution (HPLC) produced by *F. graminearum*: (S) ZEA standard; (C) control of ZEA production by *F. graminearum* grown in a medium without NO; 0.1, 0.25 and 0.5 % production of ZEA by *F. graminearum* in media respectively with 0.1, 0.25 and 0.5 % of NO.

Table 1. Effect of neem oil extract (%) in the production of ZEA (µg/g) by *F. graminearum* (isolate UEL 2118).

Treatments*	Zearalenone (µg/g rice)**	Decrease (%)
Control (0% neem)	0.240 [a] (± 0.015)	-
0.1 % NO	0.098 [c] (± 0.007)	59.05 %
0.25 % NO	0.152 [b] (± 0.080)	36.60 %
0.5 % NO	0.156 [b] (± 0.026)	34.74 %

Details of assay were described above. Means followed by the same small letter in the column do not differ between themselves according to Tukey's test (p>0.05). * Control media without NO extract; media with NO extract **Values obtained by HPLC analysis, in µg of ZEA per g of rice. Rates between brackets: standard deviation.

Highest inhibition occurred at NO concentration 0.1%, in which ZEA production decreased by 59.05 % when compared to control. When NO concentration was increased from 0.25 to 0.5 %, inhibition of ZEA production was reduced by 36.60 and 34.74 % respectively, when compared to control (Table 1). Most synthetic fungicides, which usually control fungi, produce several side effects, such as carcinogenicity, teratogenicity and residual toxicity (11). Studies with neem extracts have shown several inhibitory effects in toxigenic fungi, such as the blockage of the biosynthesis pathway of aflatoxin in *Aspergillus parasiticus* (23); decrease of citrinin in *Penicillium citrinum* (14); of penicillic acid in *P. cycplopium* (5); of patulin in *P. expansum in vitro* (13) and in contaminated apples (1). Current assay showed that concentrations 0.25 and 0.5 % of NO inhibited less ZEA production than concentration 0.1 %. Since this fact is probably due to decrease of solubility of oil extract in water, lower concentrations may have had a higher contact with and absorption of the neem oil extract by the fungus. Similar results have been obtained in assays with patulin, in which NO in less than 0.5% concentrations highly decreased the production of patulin by *P. expansum*. This event shows that liposolubility is necessary for its activity (1). Current analysis showed that NO, popularly used worldwide for a great number of uses and ailments, may be an alternative medium for the partial control of ZEA production by *F. graminearum*. Nevertheless, further studies should be undertaken to verify ZEA inhibition in *in vivo* grains.

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