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Original Article

INFLUENCE OF CARBON AND NITROGEN SOURCE ON GROWTH, DON AND NIV PRODUCTION BY TWO SPECIES OF *FUSARIUM* ISOLATED FROM FINGER MILLETS

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ABSTRACT

Objective: Influence of different carbon [C] and nitrogen [N] source on the growth and Deoxynivalenol [DON] and Nivalenol [NIV] production by *Fusarium aethiopicum* and *Fusarium culmorum* was investigated.

Methods: Seven days old monosporic cultures of *F. aethiopicum* strain GSKUMB [KJ21085] and *F. culmorum* strain GSKUMB [KJ190159] were grown in CYA broth and incubated at 27±2°C on the rotary shaker at 120 rpm for 21 days. At the end of incubation period, cultures were harvested for determination of fungal growth (biomass). The resultant culture filtrates were extracted twice with ethyl acetate and concentrated. One ml of final concentrate in methanol was employed for detection of DON and NIV with the help of RP-HPLC.

Results: The highest amount of DON and NIV were produced by *F. aethiopicum* in the presence of D-mannose and D-galactose as C source, while the highest amount of biomass was recorded on maltose and succinic acid. *F. culmorum* produced maximum amount of toxins in the presence of D-glucose, D-mannitol and D-fructose. Sodium nitrate was most favorable nitrogen source as it induced maximum amount of toxins by *F. aethiopicum*, while L-methionine, L-asparatic acid and L-tryptophan were next preferred N source. In contrast, highest biomass of fungus was obtained with L-lysine, L-glutamine and L-tyrosine. *F. culmorum* produced maximum amount of toxin and biomass with potassium nitrate and L-tyrosine respectively.

Conclusion: Present species of *Fusarium* differed varied both in toxins (DON, and NIV) and biomass production. Their response of fungi under investigation towards C and N sources is also varied.

Keywords: *F. aethiopicum, F. culmorum*, PCR, Carbon Sources, Nitrogen Sources; DON, NIV, HPLC.

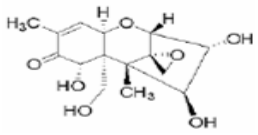
INTRODUCTION

Mycotoxins, secondary metabolites, produced by fungi on agricultural commodities both in the field and storage vary with environmental condition [1]. These mycotoxins are stable at high temperatures in processed food [2]. The contamination of food and feeds with mycotoxins are of great concern as these are responsible for many acute and chronic diseases [3]. Among these aflatoxin, ochratoxin, citrinin, patulin, trichothesene, fumonisins and zearalenone are the most common contaminants of food and feeds [4]. Species of *Fusarium* are known to be plant pathogens but also contaminate foods and feeds, and elaborate variety of mycotoxins which are health hazardous [5].

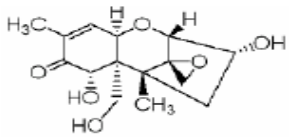
The risk due to mycotoxins in food and feed is increasing and posing a significant threat of human and animal health. Mold growth and mycotoxin contamination are likely to reduce the nutritive quality of food and feeds more than one mycotoxin in food and feed is likely to increase toxicity due to synergetic action [6]. The production of mycotoxins is likely to be influenced by intrinsic and extrinsic factors [7]. It is also clear that the mycotoxin profile of a fungal species likely to depend up on the nutritional composition of substratum [6].

Fusarium species are the major concern due to their wide spread infestation of food grains [8, 9] and elaboration of variety of trichothecene mycotoxins [10]. The trichothecene producing *Fusarium culmorum, F. graminearum, F. poae* and *F. sporotrichioides* are most common contaminants of food grains [11]. *F. graminearum* and *F. culmorum* are the main producers of potent DON and NIV (Chemical structure) which are known to be carcinogenic, genotoxic, immuno-suppressive, teratogenic and inhibit protein synthesis. *F. aethiopicum* is indistinguishable morphologically from *F. graminearum* [12].

However, very limited information is available on production of DON and NIV by *F. aethiopicum* and *F. culmorum*. Hence, the present investigations were aimed to study influence of carbon and nitrogen source on growth, DON and NIV production by two species of *Fusarium* associated with Finger millets [*Eleusine coracana* L.] was assessed with the help of liquid chromatography and the results are discussed in this communication.



Nivalenol (NIV)



Deoxynivalenol (DON)

MATERIALS AND METHODS

Isolation and Identification of *Fusarium* species

Fusarium species associated with the finger millets were isolated and identified with the help of standard keys and manuals [13, 14]. The morphologically identified *F. aethiopicum* and *F. culmorum* were further confirmed by precise molecular methods by polymerase chain reaction [PCR] and the obtained nucleotide sequences were submitted to National Center for Biotechnology Information [NCBI] with GenBank Accession number *F. aethiopicum* strain GSKUMB [KJ21085] and *F. culmorum* strain GSKUMB [KJ190159].

Influence of carbon source on growth, DON and NIV production

The influence of different C sources on growth and DON and NIV production by *F. aethiopicum* and *F. culmorum* was studied by substituting sucrose of the basal medium with different C sources [D-glucose, D-fructose, D-mannose, D-galactose, starch, D-xylose, D-sorbose, D-mannitol, sucrose, D-lactose, D-maltose, citric acid, succinic acid, tartaric acid, D-raffinose, tannic acid, melibiose and dextrin] so as to supply same amount of carbon in triplicate (*n=3*). Sucrose served as control. Ehrlenmeyer flask (250-ml) containing 100 ml broth at pH 6.5 was inoculated with 1 ml of spore suspension [10^{-5}] of *F. aethiopicum* and *F. culmorum* and incubated at 27±2°C in a rotary shaker [Yiedher LM-450D] at 120rpm for 21 days. At the ends of an incubation period, final pH of the broth, biomass and DON and NIV production was quantified by HPLC.

Influence of nitrogen source on growth, DON and NIV production

The influence of different N source on growth and, DON and NIV production by *F. aethiopicum* and *F. culmorum* was studied by adding different N source in place of sodium nitrate [ammonium chloride, ammonium molybdate, ammonium nitrate, ammonium sulphate, aluminium nitrate, barium nitrate, potassium nitrate and thiourea urea, L-glutamic acid, L-glutamine, L-glycine, L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-cysteine, L-histidine, L-leucine, L-lysine, L-tyrosine, L-tryptophan, L-methionine, p-amino benzoic acid, p-nitroaniline and p-nitrobenzoic acid and urea] so as to supply same amount of nitrogen in triplicate (*n=3*).

The final pH of the culture broth was also determined with the help of Elico pH meter. Sodium nitrate served as control. The flasks thus prepared were inoculated with 1 ml of seven-day old culture spore suspension [10^{-5}] of *F. aethiopicum* and *F. culmorum* and incubated as described above.

Determination of biomass of *Fusarium* species

At the end of 21 d incubation period, cultures of *Fusarium* species were harvested on pre-weighed Whatmann No.42 filter paper. The filter paper along with mycelial mat was dried in a hot air oven at 65-75°C for 72 hrs to obtain a constant weight in an analytical balance after cooling to room temperature in a desiccator in triplicate (*n=3*). The biomass yield per ml of medium was calculated.

Extraction and cleanup of DON and NIV

At the end of incubation period, cultures were harvested on Whatman filter paper No.42. The culture filtrate was centrifuged at 12,000g to get cell-free filtrates. The resultant culture filtrates were acidified with 0.1M *o*-phosphoric acid and extracted twice with ethyl acetate [1:1, v/v] and concentrated by rotary evaporator to get a final concentration of 1 ml in methanol and subjected to liquid chromatographic analysis.

Analysis and Quantification of DON and NIV by HPLC

Liquid chromatography [LC] analysis of OTA was carried out by using *JASCO-975*[Japan], C-18 isocratic reverse phase column [250X4.6 mm internal diameter, 5µM particle size] by injecting 20 µl of sample extract. The mobile phase for toxin consists of a mixture of methanol: water [70:30] [Sigma Aldrich, Mumbai, India]. The pH was adjusted, degassed using bath sonicator [PCI™ Analytics].

The chromatography was performed isocritically at a flow rate of 0.5 ml/min at 227 nm under UV detector. The amount of DON and NIV produced was determined by HPLC fluorometric response compared with standard DON and NIV Sigma Aldrich [Mumbai, India].

RESULTS AND DISCUSSION

Influence of C source on growth, DON and NIV production by *F. aethiopicum*

The present investigations revealed that a definite influence on growth, DON and NIV production by both the species of *Fusarium* under study. *F. aethiopicum* produced maximum amount of DON and NIV on D- mannose followed by D-galactose, D-xylose, D-maltose and L-sorbose in a descending order, while it was least in the presence of succinic acid, citric acid and starch (Table 1). Rest of the C sources supported intermediate amount of toxins production.

The highest mycelial yield was recorded in the presence of D-maltose, succinic acid D-lactose and D-fructose, while, D-mannose, D-glucose and starch were responsible for the least growth. Rest of the C sources tried induced intermediate amount of biomass. In most of the cases final pH was alkaline. However, it was near neutral in the presence of D-mannose, D-galactose and D-xylose. Final pH recorded was acidic in media containing rest of the carbon sources tried. Tartaric acid, D-raffinose, tannic acid, melibiose and dextrin failed to support growth, DON and NIV production by both the species of *Fusarium* under study.

Influence of C source on growth, DON and NIV production by *F. culmorum*

D-glucose, D- mannose, D-mannitol and D-fructose were responsible for the highest amount of DON and NIV production by *F. culmorum*, while succinic acid, citric acid and starch were poor substrates for production of both the mycotoxins under study. Rest of the C sources tried supported intermediate amount of toxin production. L-sorbose, D-mannitol, D-lactose and D-xylose supported maximum biomass, while D-mannose, D-galactose and succinic acid was responsible for least growth of the *F. culmorum*. Rest of the C sources were responsible for intermediate amount of biomass production. Final pH of the medium was alkaline in media containing D-glucose, D-mannose and D-xylose, while in D-fructose, starch and L-sorbose media it was neutral.

Influence of N source on growth, DON and NIV production *F. aethiopicum*

The highest amount of DON and NIV production was recorded in presence of sodium nitrate, L-methionine, potassium nitrate, L-asparatic acid, L-tryptophan, urea and ammonium sulphate (Table 2) while, ammonium molybdate, ammonium chloride, L-arginine, L-lysine and L-histidine were responsible for least amount of DON and NIV production. Rest of the N source supported intermediate amount toxins production. Biomass accomplished by *F. aethiopicum* was maximum in medium containing L-lysine, L-glutamine, L-tyrosine, and L-tryptophan, while it was least in medium containing L-glutamic acid, potassium nitrate, L-histidine and L-aspartic acid.

Influence of N source on growth, DON and NIV production *F. culmorum*

F. culmorum produced maximum DON and NIV when potassium nitrate, ammonium sulphate, L-aspartic acid, L-tryptophan and L-methionine were served as N source, while ammonium molybdate, ammonium chloride, L-alanine, L-arginine and L-histidine were supported poor sources for production of DON and NIV. Rest of the N sources were responsible for intermediate amount DON and NIV production. Maximum biomass production was recorded in media containing L-tyrosine, potassium nitrate, L-glycine and L-arginine. Aluminium nitrate, barium nitrate, p-amino benzoic acid, p-nitro benzoic acid, p-nitro aniline and thiourea failed to support the growth of *F. culmorum.*

Least amount of biomass was recorded in medium containing L-histidine, L-lysine, L-leucine, ammonium chloride and ammonium sulphate. Rest of the N sources supported the intermediate amount of biomass. The final pH recorded in medium containing L-alanine, ammonium sulphate, L-glutamine, L-glycine, L-asparagine, L-histidine, L-lysine, L-leucine and L-tryptophan was alkaline. On the other hand, pH was neutral in medium containing ammonium chloride, ammonium molybdate, ammonium nitrate and sodium nitrate.

Present investigations are aimed to find the nutritional composition of the medium for growth, DON and NIV production by *F. aethiopicum* and *F. culmorum* was studied. DON and NIV production was recorded maximum in media containing D- mannose, D-galactose, xylose, while it was least in media containing succinic acid, citric acid and starch in agreement with Koteswara Rao et al.

[15] who also reported critical role of C and N sources on OTA production by *P. verrucosum* and *P. nordicum*. The present investigations are also in agreement with [16] who also recorded critical influence of carbon and nitrogen source on alternariol [AOH], alternariol mono methyl ether [AME] and tenuazonic acid [TA] by *Alternaria alternata*. A positive correlation was observed between growth and DON and NIV production by both the species of *Fusarium* under investigations.

Table 1: Influence of carbon source on growth, DON and NIV production by two species of *Fusarium* isolated from finger millets

	F. aethiopicum				***F. culmorum***			
Carbon source	Final pH	Dry. wt [mg/ml]	DON [µg/ml]	NIV [µg/ml]	Final pH	Dry. wt [mg/ml]	DON [µg/ml]	NIV [µg/ml]
D-Glucose	8.13±0.25	2.40±0.15	25.20±0.89	22.79±0.75	8.36±0.25	3.26±0.47	45.00±1.71	31.29±1.07
D-Fructose	8.3±0.17	4.30±0.15	32.90±0.64	23.87±1.13	7.73±0.31	4.30±0.62	38.60±1.17	25.52±1.22
D-Mannose	7.53±0.35	2.10±0.26	48.50±0.87	31.03±0.69	8.29±0.31	2.40±0.20	43.23±1.75	31.84±0.58
Galactose	7.56±0.35	3.10±0.35	31.80±0.52	28.93±0.27	6.73±0.20	2.53±0.40	33.66±1.12	27.24±1.47
Starch	6.93±0.45	2.50±0.40	18.20±0.55	12.32±0.79	7.46±0.23	4.33±0.23	15.42±1.08	11.76±1.47
D-Xylose	7.5±0.60	3.20±0.26	33.20±0.58	25.91±0.29	8.10±0.55	4.56±0.35	30.82±0.49	28.46±0.943
L-Sorbose	8.16±0.40	3.60±0.10	41.90±0.86	23.77±0.38	7.46±0.15	5.30±0.20	28.16±0.55	25.48±0.53
Mannitol	6.93±0.83	4.20±0.10	35.80±0.31	23.71±1.17	6.30±0.17	5.00±0.56	39.20±0.65	22.92±0.43
Lactose	6.50±0.30	4.40±0.17	35.80±0.38	22.65±0.53	6.33±0.37	4.73±0.21	30.39±0.84	27.69±0.65
Maltose	7.36±0.37	5.30±0.36	33.90±0.91	24.58±0.86	7.24±0.66	3.8±0.26	34.22±0.88	28.76±0.39
Citric acid	3.43±0.25	2.90±0.40	10.80±0.33	9.726±0.53	3.63±0.15	4.03±0.21	13.72±0.41	11.51±0.69
Succinic acid	2.86±0.30	4.50±0.23	9.11±0.37	8.41±0.36	3.36±0.37	2.9±0.36	9.63±0.58	8.19±0.62
Tartaric acid	6.10±0.45	0.00±00	0.00±00	0.00±00	6.20±0.3	0.00±00	0.00±00	0.00±00
D-Raffinose	6.76±0.30	0.00±00	0.00±00	0.00±00	7.10±0.55	0.00±00	0.00±00	0.00±00
Tannic acid	6.89±0.20	0.00±00	0.00±00	0.00±00	7.10±0.55	0.00±00	0.00±00	0.00±00
Melibiose	6.30±0.26	0.00±00	0.00±00	0.00±00	5.43±0.56	0.00±00	0.00±00	0.00±00
Dextrin	5.36±0.20	0.00±00	0.00±00	0.00±00	5.76±0.80	0.00±00	0.00±00	0.00±00
Sucrose [Control]	7.96±0.40	3.90±0.43	33.50±0.87	30.03±0.62	7.59±0.49	3.23±0.35	33.89±0.80	31.20±1.47

Table 2: Influence of nitrogen source on growth, DON and NIV production by two species of *Fusarium* isolated from finger millet

	F. aethiopicum				***F. culmorum***			
Nitrogen source	Final pH	Dry. wt [mg/ml]	DON [µg/ml]	NIV [µg/ml]	Final pH	Dry. wt [mg/ml]	DON [µg/ml]	NIV [µg/ml]
Ammonium chloride	7.73±0.32	5.26±0.288	13.5±1.19	11.54±.65	7.63±0.45	3.8±0.4	15.50±0.73	13.64±1.29
Ammonium molybdate	7.03±0.49	7.4±0.1	7.98±0.90	5.61±0.67	6.76±0.30	4.53±0.351	7.81±0.73	6.35±1.05
Ammonium nitrate	7.83±0.25	4.43±0.20	15.36±0.87	14.07±0.77	7.4±0.43	5.06±0.25	17.47±0.91	15.6±0.34
Ammonium sulphate	8.33±0.25	4.4±0.43	31.46±1.26	28.17±0.43	8.1±0.26	3.96±0.73	39.29±0.89	34.63±1.23
Aluminium nitrate	5.2±0.4	0.00±0.00	0.00±0.00	0.00±0.00	5.3±0.45	0.00±0.00	0.00±0.00	0.00±0.00
Barium nitrate	5.33±0.15	0.00±0.00	0.00±0.00	0.00±0.00	5.56±0.32	0.00±0.00	0.00±0.00	0.00±0.00
L-glutamic acid	6.3±0.45	2.5±0.3	25.43±0.92	18.75±0.40	8.03±0.60	4.76±0.45	23.54±1.11	21.40±0.68
L-glutamine	8.23±0.40	6.56±0.50	28.89±0.62	20.75±0.38	8.01±0.67	5.66±0.60	27.98±0.75	24.04±0.50
L-glycine	8.06±0.51	5.3±0.2	17.34±0.81	15.37±0.86	8.04±0.89	6.1±0.4	46.23±1.89	33.99±0.94
L-alanine	8.40±0.36	3.83±0.32	15.56±0.47	13.67±1.17	8.12±0.57	5.66±0.45	14.24±0.97	10.99±0.41
L-arginine	7.16±0.35	5.1±0.26	13.66±0.56	10.93±0.59	7.44±0.29	6.1±0.26	25.36±0.83	13.89±0.98
L-asparagine	8.03±0.60	4.81±0.70	18.44±0.20	14.12±0.75	7.86±0.66	4.93±0.41	18.45±1.25	14.93±0.34
L-aspartic acid	6.83±0.30	3.66±0.60	32.1±0.74	28.79±0.44	6.8±0.3	4.8±0.26	42.21±0.19	34.27±0.66
L-cystine	6.76±0.45	4.66±0.45	28.8±0.52	20.703±0.51	7.06±0.25	5.03±0.37	25.37±0.89	21.69±1.19
L-histidine	8.26±0.15	3.06±0.32	14.09±0.81	12.12±0.62	8.4±0.36	2.06±0.37	15.68±0.62	13.03±0.49
L-lysine	8.23±0.30	7.06±0.51	14.04±0.68	10.72±0.58	7.33±0.45	3.36±0.15	17.58±0.70	15.57±0.55
L-leucine	8±0.26	4.16±0.45	15.7±0.48	12.89±1.51	7.00±0.70	3.03±0.41	17.49±0.91	15.19±0.88
L-tryptophan	8.36±0.46	5.46±0.28	31.8±1.17	28.9±1.18	8.23±0.05	5.13±0.55	42.15±0.83	32.3±0.93
L-tyrosine	6.96±0.20	6.36±0.15	21.14±0.71	18.68±0.53	7.7±0.2	6.86±0.20	23.89±0.61	22.18±0.97
L-methionine	6.66±0.51	4.03±0.30	35.9±0.91	31.62±1.21	7.03±0.60	3.16±0.30	38.57±1.27	32.37±1.71
p-amino benzoic acid	4.2±0.5	0.00±0.00	0.00±0.00	0.00±0.00	4.13±0.47	0.00±0.00	0.00±0.00	0.00±0.00
p-nitrobenzoic acid	3.53±0.30	0.00±0.00	0.00±0.00	0.00±0.00	3.3±0.45	0.00±0.00	0.00±0.00	0.00±0.00
p-nitroaniline	5.5±0.26	0.00±0.00	0.00±0.00	0.00±0.00	5.16±0.30	0.00±0.00	0.00±0.00	0.00±0.00
Potassium nitrate	8.26±0.28	2.86±0.35	34±0.78	40.61±1.15	8.06±0.55	6.26±0.47	35.26±1.12	42.58±0.44
Thiourea	6.4±0.26	0.00±0.00	0.00±0.00	0.00±0.00	7.53±0.30	0.00±0.00	0.00±0.00	0.00±0.00
Urea	6.93±0.90	4.4±0.75	32.82±0.87	29.11±0.48	6.86±0.90	5.03±0.30	38.52±1.34	31.98±0.88
Sodium nitrate [Control]	7.3±0.52	4.76±0.56	38.32±1.08	38.54±0.95	7.06±0.37	5.33±0.55	44.6±1.16	40.67±0.44

The present observation is in agreement with Narasimha Rao et al. [17] who also opined that nutritional composition of the media play a major role in production fumonisins. DON and NIV production by species of *Fusarium* differed with the nutritional composition of the medium and environmental condition [18]. DON is vomitoxin and provokes acute and chronic disease in humans and animals [19]. It is also reported to induce inhibition of protein, DNA and RNA synthesis and mitochondrial function and affects cell division [20].

Environmental factors such as humidity and water availability, temperature and nutritional availability are reported to affect vegetative growth and toxin production by species of *Fusarium* [21]. The results of the present study are positively correlated with the reports of Ferreira and Pitout [22] who also recorded highest OTA production in the presence of sucrose and considerably low on D-glucose present in the medium. Medina et al. [23] recorded significant influence of nitrogen and carbon sources on OTA

production by *Aspergillus* species. *A. ochraceus, A. carbonarius* and *A. tubingensis* are reported to produce maximum OTA in the presence of sucrose, D-glucose as C source and arabinose and phenylalanine as N source. No positive correlation was observed between DON and NIV productions by the species of *Fusarium* under investigation. Shilpa et al. (24) also reported the variability in DON and NIV production with the cultural and nutritional condition.

Present investigations *F. aethiopicum* and *F. culmorum* were grown different C and N sources at pH 6.5, after an incubation period, the final pH varied due to production of mycotoxin in the medium are positively correlated with Wheeler *et al.* [25] who also reported that of pH was ideal for mold growth and mycotoxin production.

CONCLUSION

Carbon and nitrogen sources present in the medium significantly influenced the growth and DON and NIV production by *F. aethiopicum* and *F. culmorum*. Thus, regulating these nutrients it is possible to minimize the growth of both the species of *Fusarium* and toxin production under investigations and protect the foods and feeds during their production and distribution.

ABBREVIATION

C: Carbon, N: Nitrogen, DON: Deoxynivalenol, NIV: Nivalenol , CYA: Czepak Yeast Autolysate Agar, RP-HPLC: Reverse Phase High Performance Liquid Chromatography, HPLC: High Performance Liquid Chromatography, National Center for Biotechnology Information [NCBI], OTA: Ochratoxin A

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CONFLICT OF INTERESTS

Declared None.

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