INFLUENCE OF SECONDARY METABOLIC PRODUCTS OF *ASPERGILLUS FUMIGATUS* ON THE GERMINATION RATE OF SOME LENTIL TYPES

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Résumé

L'objectif de cette étude est d'étudier l'effet des produits métaboliques secondaires de *Aspergillus fumigatus* sur la germination de quelques variérés de lentille. La technique a été réalisée en utilisant des solvants organiques, puis une chromatographie ascendante avec une colonne Al_2O_3. En fin, une chromatographie couches minces sur plaques de silice F254. Les rapports frontaux (Rf : 0.05 – 0.937) ont été déterminés après séchage. Sous lumière UV (courte =254 nm et longue = 365 nm) et sous lumière ordinaire, les taches ont donné différentes couleurs. Dix composés ont donc été déterminés. L'expérience a été menée en boites de Pétri sur milieux sablés afin de tester l'effet des concentrations des métabolites secondaires sur les différentes variétés de lentilles (Lentille (Syrie) spI, Lentille (Large Bonde Chili) spII et Lentille (Esculanta Lens Verte)spIII). Le pourcentage (60.65%) le plus faible (taux de germination) a été observé dans le cas de la variété Large Bonde Chili (LBC) et *Escualata lens* avec une concentration de 1 mg. Cependant, un taux de 70% a été atteint (pour la même concentration) dans le cas de la variété Syrienne. Les variétés *Esculata lens(verte)* et LBC ont montré le même taux, mais à la concentration de 100mg. Il faut retenir que la sensibilité des variétés aux métabolites s'est prononcée à partir de la valeur seuil de 0.25 ng et que le taux de germination était de 92.25% et 95% pour le cas des variétés ELV, LBS, *Syrie.*

Mots clés : Métabolites secondaires - *Aspergillus fumigatus* – Taux de germination – Variétés lentilles.

Abstract

Our research purpose to study the effect of the metabolic by-products of the *Aspergillus fumigatus* fungus on the germination of some types of lentil seeds. Purification has been achieved with organic solvents prior to bar chromatography using an aluminium oxide bar Al_2O_3, fallowed by thin layer chromatography transported to Silica gel F254 plates. After drying, values of the circulation rate $(0.937\text{-}0.051)R_f$ for every resulting spotwere determined. Spots occur with different colours under UV radiation with short wave 254 nm and 365 nm for long waves and likewise in regular light. It allowed determining ten close in their chemical structure. With the study of the impact of different concentrations from secondary metabolic issues secreted by *Aspergillus fumigatus* on lentil seed germination: sp. I (*Syrie*), sp. II *Large blonde (chili)*, sp. III *Esculata lens (verte)*. The experiment was designed in sterilized sandy fields inside Petri. The minimal germination percentage rate for the lentil seeds (60.65%) was recorded at concentration 1 mg for *Large blonde (chili*) and *Escualata lens (verte*), whereas *Syrie* amounted to 70% at the same concentration. Furthermore, this rate was recorded for *Esculata lens(verte)* at concentration 100 µg and for LBC type at concentration 1 µg. That means resistance capacity in *Syrie* exceeds 100, 10 times the ELV and LBC capacity respectively. It should be pointed out that sensitivity in the lentil types started at concentration 0.25 ng and germination percentage was 92.95 and 95% for ELV, LBS, *Syrie* respectively.

Keywords: Secondary Metabolic - *Aspergillus fumigatus* - germination rate - lentil seeds.

ملخص

يهدف هذا البحث إلى دراسة تأثير المنتجات الأيضية الثانوية لفطر *Aspergillus fumigats* على إنبات بعض أصناف بذور العدس . تمـت التنقيـة بالمذيبات العضوية، ثم كروماتوجرافيا العمود باستخدام عمود أكسيد الالومنيوم Al2O3 ، و أخيرا كروماتوجرافيا الطبقة الرقيقة بالسـريان علـى صـفائح Silica gel F245 . بعد التجفيف حددت قيم معامل السريان Rf(0.051-0.937) لكل البقع الناتجة. يزهر لون البقع بألوان مختلفة تحـت الأشـعة فـوق البنفسجية (UV) بطول الموجة القصيرة 254 نانومتر و الطويلة 365 نانومتر ، و كذا في الضوء العادي، و عليه حددت عشرة (10) مركبات تتقارب فـي التركيب الكيميائي . بدراسة تأثير تراكيز مختلفة من المنتجات الايضية الثانوية التي يفروها فطر *Aspergillus fumigats* علـى إنبـات بـذور العـدس Lentille (Esculanta Lens Verte)spIII ، Lentille (Large Bonde Chili) spII ، Lentille (Syrie) spI . صممت التجربة في مزارع رمليـة معقمة داخل أطباق بتري. تبين أن أقل نسبة مئوية لإنبات بذور العدس 60 ,65% سجلت عند التركيز 1 ملغ لكل من الصـنف Large Bonde Chili و Esculanta Lens Verte ، في حين بلغت 70% عند نفس التركيز في صنف Esculanta Lens Verte عند التركيز 100 ميكروجرام ، و فـي صـنف Large Bonde Chili عند التركيز 1 ميكروجرام . أي أن قدرة تحمل الصنف Syrie تفوق ب 10 ، 100 مرة تحمل كل من الصنفين Esculanta Lens Verte و Large Bonde Chili على الترتيب. و مما تجدر الاشارة اليه أن بداية حساسية أصناف العدس كانت عند التركيز 0.25 نانوجرام و بلغت النسبة المئوية للانبات (92 ، 95 ، 98) % بالنسبة لـ (Esculanta Lens Verte ، Large Bonde Chili و Syrie) على الترتيب.

الكلمات الفاتحة: *المنتجات الايضية الثانوية، فطر Aspergillus fumigatus ، نسبة الإنبات، بذور العدس.*

The lentil can endure hard environmental conditions similar to desert conditions where the rainfall is poor during the period when relatively high temperatures can affect the crop turnover. Therefore, the growth stages should meet the suitable environmental conditions in different regions.

The experimental stations managed to improve lentil seeds resistance by exploring ways to new types and stocks in order to have seeds with good cropping and trading quality, and of high cooking value as well. Lentil is subjected to fungal infection, especially stain on coastal regions. Lentil is sensitive to low temperature and dry wind, among tough environmental factors than can affect the existence of the living plants in winter.

Lentils grow very well in regions suitable for growing wheat, with a preference for damp and wet but fairly deep soils, flat, light, with little lime, with mean fertility, smooth and clean; this is rather than high fertility and very damp soils, [8]

MATERIEL AND METHODS

COLLECTION OF SAMPLES

Lentil samples:

Lentil samples of a few types were brought from a Oum-el-Bouaghi Dry Pulses and Seeds Cooperative in muslin sacks with labels on each sample according to the following **(table 1).**

Table 1: shows various types of lentil seeds produced in 2006

Serial number	Common name	Academic name	Type	Collection date	Place of collection
1	Lentil	Lentil sp. I	*Syrie*	April 2007	Oum-el-Bouaghi
2	lentil	Lentil sp. II	*Large blonde (chilli)*= (LBC)	April 2007	Oum-el-Bouaghi
3	lentil	Lentil sp. III	*Esculanta monoh (verte)*= (EMV)	April 2007	Oum-el-Bouaghi

Fungus sample

The fungus sample of *Aspergillus fumigatus* comes from the Applied Microbiology Laboratory, Faculty of Science University of Constantine. Has already been isolated and defined [8]**.**

DESCRIPTION OF FUNGUS ISOLATION

Macroscopic study: It is relied on the naked-eye observation of the fungus colony(shape, size, colour and strength) according to the method used by [17] ،[16] ,where a round piece of 0,5 cm in diameter has been taken from the fungal culture of *A. fumigatus* under favorable sterilization conditions. This small piece has been cultured in a hard Czapeck-Dox medium which is prepared in glass PETRI dishes (11 cm in diameter) and incubated at 30°C.

Microscopic study: The study was done by taking a drop of lactophenol staining that is prepared in laboratory and putting if on a clean slide and under favorable sterilization conditions, a fungal smear is transported to that drop from the fungus colony *Aspergillus fumigatus*. This latter is drayed on a light heat then a second drop of staining is added to it, and covered by a lamelle. After that, it is examined on observed under the light microscope.[9] ، ,[4], [7].

The examination was carried out using electronic microscopy in the Laboratory of Microbiologie , Faculté of Nature and Life Sciences University Clerment Ferrand II n France.

NUTRITIOUS MEDIA

Potato extract medium: Prepared out of 200g of potato, 20g sugar, and 20g agar [4]

Fermentation medium: Essentially based on liquid Czapeck-Dox added to 2.5g/l ferment extract [4],[6]

EXTRACTION AND PURIFICATION OF FUNGAL SECONDARY METABOLIC BY-PRODUCTS

Ferment medium Czapeck has been inoculated with ferment extract added at volume 50ml at a 10^6 germ/ml rate from the *A. fumigatus* isolate in a 250ml jar, and incubated at 30°C for 14 days under steady conditions. The experiment was carried out in 3 distillers[11] (Fig 1).

Extraction of organic solvents

After incubation, equal volume of chloroform (50ml) is added to each jar. The fungal culture was blended with electrical mixer and heated at 40°C, then sieved through a gauze or muslin. It must be vacuum-filtered with anhydrous sodium sulphate. Chloroform vacuum evaporation. The sediment is melted in 10ml ethyl acetate and 1 N of potassium oxalate is added at equal volume, pH solution as fixed at 2. HCl concentrated acid is dropped, solution is divided and split to two layers. Potassium oxalate and an acid layer (ethyl acetate) [11] ,[18] .

An equal volume of chloroform is added to the latter, and the pH is set at 10 in addition to sodium carbonate ($NaHCO_3$) (1N). Then the solution is divided and separated 3 times. Chloroform extract must be gathered and washed

off with refined water 3 times and separated. Chloroform is vacuum-steamed in steaming appliance, then sediment is dried through anhydrous sodium sulphate using a drying appliance (decicator) to obtain crude secondary metabolic by-products of *A. fumigatus.(* Fig 1)[11] ,[18] .

Column chromatography (CC)

The (2.5X10) cm column consists of Aluminium oxide (Al_2O_3) and a crude A. *fumigatus* sample is added to it and melted in 10ml benzene, the benzene extract is collected in a flask with 500ml capacity, illustrated in the collected crude secondary metabolic by-products (Fig 1).

Thin layer chromatography (TLC)

Circulated collected metabolic on glass sheets made out of silica (gel G F254 0.5 cm, sheets are put in an organic dissolving solution that is composed of Formic acid – Ethyl acetate – Toluene (1:4: 5) (vol/ vol/ vol).

Interpretation of the results is done by exposing the sheets to UV rays with wave length: λ = 365 mm (Fig 1).

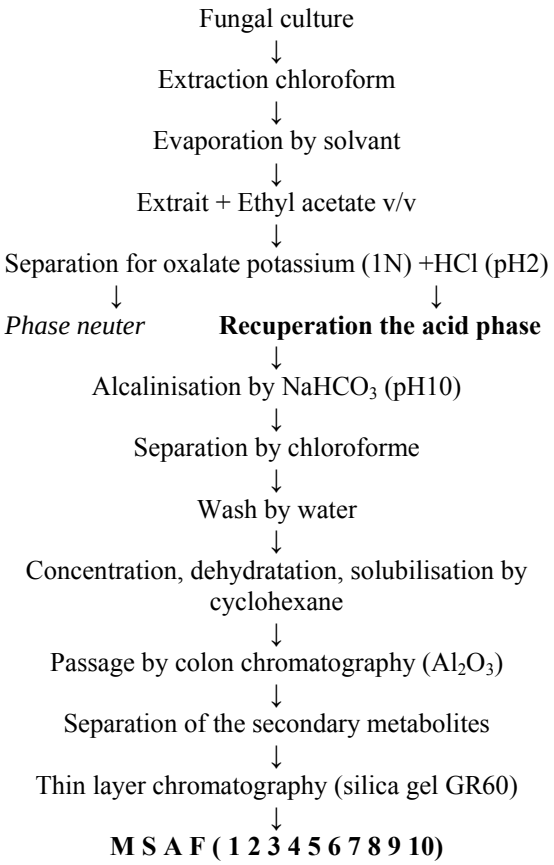

Figure 1:.Extraction and purification of secondary metabolites by products of *A. fumigatus*

EFFECT OF FUNGUS SECONDARY METABOLIC BY-PRODUCTS ON GERMINATION

Preparing the sand

Some sand is washed with 3% of HCl with concentration during 3 days, and then washed with refined water, after air drying. After that, the sand is laid on Petri dishes prior to be sterilized in autoclave at 120°C for 30mn, the sand is wetted in each dish with 10 ml of physiological water. Fixing acidity of the WTW P320 (OSI)at pH=7 [2] , [1].

Soak and planting of lentil seeds

Types of lentil seeds are soaked for an hour at different concentrations of fungal metabolic by-products (0.10, 0.25, 0.5, 1, 100, 250, 500) ng, (1, 100, 250, 500, 1000) µg. The latter are dissolved in chloroform, washed 3 times with sterile distilled water then dried with sterilized sieve paper. 100 grains are used for every type of lentil with every concentration and experiment was prepared with three distillers. Therefore, the grains are taken according to each concentration by putting 33 to 34 grains in each dish; the dishes are incubated in the dark for a week at 30°C with addition of 2.5 ml of sterile physiological water for each dish when needed [1], [3].

Estimation of germination percentage rate:

Once the incubation period under 30°C is over, the non-germinated seeds are counted in each dish for each concentration and each seed type. Then germination percentage is then estimated according to the following equation, [3],[2]

$$G\% = \frac{((TNS) - (NNGS)) * 100}{TNS}$$

Where :
TNS : Total Number of Seeds
NNGS : Number of Non-germinated seeds

RESULTS AND DISCUSSION

BARE AND MICROSCOPIC STUDY OF THE FUNGUS

The naked-eye observation of the *Aspergillus fumigatus* colony growing in PDA (Potato dextrose agar) medium (pH = 7 and temperature 37°C), showed that, the growth was evident in the early stages, the size of the colony covers up all the Petri dish surface within 3 days of incubation. The fungus appears as smooth transparent stripes, which progressively turn into bluish green.

The microscopic observation of the colony showed that, the fungus mycelium was divided into overlapped stripes carrying conidiophores with bulging upper endings. A network of stretching rowed excrescences known as phialids settle on the conidiophores along with conidia green germs which settle regularly (fig.2).

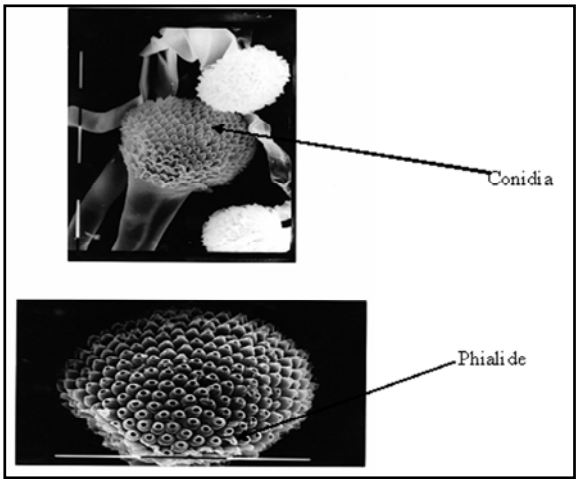

Figure 2: shows germ support of *A. fumigatus* electronic microscope magnifying 2020X [8] (Dehimat 1990)

These observations were reported by several researchers who worked on a microbiological study of the fungus, [12] [6] [10].

EXTRACTION OF FUNGAL METABOLITES

After growing *A. fumigatus* in ferment medium based on Czapek Dox medium with 2.5g/l yeast extract added, setting pH at 7, and incubating for 14 days at 30°C. Secondary metabolic by-products were extracted and purified using organic materials, column chromatography (aluminium oxide Al_2O_3), plates for thin layer chromatography (silica gel GF), solvent mixture (ethyl acetate, toluene, formic acid) vol/vol/vol. 1:4:5.

The components were displayed with UV rays. Circulation coefficient values R_f were defined for each metabolic substance and a notice was made of the existence of 10 separate components, distinct in their colour features. Further, more the similarity between the circulation coefficient values proves that the components are close in their chemical structure, (Table2). This was asserted by [9],.[18]

Table 2 : Separation of the secondary metabolites by thin layer chromatography (TLC)

R_f	Color spot	N° spot
0.051	Brown	1
0.093	Yellow orange	2
0.218	Red	3
0.375	Yellow	4
0.505	Bleu	5
0.590	Green	6
0.665	Red	7
0.697	Green bleu	8
0.821	violet	9
0.937	Brown	10

INFLUENCE OF THE SECONDARY METABOLIC BY-PRODUCTS ON THE GERMINATION RATE OF SOME LENTIL SEED TYPES

It was noticed according to the results recorded in the (Fig. 3) that, the rate of germination varies from one type to another as well as from a planting pattern to another; and is reversely influenced by the rise of the fungus metabolic substance concentration.

It was noted that, the germination percentages were close to each another. As such, in the case of seeding in dishes, it appeared that, the smallest rate 60.65% was recorded at concentration 1 mg equally for LBC and ELV as well, whereas the rate was 70% at the same concentration for the *Syrie* type.

Also, this rate recorded for *Esculanta lens* (verte) at concentration 100µg, and for LBC at 1µg. This means that resistance in *Syrie* 10,000 times resistance capacity in ELV and LBC respectively. It should also pointed out that sensitivity of the seeds started at concentration 0.25 ng and the germination rate rised went up to 92, 95, and 98% for ELV, LBC, and *Syrie* respectively.

This dissimilarity may be due to the sensitivity of the different types to variation in the anatomic and morphologic setting of the cuticles over the seeds which can delay the fungus progression in the seed or the penetration of its metabolic by-product in *Syrie* relatively to the other types. (Table 3. Fig. 3).

Table 3 : Germination rate of some lentil seeds treated at different concentrations of the *A. fumigatus* crude secondary metabolic by-products

Serial number	Concentration of the metabolic substance	Germination rate % of lentil seed types		
		Large Blonde Chili	Syrie	*Esculata Monoh* (Verte)
1	Distilled water	100	100	100
2	0.10 ng	100	100	100
3	0.25 ng	95	98	92
4	0.50 ng	88	98	92
5	1 ng	85	95	90
6	100 ng	80	94	88
7	250 ng	78	90	86
8	500 ng	74	90	80
9	1 µg	70	88	75
10	100 µg	65	88	70
11	250 µg	62	80	70
12	500 µg	62	72	68
13	1 mg	60	70	65

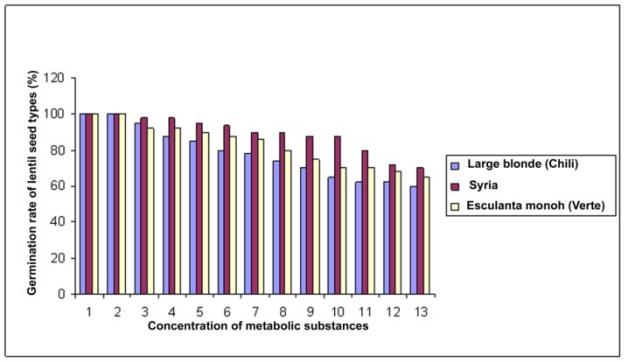

Figure 3 : The germination rate of the lentil seeds treated at different concentration with *A. fumigatus* metabolic by-products

These results are said to be consistent with conclusions made by several researchers in this field like [14];[15] for inoculated corn with Aflatoxin B1 and also [18],[5] on chickpeas soiled with fungi. Concerning the influence of fungal toxins on various legume seeds and dry beans, a notice has been made by [1] on rye seeds, and on rye wheat [8].

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