Conidia production of *Exserohilum turcicum* by a two-phase system using sponge matrix

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Key words: *Exserohilum turcicum,* Turcicum leaf blight of Maize, conidia, sponge matrix.

Zusammenfassung: Das Pilzisolat Et0005, *Exserohilum turcicum* (meiospore Morphe *Setospharia turcica*), wurde 2016–2017 während einer Erhebung der Pilzkrankheiten im Distrikt Murshidabad, Westbengalen, Indien, isoliert. In Flüssigkultur wurden jedoch keine Konidien gebildet. Myzelien von Et0005 wuchsen gut in Schwammmatrix, die in Flüssigkultur eingetaucht war (die erste Phase). Konidien wurden reichlich an den Myzelien erzeugt, wenn die Schwammmatrix der Luft ausgesetzt wurde (zweite Phase). Dieses Zweiphasensystem produzierte innerhalb von 6 Tagen konsistent über 6×10^8 Konidien pro Liter. Darüber hinaus waren die erhaltenen Konidien einheitlicher in der Größe und erreichten im Vergleich zu denen, die auf einfachen Agarmedien hergestellt wurden, höhere Prozentsätze der Keimung und Appressorienbildung. Ein zweiphasiges System mit Schwammmatrix könnte daher ein vielversprechendes Instrument sein, um die Konidienproduktion von *E. turcicum* unter Laborbedingungen zu gewährleisten.

Abstract: The fungal isolate Et0005, *Exserohilum turcicum* (meiosporic morph *Setospharia turcica*), was obtained during a 2016–2017 disease survey in Murshidabad district, West Bengal, India. However, it did not produce any conidia in liquid culture. Mycelia of Et0005 grew well in sponge matrix submerged in liquid culture (the first phase). Conidia were abundantly produced from the mycelia when the sponge matrix was exposed to the air (the second phase). This two-phase system consistently produced over 6×10^8 conidia per litre within 6 days. In addition, the conidia obtained were more uniform in size and achieved higher percentages of germination and appressoria formation compared with those produced on simple agar media. So a two-phase system using sponge matrix could be a promising tool for providing the conidia production of *E. turcicum* in laboratory condition.

Turcicum leaf blight is a common disease of maize in many parts of the world including India. As a part of disease survey during 2016–2017, infected or blighted leaves were collected from a maize field at Murshidabad district, West Bengal. The causal fungus was isolated on water agar medium and subcultured on potato dextrose agar (PDA). The isolate was capable of producing conidia on solid media such as PDA but not in liquid media at all. As this fungus might be useful for biological control purposes, similar to the use of *Exserohilum monoceras* (DRECHSLER) K. J. LEONARD & SUGGS against barnyardgrass (YAMAGUCHI 2000), we tried to overcome this for a rapid and continuous conidia production and examined the effect of a two-phase system using sponge matrix

(NAKASHIMA & al. 1988, YAMAGUCHI 2000) for the production of conidia of *E. turcicum* (PASS.) K.J. LEONARD & SUGGS under in-vitro condition.

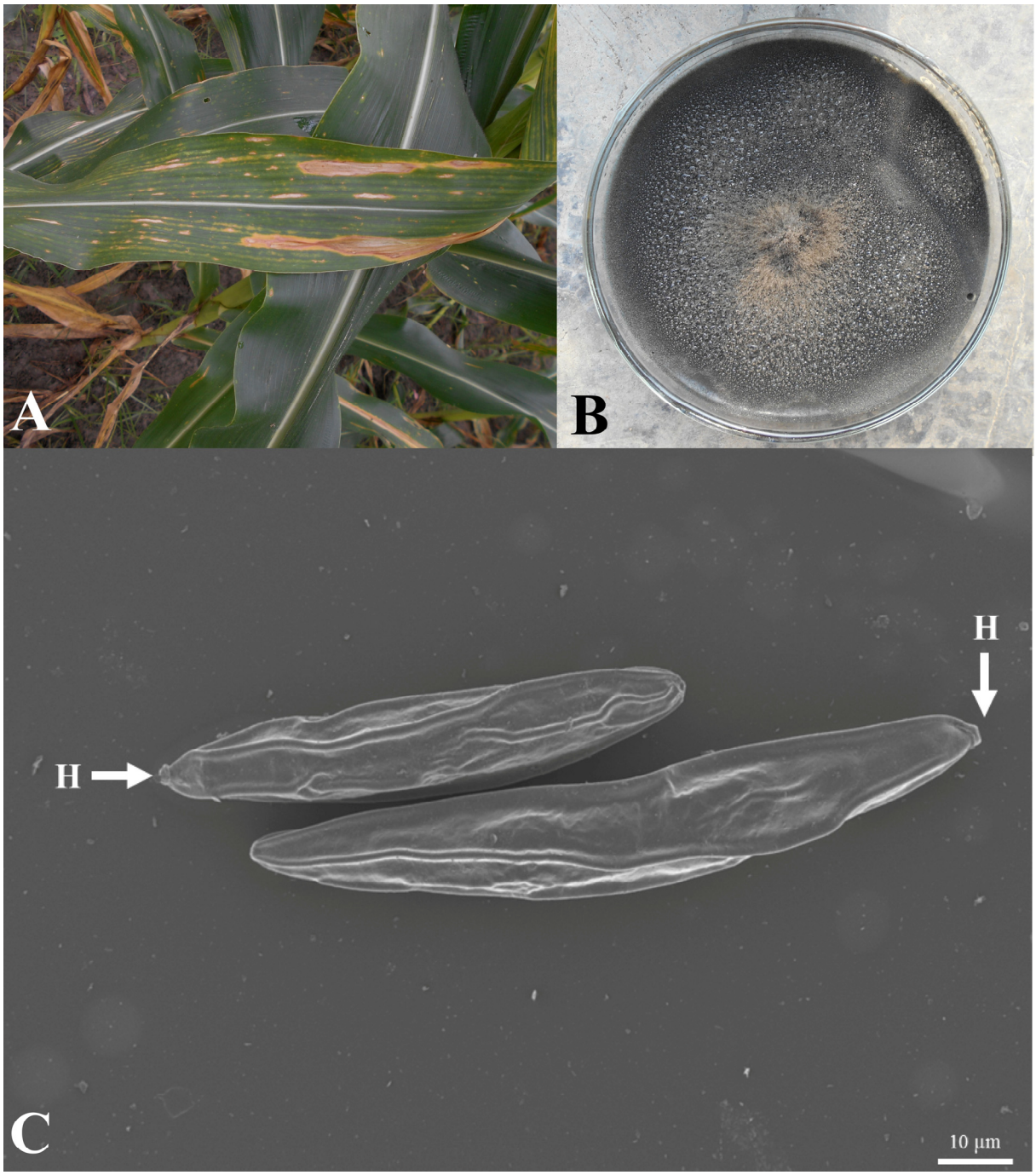

Fig. 1. *Exserohilum turcicum* Et0005 isolated from blighted leaves of maize in Murshidabad district. *A* Turcicum leaf blight disease specimen, *B* colony on PDA at 22 ˚C for 5 days, *C* scanning electron microscopy of conidia with typical hilum (H).

Materials and methods

Fungal isolate. Et0005 was used throughout the experiments. It was isolated from severely diseased leaves of maize plant in Murshidabad district, West Bengal. Based on the fact that conidia of this isolate apparently had a hilum and septa, Et0005 conidia were subjected to scanning electron microscopy (Fig.1 C), and identified as *Exserohilum turcicum*. In addition to the morphological characteristics of conidia

formed, we confirmed that the sequences of ITS 18S rDNA were highly homologous to those of a standard isolate of *E. turcicum*.

Conidia production on an agar medium. *Exserohilum turcicum* Et0005 was cultured on autoclaved PDA (potato dextrose agar), in a 90-mm petri dish and incubated at 22 ˚C in the dark for 12 days. Conidia formed on the agar plate were harvested using a plastic scrapper with sterile distilled water containing 0.1 % Triton X-100 [polyoxethylene (10) octylphenyl]. The suspension was filtrated through gauze to remove agar and mycelial debris. Conidia concentration was adjusted to 5×10^4 ml^{-1} by diluting with 0.1 % Triton X-100.

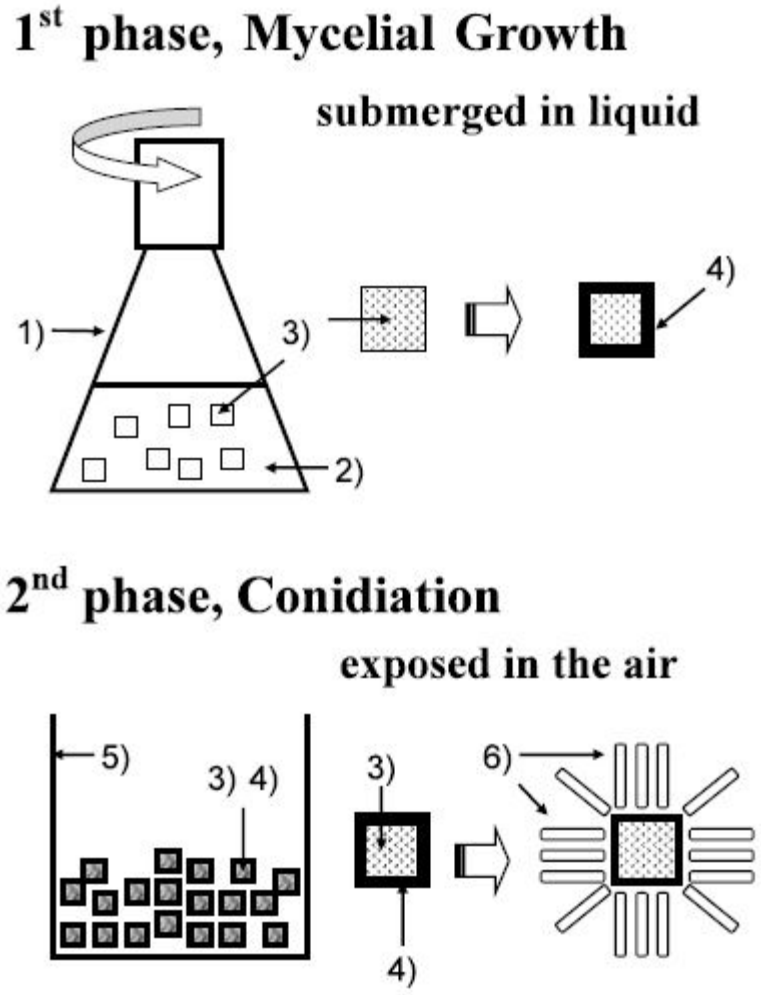

Fig. 2. Schematic illustration of a two-phase system using sponge matrix. 1) flask, 2) liquid medium, 3) polyurethane foam, 4) mycelia on the surface of polyurethane foam, 5) beaker, 6) conidia produced from mycelia.

Conidia production by a two-phase system. Procedures of a two-phase system using sponge matrix for synchronous conidia production are schematically presented in Fig. 2. In the first phase, quantitatively sufficient mycelia were cultured in submerged liquid culture. The basal medium contained 20 g glucose, 2g $NaNO_3$, 1g K_2HPO_4, 1g KH_2PO_4, 0.5g $MgSO_4$, 0.2g $CaCl_2$, 3.4g polypeptone, 3.4 g yeast extract, 10 g rice oil, and 20 g polyurethane foam (5 mm-cubes), which provide approximately 3500 cubes per 1 l of distilled water. The initial pH was adjusted to 5.5 by 0.1 N HCl. Each autoclaved 500 ml-flask with 150 ml of the medium containing polyurethane foam was inoculated with 0.1 % (v/v) conidia suspension obtained by plate culture. The flasks were incubated on a rotary shaker at 100 rpm in the dark. In the second phase, conidia were produced in the open air. After liquid culture, the sponge matrix covered with mycelia was separated from the culture broth through filtration using a sieve. To initiate conidiation, mycelia on the surface of the polyurethane foam were exposed to the air in a beaker

at 25 ˚C in a moist chamber. Conidia were harvested with distilled water containing 0.1 % Triton X-100 using a magnetic stirrer, then filtrated, and kept in suspension.

Measurements of conidia. The net weight of mycelia in the sponge matrix in the first phase was calculated based on differences in the weight of polyurethane with or without mycelia. The number of conidia in the second phase was counted under a microscope. Moreover, the length of conidia and the number of septa were measured microscopically. To assess the germination and appressoria formation of the conidia produced, suspension at 5×10^4 conidia ml^{-1} containing 0.1 % Triton X-100 was dropped on both water agar and the third expanded-leaves of maize seedlings, and the specimens were incubated at 25 ˚C in the dark. After 6 hours, the germination of approximately 100 conidia on agar plate was checked under a stereoscopic microscope. After 12 hours, the appressoria formation of approximately 50 conidia on detached leaves was observed under a light microscope following lactophenol cotton blue staining.

Pathogenicity test. Four to five leaf-stage seedlings of maize grown in a pot were sprayed with sufficient amount of suspension of 5×10^4 conidia ml^{-1} containing 0.1 % Triton X-100 using a sprayer (approximately 10 ml per seedling), and then kept in a moist chamber at 25 ˚C with 12 hours of daily illumination. The inoculated 4 leaves were collected and the number of lesions appeared was counted at 4 days after inoculation.

Results and discussion

Effect of a two-phase system on conidia production

The results of conidia production by a two-phase system using sponge matrix are summarized in Tab. 1. In the first phase, conidia of *E. turcicum* Et0005 germinated in liquid medium and then hyphae grew well and formed thin mycelia on the surface of the polyurethane foam cubes. There were few free mycelia observed in liquid medium when the polyurethane foam was inserted. The mycelia produced in the sponge matrix reached a maximum yield of over 15 dried g l^{-1} within 3 days. In the second phase, these mycelia were exposed to the air and consequently conidia were formed. The number of the conidia produced within 48 hours in a moist chamber was 6×10^8 l^{-1}, which was almost equivalent to 3 dried g l^{-1}. The estimated ratio of conidia/mycelia in dried weight was around 20 %. The results revealed that a two phase system could massively produce conidia of *E. turcicum* within 6 days, which is much shorter than conidia production by plate culture using PDA (12 days).

Tab. 1. Mycelial growth and conidia production of *E. turcicum* Et0005 by a two-phase system using sponge matrix

1^{st} phase	Days after inoculation			
Submerged in liquid	2	3	4	5
Mycelia grown (dried g l^{-1})	<10	15.8±0.3	15.4±0.6	15.2±0.8
2^{nd} phase[1)]	Hours after incubation			
Exposed in the air	24	48	72	96
Conidia produced ($\times 10^8$ l^{-1})	<1	5.3±0.2	5.3±0.3	5.2±0.3

Data present means ±SD derived from 5 replicates.
1) mycelia at 3 days after inoculation, which were used for conidia production.

There must be some technological constraints in mass production of fungal conidia of *Exserohilum*. Submerged fermentation has been preferred to solid-substrate fermentation systems due to its cost-effectiveness, availability and speed (JACKSON & al. 1996). Two-phase systems as shown by YAMAGUCHI (2000) for *E. monoceras* and in the present paper using sponge matrix could be an alternative for large-scale production of conidia, in cases when conidiation does not occur in liquid medium. It should further be

investigated if better sporulation in plate culture could be achieved with a cellulose or starch containing agar medium such as cornmeal agar or oatmeal agar, or an agar medium with filter paper or leaf tissue on the surface (SEIFERT, pers. com).

Tab. 2. Morphological characteristics of conidia produced by a two-phase system using sponge matrix compared to those produced by a plate culture

Conidia length range	Average	<70	70–89	90–109	110–129	>129	No. of septa
Two phase system[1)]	101.6	0	6	86	8	0	2–7
Plate culture[2)]	99.7	8	22	40	26	0	4–8

Morphological characteristics of the conidia

Although the averages of conidial length were almost the same in PDA plate culture and in the two-phase system, the conidia produced by the two-phase system were more uniform in size (Tab. 2). More than 85 % of the conidia produced in the sponge matrix were in the range of 90–109 µm in length. While the conidia produced on PDA showed more variation in size. Thus conidia production by a two-phase system is more synchronous than in plate culture.

Biological characteristics of the conidia

Almost all conidia produced by the two phase system were germinated and showed a higher percentage (89.2 %) of appressoria formation than those produced by plate culture (72.6 %) (Tab. 3, statistically significant $P<0.05$). The conidia of *E. turcicum* induced necrotic lesions in the leaves of maize seedlings at 4 days after inoculation (Tab. 3), which revealed the pathogenicity of the isolate.

Tab. 3. Germination, appressoria formation and infection (no. of lesions) of the conidia produced by a two phase system using sponge matrix compared to those produced by a plate culture. Data based on 5 replicates.

	Germination %	Appressoria formed	No. of lesions
Two phase system[1)]	99.4	89.2	8.1
Plate culture[2)]	75.8	72.6	7.6

1) 5-day culture used; 2) 12-day culture used.

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