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Penicillic Acid Production in Some Fungi Isolated from Barley Grown in Saudi Arabia

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Abstract. A mycological study was carried out to isolate the mycoflora of barley (*Hordeum vulgare* L.). Seventeen species of the genera *Aspergillus, Eurotium,* and *Penicillium* were isolated and tested for penicillic acid production on three different substrates: autoclaved barley grains (barley medium), yeast-extract sucrose broth and potato dextrose agar. Four of the seventeen fungal species recorded have produced penicillic acid in quantities that varied from 31 to 62.3 $\mu g\ kg^{-1}$ of barley in the various media used. The biological tests used have verified the toxicity of the barley medium extracts.

Introduction

The antibiotic penicillic acid (PA) is a mycotoxin produced by a variety of *Aspergillus* and *Penicillium* species and it has been implicated in various animal toxicosis [1]. Mycotoxins are one of the major causes of high mortality in broilers and reduction in egg production in layer hens in Saudi Arabia [2, 3]. Moreover, cereals including barley, are the main targets of penicillic acid producing fungi [4]. Barley, is the major raw material used in poultry feeds in Saudi Arabia [5, 6]. Apart from a single report by Abramson *et al.* [7], no data is available on the incidence of penicillic acid in barley. No study was carried out in this aspect on the K.S.A. earlier.

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Hence the present study has been undertaken to identify and evaluate the potentially of penicillic acid production by mold species isolated from moldy locally grown barley and its toxicity by chemical and biological means.

Materials and Methods

Sample collection

Barley (*Hordeum vulgare* L.) samples were randomly collected from 10 different poultry layer farms in Riyadh region. The samples were transferred to the laboratory in individual plastic bags and on arrival they either assayed for fungi or were stored at 2°C for fungal assessment at a later date.

Mycological examination

Six hundred intact seeds of uniform size from each sample were plated on potato dextrose agar (PDA) after surface disinfection with 1% sodium hypochlorite solution for 10 minutes [8]. The identification of the fungal isolates was carried out using the method of Raper and Fennell [9], Raper and Thom [10] and Barnett and Hunter [11]. The pure isolates of *Aspergillus* spp. *Eurotium* spp. and *Penicillium* spp. were inoculated on PDA plants and incubated for 10 to 14 days at 25°C.

Penicillic acid production

The isolated fungal species were tested for their ability to produce penicillic acid on chemically defined media [potato dextrose agar (PDA), yeast extract sucrose broth, (YES)] and on autoclaved barley grains as a neutral medium according to the method of Bullerman [12] and Tsai *et al.* [13]. The PDA and YES media were inoculated by a heavy loopful of spores of each culture grown on PDA slants at 25°C for 10 and 14 days, respectively. Barley grains were inoculated with 1 ml of a spore suspension of 10^6 – 10^7 spores/ml of each culture that was prepared using a sterile 0.05% Tween 80. The moisture was adjusted to 23% and the inoculated media were incubated for one week at 25°C. The barley cultures were shaken manually once a day to prevent clumping.

Penicillic acid detection

Following incubation, both PDA and YES broth cultures were autoclaved at 12°C for 30 seconds to kill the spores and barley grains (barley medium) were dried in the oven at 104°C for 24 hours. The PDA culture was extracted by the method of Bullerman [12] and the YES broth was analyzed according to the method of Tsai *et al.* [13] by direct spotting on TLC plates. Barley was extracted by the method described by Al-Julaifi [6] for the extraction of mycotoxins.

The extracted penicillic acid from the three different media was chemically identified by thin layer chromatography (TLC) plates on the basis of co-migration with reference standard and by their characteristic fluorescent color under UV light [14, 15, 16], and biologically by the chick embryo test. The barley medium extracts were

dissolved in 95% ethanol, and 10µl of each extract was injected into the air cell of white leghorn eggs according to the method of Verret *et al.* [17]. The concentration of penicillic acid injected ranged from 0.0003 µg to 0.0006 µg per egg depending on the extract used, and 0.0008 µg to 0.002 µg per 25 µl disc, and 0.0016 µg to 0.004 µg per 50 µl disc. The barley medium extracts were also microbiologically tested for toxicity by the method of Burmeister and Hesseltine [18] and Olivigni and Bullerman [19], using a paper disc agar technique.

Results and Discussion

Mold isolates and penicillic acid production

The most frequent fungal species isolated was *Aspergillus flavus* (80%) followed by *Aspergillus* sp. (50%) (Table 1). *Aspergillus flavus, A. fumigatus, Eurotium herbariorum* and *Eurotium* sp. produced penicillic acid on all media used. However, similar to the observation of Scott *et al.* [15] penicillic acid was detected in YES and PDA media from the same mold species with the same frequency.

On the other hand 23.5% of the tested mold have produced penicillic acid on barley medium, in quantities ranging from 31.0 – 62.3 µg/kg. *Eurotium* sp. have produced the highest amounts followed by *Aspergillus fumigatus*, and the lowest amounts were produced by *Aspergillus flavus* (Table 1).

Table 1. The prevalence of penicillic acid produced by various fungal species grown on PDA, YES broth and Barley medium for one week at 25°C using TLC plates

Isolated and tested	Detected penicillic acid in			Frequency (%)
	PDA	YES broth	Barley medium extracts (µg/kg)	
Aspergillus flavus	D	D	31.0	80
A. fumigatus	D	D	43.2	20
A. glaucus	ND	ND	ND	10
A. nidulans	ND	ND	ND	10
A. niger	ND	ND	ND	20
A. ochraceus	D	D	ND	10
A. parasiticus	ND	ND	ND	20
Aspergillus sp.	ND	ND	ND	50
A. terreus	ND	ND	ND	10
Eurotium amstelodami	ND	ND	ND	20
E. herbariorum	D	D	35.6	10
Eurotium sp.	D	D	62.3	10
Penicilium aurantigriseum	ND	ND	ND	10
P. chrysogenum	ND	ND	ND	10
P. citrinum	ND	ND	ND	10
P. corylophilum	ND	ND	ND	10
Penicillium sp.	ND	ND	ND	20

D = detectable (the amount detected was less than 20µg/kg); ND = not detectable; PA = penicillic acid; YES = Yeast extract sucrose; PDA= potato dextrose agar.

Biological test

The mortality of chick embryos and the inhibition zones of *Bacillus subtilis* caused by extracts of the tested species are shown in Table 2. For most extracts the mortality rate was 10%. The high toxicity of these extracts to chick embryos and the high inhibition zones are well correlated and similar to observations of other workers who used standard solutions of penicillic acid. Vesely *et al.* [20] found that lethal effects of penicillic acid on chick embryos occurred at 10.0 µg/egg and Olivigni and Bullerman [19] found that *B. subtilis* was inhibited by 1 µg/ml of pure penicillic acid. However, according to Mclaughin *et al.* [21] other factors including solubility of the unknown coagulating effect, pH, specific gravity and presence of unknown toxic substances must also be considered.

Table 2. Biological and microbiological toxicity confirmation tests of penicillic acid produced by various fungal species grown on barley medium

Tested fungal species	Mortality of chick embryo (%)	Inhibition zone (mm) of *Bacillus subtilis*	
		25µl	50µl
Aspergillus flavus	10	–	5.2
A. fumigatus	15	–	5.3
A. glaucus	–	–	–
A. nidulans	–	–	–
A. niger	–	–	–
A. ochraceus	–	–	–
A parasiticus	–	–	–
Aspergillus sp.	10	–	5.5
A. terreus	–	–	–
Eurotium amstelodami	–	–	–
E. herbariorum	10	5.2	5.3
Eurotium sp.	20	6.7	7.5
Fusarium sp.	–	–	–
Penicillium aurantigriseium	–	–	–
P. chrysogenum		–	–
P. citrinum	–	–	–
P. corylophilum	–	–	–
Penicillium sp.	–	–	–

However, the positive results obtained in absence of penicillic acid could be due to the factors mentioned by McLaughin *et al.* [21] or possibly to the presence of other mycotoxins.

The present study has revealed the existence of penicillic acid producing fungi on locally grown barley grains. The ability of these fungi to produce these toxins on barley medium has been verified; the incidence of the toxin produced is variable amongst the various species of fungi.

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حامض البنسلين الناتج من الفطريات المعزولة من الشعير المحلي بالمملكة العربية السعودية

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ملخص البحث. أجريت هذه الدراسة لعزل الفلورا الفطرية من الشعير المنتج محليًا بالمملكة العربية السعودية. تمّ عزل سبعة عشر نوعًا من الأجناس إسبيرجلليس، يوروشيم وبنسليام واختبارها لإنتاج حمض البنسلين في ثلاثة مستخلصات مختلفة هي بيئة الشعير، بيئة مرق سكروز مستخلص الخميرة وبيئة أجار دكستروز البطاطس. لوحظ أن أربعة عزلات فطرية قد أنتجت حامض البنسيلين بكميات تراوحت ما بين ٣١,٠٢ إلى ٦٢,٣ ميكروجرام لكل جرام شعير في البيئات المختلفة التي تم استخدامها. أثبتت الاختبارات البيولوجية التي أجريت سُمّية بيئة مستخلصات الشعير.