

XEROPHILIC MYCOPOPULATIONS ISOLATED FROM RAPESEEDS (*Brassica napus*)

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*This paper presents the results of an investigation related to mycological populations of rapeseed samples produced in the Institute of Field and Vegetable Crops in Novi Sad (location: Rimski Šančevi, Novi Sad), with a special emphasis on the potentially toxigenic mycopopulations. Mycological investigations were performed on the samples that were treated with 4% solution of Na-hypochlorite, and on the ones that were not submitted to this treatment. Isolation and determination of total mould count was carried out using Dichloran Glycerol Agar (DG18). The identification of isolated moulds was done according to modern keys for fungal determination. From 20 untreated tested samples, 17 were contaminated with moulds (10.0 to 4.7x10² cfu/g). When the samples were treated with 4% solution of Na-hypochlorite, moulds were isolated only from 4 samples, and the total mould count ranged from 10.0 to 60.0 cfu/g. In the isolated mycopopulations, xerophilic moulds dominated, especially those from the genera *Aspergillus*, *Eurotium* and *Penicillium*. In the isolated mycopopulations, high degree of isolated species belonged to toxigenic species from the genera *Alternaria*, *Aspergillus*, *Fusarium*, *Eurotium* and *Penicillium*.*

KEY WORDS: rapeseed, xerophilic moulds

INTRODUCTION

Rapeseed is the world's third most important source of vegetable oils after palm and soybean (1). The rapeseed production has been increasing during the past 30 years, and presently it contributes about 15% of the global vegetable oils. The introduction of low erucic acid varieties increased its value as edible oil, and varieties with low glucosinolates increased its value as a feed for livestock. The development of double-low varieties had made rapeseed one of the major plant oil sources, and now there is a constant tendency to increase its share in the production of oilseeds (2). Rapeseed of commercial interest is grown in the cooler areas of the world covering mainly North America, northern part of

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Europe, China, and India. It belongs to the *Brassica* genus, with around 160 species, mainly annual and biannual herbs (3).

Asia alone produced about 70% of the world's rapeseed-mustard production during 1948-1952. However, there has been a remarkable increase in the production share of European countries over the periods, and Canada and Europe together produced more than one half of the global rapeseed during 2005. Today, China, India, Canada, and Europe are the top producers, although there is a good potential for these crops to be successfully grown in Australia, United States, and South America, where both the area and production have sharply increased over the years (4).

Rapeseed (*Brassica napus* L. var. *napus*) is an important industrial plant, due to the high oil content, which ranges between 40% and 48% (5). At the same time, the rapeseed meal remains after the extraction of oil, with a crude protein content of 40% and a lysine content close to that of soybean meal (6), can be an excellent source of protein in animal feed. The food industry for domestic animals is interested in the use of full-fat canola seed, because rapeseed and/or by-products of processing are cost-effective, well-balanced source of protein, and high quality oil (7). Seeds can be used as an alternative to animal protein and fat supplements in this program. Such programs "no animal products" are becoming more popular and the use of food for domestic animals made entirely from plant products is increasing. Production of these foods is expected to continue increasing due to health problems or illnesses caused by the use of animal by-products in animal feeding.

As the other oil seeds, rapeseed can be often contaminated significantly with moulds, and also with their toxic secondary metabolites - mycotoxins. Because of that, the aim of this investigation was to examine the presence of moulds in rapeseed samples from the area of Vojvodina province, with a special emphasis on xerophilic species because the most of them are known as producers of various toxic metabolites.

EXPERIMENTAL

Samples of rapeseed

Mycological tests were carried out on samples of different genotype of rapeseed: Banačanka, Slavica, Branka, Ilia, Kata, Nena, NS-I-31, NS-I-126, NS-I-33, NS-I-128, NS-I-129, Jasna, NS-I-101, Zorica, NS-I-102, NS-I-134, NS-I-32, NS-I-136, NS-I-137, and NS-I-138. Samples of rapeseed were cultivated on plant field of the Institute of Field and Vegetable Crops on the location of Rimski Šančevi, Novi Sad, on a small parcel 48 sq. m on the soil of chernozem type. The seeding and harvesting were conducted mechanically, and standard geotechnical measures, appropriate for this crop, were applied during the vegetation. The seed was dried to 8% moisture content, and stored without cooling or heating in the store for selection materials. The analysis included 20 samples that were taken during the storage.

Isolation and determination of the total number of moulds

The isolation and determination of the total number of moulds were conducted on Dichloran Glycerol Agar, DG18 (Merck, Darmstadt). Total number of moulds was determined by the dilution method according to Koch.

The tested samples were divided in two groups: a) untreated samples and b) samples treated with 4% solution of Na-hypochlorite. Samples of rapeseed (50 g) were treated with 100 mL of this solution for 2 min. and after that, three times rinsed with 100 mL of sterile distilled water. Afterwards ensued the determination of total mould count and isolation and identification of fungal species.

Mould identification

After determining the total number, conidia and hyphal fragments of the mould colonies were transmitted on the Malt Extract Agar (MEA) (Merck, Darmstadt), Czapek Yeast Extract Agar (CYA), or on the Potato Dextrose Agar (PDA) (Merck, Darmstadt). The colonies for which, based on the macromorphological properties, was assumed to belong to the genera *Penicillium*, *Aspergillus*, and *Eurotium* were subcultured onto the CYA. The inoculated media were incubated for 7 days at 25°C. The isolates for which it was presumed to belong to the genus *Fusarium* were grown on PDA, and then subcultured on Carnation Leaf Agar (CLA), in order to obtain monosporic culture (8, 9). The monosporic cultures were incubated for 10-14 days in cyclic mode of 12 h combined light (fluorescent light and NUV - near ultraviolet light) and 12 h darkness at 25°C, for the purpose of stimulating the formation of conidiogenous structures. The other isolates for which it was assumed to belong to the genera *Alternaria*, *Cladosporium*, *Mucor*, and *Rhizopus* were subcultured on MEA and incubated for 7 days at 25°C.

The criteria described by Samson et al. (10), Samson and Frisvad (11), and Pitt and Hocking (12), were applied to identify the species of the genus *Penicillium*, while *Aspergillus* and *Eurotium* species were determined according to Klich (13), Samson et al. (10), and Pitt and Hocking (12). The *Fusarium* species were identified according to the keys for the determination described by Nelson et al. (8), and Lević (9). The other isolated species were identified according to Pitt and Hocking (12) and Samson et al. (10).

The frequency of a certain species of moulds in food samples was calculated according to the following equation:

$$\text{Frequency (\%)} = \frac{\text{Number of samples where the genus was identified}}{\text{Total number of samples}} \times 100$$

RESULTS AND DISCUSSION

The results obtained in these investigations show that a higher number of rapeseed samples which were not treated with 4% solution of Na-hypochlorite, were contaminated with moulds in comparison to the treated samples. The total mould count amounted to 4.7×10^2 cfu/g (sample NS-1-129). In three samples (Nena, NS-1-126, NS-1-138), the presence of the moulds was not observed. When the samples were treated with 4% solution of Na-hypochlorite, significantly lower contamination with moulds occurred. Mould were detected in four samples (Slavica, NS-1-31, NS-1-32; NS-1-128), and the total mould count ranged from 10.0 cfu/g (NS-1-128) to 60.0 cfu/g (NS-1-31) (Figure 1).

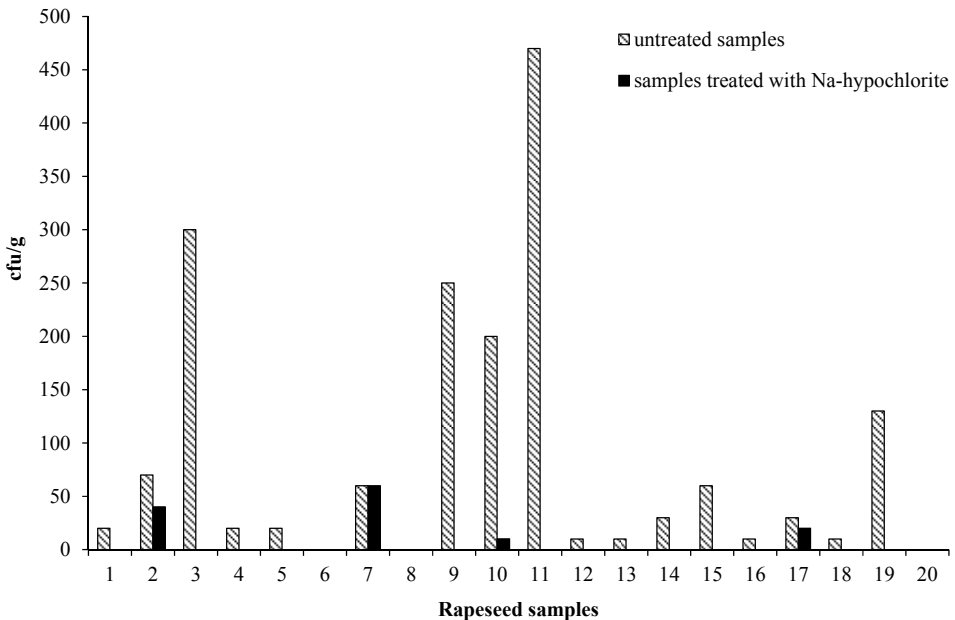


Figure 1. The total number of moulds (cfu/g) on rapeseed samples on DG18 Agar (1-Banaćanka; 2-Slavica; 3-Branka; 4-Ilia; 5-Kata; 6-Nena; 7-NS-I-31; 8-NS-I-126; 9-NS-I-33; 10-NS-I-128; 11-NS-I-129; 12-Jasna; 13-NS-I-101; 14-Zorica; 15-NS-I-102; 16-NS-I-134; 17-NS-I-32; 18-NS-I-136; 19-NS-I-137; 20-NS-I-138)

The fungi isolated from untreated rapeseed samples belonged to 8 genera and 12 species (Table 1). The most frequent were the species *Eurotium amstelodami* and *Penicillium brasilianum* that were isolated from 5 samples with a frequency of 25%. *Aspergillus niger*, *Cladosporium cladosporioides*, *Penicillium corylophilum* and *Rhizopus nigricans* had 15% of frequency in isolated mycopoultations. *Aspergillus flavus* and *F. proliferatum* were isolated from 2 samples (frequency of 10%), while other species (*Alternaria alternata*, *Aspergillus fumigatus*, *Fusarium acuminatum* and *Mucor racemosus*) were isolated only once (frequency of 5%) (Table 2).

Table 1. Mould species isolated from rapeseed

Genotype	Untreated samples	Samples treated with 4% solution of Na-hypochlorite
Banaćanka	<i>Eurotium amstelodami</i>	-
Slavica	<i>Mucor racemosus</i> <i>Rhizopus nigricans</i>	<i>Aspergillus niger</i>
Branka	<i>Eurotium amstelodami</i>	-
Ilia	<i>Aspergillus flavus</i> <i>Penicillium corylophilum</i>	-
Kata	<i>Cladosporium cladosporioides</i>	-
	<i>Alternaria alternata</i>	

Table 1. Continuation

Genotype	Untreated samples	Samples treated with 4% solution of Na-hypochlorite
Nena	-	-
NS-I-31	<i>Aspergillus niger</i> <i>Penicillium brasilianum</i> <i>Cladosporium cladosporioides</i>	<i>Alternaria alternata</i> <i>Penicillium corylophilum</i> <i>Penicillium brasilianum</i>
NS-I-126	-	-
NS-I-33	<i>Penicillium brasilianum</i> <i>Rhizopus nigricans</i>	-
NS-I-128	<i>Aspergillus niger</i>	<i>Aspergillus niger</i>
NS-I-129	<i>Aspergillus fumigatus</i> <i>Aspergillus niger</i>	-
Jasna	<i>Penicillium corylophilum</i>	-
NS-I-101	<i>Rhizopus nigricans</i>	-
Zorica	<i>Penicillium brasilianum</i> <i>Aspergillus flavus</i> <i>Fusarium proliferatum</i>	-
NS-I-102	<i>Penicillium brasilianum</i> <i>Fusarium proliferatum</i>	-
NS-I-134	<i>Fusarium acuminatum</i>	-
NS-I-32	<i>Eurotium amstelodami</i> <i>Penicillium brasilianum</i>	<i>Cladosporium cladosporioides</i> <i>Penicillium brasilianum</i>
NS-I-136	<i>Eurotium amstelodami</i>	-
NS-I-137	<i>Eurotium amstelodami</i> <i>Penicillium corylophilum</i> <i>Cladosporium cladosporioides</i>	-
NS-I-138	-	-

- no fungal growth

Table 2. Frequency (%) of mould species on untreated rapeseed samples

Mould species	Number of tested samples/number of contaminated samples	Frequency of isolated species (%)
<i>Alternaria alternata</i>	20/1	5.0
<i>Aspergillus flavus</i>	20/2	10.0
<i>A. fumigatus</i>	20/1	5.0
<i>A. niger</i>	20/3	15.0
<i>Cladosporium cladosporioides</i>	20/3	15.0
<i>Eurotium amstelodami</i>	20/5	25.0
<i>Fusarium proliferatum</i>	20/2	10.0
<i>F. acuminatum</i>	20/1	5.0
<i>Mucor racemosus</i>	20/1	5.0
<i>Penicillium brasilianum</i>	20/5	25.0
<i>Penicillium corylophilum</i>	20/3	15.0
<i>Rhizopus nigricans</i>	20/3	15.0

The mycopopulations of rapeseed samples treated with Na-hypochlorite contained the following species: *A. alternata*, *A. niger*, *C. cladosporioides*, *P. brasilianum* and *P. corylophilum* (Table 1). In these samples, the species *A. niger* and *P. brasilianum* were isolated.

ted from two samples with the frequency of 10.5% and 10.0%, while the other species were determined only once per sample (frequency 5%) (Table 3).

Table 3. Frequency (%) of mould species in the rapeseed samples treated with Na-hypochlorite

Mould species	Number of tested samples/number of contaminated samples	Frequency of isolated species (%)
<i>Alternaria alternata</i>	20/1	5.0
<i>A. niger</i>	20/2	10.5
<i>Cladosporium cladosporioides</i>	20/1	5.0
<i>Penicillium brasilianum</i>	20/2	10.0
<i>Penicillium corylophilum</i>	20/1	5.0

From total of 12 identified species, 8 were potentially toxigenic (Table 4). From the genus *Aspergillus* there were 3 potentially toxigenic species (*A. flavus*, *A. fumigatus* and *A. niger*). The species from the genus *Aspergillus* and their toxins are the most frequently found in products that were not good dried after harvest and during storage at high temperatures (14). From the *Aspergillus* species isolated in these experiments, *A. niger* is a fungal species known to be the most ubiquitously isolated from food and environment. *A. niger* represents a storage mould, but it can also be isolated from field. It is often isolated in the areas with warmer climate, because it optimally grows and develops at the temperatures from 35°C to 37°C. It is isolated from fresh and dried products (fruits, vegetables and spices), cereals, meat products, dried fish, cheese and other types of food (10, 12, 13, 15). Because of its ability to grow on substrates with low water activity (minimal $a_w=0.77$), it is often isolated from dried commodities. *A. niger* conidia are resistant to heating with microwaves, sunlight and UV rays. Some isolates can synthesize ochratoxin A and other toxic metabolites (13). *A. flavus* is also ubiquitously distributed. Its spores can be found in soil, plant material in decomposition, on peanuts and cereal grains, especially in corn. Also, *A. flavus* is isolated from spices, teas, mill, bakery and confectionary products. It grows optimally in the range of temperatures from 25°C to 42°C, minimally at 12°C, and maximally at a temperature around 48°C. *A. flavus* can grow on substrates with minimal water activity in the range from 0.78 to 0.80, which makes it a xerophilic fungal species (10). The synthesis of aflatoxin occurs at the temperatures of around 30°C and higher, at the relative humidity between 88 and 95% (16, 17). *Aspergillus fumigatus* is a thermophilic mould species that grows at the temperatures between 40°C and 42°C, with the of 12°C and maxim of 55°C. This species is marginal xerophile (0.82 a_w as the minimum for growth, near 40°C). The presence of *A. fumigatus* is principally detected on commodities in storages (stored oilseeds, cereals, stored eggs, copra, soybeans and vegetables), where lowered a_w values and high temperatures provide good conditions for growth of this species. Also, it is detected in processed meats, nuts, cashews, dried fish, baladi bread, corn snacks, rootstock snacks, melon seeds, mango pickles, dried onion, low fat buffalo milk cottage cheese, processed and kuflu cheese (12). *Aspergillus fumiga-*

tus is a fungal species associated with over 80% of all human syndroms caused by aspergilla. It causes allergic reactions in humans and animals. These diseases range from being colonization of the lung to life threatening diseases such as allergic bronchopulmonary aspergillosis (ABPA) and invasive aspergillosis (18). *Aspergillus fumigatus* produces fumitremogrens, verrucologen and gliotoxin and a role in animal disease seems likely (10).

From the *Fusarium* genus, two species were isolated, *F. proliferatum* and *F. acuminatum*, which are known as producers of various fusariotoxins, especially from the group of fumonisins and trichothecenes, as well as zearalenone. These mycotoxins and their producers are the most frequent contaminants of cereals, corn particularly, food and feed based on cereals (9, 19, 20, 21, 22).

A. alternata was the only isolated species from the genus *Alternaria* and it can produce under certain conditions several toxins (alternariol, alternariol monomethyl ether, tenuo-oxin, tenuazonic acid, altertoxins, stemfilitoxin III and altenuen) that have a harmful effect on human and animal organisms (23). *Alternaria alternata* toxins (AAT) are highly toxic metabolites similar to fumonisins. The presence of *A. alternata* is detected in tomato, wheat, barley, corn, Chinese sugar cane, rape seed, olives, apples, citruses and spices (12, 24). The most intensive production of alternariol, its monomethylether and altenuen by *A. alternata* was determined at 25°C and 0.98 a_w (25), and of tenuazoic acid at 0.90 a_w and 25°C (26).

From the genus *Eurotium*, only one species was isolated (*E. amstelodami*), which is potential producer of ehinuline and fiskion (10). Species from this genus are pronounced xerophilic moulds (minimal a_w around 0.70). They are isolated from cereals, grain fruits, dried fruits and vegetables, cheese, dried meat, fish, etc. (10, 12).

From the genus *Penicillium*, the presence was found of *P. brasilianum*, which is a producer of verrucologen and viridicatumtoxin,

Table 4. Toxigenic moulds and their mycotoxins isolated from rapeseed samples (9, 10, 12)

Mould species	Mycotoxins
<i>Alternaria alternata</i>	alternariol, alternariol monomethylether, alterotoxin I and II, altenuene, tenuazonic acid
<i>Aspergillus flavus</i>	aflatoxins, cyclopiazonic acid (few isolates)
<i>A. fumigatus</i>	fumitremogrens, verrucologen, gliotoxin
<i>A. niger</i>	naphtho- 4-pyrones, malphormins, ochratoxin A (few isolates)
<i>Eurotium amstelodami</i>	echinulin, physcion
<i>Fusarium proliferatum</i>	fumonisin B1, B2, B3, beauvericins, fusaroproliferin, fusaric acid, fusarin C, moniliformin, naphthoquinone pigments, fusapyrone
<i>F. acuminatum</i>	Enniatin B, moniliformin, trichothecenes in traces
<i>Penicillium brasilianum</i>	verrucologen, viridicatumtoxin

CONCLUSION

In 17 rapeseed samples that were not treated with 4% solution of Na-hypochlorite, total mould count was up to 4.7×10^2 cfu/g. In the case when samples were treated with 4% solution of Na-hypochlorite, significantly lower contamination with moulds was determined (10.0 cfu/g to 60.0 cfu/g), in only four samples. Mycopopulations of rapeseed samples untreated with Na-hypochlorite solution were contained the species from the genera: *Alternaria*, *Aspergillus*, *Penicillium*, *Fusarium*, *Eurotium*, *Mucor* and *Rhizopus*. The majority of the isolated species (about 70%) were typical xerophilic ones. The most frequent species were *E. amstelodami* and *P. brasilianum*. On the other hand, mycopopulations of rapeseed samples treated with Na-hypochlorite solution were contained the following species: *A. alternata*, *A. niger*, *C. cladosporioides*, *P. brasilianum* and *P. corylophilum*. From the isolated species, potential producers of mycotoxins were *A. alternata*, *A. flavus*, *A. fumigatus*, *A. niger*, *E. amstelodami*, *F. proliferatum*, *F. acuminatum*, and *P. brasilianum*.

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КСЕРОФИЛНЕ МИКОПОПУЛАЦИЈЕ ИЗ ОЛОВАНЕ ИЗ СЕМЕНА УЉАНЕ РЕПИЦЕ

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У раду су дата истраживања везана за миколошку популацију у узорцима уљане репице произведене у Институту за ратарство и повртарство (локација Римски Шанчеви, Нови Сад), са посебним освртом на потенцијално токсигену микопопулацију. Миколошка испитивања су изведена у узорцима који су третирани са Na-хипохлоритом, као и у узорцима који нису подвргнути овом третману. Изолација и одређивање укупног броја плесни изведено је на Дихлоран Глицерол агару. Идентификација изолованих врста плесни изведена је према кључевима за детерминацију који су описани у литератури. Од 20 испитаних узорка у 17 је утврђено присуство плесни и укупан број се кретао до 10 до $4,7 \times 10^2$ cfu/g. Када су узорци третирани 4% раствором Na-хипохлорита плесни су изоловане из 4 узорка, а укупни број плесни се кретао од 10,0 до 60,0 cfu/g. У изолованој микопопулацији доминирале су ксерофилне врсте из родова *Aspergillus*, *Eurotium* и *Penicillium*. Изоловану микопопулацију у високом проценту чиниле се потенцијалне токсигене врсте из родова *Alternaria*, *Aspergillus*, *Fusarium*, *Eurotium* и *Penicillium*.

Кључне речи: уљана репица, ксерофилне плесни

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