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BIOCHEMICAL REVISION OF *Penicillium hordei*

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ABSTRACT

Eight type strains belonging to *Penicillium hordei* were classified from the analysis of their crude extracts by a polyphasic approach with data processing, using the profiles of their fatty acids; secondary and volatile metabolites, as well as random amplified polymorphic DNA-polymerase chain reaction pattern (RAPD-PCR) as taxonomic markers for these strains. The study showed that with the harmony of all the four investigated markers, about all of the investigated type strains could be classified correctly at the intraspecific level using only the analysis of metabolites produced on one growth medium (YES), except in case of the volatile profile which succeeded as a cladogenetic profile but not as a strain marker. The study revealed also the ability of RAPD-PCR technique to evaluate the genetic diversity among the investigated isolates at the sub-species level, as well as a rapid and easy method than traditional characterization techniques. Other relations between type strains could be read from the dendrograms and the efficient classification showed the potential of this polyphasic approach identification system.

INTRODUCTION

Fungal taxonomy is a dynamic, progressive discipline that consequently requires changes in nomenclature. These changes are often difficult for microbiologists to understand. However, some groups of fungi, because of their economical or pathological importance, have been studied more extensively. Other features beside morphology, such as the use of molecular techniques, physiological and biochemical tests, have been used in classification and also in identification. The increased use and availability of modern techniques have opened up many new areas within systematics and have enabled more traditional ones to be developed further **(Josepa and Alberto, 1999)**.

Identification of *Penicillium* species is still never to be easy, so that, **(Pitt, 1991)** had more taxonomic handless to help *Penicillium* classification. *Penicillium hordei* was found to be one of the thirteen different allergens causative agents discovered in the study of **(Graham *et al* 1995)** in Brazil evaluating 1,410 patients with asthma and/or rhinitis. This species produces several odor and volatile metabolites such as isobutanol, isopentanol **(Larsen and Frisvad, 1995)** as well as some extrolites and mycotoxins such as terresteric acid, carolic acid, carlosic acid and roquefortineC **(Samson and Frisvad, 2004)**.

Promising results regarding the use of fatty acids (FA) for identification of filamentous fungi had been reported by many studies including **(Losel, 1989; Blomquist *et al* 1992)** which reported that with the aid of FA profile, it was possible to differentiate between various *Aspergillus*, *Mucor* and *Penicillium* species. Unfortunately, only a limited number of strains and species have been included in most of these studies, which makes the evaluation of this method as an indication parameter difficult.

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Chemotaxonomic studies of large number of isolates in *Penicillium* have shown, however, that secondary metabolites (SMs) have a potential for the characterization of its species and for phylogenetic relationships. Thus, it complements morphological data to give a fuller description of an important part of the phenotype that may be perceived by other organisms. Chemical analysis of secondary metabolites will provide more objective and comparable results than traditional description of color and odor **(Smedsgaard and Nielson, 2005).**

Fungi are known to biosynthesize a variety of metabolic products, including volatile metabolites (VMs) which can be products of both primary and secondary metabolism. Despite the advance in technology by the development of the modern analytical techniques such as (GC, GLC and GC/MS), only a few reports on the fungal chemotaxonomic studies based on the production of (VMs) were found **(Frisvad *et al* 2008).**

Some studies have evaluated a large number of primers to identify only a selected few isolates that can successfully discriminate genetic strain types. **(Kac *et al* 1999)** evaluated fifteen RAPD primers identifying merely one that was highly discriminatory for strains of *Trichophyton mentagrophytes*. So, RAPD technique was evaluated as a reliable tool with good reproducibility of the patterns for each investigated strain as affirmed by **(Stemmler *et al* 2001)**. But problems of interpretation due to inconsistent intensity of bands in different PCR runs may arise for less experienced personnel. RAPD analysis can be performed within one working day and needs less DNA compared with RFLP, so, costs will be reduced.

Most of the known established techniques and designing options of fungal taxonomy have been validated for only a few dozen of fungal strains and the lack of efficient genetic engineering strategic forms still an obstacle for a multitude of identifying fungi producing commercially interesting metabolites. To fully explore their biotechnological capacities, these constraints have to be solved **(Vera, 2008).**

MATERIALS AND METHODS

Fungal strains: all of the tested *Penicillium hordei* type strains were purchased from the International Mycological Institute (IMI) culture collections and coded here as; (30) *P.hordei* (IMI 246204), (31) *P.hordei* (IMI 286971), (32) *P.hordei* (IMI 297900), (33) *P.hordei* (IMI 284723), (34) *P.hordei* (IMI 264173), (35) *P.hordei* (IMI 223651), (36) *P.hordei* (IMI 151748) and (37) *P. hordei* (IMI 040213).

Media: two types of media were used; Malt Extract Agar (MEA) medium was used for maintenance of the strains according to **(Smith and Onions, 1983)**. The other medium type was Yeast Extract Sucrose (YES). This semi-synthetic medium was used in liquid form for the production of intracellular fatty acids from the cultivated strains **(Peter and Michael, 1996)**, intracellular secondary metabolites **(Frisvad and Samson, 2004)** and intracellular volatile metabolites **(Larsen and Frisvad, 1995b; Kristian and Thomas, 2005)** as well as for the DNA study **(Zhou and Linz, 1999).**

Media were sterilized by autoclaving at 121°C for 20 min. Mycelia growth from 7 days old cultures on MEA slopes were scraped by using 2 ml of sterile distilled water. Then, 2.0 ml of $4x10^2$ cells/ml spore suspension of each type strain were used to inoculate a 100 ml YES medium in a universal 250 ml flask, and then incubated at 25°C for 7 days, except in case of the DNA analysis that all flasks were incubated with a gentle shaking at 180 rpm. at 25°C for 2 days.

Fungal mycelia and pellets were harvested by filtration under aseptic conditions using microcloth and washed thoroughly with sterile distilled water then weighed, decanted in sterile containers and stored at -4°C for further analysis. While for complete DNA analysis, fungal pellets were lyophilized using a freeze dryer system (Heto lyophilizer model Maxi Dry plus). The lyophilized pellets were grounded in a sterile cold mortar using sterile pestle and decanted in a sterile 1.5 ml microfuge tube.

It is worthy to mention that all of the experimental work throughout this research was carried out at the Regional Center for Mycology and Biotechnology (RCMB), at Al-Azhar University except for the fatty acids analysis which was achieved at the Central Lab of the Ain Shams University.

Volatile Metabolite Analysis: Intracellular volatile metabolites were extracted from fungal mycelia according to **(Evans, 2002)** then analyzed using Shimadzu QP 5050A GC/MS supported with a Class 5000 software and Whiley mass spectral data base searchable library.

Fatty Acid Analysis: Intracellular fatty acids were extracted according to **(Peter and Michael, 1996).** Gas chromatographic analysis was achieved using Dani GLC-FID 1000. For the complete identification of the resulted compounds, a fatty acids standard was used. This standard was manufactured by Supelco^{tm}, containing mixture of 37 fatty acids methyl ester (C_4- C_{24}) dissolved in methylene chloride.

Secondary Metabolite Analysis: Extraction, analysis and identification of intracellular secondary metabolites were carried out using the TLC plate technique of the automatic scanner system (HPTLC Scanner 3 -CAMAG, Switzerland) using griseofulvin as reference standard. The identity of the metabolites was performed by comparing shape, color and R_f values of the recorded spots with those given at **(Paterson and Bridge, 1994).**

Fungal DNA Extraction: DNA extraction was conducted using DNeasy kit (Qiagen, Germany).

RAPD-PCR: Amplification reaction mixture solution was prepared in a final volume of 50 µl containing: 3 µl (200 ng) of genomic DNA; 1 µl of 50 pmole of each desired primer; 25 µl of the Go Taq Green Master Mixture (Promega Co.) and deionized RNase-DNase free water in sufficient amount to give the total reaction mixture volume of 50 µl. The amplification was performed using Research Programmable Thermal Cycler (gradient Robocycler 96 Stratagene, USA) where the applied program was as follows: universal denaturation cycle (5 min. at 94°C), 45 cycles of annealing/extension reactions (30 sec. at 94°C, 1 min. at an optimum annealing temperature 36°C for each used universal primer and 2 min. at 72°C) and cycle of final extension step (5 min. at 72°C) was followed by soaking at 4°C.The sequence of six oligonucleotide universal primers used in the current search were: primer 1: (5'-GGTGCGGGAA-3'), primer 2: (5'-GTTTCGCTCC-3'), primer 3: (5'-GTAGACCCGT-3'), primer 4: (5'-AAGAGCCCGT-3'), primer 5: (5'-AACGCGCAAC-3') and primer 6: (5'-CCCGTCAGCA-3'). These applied primers were of HPSF grade and obtained from the MGW Biotech. Ag. Co.

Horizontal Gel Electrophoresis: The amplified products were separated by agarose gel electrophoresis using a horizontal submarine gel system (Agagel Maxi, Biometra) as well as agarose (Gibco BRL Life Technologies) at a concentration of 2% (w/v). Electrophoresis was conducted in 0.5XTBE buffer (5.4 g of Tris base, 2.75 g of Boric acid and 2 ml of 0.5 M EDTA [pH 8.0] in 1 liter of distilled water) at 10 v/cm for various times, depending on the size of the gel unit **(Weising *et al* 1995).**

DNA bands were stained with ethidium bromide (10 mg/ml) then visualized and photographed under a UV Transilluminator system using a Gel Doc. 2000 (Bio-RAD).

Statistical Cluster Analysis of the Phylogenetic Relationships: The role of the RAPD-PCR patterns as well as the fatty acid, volatile metabolite and secondary metabolite profiles as useful criteria for studying phylogenetic relationships among the investigated penicillia strains was evaluated by using statistical cluster analysis with joining (tree clustering) being the clustering method. Genetic relationships and divergence between RAPD – PCR patterns of the investigated strains were calculated from the decimal coefficient using the Quantity One (4.0.3) software and were illustrated in dendrograms constructed using the unweighed pair-group method with arithmetic averages (UPGMA). While, each of fatty acid, volatile metabolite and secondary metabolite profiles were amalgamated by a complete linkage using the Elucidation distance as the distance metric as well as the dice coefficient as the calculation method using the Statistica software for Windows release (4.5 F, State Soft. Inc. 1993).

RESULTS

Volatile Metabolite Profile: The available results from the volatile metabolite (VM) profile of the investigated *P.hordei* type strains (eight type strains), revealed twelve distinct types of volatile compounds, which studied effectively to clear up a specific dilemma tied to the morphological grouping of the examined strains, **(Table 1).** From the pointed out results, no single volatile compound could be used as a chemotaxonomic marker due to their widely distribution among the tested strains. However, the VM profile, as a whole, succeeded in differentiation between the tested strains into two groups. On the other hand, the aforementioned illustration of the VM profile gives the impression to be perplexing. For that reason, the cluster analysis tree dendrogram **(Figure 1)**, revealed the grouping of the investigated strains into two clades based on their volatile metabolite content. The first clade grouped *P.hordei* numbers 33, 34, 32 and 30 together at a linkage distance ranged between zero and 1.0, through which, each of *P.hordei* numbers 33, 34 and 32 were linked together at a linkage distance of zero, which looked as if they are the same subspecies. While, *P.hordei* number 30 appeared to be more likely the same subspecies also, since it connected to the rest strains of this group at a linkage distance of 1.0, which is so close to the similarity matrix value of the rest strains across this clade (zero). Similarly, the second clade included *P.hordei*

Table 1. Volatile metabolite profile of the investigated *P.hordei* type strains

Compound	*P. hordei* type strain numbers							
	30	31	32	33	34	35	36	37
Isobutanol	*	*	*	*	*	*	*	*
Isopentanol	*	*	*	*	*	*	*	*
L-menthol	*		*	*	*			
3-Octanone	*	*	*	*	*	*	*	*
3-Heptanone		*				*	*	*
Bornyl Acetate	*	*	*	*	*	*	*	*
Methyl Palmitate	*		*	*	*			
Linalool		*				*	*	*
Limonene	*		*	*	*			
α-Selinene	*	*	*	*	*	*	*	*
β-Elemene	*	*	*	*	*	*	*	*
3-Methyl Furan	*		*	*	*			

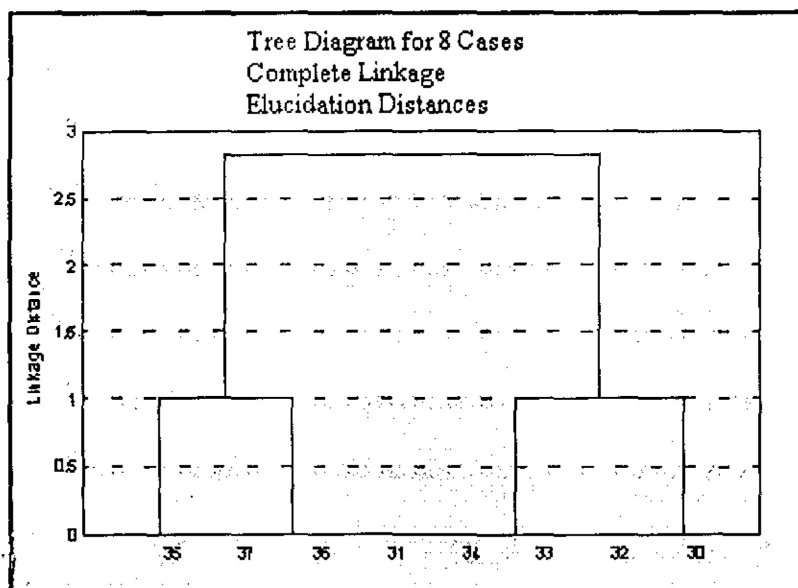

Figure 1. The phylogenetic tree of the considered *P.hordei* type strains based on their similarity matrix values of the volatile metabolite profile

numbers 31, 36, 35 and 37 fixed them together at a linkage distance ranged from zero to 1.0 also, fusing each of *P.hordei* numbers 31, 36, and 37 as one subspecies that linked altogether at a linkage distance of zero. Whilst, *P. hordei* number 35 appear to be more apparently the same subspecies also since it linked to the rest strains at 1.0. It is worthy to mention that both clades were connected at a linkage distance of 3.1, which appeared to be not so close. The phylogenetic relationships of the studied penicillia raised from their VM profile were consistent in some cases and detracted in others. So, this marker alone is not enough and needs further assessment of another marker, thus, fatty acid (FA) profile as a chemotaxonomical marker had been applied.

Fatty Acid Profile: The presented results from the fatty acid (FA) profile of the surveyed *P.hordei* type strains, discovered eighteen different types of fatty acids, which were studied to resolve certain problems associated with the morphological classification concerning the investigated species (**Table 2**).

It is admirable to state that each of caprilic, lauric, myristic, *cis-heptadecenoic*, arachidonic, capric, linoleic and henicosanoic acids may be useful as a chemotaxonomic markers specially designed for the investigated *P.hordei* strains based on the FA profile thanks to their limited dispense through the studied strains and could be used effectively to clarify the type strains possessed these fatty acids during identification using these conditions from medium, extraction to analysis. On the other side, the previous representation of the FA profile looked confusing. So, the phylogenetic tree of the cluster analysis differentiated the combination of the inspected type strains into three clades based on the fatty acid content (**Figure 2**). The first one included entirely *P.hordei* numbers 33 and 32 joining them together at a linkage distance of 2.4. Furthermore, the second clade involved *P.hordei* numbers 30 and 35 joining them together at a linkage distance of 1.7, which joined to the first clade at a linkage distance of 2.5, Besides, the third clade contained *P.hordei* numbers 37, 36 and 31 joining them together at a linkage distance of 1.7 as well, while the closest two strains across this clade appeared to be *P.hordei* numbers 37 and 36 that joined together at a linkage distance of 1.4. It is commendable to mention that the three groups were spaced out from each other by the linkage distance of 3.1. Finally, *P.hordei* number 34 was separated alone that linked with the others by a linkage distance of 3.8. Conclusively, it materialized more supportive considering the use of fatty acid outline in penicillia chemotaxonomy, since this marker confirmed the former results obtained from the VM marker, however, *P.hordei* number 35 appeared to be located at different clades throughout both profiles as illustrated before.

So, the FA profile provided us a valuable chemo-categorization indicator for the studied strains. But it wouldn't be possible to substantiate such relationships without a supplementary assessment using other marker, such as the application of the secondary metabolite (SM) profile.

Secondary Metabolite Profile: The readily available results from the secondary metabolite (SM) profile of the considered *P.hordei* type strains, showed out twenty one discrete types of secondary metabolites, which inspected to resolve certain crisis associated with the morphological establishments of the surveyed isolates of this species, (**Table 3** and **Figure 3**). It is remarkable to declare that due to their restricted distribution throughout the studied *P.hordei* strains, all of (-)flavoskyrin, cinnamic acid, dehydrocarolic acid, cyclopenin, viridicatin and terresteric acid could be advantageously accepted as chemotaxonomical markers as well for those species.

Fortunately, four unidentified compounds with different R_f values of 11, 38, 46 and 54 that regained from the tested type strains were might be considered to be good taxonomical markers also, owing to their minor and restricted spreading throughout the tested strains that ranged from one to two strains at maximum for all and need further categorization later on. Alternatively, the prior illustration of the SM profile seemed to be puzzling. Hence, the cluster analysis tree (**Figure 4**), revealed the federation of the considered strains into three clades based on their SM content.

The first one included *P.hordei* numbers 33 and 32 joining them together at a linkage distance of 2.4. While, the second clade fusing *P.hordei* numbers 30 and 35 together at a linkage distance of 1.7, then joined them closely to the first clade at linkage distance of 2.5. Besides, the third clade contained *P.hordei* numbers 37, 36 and 31, joining them together at a linkage distance between 1.7 and 1.46, in which the closest two strains were *P.hordei* numbers 36 and 37 that joined together at a linkage distance of 1.4. It is commendable to mention that the three groups were joined together at a linkage distance of 3.1 which considered apart from each other. Only *P.hordei* number 34 was discarded alone from the rest groups, indicating the uniqueness of it as a separate subspecies that a parted from the rest clades by a linkage distance of 3.8.

Conclusively, it looked more supportive considering the use of secondary metabolite profiling in *P.hordei* chemotaxonomy, which was not an easy task. Since, the results of secondary metabolite marker confirmed the results gained by both of volatile and fatty acid markers concerning the grouping of each of *P.hordei* numbers 37, 36 and 31 together as well as *P.hordei* numbers 32 and 33 in their own groups, but not supporting them in the rest of clades speciation.

RAPD-PCR Profile: Six oligonucleotide primers were tested for their ability to generate RAPD-PCR pattern from the genomic DNA of the eight studied

Table 2. Fatty acid profile of the investigated *P.hordei* type strains

Fatty Acid	*P.hordei* type strain numbers							
	30	31	32	33	34	35	36	37
Butyric	*	*	*	*	*	*	*	*
Caproic	*	*	*	*		*	*	*
Caprilic	*				*	*		
Capric	*					*		
Lauric	*			*		*		
Myristic	*		*		*			
Pentadecanoic	*	*				*	*	*
Palmitic		*	*		*	*		*
Palmitoleic	*	*	*	*	*	*	*	*
Margaric	*	*			*	*	*	*
Cis-Heptadecenoic	*	*			*			
Stearic	*	*	*	*	*	*	*	*
Oleic	*	*	*	*	*	*	*	*
Linoleic					*			
Linolelaidic		*	*				*	*
γ-Linolenic	*	*		*	*	*		*
Arachidonic			*	*	*			
Henicosanoic			*					

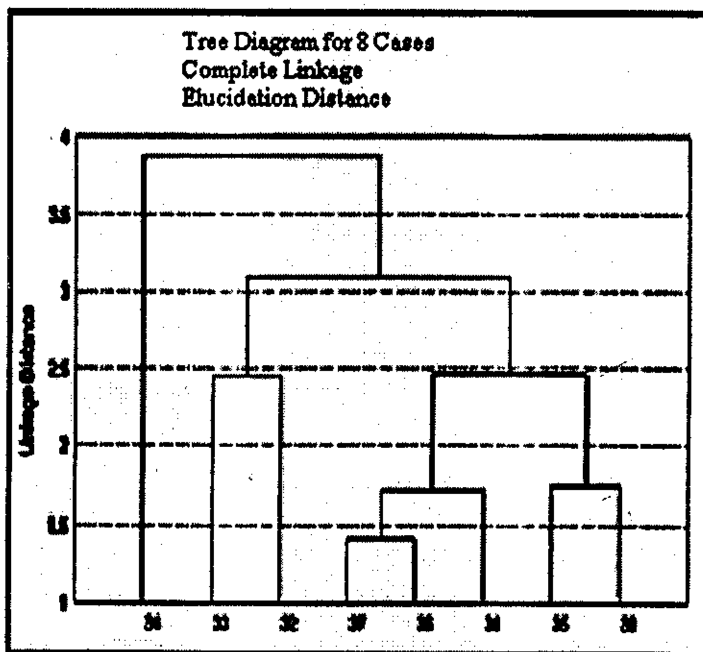

Figure 2. The phylogenetic tree of the considered *P.hordei* type strains based on their similarity matrix values of the fatty acid profile

Table 3. Secondary metabolites profile of the investigated *P.hordei* type strains.

Compound Name	*P.hordei* type strain numbers							
	30	31	32	33	34	35	36	37
Acetyl Carbinol	*	*		*	*			
Carlosic Acid	*		*	*	*		*	*
Carolic Acid	*	*	*	*	*	*	*	
Chaetoglobosin C	*	*	*	*	*	*	*	*
Cinnamic Acid			*	*	*			
Citrinin	*	*	*	*	*	*	*	*
Cyclopenin	*	*						
Dehydrocarolic Acid			*	*	*			
Fulvic Acid	*	*	*	*	*	*	*	*
Hevalonic Acid		*		*	*	*	*	*
Patulin	*	*	*	*	*			
Physodic Acid	*	*	*	*	*	*		*
Roquefortine B		*		*	*	*		*
Terresteric Acid				*	*			
Viridicatin	*	*	*					
Xanthocillin	*	*		*	*	*	*	*
(-)Flavoskyrin			*	*	*			
Unknown (R_f of 38)			*					
Unknown (R_f of 54)			*					
Unknown (R_f of 11)				*				
Unknown (R_f of 46)				*	*			

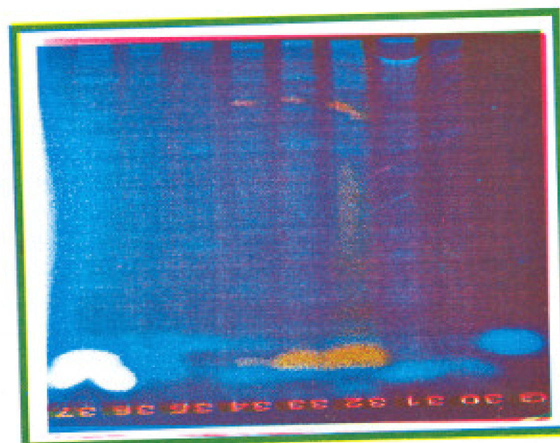

Figure 3. TLC plate of *P.hordei* type strains secondary metabolites under long UV. Lane G; griseofulvin, lanes 30-37; *P.hordei* type strains

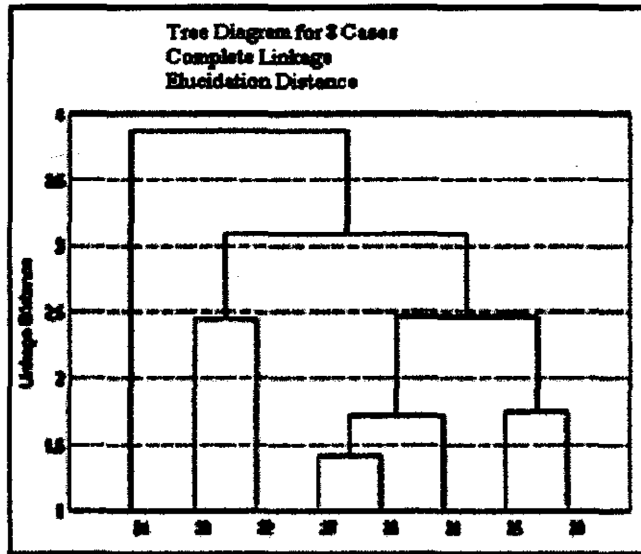

Figure 4. The phylogenetic tree of the considered *P.hordei* type strains based on their similarity matrix values of the secondary metabolite profile

P. hordei type strains. **Figures (5, 7, 9, 11, 13 and 15)** entail the DNA bands of the eight investigated *P.hordei* type strains amplified by all primers, which explained as the tree dendrograms represented at **Figures (6, 8, 10, 12, 14 and 16)**.

These tree dendrograms categorized the tested *P.hordei* type strains into one big clade, except for *P.hordei* number 34 by primer one. It is worthy to mention that the separation of *P.hordei* number 34 alone away from the rest strains by primer one was in consistent merely with what obtained from both the FA as well as the SM markers of the tested *P.hordei* strains, while argued with those of the VM marker. Therefore, further assessment using several primers was proposed. The relationships among the *P.hordei* strains developed by primer six were seeing as close to that developed by most of the tested primers except for primer one. Since; most of the studied primers agreed in grouping the eight examined *P.hordei* type strains into two clades a part from primer one that grouped them into one big clade. Also, all the studied primers, a part from primer one, agreed in grouping *P.hordei* numbers 30, 32 and 33 as a one strain. At the same time, *P.hordei* numbers 34 and 35 were considered to be two copies of the same subspecies due to the harmony of most of the tested primers except for primers one and two. Additionally, only primers three and five argued with the rest primers concerning the separation of *P.hordei* number 37 alone in its own group as a matchless strain. Finally, both of *P.hordei* numbers 31 and 36 were the reminder strains of great argument throughout the studied primers, being in consistent with some and detracted in others.

Conclusively, four markers in total; namely; VM, FA, SM profiles, as well as six oligonucleotide primers of the RAPD-PCR pattern, agreed in classifying the eight studied *P.hordei* type strains into three major groups. The first group separated *P.hordei* numbers 30, 32 and 33 being that these strains were the same subspecies of this *Penicillium* species, according to the harmony of all the tested markers except for primer three as well as both of the FA and SM profiles. It is worthy to state that all of the examined markers agreed in gathering both *P.hordei* numbers 32 and 33 together as an identical versions of one subspecies.

Concurrently, at the second group, either of *P.hordei* numbers 34 and 35 were said to be alike subspecies as a results of the harmony of all markers apart from primers two and one as well as the VM profile. The third group gathering *P.hordei* numbers 31, 36 and 37 as one strain according to the synchronization of all studied markers despite primers three, five and six. It is precious to point out that all the studied markers, except for primers three and five, harmonized in separating *P.hordei* numbers 31 and 36 as a duplicate of one strain. Thus it is promising to handle the examined four markers exclusively or with each other to

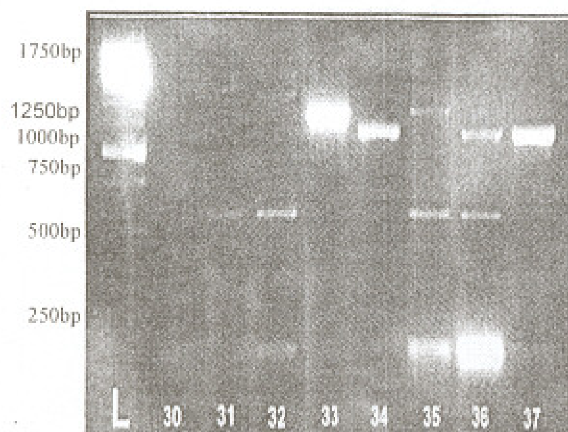

Figure 5. Banding pattern of RAPD-PCR of *P.hordei* type strains using primer-1. Lane L; molecular weight marker 250bp ladder; lanes 30-37 *P.hordei* type strains

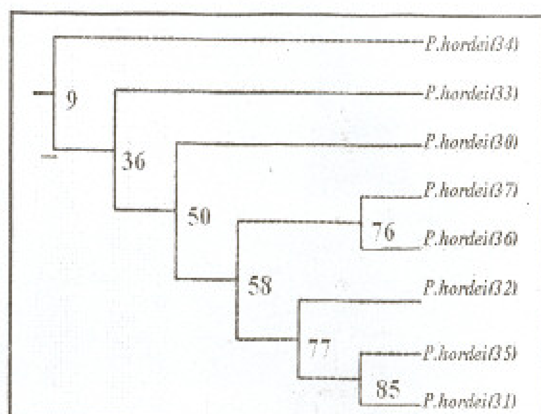

Figure 6. The phylogenetic tree developed from the similarity matrix values of the *P.hordei* type strains RAPD-PCR pattern using primer-1.

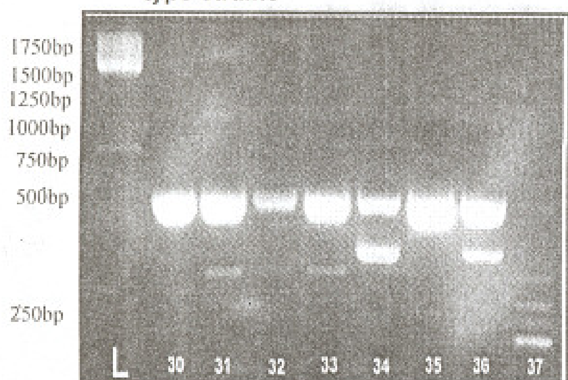

Figure 7. Banding pattern of RAPD-PCR of *P.hordei* type strains using primer-2. Lane L; molecular weight marker 250bp ladder; lanes 30-37 *P.hordei* type strains

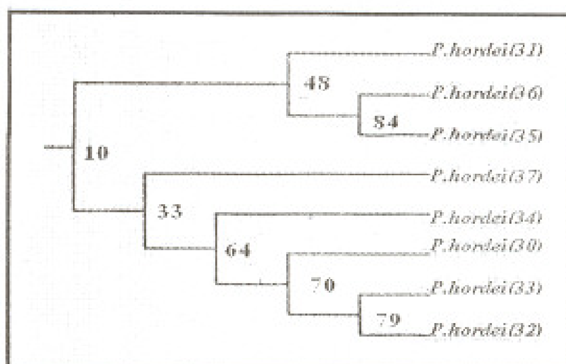

Figure 8. The phylogenetic tree developed from the similarity matrix values of the *P.hordei* type strains RAPD-PCR pattern using primer-2.

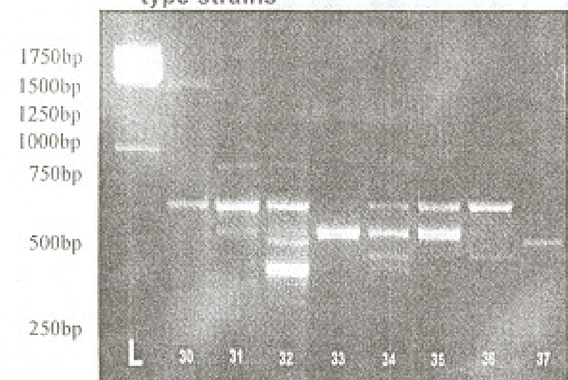

Figure 9. Banding pattern of RAPD-PCR of *P. hordei* type strains using primer-3. Lane L; molecular weight marker 250bp ladder; lanes 30-37 *P.hordei* type strains

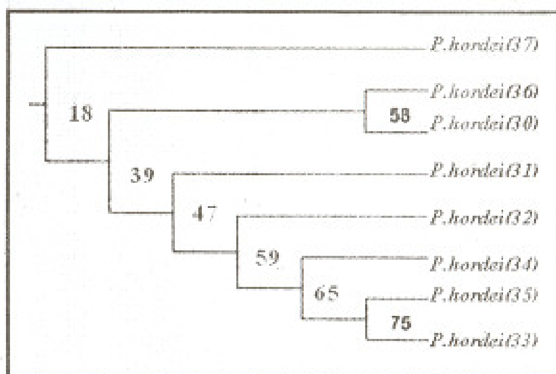

Figure 10. The phylogenetic tree developed from the similarity matrix values of the *P.hordei* type strains RAPD-PCR pattern using primer-3

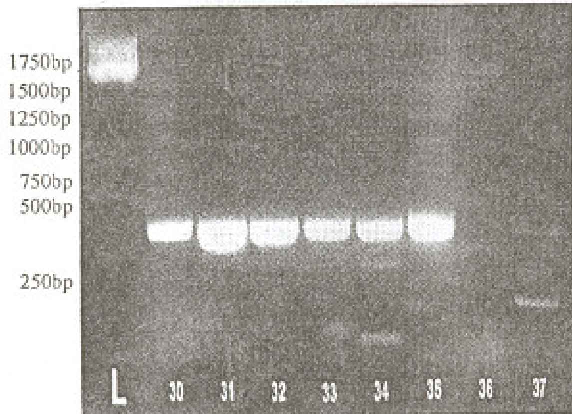

Figure 11. Banding pattern of RAPD-PCR of *P. hordei* type strains using primer-4. Lane L; molecular weight marker 250bp ladder; lanes 30-37 *P. hordei* type strains

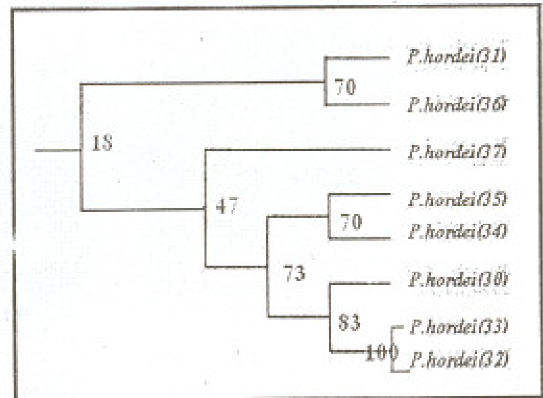

Figure 12. The phylogenetic tree developed from the similarity matrix values of the *P.hordei* type strains RAPD-PCR pattern using primer -4

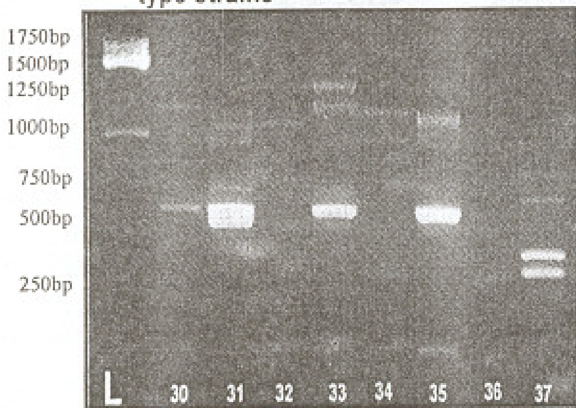

Figure 13. Banding pattern of RAPD-PCR of *P. hordei* type strains using primer-5. Lane L; molecular weight marker 250bp ladder; lanes 30-37 *P.hordei* type strains

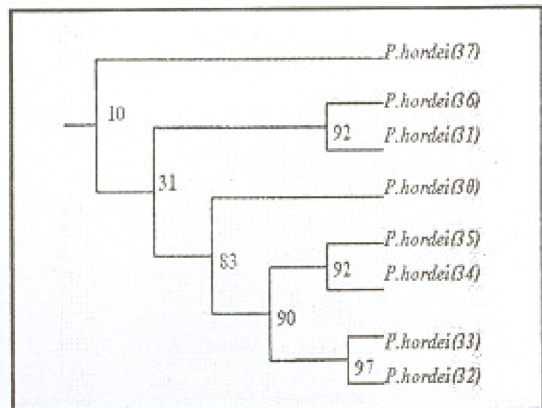

Figure 14. The phylogenetic tree developed from the similarity matrix values of the *P. hordei* type strains RAPD-PCR pattern using primer-5

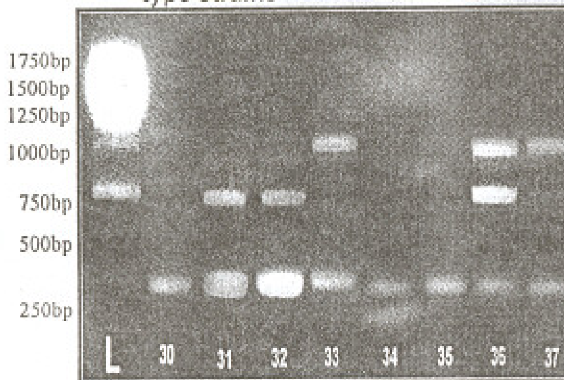

Figure 15. Banding pattern of RAPD-PCR of *P. hordei* type strains using primer-6. Lane L; molecular weight marker 250bp ladder; lanes 30-37 *P. hordei* type strains

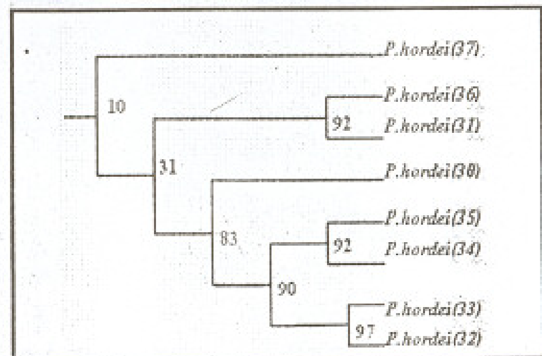

Figure 16. The phylogenetic tree developed from the similarity matrix values of the *P.hordei* type strains RAPD-PCR pattern using primer -6

categorize the investigated *P.hordei* ensuring the value of these markers as exceptional techniques in the intraspecific grouping of these penicillia.

DISCUSSION

In recent years, there has been substantial progress in the development of innovative methods to analyze fungi (and other organisms) at the molecular level as well as at biochemical level **(Jianping, 2008)**.

Referring to the pointed out results of the VM profile as a chemotaxonomic marker for the eight studied *P.hordei* type strains of the present work, since, no specific volatile compound was unique to a specific isolate of the studied *P.hordei* strains. These fallouts could be consistent with **(Borjesson, 1993)**, who concluded that VM couldn't be used in the classification of penicillia if few isolates of the same species investigated since significant differences could be observed between isolates of the same species. As well, a major reason why some literatures reports question the use of fungal metabolites in taxonomy is probably the use of only one or very few isolates of the species studied. Furthermore, some of these few isolates might be incorrectly identified **(Frisvad and Samson, 2004)**. These results might be concurrent with the theory that "if some of the few isolates used, it was found to be misidentified, then false taxonomic conclusions might be made" stated by **(Lund and Frisvad, 1994 and 1995)**.

However, the volatile profile might be useful to differentiate among these strains merely at the clade (group) level. The succession of the VM profile of summation of some isolates of the investigated strains as one species was noted.

It materialized more supportive considering the use of fatty acid (FA) outline in the chemotaxonomy of the studied eight type strains of *P.hordei*. These results were harmonized with the promising results regarding the use of FAs for the identification of filamentous fungi that have been reported for several years by many authors including **(Losel, 1989; Blomquist *et al* 1992)** they declared that "with aid of FA profile, it was possible to differentiate between various *Aspergillus*, *Mucor* and *Penicillium* species". Also, current results were in agreement with those of the FA composition of eighteen species of *Penicillium* studied by **(Smedsgaard and Nielsen, 2005)** to investigate its taxonomic usefulness. Pertaining to the perceived FAs from the eight examined *P.hordei* type strains, all which are harmonized with the results of **(Kock and Botha, 1998)** as well as **(Abu-Seidah, 2002)** who reported that the most abundant FAs produced by fungi are palmitic, palmitoleic, stearic, oleic, linoleic and linolenic acids. Relating to what previously stated at the present work concerning each of caprilic, lauric, myristic, *cis*-heptadecenoic, arachidonic, capric, linoleic and henicosanoic acids as they were useful chemotaxonomical markers specially designed for the investigated *P.hordei* strains based on the FA profile, their limited dispense throughout the studied strains could be used effectively to clarify the type strains possessed these fatty acids during identification applying these conditions from medium, extraction to analysis. These findings were in conformity with what affirmed by **(Larsen and Frisvad, 1995a)** that "those compounds for being of restricted distribution might be considered as taxonomic markers for the rapid identification of the species, however to ensure their species specificity, a number of isolates belonging to the same species needed to be analyzed".

It looked more supportive considering the use of secondary metabolite (SM) profiling in *P.hordei* chemotaxonomy. These outcomes were supported by the statement of **(Frisvad *et al* 2008)** that it was promising to use the SM profile in fungal phylogeny, because the individual metabolites have a limited distribution throughout the fungal kingdom. Hence, this is the very quality that makes SMs so useful in classification and identification. The results of the SM profile especially, (-)flavoskyrin, cinnamic acid, cyclopenin, terresteric acid, dehydrocarolic acid and viridicatin as well as the four unidentified compounds with different R_f values, that were considered to be useful as chemotaxonomical markers thanks to their limited distribution range among the explored *P.hordei* type strains, were in consistency with what stated by **(Larsen and Frisvad, 1995a)** that "those compounds for being of restricted distribution might be considered as taxonomic markers for the rapid identification of the species, however to ensure their species specificity, a number of isolates belonging to the same species needed to be analyzed". Similarly, these findings were of the harmony with the results of **(Mokhtar, 2001)** who had been reported that *P.hordei* produced a set of SMs that helped in classifying this species such as citrinin, hevalonic acid, (-)flavoskyrin, xanthocillin, cyclopenin, patulin, carlosic acid, physodic acid and palitantin.

Referring to all of the SMs detected from all of the investigated *P.hordei* type strains, they couldn't be considered here to be useful chemotaxonomical markers due to wide spreading among the testes strains, which was coherently accepted with what concluded by **(Ciegler *et al* 1973)** that "production of similar metabolic products does not provide an adequate basis for recognition of a new taxon", based on the advice of **(Frisvad and Filtenborg, 1990)** whom was the first to suggest that "only unique extrolites could be used directly in *Penicillium* taxonomy" and this was followed up by a study on many of the species in subgenus *Penicillium* by **(Frisvad *et al* 1998)**, where it was shown that only restricted extrolites were of particularly high value in the taxonomic sense. Conclusively, the succession of each of the FA and SM profiles as chemotaxonomical markers was noticed. While, the VM profile was failed at this level but considered as a good strain profile. So, the use of chemotaxonomy was not a replacement of morphology but for support, aid and enhancement.

RAPD pattern produced a profile of bands that allowed the identification of intraspecific polymorphisms among the investigated penicillia. Each primer yielded a strong distinctive pattern for the studied strains, while the number and the size of the generated fragments were entirely different from each other.

The relationships among the *P.hordei* strains developed by all of the six studied primers seemed as close to each other except for primer one. The presented results regarding the generated RAPD-PCR pattern, using six oligonucleotide primers of the genomic DNA of eight studied *P.hordei* type strains, that explained as the phylogenetic relationships and categorized the tested type strains into one big clade, except for *P.hordei* number 34, using primer one and into two clades thanks to the rest five primers are in harmony with those stated by **(Pina *et al* 2005)**, they used the combination of PCR-fingerprinting and RAPD assays to discriminate fifty eight yeast isolates from carbonated orange juice factory that showed to be very useful in tracking the route of contamination in a carbonated juice production chain. Current study and those of others **(Gil *et al* 2003, Vasdinyei and Deak, 2003; Fadda *et al* 2004)** have shown that RAPD-PCR methods performed with different oligonucleotide primers basically generated consistent patterns, with several shared fragments unique to each species. According to the current results obtained from the RAPD-PCR fingerprinting pattern and those of **(Abulhamd *et al* 2007)**, it was useful when discriminating similar organisms to consider RAPD assay as an important tool to identify as well as study the intra-specific genetic variability among several yeast isolates. Their RAPD-PCR results detected genetic diversity between related representative isolates of the same species. In addition, presented RAPD marker results evolved more rapidly than other studied markers such as fatty acid or volatile and non volatile secondary metabolites. Considering the total four markers, they all agreed in classifying the eight studied *P.hordei* type strains into three major groups. Thus, it is promising to handle the examined four markers exclusively or with each other to categorize the investigated *P.hordei* type strains ensuring the value of these markers as exceptional techniques in the intraspecific grouping of these penicillia on one growth medium. These fallouts are in consensus with the obtained results by **(Zain, 2004)** who confirmed the rationale for using more than one tool in fungal taxonomy, such as the profiles of either of secondary metabolites and fatty acids which referred to be appropriate tools for this purpose. Also, it harmonized with what declared by **(Johnson *et al* 2006)** that the fatty acid profile can confirm the molecular classification of microbes.

Considering, the entirety of the four investigated markers that fall in classifying the eight studied *P.hordei* type strains into four chief groups, it concurred with the results of **(Svendsen and Frisvad, 1994)** that a correlation in the biosynthesis of volatile and non volatile secondary metabolites probably exists for many mycotoxins-producing fungi such as penicillia. This hypothesis is strongly supported by the successful use of both non-volatile **(Lund and Frisvad, 1994; Lund and Frisvad, 1995; Lund, 1995)** and volatile secondary metabolites in the chemosystematics of penicillia **(Larsen and Frisvad, 1995a and 1995b)**.

Hence, it was possible by using the investigated four markers solely or with each other to discriminate between the eight investigated *P.hordei* type strains ensuring the usefulness of these markers as extraordinary techniques in the intraspecific taxonomy of these penicillia.

This work revealed that with the harmony of all the four investigated markers; the profiles of the fatty acid, secondary and volatile metabolites, as well as RAPD-PCR pattern, about all of the investigated type strains could be classified correctly at the intraspecific level using only the analysis of metabolites produced on one growth medium (YES), except in case of the volatile profile which succeeded as a cladogenetic profile but not as a strain marker.

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حوليـات العلـوم الزراعية
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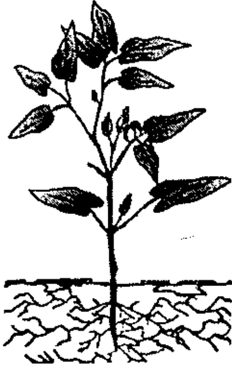

مراجعة بيوكيميائية لفطرة *Penicillium hordei*

[٢]

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الموجـــز

اهتم هذا البحث بدراسة عدة دلالات بيوكيميائيـة وجزيئية لثمانيـه سـلالات نموذجيـة مـن جـنس *Penicillium* والتى تنتمى الى نوع *P.hordei* وذلك على بيئة غذائية موحدة وهى بيئة مستخلص الخميرة والسكروز (YES) وذلك لمعرفة مدى الاستفادة مـن هذه الخصائص كدلالات تصنيفية لتعريف هذا الجنس على وجه العمـوم وهـذه السـلالات علـى وجـه الخصوص.

وكانت الأربع دلالات التصنيفية هـم : محتـوى المركبات الأيضية الطيارة ومحتوى الأحماض الدهنية والمحتوى الأيضي الثانوى بالاضافة الى دراسة تقنية التضخم العشوائى عديد الأوجه المعتمد على تفاعـل البلمرة المتسلسل RAPD-PCR وذلك باستخدام سـتة أنواع متابينة من البادئات عشوائية التتابع لاستخدامهم كنموذج بصمة الإصبع للتفرقة بين السـلالات محـل الدراسة .

وقـد تبين نجـاح الأربـع دلالات المـستخدمة كـدلالات تصنيفية قويـة للتفرقـة بـين العـزلات المدروسة من هذا الجنس على منبت غـذائى موحـد لهذه الدلالات . وذلك فيمـا عـدا محتـوى المـواد المتطايرة وذلك نظراً لقلـة عدد العزلات المتاحة من هذا النوع.

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