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

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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. Cochliobulus sativus* 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. sorokiniana* (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.

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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 mm²) was scrambled 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 interval. The AUDPC was calculated using percent disease

\[ \text{AUDPC} = \frac{\sum_{i=1}^{n} \{Y_i+Y_{i+1}\}[t_{i+1}-t_i] }{n} \]

where,

- \( Y_i \) = disease level at time \( t_i \)
- \( t_{i+1}-t_i \) = 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...
black, mixed and white isolates was similar as recorded 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, 0.17cm² in 1998 and 0.30cm² in 2011, whereas, lesion size produced by isolate 14B was the smallest before the storage (0.03cm²) as well as after the storage (0.04cm²). 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 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.
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 population represented in highest frequency with higher number of nuclei (Jaiswal et al., 2007; Pandey et al., 2008; Bashyal et al., 2010). Association of melanin content with conidiogenesis in Bipolaris sorokiniana of barley (Hordeum vulgare L.). World J. Microbiol. Biotechnol. 26: 309-316.


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.

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REFERENCES


