# VIABILITY OF *COCHLIOBOLUS SATIVUS* CULTURES AFTER STORAGE UNDER DIFFERENT CONDITIONS

**M.I.E. Arabi, M. Jawhar and A. Al-Daoude**

*Department of Molecular Biology and Biotechnology, AECS, P.O. Box 6091, Damascus, Syria*

## SUMMARY

Different storage methods were tested to examine their usefulness in viably maintaining field isolates of *Cochliobolus sativus*. Fungal isolates were stored at (*i*) 4°C in sand, (*ii*) in silica gel at 4°C (*iii*) on potato dextrose agar (PDA) medium at 4°C, or (*iv*) frozen as conidia at -20°C. The cultures were assessed after 12 and 24 months of storage. Cultures of all isolates were viable after two years of storage in sand and silica gel at 4°C or dry at -20°C, whereas, isolates stored on PDA were not viable and heavy contamination with bacteria was frequently observed. After two years storage, isolates were able to infect barley leaves but sporulation was reduced dramatically, depending on the isolate and the storage method employed. AFLP analysis revealed several genetic changes between stored and non-stored isolates. A total of 444 scorable DNA bands were obtained; 324 of these (71%) were polymorphic. However, the proportion of DNA alterations varied according to the storage method.

*Key words*: *Cochliobolus sativus*, barely, spot blotch, storage methods, AFLP markers.

## INTRODUCTION

Fungal collections conserve living specimens (Karen *et al.*, 2004) without which there would be no stocks of important or rare strains that are so valuable for biotechnology and biomedical research. Therefore, the discovery of new fungi of relevance to plants requires that isolates should be preserved in stable condition for present and future use.

There are many methods available to preserve fungal isolates. Continual sub-culture is the most common but is not suitable for extended storage because of strain drift. Longer-term methods involve storage under mineral oil or water (or as mycelial plugs in water) and freezing at -20°C. Alternatively, fungi can be dehydrated in the presence of silica gel or stored in sterile sand or soil (Smith and Onions, 1994). However, although lyophilization and cryopreservation of living cells provide efficient mechanisms for stabilizing cells over long periods of time, these procedures are technically complex, lengthy and require expensive equipment (Stalpers *et al.*, 1987, Smith and Thomas, 1998). Recent research has suggested that some strains subjected to preservation may be damaged. Furthermore, long-term stability is critical if the isolate is to be used by the biotechnology industry (Ryan *et al.*, 2000).

*Cochliobolus sativus* (Ito and Kuribayashi) Drechs. ex Dastur [anamorph: *Bipolaris sorokiniana* (Sacc.) Shoem], the causal agent of spot blotch of barley (*Hordeum vulgare*) is a common foliar pathogen worldwide. It has a wide host range and it is economically important also on wheat (Mathre, 1982; Nutter *et al.*, 1985). Since the variability of the fungus is well documented (Fetch and Steffenson, 1994; Arabi and Jawhar, 2003; 2004) a storage method which conserves the isolate's characteristics should be used.

The objective of the current research was to estimate the viability and genetic changes of some *C. sativus* isolates after being stored for two years in sand, silica gel, or potato dextrose agar (PDA) medium at 4°C, or frozen at -20°C.

## MATERIALS AND METHODS

**Fungal isolates**. The *C. sativus* isolates used in this study were collected in 2000 and 2001 from naturally infected barley leaves from different locations in Syria. The fungus was grown separately in 9 cm Petri dishes containing potato-dextrose agar (PDA, Difco, Detroit, MI, USA) for 10 days at 22 ± 1ºC in the dark to allow mycelial growth. After the positive isolation of *C. sativus*, three different isolates based on morphological (mycelial growth rate, colour) and physiological criteria (virulence) were selected. Colonies produced from single spores were used as pure isolates.

*Corresponding author*: M.I.E. Arabi
Fax: +963.11.612289
E-mail: miaarabi@aec.org.sy

**Storage methods.** Mycelium and conidia of each selected isolate were produced on PDA. Four different methods of storage were tested for the first three of which, we used Pyrex glass tubes (Duran, Germany, 150 × 20 mm) subsequently sealed with cotton plugs and parafilm, and stored at 4°C. Subsequently: (i) aliquots of 1 ml of aqueous concentrated spore suspension were added to tubes containing fine sand (sea sand was passed through a sieve 1mm diam) up to 1/2 volume of the tubes; (ii) a concentrated spore suspension of each isolate was slowly added to tubes containing sterile silica gel; (iii) slants of PDA (15 ml, supplemented with 13 mg/L kanamycin sulphate) were inoculated with each isolate, incubated at 23 ± 1°C in the dark for 5 days prior to storage; (iv) conidia of each isolate were taken from PDA cultures with a sterile needle and placed in sterile 2 ml Eppendorf tubes containing 1ml of 20% glycerol. Tubes were then stored at -20°C.

**Pathogenicity tests.** Pathogenicity tests of all stored isolates and controls (non-stored) were performed on WI 2291, the universal susceptible cultivar from Australia, using the method described by Arabi and Jawhar (2003). Disease ratings were recorded 10 days after inoculation using a 0-9 scale of Fetch and Steffenson (1999). This scale considers both the size and the type (degree of chlorosis/necrosis) of lesions. It has been useful for classifying infection responses in many types of study in the barley spot blotch pathosystem (Fetch and Steffenson, 1999). The experiments were replicated three times. The STAT-ITCF program (Anonymous, 1988) was employed to determine significant differences among mean disease rating values.

**Viability assessment.** Viability of each isolate was assessed after 1 and 2 years of storage. Viability was determined by inoculating each culture onto 9-cm plastic Petri dishes containing PDA followed by incubation at 23 ± 1°C in the dark for three days. Mycelium growth indicated that the culture was viable.

To quantify conidial production on unit leaf area, five leaf disks of the barley susceptible genotype WI 2291 were placed in one Petri dish containing agar medium. Disks were inoculated with actively-growing mycelial plugs (5mm diameter) and incubated at 23 ± 1°C in the dark. Each isolate/storage method was tested three times. Spore production was quantified after 12 days by immersing the leaf disks of each plate in individual flasks containing 100 ml distilled water. The number of conidia was estimated using a haematocytometer. Five sub-samples per flask were counted, and the number of conidia per $cm^2$ of lesion was determined.

**AFLP.** Amplified fragment length polymorphism (AFLP) analysis was done as described by Vos *et al.* (1995) with a few modifications. Pre-amplification was performed using E+A and M+C primers (*Eco*RI and *Mse*I) containing one selective nucleotide at the 3` end to reduce the number of amplified fragments. The sequence of oligonucleotide adapters and primers used shown in Table 3. Pre-amplification was carried out in an 8.5 µl volume containing 125 ng of DNA using the following cycling parameters: 20 cycles of 30s at 94°C and 40 sec at 56°C and 50 sec at 72°C. The pre-amplified DNA was diluted 10 times by adding $ddH_2O$ and 2.5 µl was used as a template for the consequent selective amplification in which *Eco*RI and *Mse*I primers with three selective nucleotides were deployed. DNA was amplified under the following conditions: 12 cycles with annealing from 68°C to 59.6°C (decreasing 0.7ºC each cycle), then 23 cycles at 59°C annealing temperature. In each cycle, the denaturation and elongation steps were the same: 94ºC for 30 sec and 72°C for 1 min. AFLP analysis was repeated twice. PCR products were mixed with 4 µl loading buffer (98% formamide, 10 mM EDTA, 0.025% xylene cyanol, and 0.025% bromophenol blue) and denatured for 3 min at 95°C. AFLP fragments were separated on 6% polyacrylamide gel with 7 M urea, and 1X TBE buffer (12.1 g Tris, 5.11 g boric acid, 0.37 g EDTA). Gels were run at a constant 1500 V, 80 W, 100 mA, for 3 h and 20 min, and visualized using silver staining method (Silver Sequence kit, Promega, Corp, Madison, WI, USA).

All AFLP gels of stored and non-stored (controls) isolates were scored as presence (1) or absence (0) of a specific band for every fungal isolate. The data for all 12 different primer combinations were used to estimate similarity on the basis of number of shared amplification products according to Nei and Li (1979).

## RESULTS AND DISCUSSION

Cultures of all isolates were viable after two years of storage in sand and silica gel at 4°C or dry at -20°C. Isolates stored on PDA were not viable and heavy contamination with bacteria was frequently observed (Table 1).

**Table 1.** Viability of *C. sativus* isolates stored under different conditions for one or two years.

| Storage method[a] | Isolate | | | | | |
|---|---|---|---|---|---|---|
| | Cs16 | | Cs 15 | | Cs5 | |
| | 2 Year | 1 Year | 2 Year | 1 Year | 2 Year | 1 Year |
| PDA | C | C | - | - | - | -[a] |
| Silica gel | + | + | + | + | + | + |
| Sand | + | + | + | + | + | + |
| Dry | C | + | + | + | C | + |

[a]: No mycelial growth; +: mycelial growth; C: contaminated culture.

**Table 2.** Mean disease rating of stored and non-stored isolates of *Cochliobolus sativus* on barley cv. WI 2291.

| Isolate | Storage method | | | |
|---|---|---|---|---|
| | Non-stored (con.) | Silica gel | Sand | Dry |
| Cs5 | A7.53a[a] | B4.64a | B5.00a | B4.00a |
| Cs15 | A6.24b | B2.33c | B5.00a | B3.33b |
| Cs16 | A4.67c | B3.00b | B2.67b | B3.33b |

[a] Means proceeded by the same capital letter (line) and followed by the same small letter (column) are not significantly different (P<0.05) according Newman-Keuls test.

All isolates continued to infect barley leaves, even after 2 years of storage. However, the pathogenicity was significantly ($P$<0.05) reduced in isolates stored in different ways as compared with controls (Table 2). Figure 1 shows that reduction in conidia production of isolates depended on both storage method and isolate. However, isolate Cs5 stored for 2 years in silica gel produced fewer conidia compared with its original and with other storing methods. The number of conidia produced by isolates was reduced by storage, as observed after 1 and 2 years (Fig. 1).

Complex AFLP patterns were obtained using 12 primer combinations, and genomic similarity analyses are summarised in Fig. 2. A total of 444 DNA bands were scored with an average of 37 bands per primer combination; 324 bands (71%) were polymorphic. Some differences were found between stored and non-stored isolates. The silica gel and dry methods had little effect on genomic stability but storage in sand caused more DNA changes than other methods tested (Table 4). These results are in good agreement with those obtained by Holden and Smith (1992) who reported a risk

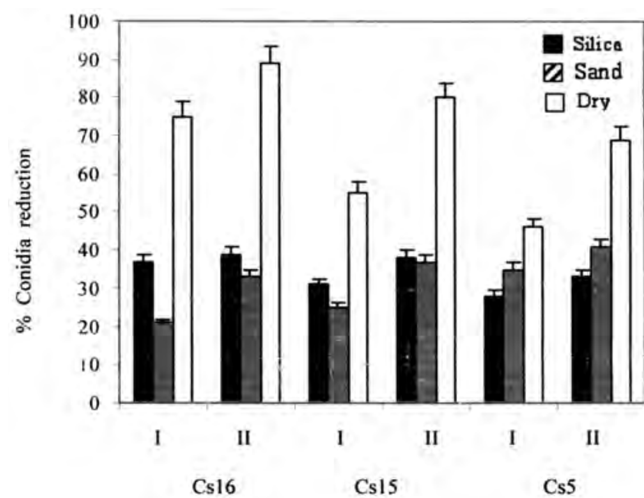


**Fig. 1.** Percent reduction in conidia production of *C. sativus* on an infected leaf over initial values after 1 and 2 years storage using different methods. Data significant at $P > 0.05$.

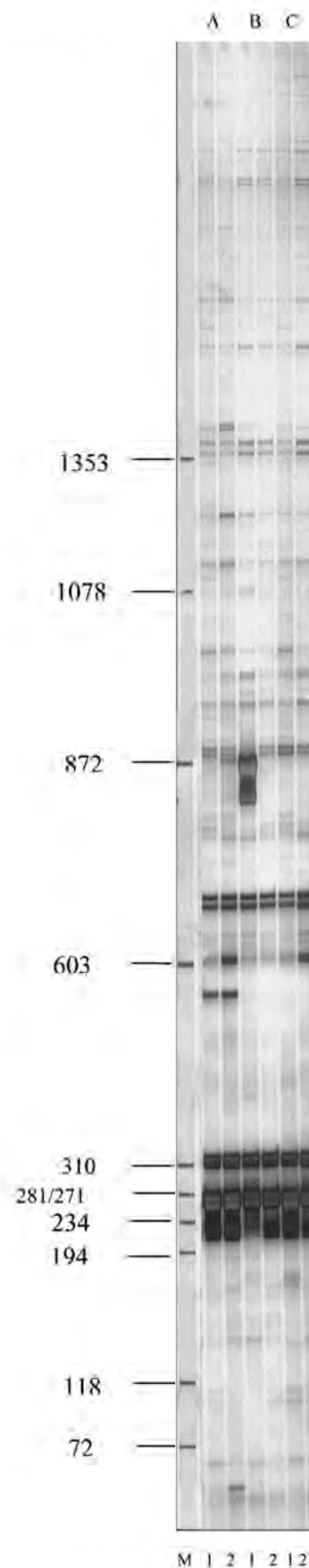


**Fig. 2.** A portion of silver stained AFLP gel generated from Cs5 isolate of *C. sativus* stored under different methods (A: Silica gel, B: Sand and C: Dry) using *Eco*RI+ACA/*Mse*I+CTA primer combination. 1 = stored; 2 = non-stored isolate. M: IX marker (Roche Diagnostic, Indianapolis, USA).

**Table 3.** Sequences of oligonucleotide adapters and primers used in the pre amplification step and the selective AFLP primer combinations.

| Name | Reaction | Code | Sequence |
|---|---|---|---|
| *Eco*RI adapter | Ligation | | 5′-AATTGGTACGCAGTCTAC-3′ |
| | | | 3′- CCATGCGTCAGATGCTC-5′ |
| *Mse*I adapter | | | 5′-TACTCAGGACTCAT-3′ |
| | | | 3′-GAGTCCTGAGTAGCAG-5′ |
| *Eco*RI + A | Pre-amplification | E-A | 5′-GACTGCGTACCAATTC**A**-3′ |
| *Mse*I + C | | M-C | 5′-GATGAGTCCTGAGTAA**C**-3′ |
| *Eco*RI +ACG | Selective amplification | E-ACG | 5′-GACTGCGTACCAATTC**ACG**-3′ |
| *Eco*RI +ACT | | E-ACT | 5′-GACTGCGTACCAATTC**ACT**-3′ |
| *Eco*RI +AAG | | E-AAG | 5′-GACTGCGTACCAATTC**AAG**-3′ |
| *Eco*RI+ AGC | | E-AGC | 5′-GACTGCGTACCAATTC**AGC**-3′ |
| *Eco*RI+ AGG | | E-AGG | 5′-GACTGCGTACCAATTC**AGG**-3′ |
| *Mse*I + CAG | | M-CAG | 5′-GATGAGTCCTGAGTAA**CAG**-3' |
| *Mse*I + CTG | | M-CTG | 5′-GATGAGTCCTGAGTAA**CTG**-3' |
| *Mse*I + CTC | | M-CTC | 5′-GATGAGTCCTGAGTAA**CTC**-3' |
| *Mse*I + CAT | | M-CAT | 5′-GATGAGTCCTGAGTAA**CAT**-3' |
| *Mse*I + CTA | | M-CTA | 5′-GATGAGTCCTGAGTAA**CTA**-3' |
| *Mse*I + CAC | | M-CAC | 5′-GATGAGTCCTGAGTAA**CAC**-3' |

of mutation upon storage in silica gel or sand. Dry storage is reported to carry less risk of mutation (Windels *et al.*, 1993).

Biotechnologists are always looking for new and interesting organisms. There is an increased need to preserve more strains, whereas few new collections are being established, and many existing collections are at full capacity. However, in spite of the effectiveness of the dry method in storing isolates of some fungal species, viability of *C. sativus* was not best maintained using this technique (Table 1). Karen *et al.* (2004) reported that larger fungal spores (such as those of *C. sativus*) tend to collapse during cryopreservation, and the damage is not reversible by hydration. Also, a significant number of spores of appropriate size were physically damaged and killed during freezing by formation of ice crystals. Mazer *et al.* (1977) reported that distortion of membranes, leading to injury, develops not only by osmotic shrinkage of cells due to freezing-induced dehydration, but also by mechanical deformation of cells due to growth extracellular ice. On the other hand, our results show that the storage of *C. sativus* in PDA resulted in contamination problems. This is in a good agreement with results of Holden and Smith (1992).

*C. sativus* isolates continued to be pathogenic after 2 years storage in sand or silica gel and the fungus was able to produce conidia (Table 2). The reduction in numbers of conidia observed among isolates after storage may be attributed to the fact that any change in nutrition or environment would allow to different patterns of intermingling of the nuclei and hence would alter morphological expression (Delcan *et al.*, 2002). Perhaps the reduction of conidia of *C. sativus* could be a transient phenomenon induced by the methods of preservation employed. Like other fungi, *C. sativus* easily adapts to dryness and abnormal osmotic conditions and so it may change its behavior temporarily. This might be contributed to the fact that the normal cytological condition of hyphal cells in this fungus is multinucleate and frequently heterokaryotic (Day, 1974).

All the storage methods tested had previously been found appropriate for fungi such as *Botrytis cinerea* (Delcan *et al.*, 2002), *Arthrobotrys robusta* and *Monacrosporium thaumasium* (Mota *et al.*, 2003). Our study showed that problems can arise when trying to preserve *C. sativus*. Poor viability and frequent contamination were common when the fungus was stored on PDA. In spite of this, it retained viability and virulence during 2 years of storage in sand and silica gel, but a risk of mutation was found using these methods. A lower mutation rate was observed using the dry method, however, to avoid risk of mutation in this method a temperature lower than -20°C might be used.

**Table 4.** AFLP similarity (%) of *C. sativus* stored for 2 years using different storage methods.

| Storage methods | Isolate | | |
|---|---|---|---|
| | Cs16 | Cs15 | Cs5 |
| Silica gel | 98.73 | 96.66 | 99.28 |
| Sand | 81.18 | 89.37 | 68.47 |
| Dry | 99.98 | 98.08 | 97.31 |

Data are the AFLP similarity over initial data (non-stored isolates.

**ACKNOWLEDGMENTS**

The authors thank the Director General of the Atomic Energy Commission of Syria and the Head of the Molecular Biology and Biotechnology Department for their support.

**REFERENCES**

Anonymous, 1988. STAT-ITCF, Programme, MICROSTA, realized by ECOSOFT, 2nd Ver. Institut Technique des Cereals et des Fourrages, Paris, France.

Arabi M.I.E., Jawhar M., 2003. Pathotypes of *Cochliobolus sativus* (spot blotch) on barley in Syria. *Journal of Plant Pathol*ogy **85**:193-196.

Arabi M.I.E., Jawhar M., 2004. Identification of *Cochliobolus sativus* (spot blotch) isolates expressing differential virulence on barley genotypes in Syria. *Journal of Phytopathology* **152**: 461-464.

Day P.R., 1974. Genetics and Host-Parasite Interaction. W.H. Freeman and Company, San Francisco, CA, USA.

Delcan J., Moyano C., Raposo R., Melgarejo P., 2002. Storage of *Botrytis cinerea* using different methods. *Journal of Plant Pathology* **84**: 3-9.

Fetch T.C., Steffenson B.J., 1994. Identification of *Cochliobolus sativus* isolates expressing differential virulence on two-row barley genotypes from North Dakota. *Canadian Journal of Plant Pathology* **16**: 202-206.

Fetch T.C., Steffenson B.J., 1999. Rating scales for assessing infection responses of barley infected with *Cochliobolus sativus*. *Plant Disease* **83**: 231-217.

Holden A.N.G., Smith D., 1992. Effects of cryopreservation methods in liquid nitrogen on viability of *Puccinia abrupt* var. *partheniicola* urediniospores. *Mycological Research* **96**: 473-476.

Karen K.N., Stephen W.P., Shung C.J., 2004. Preservation and distribution of fungal cultures. In: Müller G.M. (ed). Biodiversity of Fungi, pp. 3-11. Elsevier Academic Press, San Diego, USA.

Mazer I., Hiroyuki I., William S.P., Yoji A., Tomomi K., 1977. Freezing behaviours in plant tissues as visualized by NMR microscopy and their regulatory mechanisms. In: Cryopreservation of Tropical Plant Germplasm, pp. 22-56. National Institute of Agrobiological Resources, Japan.

Mathre D.E., 1982. Compendium of Barley Diseases. APS Press, St. Paul, MN, USA.

Michael B., Zohra F., Zouaoui I.E., 2004. Pathogenic and genetic characterization of Algerian isolates of *Fusarium oxysporum* f. sp. *lentis* by RAPD and AFLP analysis. *African Journal of Biotechnology* **3**: 25-31.

Mota M.A., Artur K., Campos A.K., Araújo J.V., 2003. Influence of different storage methods on the predatory capacity of the fungi *Arthrobotrys robusta* and *Monacrosporium thaumasium* after passage through the bovine gastrointestinal tract. *World Journal of Microbiology and Biotechnology* **19**: 913-916.

Nei M., Li W.H., 1979. Mathematical model for studying genetic variation in terms of restricting endonucleases. *Proceedings of the National Academy of Sciences, USA* **76**: 5269-5273.

Nutter F.W., Pederson V.D., Foster A.E., 1985. Effect of inoculations with *Cochliobolus sativus* at specific growth stages on grain yield and quality of malting barley. *Crop Science* **25**: 933-938.

Ryan M.J, Smith. D., Jeffries P., 2000. A decision-based key to determine the most appropriate protocol for the preservation of fungi. *World Journal of Microbiology and Biotechnology* **16**: 183-186.

Smith D., Onions A.H.S., 1994. The Preservation and Maintaintenance of Living Fungi, 2nd Edition. IMI Technical Handbooks, no. 2. International Mycological Institute, Kew, UK.

Smith D., Thomas V., 1998. Cryogenic light microscopy and the development of cooling protocols for the cryopreservation of filamentous fungi. *World Journal of Microbiology and Biotechnology* **14**: 49 -57.

Stalpers J.A., De Hoog A., Vlug I.J., 1987. Improvement of the straw technique for the preservation of fungi in liquid nitrogen. *Mycologia* **79**: 82-89.

Vos P., Hogers R., Bleeker M., Reijans M., Lee T., Hornes M., Frijters A., Pot J., Peleman J., Kuiper M., Zabeau M., 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23**: 4407-4414.

Windels C.E., Burnes P.M., Kommendahl T., 1993. *Fusarium* species stores on silica gel and soil for ten years. *Mycologia* **85**: 21-23.

Received May 12, 2006
Accepted September 29, 2006