Indian Phytopath. 65 (4) : 0-0 (2012)

RESEARCH ARTICLE

Technique for the maintenance of heterokayotic isolates of *Bipolaris sorokiniana* under ordinary conditions

RAMESH CHAND[1]*, O.P. YADAV[1], B. M. BASHYAL[2], L.C. PRASAD[3] and A. K. JOSHI[4]
[1]*Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi 221 005*
[2]*Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi 110 012*
[3]*Department of Genetics and Plant Breeding, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi 221 005*
[4]*Department of Genetics and Plant Breeding, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi 221 005 and International Maize and Wheat Improvement Center (CIMMYT), South Asia Regional Office, Singh Durbar Road, Kathmandu, Nepal*

ABSTRACT: One hundred heterokaryotic isolates of *Bipolaris sorokiniana,* collected from wheat during 1997-1998, were evaluated for their morphology, growth, number of nuclei/cell and disease causing ability. Autoclaved sorghum grains were used to colonize these isolates in a culture tube plugged with non- absorbent cotton and stored under room temperature in air tight plastic boxes. After 10 years, 92% isolates were recovered. The distribution of nuclei in different groups varied from 1.14-4.14. The host range and of isolates remained unchanged and area under disease progress curve (AUDPC) of isolates ranked similar as prior to the storage. The proposed technique is simple and cost - effective, and can be utilized for the maintenance of heterokaryotic fungi.

Key words: *Bipolaris sorokiniana*, heterokaryon, technique, storage

Bipolaris sorokiniana (Sacc.) Shoemaker (Syn. *Cochliobolus sativu*s telomorph) is a well known cause of spot blotch disease of barley and wheat. This pathogen induces head blight, seedling blight, foliar blight/spot blotch, common root rot and black point of wheat, barley, other small cereal grains and grasses (Zillinsky, 1983; Wiese, 1998). Losses due to spot blotch are reported to be 16% in India, 20% in Nepal, and 23% in Bangladesh (Dubin and Ginkel, 1991; Saari, 1998). The phytopathogenic fungus *B. sorokiniana* carries high morpho-pathological variability, which has been confirmed by many workers (Nelson, 1960; Oliveira *et al.,* 1998; Chand *et al.,* 2003). Parasexual recombination and anastomosis have been reported as one of the causes of variability in the natural populations of *B. sorokininana* (Tinline, 1962; Pandey *et al.,* 2008). Many fungi are heterokaryotic and carry two or more genetically different nuclei in the same cell. Heterokaryon formation in such fungi is facilitated by anastomosis, wherein two vegetative cells get fused and bring two or more than two different nuclei within one cell. These new heterokaryotic cells grow, reproduce and ultimately form a new population. Heterokaryon formation has potential benefits of functional diploidy and mitotic genetic exchange-parasexual recombination (Pontecorvo, 1956).

Fungal collections conserve living specimens of important or rare strains (Karen *et al.,* 2004) that are so valuable for biotechnology and biomedical research. In such cases, long-term stability of fungi is valuable for the higher recovery of enzymes, antibiotics and other important metabolites (Ryan *et al.,* 2000). An ideal storage method maintains viability of the fungus, causes neither loss of virulence in pathogenic isolates nor alterations to physiological or morphological characteristics (Day and Stacey, 2008; World Federation for Culture Collections, 2010). Heterokaryons are generally unstable and can change morphological and bio-chemical properties of fungi during sub-culturing (Butler, 1980; Bacon, 1988; Hajek *et al.,* 1990; Sneh and Adams, 1996). Pathogenicity of some fungi to host plants has also been altered after long-term storage (Hajek *et al.,* 1995). Poor viability and frequent contamination are quite common when the fungus is stored on potato dextrose agar (PDA). Lyophilization and cryopreservation of living cells provide efficient mechanism for stabilizing cells over long periods of time. However, these procedures are technically complex, lengthy and require expensive equipment (Stalpers *et al.,* 1987; Smith and Thomas, 1998). Therefore, an easy and cost effective technique would be of great help to many institutions and countries, especially developing countries, to maintain commercially valuable heterokaryons in fungi.

The present work was undertaken on the model organism *B. sorokiniana* that is heterokaryotic and keeps on changing its characters, when sub-cultured (Pandey *et al.,* 2008). The objective was to develop a cost - effective technique for the medium term storage of pure culture under ordinary conditions.

MATERIALS AND METHODS

B. sorokiniana isolates

One hundred isolates of *B. sorokiniana* isolated from the wheat cultivars in the year 1997-98 at Banaras Hindu University, Varanasi which were maintained the present studies. These isolates were purified by monoconidial isolation. Isolates were characterized for the colour morphology, number of nuclei, aggressiveness and host range before storage.

*Corresponding author: rc_vns@yahoo.co.in

Inoculation, colonization and storage of the selected isolates

Healthy and bold sorghum grains (cv. Phule Yashoda) were collected. These grains were washed thrice in running water and soaked in sterile water for 7 hrs. Water was drained after softening of grains. Twenty grains were filled in a 5 ml screw cap tube. Five screw cap tubes were used to store the individual isolate for 10 years. Cap of the tube was replaced by non- absorbent cotton plug for gaseous exchange. Tubes with grains were sterilized in an autoclave at 15 lb pressure for 30 minutes. Single 5mm diameter mycelial disc of *B. sorokiniana* was taken from the 72 hr old growth of pathogen and grains in individual tube were inoculated. The inoculated tubes were properly labeled and incubated at 25 °C for 6 days for grain colonization. When grains were fully colonized, these tubes were stored in air tight plastic boxes at room temperature (5 – 40 °C) for 10 years.

Sub-culturing of isolates

After ten years of storage, individual isolates were sub-cultured for their viability, colony morphology, aggressiveness and nuclear condition. Stored tubes were opened aseptically and two grains from each tube was taken out aseptically and inoculated in Petri plate containing PDA medium. The inoculated plates were incubated at 25 °C for a week.

Nuclear characterization of *B. sorokiniana* isolates

The fungal isolates were characterized for their colony morphology (Chand *et al.,* 2003; Bashyal *et al.,* 2010) and number of nuclei per cell in different groups (Chand *et al.,* 2003). Fifty isolates belonging to different morphological groups were screened for the presence of their nuclear conditions. The number of nuclei per cell was resolved by the fluorescent microscopy (Reichert Diastrar- 1762, made in Austria). A small portion of the fungal mycelium (1 mm^2) was scrapped from 7 day old culture, placed in centrifuge tube and washed with distilled water. The piece of mycelium was shifted to a glass slide having 3:1 ethanol: acetic acid for ten minutes. The fungal cells were washed in sterile distilled water for 10 minutes and suspended in dilute fluorochrome (DAPI) 0.1 µg ml^{-1} for ten minutes at room temperature. The mycelium was washed twice with distilled water for five minutes each. Mycelia were placed on clean glass slide in 25% glycerol solution, squashed under a cover glass and observed under the fluorescent microscope.

Host range

Five fungal isolates from each morphological group (black, mixed and white) were selected for their host range. The isolates were multiplied on PDA and allowed to grow for 8 days at 25 °C. A spore suspension was prepared by pouring 10 ml sterile water in each plate and gently rubbed by the sterile glass rod. Spore suspension was adjusted to 10^4/ml and 100µl was spotted on wheat (cv. Sonalika), barley (cv. RD 2503), paddy (cv. MTU 7029), maize (cv. Ganga 4) and sugarcane (cv. CoS 687) leaves. Five leaves of each of the host were inoculated when the plants were 60 days old. Appearance of symptoms was observed after 10 days when most of the inoculated spots on wheat and barley showed their symptoms.

Aggressiveness of isolate on wheat

Five isolates were randomly selected from each morphological group (black, mixed and white) for their aggressiveness on the wheat (cv. Sonalika). The aggressiveness of the representative isolate was tested on the leaf below the penultimate. Five leaves of wheat were spray inoculated (10^4 spore/ml) when the plants were at growth stage 57 (Zadok *et al.,* 1974). The number and size of lesions per inoculated leaf were counted. Data were taken after 10 days from the date of inoculation. The experiment was laid in randomized block design in three replications. Each replication carried five leaves.

Area under Disease Progress Curve (AUDPC*)*

Seeds of wheat (cv. Sonalika) were treated with Thiram 2g/kg and grown in earthen pots (40 cm diameter) filled with a mixture of garden soil and compost under poly house conditions. Spore suspension (10^4/ml) was sprayed at complete tillering (Growth stage 29) and flag leaf (Growth stage 49) stage (Zadoks *et al.,* 1974). Spot blotch severity was scored in double digit scale (DD, 00-99), modified by Saari and Prescott (1975) and Eyal *et al.* (1987). The first digit (D1) indicates vertical disease progress on the plant while the second digit (D2) indicates severity measured in diseased leaf area, eg., for a score of 59, 5 represents 50 % of plant height (from ground up) and 9 represents the 90% spot blotch severity up to that height. For each score the disease severity percentage was based on the following formula:

% Severity = (D1/9) (D2/9)100

The disease rating was recorded three times at 8 days interval. The AUDPC was calculated using percent disease severity estimations as outlined by Shaner and Finney (1977) and Roelfs *et al.* (1992):

$$AUDPC = \sum_{i=1}^{n} \{(Yi+Yi+1)/2[ti+1-ti]\}$$

where,

Yi = disease level at time ti
ti+1-ti= time (days) between two disease scores,
n = number of dates at which spot blotch was recorded

RESULTS

Survival of the isolates

Out of 100 isolates stored on grains, 92 were recovered without any contamination. The remaining eight isolates could not be revived due to contamination of *Penicillium* spp**.**

Frequency of *B. sorokiniana* colour morphology before and after storage

Colony morphology of all isolates was similar as recorded before storage of isolates. Also, the proportion of

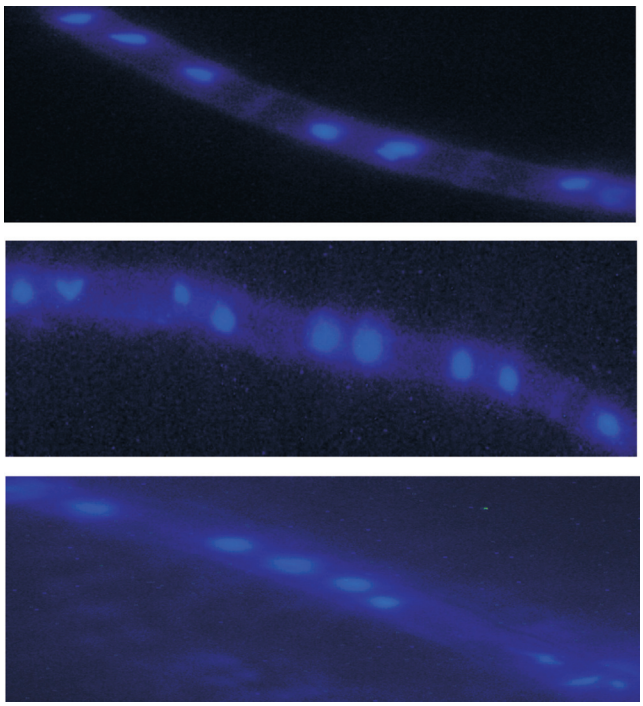

Fig. 1. Frequency distribution of different sub-populations of *B. sorokiniana* of wheat after storage of ten years

black, mixed and white isolates was similar as recoded before storage. The percent of mixed sub-population was highest (48.91%), while lowest (21.73%) for the white isolates (Fig.1).

Distribution of number of nuclei per cell

Mean distribution of nuclei/cell in mycelium varied from 1.14 to 4.14 (Table 1; Fig. 2) and was similar to the population before storage. The three populations differed significantly for number of nuclei/cell. Black isolates carried the least (1.14) nuclei per cell, whereas the white isolates carried highest (2.43) number of nuclei per cell.

Host range

Necrotic and restricted lesions were produced on paddy and maize. On the other hand, necrotic lesions on barley and wheat were expanded to several centimeters from the point of inoculation. None of the isolates was able to infect sugarcane leaves before storage of the pathogen in 1998 and after the storage.

Aggressiveness of isolate on wheat

Isolates of *B. sorokiniana* showed variation for lesion size on wheat plants (Table 2). White isolate (4W) of recorded largest lesion size, 0.17cm^2 in 1998 and 0.30cm^2 in 2011, whereas, lesion size produced by isolate 14B was the smallest before the storage (0.03cm$^{2)}$ as well as after the storage (0.04cm^2). Different isolates produced variable numbers of lesions on wheat plants. Maximum number of lesions were produced by the isolate 1W, which were 25.33 and 28.22 for the year 1998 and 2011 respectively. Whereas the isolate 15B produced minimum number of lesions (Table 2). AUDPC of the isolates ranked similar before and after

Table 1. Mean frequency of nuclei in three different subpopulation of *B. sorokiniana*

Group	Mean frequency of nuclei /cell
Black	1.14
White	2.43
Mixed	4.14
LSD (0.01)	1.42

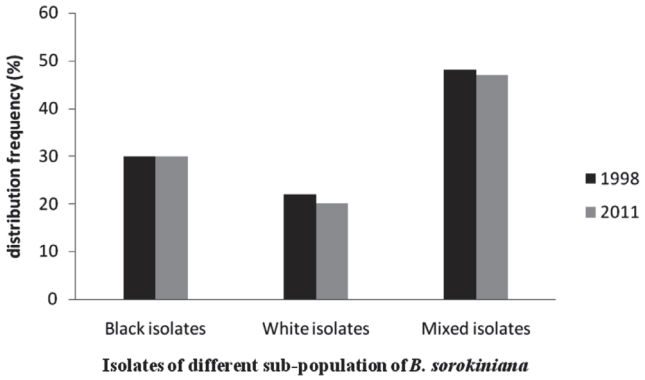

Fig. 2. Arrangement of nucleus in mycelium of three different sub-populations of *B. sorokiniana* of wheat: a. black; b. white and c. mixed (bar = 10µm)

Table 2. Aggressiveness of *B. sorokiniana* isolates on wheat before and after storage

Isolate	1998		2011	
	Lesion size (cm^2)	Number of lesions/leaf	Lesion size (cm^2)	Number of lesions/leaf
1W	0.13	25.33	0.15	28.22
2W	0.13	21.00	0.14	23.00
3W	0.1	21.67	0.20	24.00
4W	0.17	19.53	0.30	21.00
5W	0.08	23.33	0.09	23.00
6M	0.09	16.33	0.10	19.00
7M	0.07	17.67	0.08	20.00
8M	0.07	21.00	0.08	25.00
9M	0.10	18.67	0.12	19.00
10M	0.07	15.00	0.05	17.00
11B	0.06	14.50	0.04	15.00
12B	0.04	13.91	0.06	15.00
13B	0.08	23.57	0.09	22.00
14B	0.03	19.53	0.04	21.00
15B	0.08	11.00	0.11	11.67
LSD (0.05)	0.05	1.17	0.07	2.20

Where: B= black isolate; M= mixed isolate; W= white isolate

the storage. It was highest for the isolate 13W in 1998 (998.00) and 2011 (1038.74), and (440 and 478.29) for the isolate 4B in 1998 and 2011, respectively (Table 3).

DISCUSSION

Information about the pathogenic variability in the *B. sorokiniana* is significant for the development of spot blotch

Table 3. Area under disease progress curve of different subpopulation of *B. sorokiniana* on wheat before and after storage

Isolate	AUDPC		Mean*	Rank
	1998	2011		
1W	998.00	1081.74	1039.87	1
2W	879.60	913.33	896.46	2
3W	832.00	888.89	860.44	3
4W	768.00	799.22	783.61	4
5W	750.00	793.32	771.66	5
6M	741.00	784.81	762.90	6
7M	737.22	779.4	758.30	7
8M	720.00	751.87	735.93	8
9M	677.60	703.72	690.66	9
10M	683.00	696.11	689.55	10
11B	650.00	674.99	662.49	11
12B	647.40	670.59	658.99	12
13B	622.60	640.71	631.65	13
14B	617.60	638.00	627.80	14
15B	440.00	474.29	457.14	15
LSD (0.05%)	60.25	123.50		

Where: B= black isolate; M= mixed isolate; W= white isolate; *= Mean of 1998 and 2011

resistant cultivars in wheat and barley. Selection of aggressive strain on the resistant wheat genotype has been reported (Maraite *et al.,* 1998) which might be of importance to develop breeding populations that can be screened by the new aggressive isolate. Anastomosis is one of the main mechanisms of the heterokaryon formation in the *B. sorokiniana* (Tinline, 1962; Pandey *et al.,* 2008). The number of nuclei per cell generally varied from 1 to 4 and this keeps on changing the morphological and pathological behavior of the pathogen (Chand *et al.,* 2003). During sub-culturing, variability has also been recorded in mono-conidial isolates of the pathogen (Pandey *et al.,* 2008). This kind of variability is one of the major problems encountered during handling and maintenance of pure culture for various studies.

Arabi *et al.* (2007) tested isolates of *Cochliobolus sativus* after 2 years of storage in sand and silica gel at 4 °C or dried at -20 °C and found these to be infective. Webb *et al.* (2011) also maintained isolates of *Rhizoctonia solani* and tested their overall pathogenicity on sugar beet after 10 years of storage in cryopreservation (storage in liquid nitrogen). However, loss of virulence for some of the isolates was observed. Three sub-population of *B. sorokiniana viz.*, black, white and mixed were recovered from stored culture. The reports on frequency distribution suggested that mixed sub-population represented in highest frequency with higher number of nuclei per cell. White sub-population was in lowest frequency with lower number of nuclei (Jaiswal *et al.,* 2007; Pandey *et al.,* 2008; Bashyal *et al.,* 2010). The present method of storage showed good stability in the morphology and number of nuclei per cell even after 10 years of storage. The mean numbers of nuclei present in different sub-populations of the isolates were almost same as before storage. Probably this may be the reason for the stabilization of most of the characters investigated.

Repeated transfer of pathogenic fungi on artificial media usually results in the loss of its pathogenicity, sporulation or both (Dhingra and Sinclair, 1995). Further genetic degeneration or attenuation of strains has also been recorded. Mutations during storage may also change an individual isolate(s) identity with some mutations potentially causing the loss of virulence (Arabi *et al.,* 2007). In the present technique, isolates were pathogenic, aggressive and showed similar host range even after the storage for ten years. Windels *et al.* (1993) reported the less risk of the occurrence of mutations in dry storage which might be helpful for the stability of *B. sorokiniana* as well. Present finding corroborate the report of Windels *et al.* (1993).

The proposed technique is more effective, inexpensive and technically simpler for the storage of heterokaryotic fungi. It displayed apparently no loss in aggressiveness, host range and also maintained similar number of nuclei per cell which could be utilized for the storage of *B. sorokiniana.*

ACKNOWLEDGEMENT

Senior author Ramesh Chand is thankful to Council of Scientific and Industrial Research for providing financial support.

REFERENCES

Arabi, M.I.E., Jawhar, M. and Al- Daoude, A. (2007). Viability of *Cochliobolus sativus* cultures after storage under different conditions. *J. Plant Pathol.* **89**: 79-83.

Bacon, C.W. (1988). Procedure for isolating the endophyte from tall fescue screening isolates for ergot alkaloids. *Applied Environ. Microbiol.* **54**: 2615-2618.

Bashyal, B.M., Chand, R., Kushwaha, C., Sen, D., Prasad, L.C. and Joshi, A.K. (2010). Association of melanin content with conidiogenesis in *Bipolaris sorokiniana* of barley (*Hordeum vulgare* L.). *World J. Microbiol. Biotechnol.* **26**: 309-316.

Butler, E.E. (1980). A method for long-time culture storage of *Rhizoctonia solani*. *Phytopathology* **70**: 820-821.

Chand, R., Pandey, S.P., Singh, H.V. and Joshi, A.K. (2003). Variability and its probable cause in the natural population of spot blotch pathogen *Bipolaris sorokiniana* of wheat (*T. aestsivum* L.*)* in India. *J. Plant Dis. Protect.* **110**: 27-35.

Day, J.G. and Stacey, G.N. (2008). Biobanking. *Mol. Biotechnol.* **40**: 202-213.

Dhingra, O.D. and Sinclair, J.B. (1995). Basic Plant Pathology Methods. 2nd (Edn). Lewis Publishers (CRC Press), UK.

Dubin, H.J. and Ginkel, V. (1991). The status of wheat diseases in warm areas of south Asia: An update. In: Wheat in Heat Stressed Environments: Irrigated Dry Areas and Rice–Wheat Farming Systems (Saunder, D.A. and Hettel, G.P., eds). Mexico, D.F., Mexico: CIMMYT, pp. 353-359.

Eyal, Z., Scharen, A.L., Prescott, J.M. and van Ginkel, M. (1987). The Septoria disease of wheat: concept and methods of disease management. Mexico, DF, Mexico, CIMMYT.

Hajek, A.E., Humber, R.A. and Griggs, M.H. (1990). Decline in virulence of *Entomophaga maimaiga* (Zygomycetes: Entomophthorales) with repeated *in vitro* subculture. *J. Invert. Pathol.* **56**: 91-97.

Hajek, A.E., Shimazu, M. and Humber, R.A. (1995). Instability in pathogenicity of *Entomophaga maimaiga* after long-term cryopreservation. *Mycologia* **87**: 483-489.

Jaiswal, S.K., Sweta, Prasad, L.C., Sharma, S., Kumar, S., Prasad, R., Pandey, S.P., Chand, R. and Joshi, A.K. (2007). Identification of molecular marker and aggressiveness for different groups of *Bipolaris sorokiniana* isolates causing spot blotch disease in wheat (*Triticum aestivum* L.). *Curr. Microbiol.* **55**: 135-141.

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

Maraite, H., Di Zinno, T., Longrée, H., Daumerie, V. and Duveiller, E. (1998). Fungi Associated with Foliar Blight of Wheat in Warm Areas. In Duveiller E, Dubin HJ, Reeves J, McNab, eds. Proc Int Workshop Helminthosporium Blights Diseases of Wheat: Spot Blotch and Tan Spot, CIMMYT, El Batan, Mexico, pp. 293-300.

Nelson, R.R. (1960). Evolution of sexuality and pathogenicity in interspecific crosses in the genus *Helminthosporium*. *Phytopathology* **50**: 375-377.

Oliveira, A.M.R., Matsumura, A.T.S., Prestes, A.M., Matos, G.S. and Van Der Sand, S.T. (1998). Variabilidade patogeˆnica e morfolo´gica em isolados de *Bipolaris sorokiniana*. *Fitopatol. Bras.* **23**: 349-353.

Pandey, S.P., Sharma, S., Chand, R., Shahi, P. and Joshi, A.K. (2008) Clonal variability and its relevance in generation of new pathotypes in the spot blotch pathogen, *Bipolaris sorokiniana*. *Curr. Microbiol.* **56**: 33-41.

Pontecorvo, G. (1956). The parasexual cycle in fungi. *Ann. Rev. Microbiol.* **10**: 393-400.

Roelf, A.P., Singh, R.P. and Saari, E.E. (1992). *Rust Diseases of Wheat: Concepts and Methods of disease management* CIMMYT, Mexico DF, pp 81.

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

Saari, E.E. (1998). Leaf Blight Disease And Associated Soil Borne Fungal Pathogens of Wheat in South and Southeast Asia. In: *Helminthosporium blights of wheat: spot blotch and tan spot.* Duveiller E, Dubin HJ, Reeves J, McNab A (ed) CIMMYT, Mexico, DF, pp 37-51.

Saari, E.E. and Prescott, J.M. (1975). A scale for appraising the foliar intensity of wheat disease. *Pl. Dis. Reptr.* **59**: 377-380.

Shaner, G. and Finney, R.E. (1977). The effect of nitrogen fertilization on the expression of slow-mildewing resistance in Knox wheat. *Phytopathology* **67**: 1051-1056.

Smith, D. and Thomas, V. (1998). Cryogenic light microscopy and the development of cooling protocols for the cryopreservation of filamentous fungi. *World J. Microbiol. Biotechnol.* **14**: 49-57

Sneh, B. and Adams, G.C. (1996). Culture preservation methods for maintaining the genetic integrity of *Rhizoctonia* spp. isolates. In *Rhizoctonia Species:* Taxonomy, Molecular Biology, Ecology, Pathology, and Disease Control, pp. 139-145.

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

Tinline, R.D. (1962). *Cochliobolus sativus* V. Heterokayosis and parasexuality. *Canadian J Bot.* **40**: 425-437.

Webb, K.M., Hill, A.L., Laufman, J., Hanson, L.E. and Panella, L. (2011). Long-term preservation of a collection of *Rhizoctonia solani* using cryogenic storage. *Annl. Appl. Biol.* **158**: 297-304.

WFCC (2010). World Federation for Culture Collections guidelines for the establishment and operation of collections of cultures of microorganisms. 3rd ed. London, UK: The Welcome Trust.

Wiese, M.V. (1998). Compendium of wheat diseases. In: Duveiller E, Dubin HJ, Reeves J, McNab A (eds) Proc. Int. Workshop *Helminthosporium* Disease of Wheat: Spot Blotch and Tan Spot. 9-14 Feb. 1997, CIMMYT, El Batan, Mexico, DF, pp 114-118.

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

Zillinsky, F. (1983). Common diseases of small grain cereals, a guide to identification. CIMMYT, Mexico, DF, pp141.

Zadoks, J.C., Chang, T.T. and Konjak, C.F. (1974). A decimal code for the growth stages of cereals. *Weed Res.* **14**: 415-421.

Received for publication:
Accepted for publication: