ECOLOGIA DOS FUNGOS

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Metabolic response of filamentous fungi to preservation stress

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Introduction

Important fungal isolates, for example, ex-type strains and isolates important to academia and industry are routinely preserved and stored in national or "in-house" collections. This serves a number of purposes, it provides a permanent reference for other scientists; provides a back-up in case of difficulty with manufacturing strains; satisfies patent and legal considerations concerning the requirement to deposit strain(s) as part of patent publication under the terms of the Budapest treaty and allows "traceability of sovereign rights" respecting the convention on Biological Diversity (CBD) (Smith *et al.*, 2001).

The main aim of the long-term preservation of microorganisms is to secure not only the viability of the organism preserved but also to retain its physiological and genetic stability throughout storage. The basis of preservation procedures is to bring the organism to a state of dormancy, where the metabolic activity is minimal. There are various methods available. The rate of metabolism can be slowed either by cold (*e.g.* maintenance at 4 °C), reduced oxygen tension (*e.g.* storage under mineral oil) or water removal (*e.g.* drying over a desiccant such as silica gel). Freeze-drying relies on the removal of water under vacuum by the sublimation of ice from the frozen material. Freezing and storage at ultra-low temperatures (*e.g.* at –196 °C in liquid nitrogen) will suspend biochemical reactions and therefore, metabolism. In all these processes the microorganism is placed under stressful conditions and damage may occur. Individual fungi will respond differently depending on the taxonomic group and physiological condition. To achieve optimum survival and stability it is essential to understand the response of the fungus cells to the stresses introduced by preservation.

Retention of metabolic stability is necessary for many scientific and industrial applications and is imperative for isolates used in the commercial production of

biochemicals, chemotaxonomy investigations, initiation of pathogenisis by biological control agents and in bioassays. Therefore, it is essential that back-up strains are available and that isolates that have been preserved and stored in culture collections have retained their metabolic integrity. However, despite the relative security provided by collections, there have been few attempts to determine the subsequent physiological stability of characters following resuscitation from preservation.

Examples of changes in secondary metabolite production following preservation have been made on chance observations. For example, Svendsen and Frisvad, (1994) found that two strains of *Penicillium camembertii* that were reported by Bridge *et al.*, (1989) to produce citrinin, did not produce citrinin in their investigation. It has also been suggested that strain degradation of cultures subjected to continual sub-culture may be correlated with changes in secondary metabolite production. Here, we present two case studies concerning the detection of the mycotoxins patulin and citrinin in *Penicillium expansum*, before and after preservation, and the effects of preservation regime on secondary metabolite production in *Metarhizium anisopliae* and *Fusarium oxysporum*.

Patulin and citrinin production by *Penicillium expansum*

The mycotoxins patulin and citrinin have been reported as characteristic secondary metabolites of *Penicillium expansum* (Frisvad and Filtenborg, 1989). This organism is considered a broad-spectrum pathogen on fruits (Snowdon, 1990), causing extensive rot especially in apples and pears. During a study of mould contamination of grapes and the assessment of the mycotoxin production capability of the isolates, Abrunhosa *et al.* (2001) isolated and screened 51 *Penicillium expansum* strains. These strains exhibited different profiles according to the ability to produce patulin, citrinin or both in relation to the culture media used for growth and assessment. In order to evaluate the influence of different culture preservation techniques on the production of patulin and citrinin, we studied 10 representatives of the isolated strains before and after being submitted to subculture and maintenance at 4 °C, preservation under mineral oil, drying on silica gel and freeze-drying.

Culture conditions and mycotoxin detection

The *Penicillium expansum* Link isolates (MUM 99.19, 99.20, 99.22 to 99.24 and 00.01 to 00.05), were cultured on malt extract agar (MEA) for 7 d at 25 °C for preservation. Preservation under mineral oil, drying on silica gel and freeze-drying are fully described in Smith and Onions (1994). The preserved cultures were revived at defined time periods, of 0.5, 2-3, 6, and 12 months, using MEA, yeast extract sucrose agar (YES), and grape juice agar (GJ). After incubation for 7 d at 25 °C, the cultures were transferred to YES and GJ for mycotoxin detection. Mycotoxin profiles were analysed, before and after the application of the preservation techniques, using thin layer chromatography (TLC) as described by Singh *et al.* (1991). Agar plugs were cut from 14 day old cultures on YES and GJ, and applied to silica gel 60 TLC plates without indicator (Merck). The solvent system used was TEF (toluene: ethyl acetate: formic acid; 5:4:1, v/v/v). For patulin detection the plates were sprayed with 5 gl^{-1} MBTH (3-methyl-2-benzothiazoline hydrazone hydrochloride), dried for 15 min and heated at 110 °C for another 15 min. Standards used were griseofulvin and patulin. GJ medium was also assessed for the mycotoxins, as a control.

Citrinin and patulin profiles

Citrinin could be visualised under ultra-violet light (366 nm) as a yellow green streak with a medium Rf of 42 (Fig. 1L), while patulin was visible after treatment with MBTH as a yellow spot under white light with a medium Rf of 35 (Fig. 1R). Citrinin, was detected in all cultures by all techniques on YES agar but not repeatably on GJ. Before preservation, only strain MUM 00.01 produced citrinin on GJ as previously observed by Abrunhosa *et al.* (2001). After preservation citrinin was detected on GJ also for strains MUM 99.19, 99.20, 99.23, 99.24 and 00.04, but citrinin production was not very consistently expressed. The more steady behaviour seems to have been observed for the freeze-dried strains MUM 99.19 and 99.20. Strain MUM 99.19 failed to produce detectable levels of citrinin on YES after 12 months preservation by subculture and maintenance. Nevertheless, citrinin production seems to be a stable character for the studied strains.

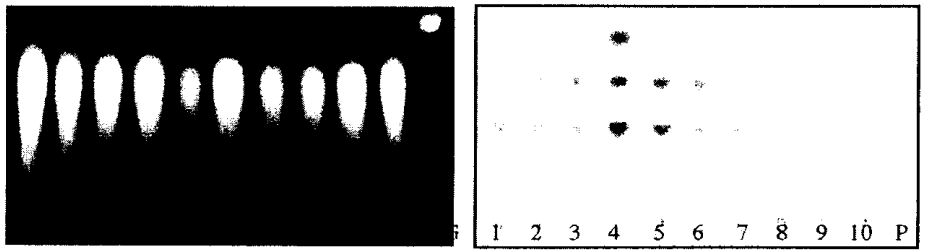

Figure 1. TLC plates of the 10 studied strains: 99.24, 99.22, 99.19, 00.01, 99.23, 99.20, 00.02 to 00.05; O: Ochratoxin A, G: griseofulvin, P: patulin. Left (L) – after 3 months preservation on silica gel; right (R) – after 0.5 months freeze-drying.

Three different profiles were encountered regarding to patulin production, represented in Table 1 by strains MUM 99.19, 99.23 and 00.05 respectively.

Table 1. Detection of patulin for strains MUM 99.19, 99.23 and 00.05 before and after 0.5, 2-3, 6 and 12 months (M) preservation by subculture (SC), mineral oil (MO), silica gel (SG) and freeze-drying (FD).

		Culture medium																			
		MEA/YES					MEA/GJ					YES/YES					GJ/GJ				
Strain	Time (M) Method	0	0.5	2*-3	6	12	0	0.5	2*-3	6	12	0	0.5	2*-3	6	12	0	0.5	2*-3	6	12
99.19	SC	+	nd	+	+	-	+	nd	+	+	-	+	nd	+	+	-	+	nd	+	+	-
	MO	+	+	-	+	-	+	+	-	+	-	+	+	+	+	-	+	+	+	-	-
	SG	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	FD	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
99.23	SC	-	nd	+	+	-	+	nd	+	+	+	-	nd	+	+	-	+	nd	+	+	+
	MO	-	-	-	-	-	+	+	+	+	-	-	-	+	+	-	+	+	+	+	-
	SG	-	-	+	+	-	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+
	FD	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
00.05	SC	-	nd	+	+	-	-	nd	-	+	+	-	nd	-	-	+	-	nd	+	+	+
	MO	-	-	-	+	-	-	+	+	-	-	-	+	-	+	-	-	-	+	-	-
	SG	-	+	-	+	-	-	+	-	+	-	-	-	-	+	+	-	+	+	+	+
	FD	-	+	-	+	+	-	+	-	+	+	-	+	-	+	+	-	-	+	+	+

* Subculture only

Both YES and GJ gave positive results for some strains, represented in Table 1 by strain MUM 99.19, and this behaviour is maintained in a quite consistent manner throughout the experiment, regardless of the preservation technique. Nevertheless, silica gel and freeze-drying seem to give more stable results, particularly if the strains are grown on GJ for mycotoxin assessment. In the particular case of strain MUM 99.19 patulin was not detected in either culture media after 12 months of subculture and maintenance at 4 °C. A tendency towards non-detection of patulin also applies to storage under mineral oil. In contrast, strain MUM 99.23 (Table 1) was originally patulin negative in YES medium whereas in GJ patulin was always detected with the exception of 12 months preservation under mineral oil. In YES medium the tendency is towards patulin detection with time. This is very consistent for the freeze-dried samples. Finally, in the case of strains MUM 00.05 and 00.02 to 00.04, patulin was not detected in either culture media before preservation. Patulin is however detected after preservation but with alternating patterns. A more homogeneous behaviour seems to be found when

cultures are revived and cultured on GJ in the case of freeze-drying and silica gel storage. All the 10 strains studied failed to produce detectable levels of patulin after 12 months preservation under mineral oil. Neither patulin nor citrinin were detected on the GJ control. Furthermore, all the strains were viable after one-year preservation for all the tested methods.

Considering the three distinct behavioural groups found for patulin and the overall results obtained, variability in the profiles of the mycotoxins tested seems to be more strain specific than dependent on the preservation technique used. Evidence supporting the assumption that response to preservation may be strain specific was also found by Ryan *et al.* (2001). Nevertheless, and although there is not a marked evidence, higher consistency seems to be found for freeze-drying and silica gel storage, especially, in the case of patulin, if the strains are cultured and/or revived on GJ.

Secondary metabolite synthesis is often related to the depletion of nutrients (Griffin, 1994) and with possible ecological roles in nature, as well as in, or at least coincident with, differentiation (Betina, 1989). Here, mycotoxin synthesis seems to appear as a response to preservation. This may account for the fact that long-term deposits of *Penicillium expansum* strains in culture collections, which have not become atypical with possible loss of mycotoxin production, are often regarded as patulin and citrinin producers.

The results also show that there is a potential for patulin detection on natural culture media. Some of the strains showed a marked preference for GJ. This is important when assessing the probability of mycotoxin production in natural substrates. It has been shown that grapes are able to support patulin production by natural fungal populations (Scott *et al.*, 1977). Abrunhosa *et al.* (2001) reported on 65% *Penicillium expansum* isolates positive for patulin on GJ against 20% positive on YES. The fact that citrinin is preferentially produced in YES is probably related to the composition of the culture media, as citrinin is not generally detected in grape products or is unstable (Scott *et al.*, 1977).

The best preservation method is considered to be the one where no growth and reproduction can take place, but where all the structural and functional characteristics are retained. While maintenance by subculturing and mineral oil storage allow for growth and metabolism, to a higher or lesser degree, drying on silica gel, freeze-drying and cryopreservation below –140 °C are recommended as the most appropriate methods for the long-term preservation of sporulating filamentous fungi (Smith, 1993). In this study, loss of secondary metabolite production occurred in subculture and maintenance at 4 °C, and storage under mineral oil, whereas silica gel storage and freeze-drying are the methods that present higher consistency in the cases where secondary metabolite production appears as a

response to preservation. Taking into account the different responses that may be obtained when using different preservation techniques it is always advisable to use more than one method, particularly if industrial or test strains are at stake.

Secondary Metabolite Production in *Fusarium oxysporum* and *Metarhizium anisopliae*

The effects of five commonly used preservation regimes on secondary metabolism of two genera of economically important fungi, *Fusarium oxysporum*, a phytopathogenic, mitosporic fungus, and *Metarhizium anisopliae*, an entomopathogenic, mitosporic fungus, were assessed over a two year testing period. The tested presevation methods were continual sub-culture, freeze-drying, storage of mycelial plugs in water, storage at –20 °C and cryopreservation at –196 °C. Physiological stability was assessed using thin layer chromatography and high performance liquid chromatography of secondary metabolites.

Culture maintenance and selection

Three isolates each of *Fusarium oxysporum* Schlecht., IMI370367, UKCK19, UKCK54 and *Metarhizium anisopliae* (Metschnikoff) Sorokin., CCMa9107, CCMa9233, IMI382472, were studied. Each isolate was obtained specifically for the programme and had undergone no more than 2-3 previous sub-cultures on an agar medium. Archive material of all isolates is now stored in liquid nitrogen at CABI Bioscience.

Preservation

Five preservation methods were selected: continual sub-culture, freeze-drying [two-stage centrifugal with 10% (w/v) skimmed milk / 5% meso-inositol (w/v) (BDH) as a lyoprotectant], storage of mycelial plugs in water, storage at –20 °C and cryopreservation (with controlled cooling at –1 °C per min and 10% glycerol (BDH) as a cryoprotectant). The methods for freeze-drying and cryopreservation are fully described in Smith and Onions (1994). Continual sub-culture was by mycelial transfer onto fresh media in Petri dishes every two months from cultures maintained on a maintenance medium which did not promote excessive sporulation [Synthetic Nutrient Agar (SNA, Nirenberg, 1981) for *Fusarium* isolates and Malt Agar (MA, Smith and Onions, 1994) for *Metarhizium* isolates]. Storage at –20 °C involved the storage of 35-day old slope cultures on maintenance media in a freezer at –20 °C without cryoprotectants. Storage of mycelial plugs in water involved cutting mycelial plugs with a 5 mm diameter cork-borer from plate cultures maintained on maintenance media for 28 d and then submersing them in 10 ml of sterile distilled water contained in glass universal bottles.

Testing periods

Up to 5 replicates of each isolate and preservation treatment were removed from storage for secondary metabolite analysis after 1 week, 16 weeks, 1 year and 2 years preservation. After removal from storage replicates were not re-preserved. All biochemical tests were duplicated.

Thin Layer Chromatography

The methods used were followed from Paterson and Bridge (1994). Except, isolates were grown in the dark on yeast extract-sucrose (YES) (Scott *et al.*, 1970) agar for 21 d at 21°C for *Fusarium* isolates and 28 d at 25 °C for *Metarhizium* isolates. Agar plugs were randomly cut from the colony of 21 d old cultures of representative cultures of each line with a cork borer (5 mm diam.) in reduced light conditions to prevent photo-oxidatio). Griseofulvin (1 µg/ml; Sigma) in a 2:1 (v/v) mixture of chloroform/methanol was also applied to the plate as a reference compound of known Rf. To ensure correct classification of metabolites, profiles were compared after the mean and standard error Rf values were calculated for individual spots showing similar properties.

High Performance Liquid Chromatography

Isolates were grown as described for TLC. The contents of each plate were extracted into 50 ml of HPLC grade methanol (BDH) using a vertical macerator at high speed. Extracts were then rotary evaporated and re-suspended in 5 ml of HPLC methanol and stored at 4 °C until required. Extracts were run on an HPLC (Varian 5000) with a wavelength detector at 210 nm through a Spherisorb C18 column. A volume of 50 ml was injected at a flow of 1.5 ml/min. The gradient solvent system was solution A (0.1M Ammonium dihydrophosphate: 3 ml/l Phosphoric acid) and solution B (75% Acetonitrile: 25% 0.1M Ammonium dihydrophosphate: 3 ml/l Phosphoric acid). The solvent programme for the A/B mixture was: A at 100% initially, lowered to 0% in 20 min, held for 10 min, raised to 100% in 2 min and held for 5 min. Mobile phases were prepared with MilliQ 18 ohm water, filtered through a Noorganic cartridge (Whatman, UK).

Statistical analysis

HPLC and TLC profiles were visually compared and a binary matrix constructed by scoring peaks/spots (1 when peak present, 0 when peak absent). Dendrograms were then constructed using hierarchical cluster analysis and unweighted average linkage on a percentage scale, with the statistical package Statistica5.

Fusarium oxysporum

Long-term stability of secondary metabolite profiles was influenced by preservation regime in all three isolates of *Fusarium oxysporum*, throughout the two-year investigation. Some strains were more severely influenced by preservation than others. For example, no replicates of isolate UKCK54 exhibited the secondary metabolite profile exhibited by the original isolate at any time following preservation by any of the five preservation methods used. Whereas, 89% of replicates of isolate IMI 370367 retained the secondary metabolite profile exhibited by the original isolate after preservation for one year. However, after 2 years of preservation, the number of replicates of isolate IMI370367 exhibiting a secondary metabolite profile typical of the original isolate was reduced (45%) as compared to 89%.

Table 2. Stability of secondary metabolite profiles after 1 year of preservation of *Fusarium oxysporum* (number of replicates exhibiting typical and atypical secondary metabolite profiles)

	CS		*FD*		*MP*		*FZ*		*LN*	
Isolate code	T	A	T	A	T	A	T	A	T	A
IMI370367	5	0	3	0	4	1	3	0	2	1
UKCK19	1	4	0	3	3	2	3	0	1	2
UKCK54	0	5	0	5	0	5	0	5	0	5

T = Typical profile (i.e. identical to the metabolite profile exhibited by the original 'unpreserved' isolate)

A = Atypical profile (i.e. different to that exhibited by the original isolate)

(CS, continual sub-culture; FD, freeze dried; MP, mycelial plugs in water; FZ, freezing at -20 °C; LN, cryopreservation.)

Metarhizium anisopliae

Preservation method affected secondary metabolite production in replicates of all *Metarhizium anisopliae* isolates throughout the 2 year investigation. For example, after one year of preservation (Table 3) only replicates of one isolate from one preservation treatment did not exhibit differences in the characteristic 'typical' secondary metabolite profile exhibited by the original 'un-preserved' isolate.

Table 3. Stability of secondary metabolite profiles after 1 year of preservation of *Metarhizium anisopliae* (number of replicates exhibiting typical and atypical secondary metabolite profiles)

	CS		*FD*		*MP*		*FZ*		*LN*	
Isolate code	T	A	T	A	T	A	T	A	T	A
CCMa9107	1	4	2	2	1	4	–	–	3	1
CCMa9233	0	5	3	0	1	4	2	1	2	1
IMI382472	4	1	4	1	0	2	5	0	3	1

T = Typical profile (i.e. identical to the metabolite profile exhibited by the original 'unpreserved' isolate)

A = Atypical profile (i.e. different to that exhibited by the original isolate)

(CS, continual sub-culture; FD, freeze dried; MP, mycelial plugs in water; FZ, freezing at -20 °C; LN, cryopreservation.)

A 'one-off' HPLC assessment of secondary metabolite production by replicates of *Metarhizium anisopliae* IMI 382472 after 16 weeks of storage (Fig. 2) yielded some interesting results. Replicates of four of the preservation treatments clustered together on the dendrogram (Fig. 2). The most homology between the profiles within preservation treatments was amongst those that had been stored by cryopreservation, the least homology was exhibited by replicates that had been stored by mycelial plugs in water and by replicates maintained by continual subculture.

The secondary metabolite profiles of *Fusarium oxysporum* and *Metarhizium anisopliae* were changed by preservation regime throughout the investigation. Secondary metabolism appears to be extremely sensitive in response to different preservation regimes. Even after relatively short storage times (< 1 week) the profiles of many replicates preserved by various preservation treatments were

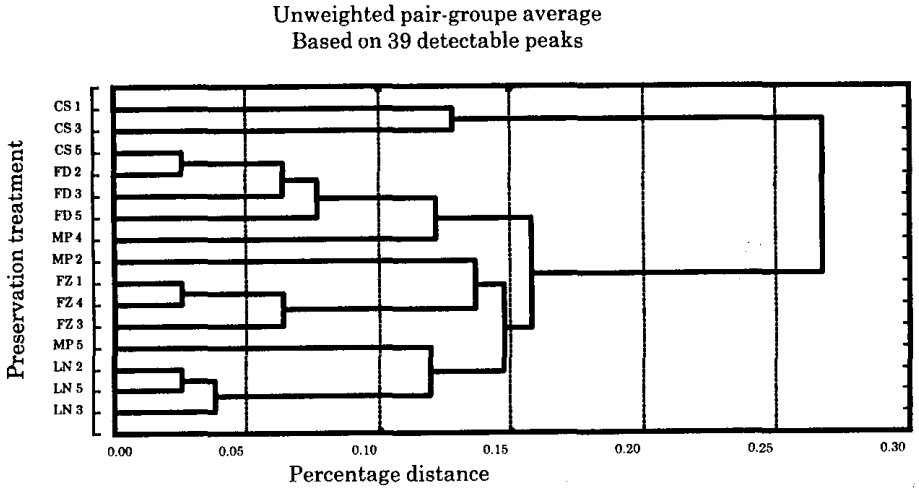

Figure 2. Dendrogram compiled from HPLC profiles of *Metarhizium anisopliae* IMI 382472 after 16 weeks of storage. CS, continual sub-culture; FD, freeze-dried; MP, mycelial plugs in water; FZ, freezing at -20 °C; LN, cryopreservation.

different from the profiles that were exhibited by the original isolates before preservation. In replicates stored by methods that do not totally suppress metabolism (continual sub-culture, storage under water or in a domestic-type freezer at –20 °C) there is an increasing deterioration from the characteristic profiles exhibited by the original un-preserved isolates. This was apparent in all replicates of all isolates of fungi preserved by these methods during this investigation. In replicates stored by methods that apply an almost total degree of metabolic suspension (cryopreservation and freeze-drying) many of the replicates exhibited a characteristic profile, irrespective of the length of storage. However, in some replicates disruption was evident after only short storage times. This would suggest that it is the actual physical preservation process that can exert damage rather than the length of storage *per se*.

The results suggest that storage in water and storage at –20 °C may provide the best short-term (<16 weeks) preservation of secondary metabolite activity. However, these protocols are not at all suitable for long-term preservation (>16 weeks) as the stability of secondary metabolite profiles rapidly deteriorates. Although both cryopreservation and freeze-drying can induce changes in secondary metabolite production, they are the most suitable long-term protocols for maintaining the stability of metabolite profiles in the long-term.

The consequences of compromised metabolic stability are potentially disastrous. For example, if metabolically 'unstable' cultures are used in chemotaxonomic investigations, 'false' results could result in an unreliable characterisation. Recent research suggests that cryopreservation regimes can be optimised for individual strains (Smith and Thomas 1998) and this may reduce the prospects of instability in metabolite production, indeed fungal gene function generally (Ryan *et al.*, 2001).

Conclusions

The preservation regime can influence secondary metabolite production in the test fungi. Changes in secondary metabolite profiles can occur after relatively short storage periods, irrespective of the preservation treatment used. The results also indicate that response to preservation and storage can be different among strains of the same species. It is also apparent that variability in secondary metabolite profiles after preservation may be strain specific and therefore less dependent on the preservation technique used. No preservation treatment can be guaranteed to provide total stability of secondary metabolite production. Although higher homology and greater consistency in profiles was found for cryopreservation at –196 °C and freeze-drying, there is still a need to develop new and existing preservation criteria with emphasis on strain-specific criteria to reduce the prospects of instability in secondary metabolite production. Culture collections are committed to improving procedures and standards through a continuing programme of research and development.

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