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# EFFECT OF SUBCULTURING AND CULTURE AGE ON SPORULATION AND MORPHOLOGICAL CHARACTERISTICS OF *Bipolaris sorokiniana*

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**Abstract**: Two *in vitro* experiments were conducted to evaluate the effect of successive sub-culturing on sporulation, morphological characteristics and culture age for the production of conidia of three isolates of *Bipolaris sorokiniana* of which two were from wheat (BS-1 and BS-2) and one from barley (BS-3). The experiments were conducted following standard procedure. After every successive sub-culturing, number of spores gradually declined in all three isolates. In first two isolates spores were produced up to fourth subculture, but no spore was observed at fifth subculture. In isolate BS-3 the spores were produced up to second subculture and no spore was found at third subculture. There was significant variation among these three isolates for the production of spores in each subculture. Variation was found in margin, shape, color, texture, zonation and arial mycelium in top view and reverse view after 12 days of inoculation. Variation was also observed in conidia and conidiophore of the three isolates at 12 days age culture. In isolates BS-1, BS-2 and BS-3 the highest conidia production were obtained in 14 days (10620 x$10^3$ conidia/ml), 18 days (578 x$10^3$ conidia/ml) and 10 days (7.8 x$10^3$ conidia/ml) old culture, respectively. In the isolates BS-1 and BS-2, number of conidia was increased up to 14 days and 18 days, respectively and then decreased. In isolate BS-3 the highest number of conidia was found at 10 days old culture but decreased gradually with the age.

**Keywords**: Subculture, culture age, sporulation, *Bipolaris sorokiniana*

## Introduction

Wheat and barley are considered as two important cereal crops in the world. Both these two crops are vulnerable to many diseases. Among them leaf blotch or spot blotch caused by *Bipolaris sorokiniana* is the major and devastating one in Bangladesh (Hossain and Azad, 1992). *Bipolaris sorokiniana* (Sacc.) Shoemaker, teleomorph *Cochliobolus sativus,* Drechsier ex Dastur can incite any parts of wheat and barley plants (Sivanesan, 1990) and causes foot rot/root rot/leaf spot/leaf blotch/leaf blight/ black point. It is the major cause hampering commercial wheat production in warm and humid tropical areas (Lapis, 1985; Kulkarni and Nargund, 1986; Dubin and Ginkel, 1991, Kohli *et al.,* 1991 and Saari, 1998). It can attack a wide range of gramineous species and is common on rice, wheat and barley (Ma Quxiang and Jiabi, 1987; Nema, 1986; Raguchander *et al.,* 1988; Reis, 1991 and Adhikary and Mian, 2004), maize, rye, triticale (Saari, 1985).

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Alam *et al.* (1998) observed that spot blotch is the most destructive diseases of wheat and barley in the rice based cropping system of Bangladesh. Almost all released varieties showed varying degrees of spot blotch susceptibility. In farmers' fields in Bangladesh, the average loss due to spot blotch was estimated to be 15 percent (Alam *et al.*, 1998). *In vitro* experiments are routinely used to study the nature and control of plant diseases caused by fungal pathogens. Sporulation is a key component for several purposes because fungal spores are frequently used as propagules to infect plants (Clinton *et al.*, 1975).

The necessity of periodic subculturing results in problems as loss of the viability, morphologic and physiologic, changes (aspect and color of all the cultures), decrease and/or loss of the capacity of sporulation and later in most cases, the loss of the pathogenicity of the isolates. These are caused by accumulation of metabolites excreted during the development of the microorganism in the substrate (Bueno *et al.,* 2006; Meyer *et al.*, 2006; Vechiato *et al.,* 2003; Ignoffo *et al.*, 1982, Aparecido *et al.*, 2007).

In practice the changes that occur in the isolates come from the constant manipulation demanded due to the method of conservation which demands successive subculturing (Aparecido *et al.*, 2007). Therefore, the necessity of maintenance and manipulation of the isolates demand studies which characterize the effect of the preservation *in vitro* of the pathogenic and reproductive characteristics of the isolate of *B. sorokiniana.*

Considering the above facts, this study was undertaken to evaluate the effect of successive subculturing over sporulation and morphological characteristics of isolates of *B. sorokiniana* and to determine the effect of culture age on production of conidia.

**Materials and Methods**

The location, year of collection and relevant information of the cultivars are presented in Table 1.

Table 1: General profile of the isolates of *Bipolaris sorokiniana*

| Isolates | Regional Origin | | Year of Isolation | Wheat and Barley Cultivars | Reaction |
|---|---|---|---|---|---|
| | Districts | Upazila | | | |
| BS-1 | Magura | Simakhali | December,2012 | Wheat ( Bijoy) | + |
| BS-2 | Magura | Simakhali | December,2012 | Wheat ( Local) | + |
| BS-3 | Khulna | Batiaghata | December,2012 | Barley (BARI barley-3) | + |

BS = *Bipolaris sorokiniana*, + = Pathogenic

*Bipolaris sorokiniana* was isolated following seed or tissue planting method (Dhingra and Sinclair 1985; Mian 1995). The pathogen was identified by placing the conidia adhered to a sterilized needle on a drop of Potato Dextrose Agar (PDA) (2%) kept in a sterilized petridish. After four days the slide was examined for germination of conidia. After that the fungal growth was observed and then identified. Purification of all isolates was made using the single spore method as stated by Ruppel (1972) with some modifications (Adhikary *et al.,* 2004). Pathogenicity of the isolates was tested on excised leaves as in Dhingra and Sinclair (1985) and multiplication was done by inoculating the plates of three days old PDA culture of *B. sorokiniana* (5 mm discs) and keeping it in the growth chamber at a temperature of

26±2°C for few days. Test tube slants with five to seven days old profuse mycelium and conidia of *B. sorokiniana* were stored in refrigerator at 4°C.

Two experiments were conducted to achieve the objective of the present study. Experiment-I continued of five treatments with five replications. Each petridish was considered as a replication. The treatments were $T_1$ –first subculturing obtained from single spore isolate, $T_2$ – sporulation obtained from the first subculturing of the original culture; $T_3$ – sporulation obtained from the second subculturing; $T_4$ – sporulation obtained from the third subculturing; $T_5$ – sporulation obtained from the fourth subculturing. From first subculturing ($T_1$) to second subculturing ($T_2$) was done after 12 days of interval. Successively each subculture was done at 12 days interval.

For measuring sporulation ability, twelve days old culture grown on PDA was flooded with 100 ml of sterilized distilled water. Conidia along with mycelial mass were separated from the substratum by scrapping with a narrow edged sterilized glass slide. The suspension was sieved through double layer cheese cloth to discard mycelial mass. The volume was adjusted up to 100 ml using distilled water. It was then diluted 10 times by adding distilled water. The number of spore/ml was counted standard haemocytometer with Neubauer rulings and seen at 40x (4x objective). Counting was done five times for each replicated plate; morphological characteristics were recorded based on visual observation. Data were recorded on number of spores at 12 days intervals for each subculturing and morphological characteristics of mycelium and conidia of *Bipolaris sorokiniana* after 12 days of inoculation.

The experiment-II was laid out with six treatments and five replications using three single spore isolates of *B. sorokiniana.* The treatments were: $D_{10}$ – colonies with 10 days of storage; $D_{14}$ – colonies with 14 days of storage; $D_{18}$ – colonies with 18 days of storage; $D_{22}$ – colonies with 22 days of storage; $D_{26}$ – colonies with 26 days of storage; and $D_{30}$ – colonies with 30 days of storage. Number of spores was counted following the procedure of Experiment I and recorded at four days interval starting from 10 days up to 30 days. The both experiments were laid out under completely randomized design (CRD). Recorded data were transformed by using Square Root Method (Zaman, 1982) for exactly analyzing the data avoiding statistical complication. The data were analyzed statistically using MSTAT-C computer program and means were compared following Duncan's Multiple Range Test (DMRT) and Least Significant Difference test (LSD).

## Results and Discussion

After isolation and purification, dictyospore (spore with many transverse septa) were observed. The conidia / dictyospore were subjected to germination and found germ tube from both ends which confirmed the fungus as *Bipolaris sorokiniana*. All the three isolates were found to produce typical leaf spot/ leaf blotch symptoms and confirmed as pathogenic.

The effect of subculturing on three isolates of *B. sorokiniana* over the sporulation and morphology was examined. Number of spores significantly varied ($p \leq 0.01$) from subculture to subculture. No sporulation was observed at 3rd subculture in the isolate BS-3 but in case of other two isolates (BS-1 and BS-2) at 5th subculture sporulation was noticed to be stopped (Table 2).

Table 2: Effect of number of subculturing over the production of conidia of *B. sorokiniana*

| Treatments (number of subculturing) | BS-1 (Conidia/ml) | BS-2 (Conidia/ml) | BS-3 (Conidia/ml) |
|---|---|---|---|
| $T_1$ | 382.8 a (15.0 x $10^4$) | 357.5 a (12.78 x $10^4$) | 255.3 a (6.52 x $10^4$) |
| $T_2$ | 143.9 b (2.07 x $10^4$) | 159.4 b (2.54 x $10^4$) | 23.21 b (0.054 x $10^4$) |
| $T_3$ | 87.72 c (0.77 x $10^4$) | 52.45 c (0.28 x $10^4$) | 0.7071 c (0.0000) |
| $T_4$ | 17.85 d (0.032 x $10^4$) | 13.14 d (0.28 x $10^4$) | |
| $T_5$ | 0.7071 d (0.0000) | 0.7071 e (0.0000) | |
| CV (%) | 17.17 | 3.66 | 1.44 |
| Level of significance | 0.01 | 0.01 | 0.01 |
| Upper limit | | | Transformed value |
| Lower limit | | | Original value |

Number of spores in the isolate BS-1 was 15.0 x $10^4$ conidia / ml ($T_1$), 2.07 x $10^4$ conidia / ml ($T_2$), 0.77 x $10^4$ conidia / ml ($T_3$), 0.032 x $10^4$ conidia / ml ($T_4$) and 0.0000 conidia / ml ($T_5$). Number of spores in the isolate BS-2 was 12.78 x $10^4$ conidia / ml ($T_1$), 2.54 x $10^4$ conidia / ml ($T_2$), (0.28 x $10^4$ conidia / ml ($T_3$), (0.018 x $10^4$ conidia / ml ($T_4$) and (0.0000 conidia / ml ($T_5$). Number of spores in the isolate BS-3 was 6.52 x $10^4$ conidia / ml ($T_1$), 0.054 x $10^4$conidia / ml ($T_2$) and0.0000 conidia / ml ($T_3$).

Number of spores and the pattern of sporulation varied with the subculture and also with the isolates. A gradual declination in the number of spores was noticed as successive subcultures proceeded. In case of the isolate BS-3 sporulation was stopped at 3rd subculture, whereas in the other isolates this phenomenon was happened at 5th subculture.

The regression analysis considering number of subculture as independent variable and conidia formation as dependent variable revealed that there was an immense effect of subculturing over conidia formation (Fig. 1, 2 and 3). In the isolates of BS-1, BS-2 and BS-3 the regression equation revealed that more than 83%, 57% and 84% of the variation, respectively could be explained by the increase of subculturing number. Rest of the variation occurred due to other causes.

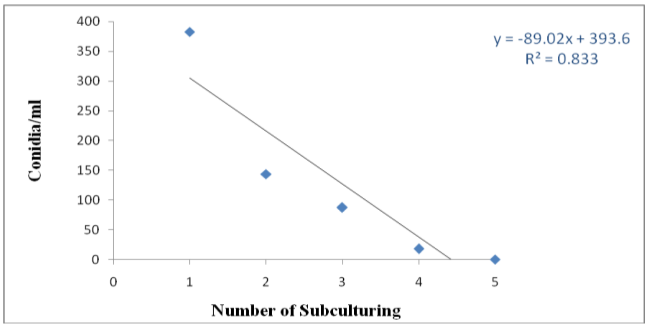


Fig. 1: Relationship between number of subculturing and production of conidia of BS-1 isolate.

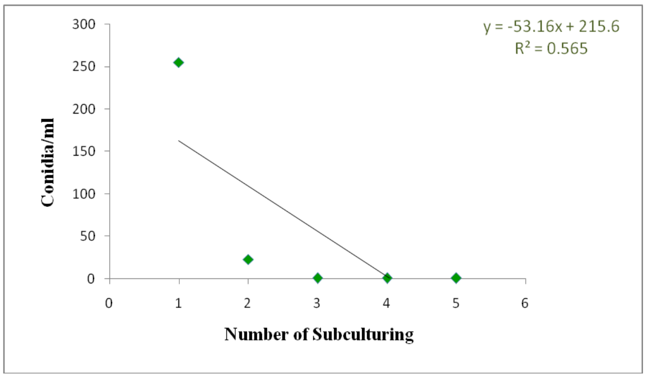


Fig. 2: Relationship between number of subculturing and production of conidia of BS-2 isolate

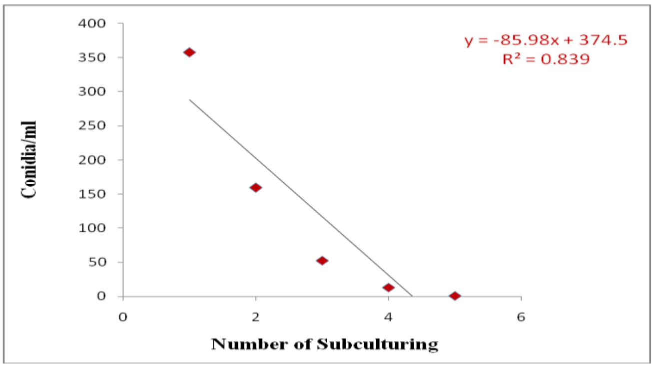


Fig. 3: Relationship between number of subculturing and production of conidia of BS-3 isolate

It was observed that conidia production was sharply decreased when the number of subculture increased and in case of isolates BS-1 and BS-2 at five subcultures, the number of conidia was reduced to 100% and in isolate BS-3 at three subcultures this number of conidia was reduced to 100%. In another experiment conducted by Cruz *et al.* (2011) with *Magnaporthe oryzae* (*Pyricularia oryzae*), they found that conidia production was greatly decreased when the number of subcultures increased and after eight subcultures this number of conidia was reduced in 80%. Similar results were found by Cruz *et al.* (2011) when he conducted similar experiment with the fungus but in different laboratory. The best sporulation was recorded in first subculture ($T_0$) in all isolates compared to subsequent subcultures. The higher sporulation in ($T_0$) might be related to the adaptation of the colony of the fungus to the culture medium (Gil *et al.*, 2010). The results of the present experiment varied with the results of (Camargo, 2004). It might be owing to different fungus, host, media composition and laboratory. These results also corroborate with the results of Gil *et al.* (2010) who reported that low virulence attributed to the loss of pathogenecity due to procedures of successive subculturing during the period of maintaining the fungus in tubes containing PDA medium. Gil *et al.* (2010) also emphasized that, normally samples maintained by period subplanting are stored in room temperature, and they are exposed to the alterations that it may occur. These alterations increase the frequency of mutation (environment pressure / selection pressure) and, as a result the culture may be lost (Aparecido and Figueiredo, 1997).

Colony characters revealed that colony margin of both upper and lower surface of BS-1 were wavy and in case of BS-2 and BS-3 were regular, respectively. The shape in the upper surface of BS-1, BS-2 and BS-3 was oval, round and irregular, respectively.

In BS-1 the color in the upper surface was dark color but in the lower surface was dark brown color. In BS-2 and BS-3 the color of upper surface was ash-gray but in the lower surface was light brown and dark brown color, respectively. The texture of BS-1 and BS-2 in the upper surface was stiffy, but in BS-3 was puffy. In the present study, zonations observed in fungal colonies were found to be different in three isolates. The concentric zonation was observed in BS-1 and BS-3, but in BS-2 was non-concentric. The Arial mycelium of BS-1 in the upper surface was wiry and for BS-2 and BS-3 was floccose (Table 3).

Table 3: Morphological characteristics of mycelium of *B. sorokiniana* after 12 days of inoculation

| Qualitative characters of mycelium of isolates | Margin | | Shape | Color | | Texture | Zonation | Arial Mycelium |
|---|---|---|---|---|---|---|---|---|
| | Top View | Reverse View | Top View | Top View | Reverse View | Top View | Top View | Top View |
| BS-1 | Wavy | Wavy | Oval | Dark | Dark brown | Stiffy | Concentric | Wiry |
| BS-2 | Regular | Regular | Round | Ash-grey | Dark brown | Stiffy | Non-Concentric | Floccose |
| BS-3 | Regular | Regular | Irregular | Ash-grey | Light brown | Puffy | Concentric | Floccose |

In BS-1, margin, shape, color, texture, zonation and Arial mycelium were wavy (both upper and lower surface), oval (upper surface), dark and dark brown (upper and lower surface), stiffy, concentric and wiry, respectively. In BS-2, margin, shape, color, texture, zonation and arial mycelium were regular (both upper and lower surface), round shape (upper surface), ash-grey and dark brown (upper and lower surface), stiffy, non-concentric and floccose, respectively. In BS-3 the margin, shape, color, texture, zonation and arial mycelium were regular (both upper and lower surface), irregular (upper surface), ash-grey and light brown (upper and lower surface), puffy, concentric and floccose, respectively. Morphological variation is one of many properties that have been shown to contribute to fungal virulence (Heitman *et al.*, 2006). Changing cell shape or size is likely an adaptive trait that is also advantageous when these pathogens encounter the host. Accordingly, signaling pathways that play important roles in coordinating morphological changes and virulence are typically involved in sensing environmental stresses (Wang and Lin, 2012). So, morphological characteristics of mycelium were dissimilar within the isolate and among the isolate.

Radial mycelial growth in all isolates was significantly varied ($p \leq 0.01$). Irrespective of media, maximum and significantly more growth was attained by isolate BS-2 (53.3mm). In contrast, minimum growth was recorded in isolate BS-1 (45.6 mm) and BS-3 (44.3 mm) after 12 days of inoculation (Table 4). So, the relative growth of *Bipolaris* isolates on PDA showed variation in their mycelial growth.

Table 4: Radial mycelial growth of *B. sorokiniana* after 12 days of inoculation

| Name of the isolates | Radial Growth (mm) |
|---|---|
| BS-1 | 45.6 |
| BS-2 | 53.3 |
| BS-3 | 44.3 |
| LSD value | 5.80 |
| Level of significance | 0.01 |

Conidia characteristics revealed that the color of conidia of BS-1was light brown but in case of BS-2 and BS-3 was dark brown. The color of conidiophore BS-1 and BS-2 was dark brown and BS-3 was light brown. For of BS-1 and BS-3 the conidial shape was oval to elongate and for BS-2 the shape was elongated. For BS-1and BS-2 the Conidiophore shape was club shaped and for BS-3 the shape was hyphe like. The geniculation of conidiophore was present in all isolates (Table 5).

Table 5: Morphological characteristics of conidia of *B. sorokiniana* after 12 days of inoculation

| Qualitative characteristics of conidia/ conidiophores of isolates | Color | | Shape | | Geniculation |
|---|---|---|---|---|---|
| | Conidia | Conidiophore | Conidia | Conidiophore | Conidiophore |
| BS-1 | Light brown | Dark brown | Oval to Elongate | Club shaped | present |
| BS-2 | Dark brown | Light brown | Elongate | Club shaped | present |
| BS-3 | Dark brown | Dark brown | Oval to Elongate | Hyphae shaped | present |

For BS-1 the conidial and conidiophore color was light brown and dark brown and shape was oval to elongate and club shaped respectively. For BS-2 the conidial and conidiophore color was dark brown and light brown and shape was elongate and club shaped respectively and for BS-3 the conidial and conidiophore color was dark brown and shape was oval to elongate and hyphe shaped respectively. In all isolates geniculation was present in conidiophore. From the findings (Table 5) it may be said that conidia and conidiophore varied within the isolates and between the isolates. Li *et al.* (2011) reported that when small spores were allowed to germinate and develop into large cells *in vitro* the virulence of the inoculums increased prior to the infection. So, it seemed that morphologically the isolates were dissimilar. For confirmation of dissimilarities among the isolates further experiments may be undertaken on pathogenicity and genetic study with a large number of isolates.

Number of cell/conidium was significantly varied ($p \leq 0.01$) with all isolates. The number of cell/conidium of isolate BS-1, BS-2 and BS-3 was 3.6, 8.6 and 12.6, respectively which means size of conidia in isolate BS-1 and BS-2 was smaller than isolate BS-3 (Table 6).

Table 6: Number of cell/conidium of *B. sorokiniana* after 12 days of inoculation

| Name of the isolates | Number of cells/Conidium |
|---|---|
| BS-1 | 3.6 |
| BS-2 | 8.6 |
| BS-3 | 12.6 |
| LSD value | 3.88 |
| Level of significance | 0.01 |

Effect of the culture age over the production of *B. sorokiniana* was examined after spore formation with 4 days (10, 14, 18, 22, 26 and 30 days) interval. The effect was varied significantly from age to age ($P \leq 0.01$) (Table 7). In the isolates BS-1, BS-2 and BS-3 the highest conidia production obtained in 14 days (10620 x $10^3$ conidia / ml), 18 days (578 x $10^3$ conidia / ml) and 10 days (1.8 x $10^3$ conidia / ml) old culture, respectively compared to other culture ages. Number of spores in the isolate BS-1 was 3320 x $10^3$ conidia / ml ($D_{10}$), 10620 x $10^3$ conidia / ml ($D_{14}$), 2400 x $10^3$ conidia / ml ($D_{18}$), 1500 x $10^3$ conidia / ml ($D_{22}$) , 626 x $10^3$ conidia / ml ($D_{26}$) and 44 x $10^3$ conidia / ml ($D_{30}$). Number of spores in the isolate BS -2 was 98 x $10^3$ conidia / ml ($D_{10}$), 304 x $10^3$ conidia / ml ($D_{14}$), 578 x $10^3$ conidia / ml ($D_{18}$), 300 x $10^3$ conidia / ml ($D_{22}$), 290 x $10^3$ conidia / ml ($D_{26}$) and 78.2 x $10^3$ conidia / ml ($D_{30}$).

Table 7: Effect of culture age over the production of conidia of *B. sorokiniana*

| Culture age | BS-1 (Conidia/ml) | BS-2 (Conidia/ml) | BS-3 (Conidia/ml) |
|---|---|---|---|
| $D_{10}$ (10 days) | 1815 b<br>(3320 x $10^3$) | 309.7 c<br>(98 x $10^3$) | 88.06 a<br>(7.8 x $10^3$) |
| $D_{14}$ (14 days) | 3245 a<br>(10620 x $10^3$) | 554.9 b<br>(304 x $10^3$) | 41.50 b<br>(1.8 x $10^3$) |
| $D_{18}$ (18 days) | 1547 b<br>(2400 x $10^3$) | 759.1 a<br>(578 x $10^3$) | 25.06 c<br>(0.70 x $10^3$) |
| $D_{22}$ (22 days) | 1223 c<br>(1500 x $10^3$) | 545.0 b<br>(300 x $10^3$) | 19.51c<br>(0.42 x $10^3$) |
| $D_{26}$ (26 days) | 790.1 d<br>(626 x $10^3$) | 535.8 b<br>(290 x $10^3$) | 19.68 c<br>(0.40 x $10^3$) |
| $D_{30}$ (30 days) | 182.3 e<br>(44 x $10^3$) | 279.5 c<br>(78.2 x $10^3$) | 0.7071d<br>0.000 |
| CV (%) | 11.40 | 10.15 | 18.25 |
| Level of significance | 0.01 | 0.01 | 0.01 |
| Upper limit | | | Transformed value |
| Lower limit | | | Original value |

The number of spores in the isolate BS -3 was 7.8 x $10^3$ conidia / ml ($D_{10}$ ), 1.8 x $10^3$ conidia / ml ($D_{14}$ ), 0.7 x $10^3$ conidia / ml ($D_{18}$), 0.42 x $10^3$ conidia / ml ($D_{22}$) , 0.4 x $10^3$ conidia / ml ($D_{26}$ ) and 0.000 conidia / ml ($D_{30}$). In the isolates BS -1 and BS -2 spores/ml increased gradually and then decreased. But in isolate BS-3 the highest number of spores/ml was recorded at 10 days then decreased gradually. Conidia productions were significantly different among the culture age.

It was observed that the conidia production was increased in isolates BS -1 and BS -2 up to $14^{th}$ and $18^{th}$ days after inoculation after that conidia production was decreased. But in isolate BS -3 conidia production was increase at $10^{th}$ days after inoculation then number of conidia was decreased. In all isolates spore productions were dropped significantly and the spore capacity was decreased at 77%, 48% and 77% in 18, 22 and 14 days of culture age of isolate BS -1, BS -2 and BS -3. This fact might be related to the culture aging, which causes losses of vigor of the conidiophores and reduction of the sporulation due to the consumption of the medium of the culture and/or to the accumulation of metabolites excreted during the development of the microorganism in the substrate. Aparecido *et al.* (2007) and his co-workers reported that after 14 days, the production of conidia dropped significantly, considering that at 18 days, the spore capacity decreased 95% in *Magnaporthe oryzae*. This might also be caused by germination of conidia due to maturity. Isacc (1992) reported that in some fungi, conidia must attain maturity prior to germination. Higher conidia germination occurs due to dark colored and the cells contained numerous granules. These granules may contain large amounts of storage materials such as glycogen and trehalose. A similar finding has been reported on *Pyricularia oryzae*, a pathogen of rice (Abe, 1936).

The aged conidia might contain a high level of toxic or waste materials, which eventually resulted in autolysis or self digestion. It might also decrease sporulation of isolates. Aged conidia may become nutrient depleted, especially trehalose, which quickly lead to a decrease in physiological activity. Trehalose is the most abundant sugar in spores and it seems to be involved in germination. The result of the present experiment corroborate with the result of Undeen (1990). Hailmi *et al.* (2011) observed in *Dactylaria higginsii* low conidia counting in 22, 26 and 30 days old culture attributed to the higher number of presumably empty conidia as indicated by the absence of granulation in the cells and also the appearance of colorless cell content. This could be due to the aging process of the conidia.

## Conclusion

The best sporulation was found in $T_1$ stage in all isolates and no conidia were found in $T_5$ stage for BS-1 and BS-2 and in $T_3$ stage for BS-3 after successive subculturing. Remarkable variability was found in morphological characteristics of three isolates of *Bipolaris sorokiniana* after 12 days of inoculation. The highest conidia production was obtained in 14 days, 18 days and 10 days old culture in isolates of BS-1, BS-2 and BS-3, respectively after inoculation compared to other culture ages.

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