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Improved procedure for mass inoculum production of *Fusarium* species in a short period of time

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Abstract

The induction of sporulation on artificial media is a critical factor for the production of inoculum in *Fusarium* species. An improved method of conidia production is described and compared to a conventional method growing *Fusarium* species on low-strength potato dextrose agar (LSPDA) under near-ultraviolet (NUV) light for 21 days. In the improved procedure, *Fusarium* spp. were grown in potato dextrose broth (PDB) for three days and subsequently spread as aliquots onto LSPDA; synthetic nutrient poor mineral agar (SNPMA) and water agar (WA) media. To remove excess water, the cultures were dried in a laminar flow cabinet for 20 to 30 min. Air-dried plates were incubated under NUV light at 25 ºC for 3 days. To compare aggressiveness of conidia produced by either method, wheat ears were inoculated at the mid-flowering. Disease incidence (% infected kernels) and Fusarium head blight (FHB) severity as well as fungal biomass produced in the infected kernels were determined. The improved method effectively triggered sporulation of *Fusarium* spp. and gave high yields of conidia per unit of area within two days of incubation on solid media which is significantly higher compared to spore quantities produced with the conventional method. The average number of conidia produced by *Fusarium* spp. using the improved and conventional methods ranged from 56 to 156 and 0.8 to 38.6×10^6 (per plate), respectively. Spore production quantity was highly variable with the conventional method using different *Fusarium* species. However, morphology of conidia was similar in both assays. The aggressiveness of *Fusarium* inocula produced by both methods was not significantly different with respect to the ability to cause FHB and to colonize wheat kernels ($P\leq0.05$). Microscopy examination showed the high conidiation rate from phialides on hypha. The method will facilitate studies of pathogens causing FHB requiring large quantities of conidia.

Key words: Conidia sporulation, conidiation, Fusarium head blight, *F. graminearum*.

بهینه سازی تولید انبوه زادمایه گونه های فوزاریوم در زمان کوتاه

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چکیده

در تحقیق حاضر روشی بهبود یافته برای تولید کنیدیوم در تعدادی از گونه‌های فوزاریوم عامل بلایت خوشه گندم بررسی و با روش رایج آن مقایسه شده است. در این روش، گونه هایی از *Fusarium* در محیط مایع سیب‌زمینی دکستروز (PDB) به مدت سه روز کشت و پس از آن در تشتک‌های پتری حاوی محیط‌های کشت آگاردار پخش شدند. برای حذف آب اضافی، تشتک‌های پتری به مدت ۲۰ تا ۳۰ دقیقه در هود لامینار خشک شد. پس از آن در دمای ۲۵ درجه سلسیوس به مدت سه روز برای تولید اینوکولوم نگهداری شدند. برای سنجش توانایی اینوکلوم در ایجاد بیماری، خوشه‌های گندم در اواسط گلدهی مایه‌زنی و فراوانی آلودگی، شدت بیماری زایی و همچنین زیست توده قارچی سنجش شد. روش بهبود یافته باعث القاء تولید کنیدی در مقادیر زیاد در واحد سطح و تا ۷۰ برابر بیشتر از روش رایج گردید. متوسط تعداد کنیدیوم تولید شده در گونه‌های فوزاریوم مورد استفاده در روش رایج و بهبود یافته به ترتیب از ۰/۸ تا ۳۸/۶ و $۱۰^۶\times$ ۱۵۶ تا ۵۶ (در تشتک پتری) متغیر بود. تفاوتی از نظر مورفولوژی تولید کنیدیوم در هر دو روش مشاهده نگردید. از نظر آماری تفاوتی در میزان بیماری تولید شده ناشی از زادمایه دو روش مشاهده نگردید ($P\leq0.05$). مشاهدات میکروسکوپی حاکی از نرخ بالای تولید کنیدیوم از فیالیدها روی میسلیوم‌ها بود. مطالعه حاضر باعث تسهیل تحقیقات روی جنبه های مختلف گونه‌های فوزاریوم زمانی که میزان زیادی مایه تلقیح مورد نیاز است می‌گردد.

واژه‌های کلیدی: اسپورزایی، بلایت فوزاریومی خوشه گندم، کنیدیوم، کنیدیوم‌زایی.

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Introduction

Reproduction and dispersal via production of asexual spores is an important part life cycle of fungi (Jung *et al*., 2014). Most fungi may produce large quantities of conidia in a very short time period after induction of conditions conducive for sporulation such as high temperature, nutrient depletion, high density of inoculum and other factors inhibiting vegetative development (Hanlin, 1994; Lapaire and Dunkle, 2003; Sekiguchi *et al*., 1975). Inducing spore production in the laboratory is a common procedure since fungal spores are used in various fungus-plant interaction studies.

Microcycle conidiation (MC), i.e. sporulation directly after spore germination without or with strongly reduced mycelial growth, has been most commonly used through the manipulation of environmental conditions (Hanlin, 1994; Smith *et al*., 1981). This type of conidiation has been reported in more than 100 fungal species (Jung *et al*., 2014). The ability to induce MC in various fungi has been also a useful tool in biochemical and physiological studies of sporulation, as it permits the synchronization and simplification of the conidiation process (Hanlin, 1994; Leslie and Summerell, 2006). Based on morphological differences during MC among fungal species, MC has been separated into four distinct categories (Jung *et al*., 2014). In the second type of MC, which has been reported for plant pathogenic fungi, most conidiophores produce conidia without specialized phialides as well as conidia frequently produced from intercalary phialides.

The infection of cereal kernels with *Fusarium* species and contamination with their mycotoxins are a threat to food and feed supply throughout the world (Bottalico and Perrone, 2002; Logrieco *et al*., 2003). *Fusarium* head blight (FHB) is often a complex disease, with several *Fusarium* species contributing to the disease (Reid *et al*., 1996). Isolates of the *Fusarium* species differ greatly in biological and ecological features, e.g. aggressiveness to cereals, host range, mycotoxin production, optimum of growth conditions, and survival on crop debris (Akinsanmi *et al*., 2004; Desjardins, 2006; Hörberg, 2002; Kohl *et al*., 2007; Logrieco *et al*., 2003; Parry *et al*., 1995; Rossi *et al*., 2002).

Fusarium species also differ in their thermal requirements for optimal growth on potato dextrose agar (Brennan *et al.*, 2003; Cook and Christen, 1976; Pettitt *et al*., 1996). Rossi *et al.* (2002) reported that *Fusarium* species are able to produce macroconidia at 5 to 35 °C.

Different procedures of spore production have been previously developed for *Fusarium* species either in liquid or solid cultures, but they are often time consuming and do not lead to the production of large number of spores in less than two weeks. Reid *et al.* (1996) described a low-sugar liquid medium with spore production up to 2×10^6 conidia mL^{-1} during two weeks, depending on the strain in some *Fusarium* species. Evans *et al.* (2000) developed an inoculum production technique using mung bean agar to attain high yields for different isolates of *F. graminearum*. Macroconidia, however, could only be harvested after two weeks of incubation.

Gale *et al.* (2005) described conidia production in mung bean broth (extract of 40 g of mung beans in 1 liter of water for 10 min) followed by shaking on a rotary shaker for 3–4 days at 20 –25 . The aim of this study was to develop an improved and rapid method for mass inoculum production in *Fusarium* species.

Materials and methods

Fungal strains: Strains of different *Fusarium* species including *F. avenaceum* (Fr.) Sacc. (= *Gibberella avenacea* R.J. Cook) (isolate 1.7), *F. culmorum* (W.G. Sm.) Sacc. (isolate 3.2), *F. graminearum* Schwabe (= *G. zeae* (Schwein) Petch) (isolate 5.1), *F. poae* (Peck) Wollenw. (isolate 7.8) and *F. tricinctum* (Corda) Sacc. (= *G. tricincta* El-Gholl, McRitchie, Schoult& Ridings) (isolate 10.11) were used after purification via single spore method. All isolates were from the Institute of Crop Science and Resource Conservation, University of Bonn, Germany.

Media composition: Laboratory grade chemicals and ultra-pure water were used for media preparation. Cultural media were: Low-strength potato dextrose agar (LSPDA; per 1 L: potato dextrose agar 12.5 g, agar-agar 19.5 g; Merck, Darmstadt Germany); synthetic nutrient poor mineral agar (SNPMA; Nirenberg 1981)**,** potato dextrose broth (PDB; per

1 L: 24 g; Merck, Darmstadt, Germany) and water agar (WA; per 1 L: agar-agar 20 g).

Inoculum production

Conventional method: The *Fusarium* species including *F. avenaceum*, *F. culmorum*, *F. graminearum*, *F. poae* and *F. tricinctum* were grown on LSPDA medium under constant near-ultraviolet (NUV) light for 21 days at 25°C. The cultures were checked daily during the period for conidia production. After 21 days, conidia were harvested by adding 20 ml sterile distilled water including 0.01% Tween 20 (Merck, Hohenbrunn, Germany) and slightly scraping with a spatula. The suspension was passed through double-layers of sterile cheesecloth. The number of conidia per 20 mL was determined using a haemocytometer (Bright-Line™, Sigma-Aldrich®, Germany) and then adjusted to the plate size (9 cm in diameter). The experiments were repeated five times for each species and representative data have been shown.

Improved method: The *Fusarium* species were cultured on PDA, after five days, three mycelial plugs (0.5 cm) from the edge each colony growth were added into 250 mL Erlenmeyer flasks containing 50 ml PDB. The flasks were incubated for three days at 25 ºC in darkness and 200 rpm shaking conditions. After three days of incubation, 1 ml of suspension was spread over the surface of Petri dishes containing LSPDA, WA and SNA, respectively, using a sterile bent-glass rod in five replications. To remove excess water, the cultures were dried under a laminar flow cabinet for 20-30 min. The plates were incubated under NUV light at 25 ºC for two days. The number of conidia per plate was determined as described above for the conventional method. Other media such as SNA and WA were included in the experiments to assess the ability of conidia production on different media. The experiments were repeated five times for each species and representative data have been shown.

Inoculation of wheat ears: The quality of conidia produced by *F. avenaceum, F. culmorum, F. graminearum* and *F. poae* on LSPDA using the two methods were assessed in pathogenicity tests. Conidia concentrations were adjusted to 5×10^4 conidia mL^{-1} for inoculations of wheat ears. At the mid flowering stage (GS 65, Meier) sixty wheat ears (*Triticum aestivum*, cv. Munk) were spray-inoculated with 120 mL spore suspension of the *Fusarium* isolates 1.7 (*F. avenaceum*), 3.11 (*F. culmorum*), 5.1 (*F. graminearum*), and 7.8 (*F. poae*), respectively. Control plants were sprayed with sterile distilled water in the same way. After inoculation, the plants were covered with plastic bags for 48 hours to provide high relative humidity for optimum infection conditions. The experiments were conducted in a completely randomized design with 5 replicates (5 pots with 12 ears) for each *Fusarium* species.

Disease assessment

Disease severity and kernel colonization: Disease severity was assessed as percentage of bleached spikelets 14, 21 and 28 days after inoculation using a nine-class rating scale (Miedaner *et al*., 1997) in which 1 = no infection, 2 = <5%; 3 = 5-15%; 4 = 16 - 25%; 5 = 26 -45%; 6 = 46-65%; 7 = 66-85%; 8 = 86-95%, and 9 = 96-100% of bleached spikelets. The mean value of the three disease severity ratings was determined.

To assess kernel colonization for each replicate, 50 kernels were sterilized in 0.5% sodium hypochlorite (NaOCl, Applichem, Darmstadt, Germany) for 2 min, rinsed with sterile water twice, dried, and cultured on Czapek-Dox-Iprodione-Dicloran agar (Abildgren *et al.*, 1987) for re-isolation of *Fusarium* spp. Synthetic nutrient-poor mineral agar (Nirenberg 1981) and Banana leaf agar were used to grow the isolates for morphological identification (Seifert, 1996).

Assessment of fungal biomass: A CTAB method (Brandfass and Karlovsky, 2006; Stewart and Via, 1993) with some modifications was used in all experiments for DNA extraction. The modification entailed the use of 20 ml of CTAB-extraction buffer (10 mM Tris, 20 mM EDTA, 0.02 M CTAB, 0.8 M NaCl, 0.03 M N-laurylsarcosine, 0.13 M sorbitol, 1%(w/v) polyvinylpolypyrrolidone, pH set to 8.0 (with NaOH), 40 µl mercaptoethanol and 50 µl proteinase K (from a 10 mg/ml stock solution). The solution was added to 250 mg ground grains or 50-100 mg fresh mycelia (growing on the surface of agar media) contained in 50–ml centrifugation tubes and mixed vigorously. The mixture was incubated at 65 °C for 60 min and mixed every 10 min. Eight hundred µl of the upper phase was transferred to a new 2 ml

tube containing 5 µl of RNAase (50 mg / ml) and incubated for 10 min at 65°C. Following incubation, 800µl of chloroform-isoamyl alcohol (24:1) was added to each tube. The samples were mixed by inverting the tubes and subsequently centrifuged for 10 min at 5,000g at room temperature. The aqueous phase was transferred into a 1.5 ml tube containing 500 µl isopropanol, mixed and incubated for 20 min at room temperature and centrifuged for 15 min at 15,000 g at room temperature. The pellet was washed with 70% (v/v) ethanol, dried and dissolved in 200 µl TE buffer and incubated at 4 °C overnight and kept at -20 °C.

All primers (Table 1) were synthesized by Carl Roth Company (Karlsruhe, Germany). Fungal biomass was quantified using a SYBR®-Green real-time PCR assay (ABI Prism® 7000 SDS; Applied Biosystems, Foster City, USA) as described by Moradi *et al*. (2010). Fungal biomass was calculated as ng fungal DNA per mg kernel dry weight.

Table 1. Sequences of species-specific primers for detecting *Fusarium* species using real-time PCR

Fragment name	Sequence of primers	Target	Size (bp)	Annealing temperature
F. graminearum [A]	GGCGCTTCTCGTGAACACA TGGCTAAACAGCACGAATGC	*F. graminearum*	94	55ºC
F. culmorum [A]	TCACCCAAGACGGGAATGA GAACGCTGCCCTCAAGCTT	*F. culmorum*	60	55ºC
F. avenaceum [A]	CAAGCCCACAGACACGTTGT CCATCGCCGTGGCTTTC	*F. avenaceum*	58	57ºC
F. poae [B]	CAAGCAAACAGGCTCTTCACC TGTTCCACCTCAGTGACAGGTT	*F. poae*	220	60ºC

A, Waalwijk *et al.* (2004); B, Parry *et al*. (1995)

Microscopy: Fungal growth and conidia production of *Fusarium* species were observed without slide preparation with a stereomicroscope (MZ16 F, Leica Microsystems, Bensheim, Germany). For image processing the microscope was equipped with a KY-F75 digital camera (Sony, Munich, Germany) and Diskus software (TB Hilgers, Königswinter, Germany). Microscopic observations were performed either with slide preparations in sterile distilled water or directly from the cultures using a Nikon Eclipse 80i light microscope (Nikon USA, Milville, NY) equipped with a Nikon digital camera DS-L3.

Statistical analysis: For all *Fusarium* species the average quantity of conidia production, disease severity ratings, frequency of re-isolation and the content of fungal DNA were separately determined for each replication. When necessary, data were log-transferred prior to analysis. Data were analysed using the Proc GLM procedure [SAS 9.0, SAS Institute, Inc. Cary, NC). Mean comparisons were made using Student Newman-Keuls multiple comparison test at 5% of error probability. Pairwise comparisons of mean for disease severity ratings, frequency of re-isolation and the content of fungal DNA values between data obtained from the conventional and the improved method were made using Student's t-test (Elliott, 2014). Pearson's Chi-squared test with Yates' continuity correction was applied to compare the conidia production and aggressiveness between and within *Fusarium* species using the statistical package for social sciences (SPSS) and as described by Preacher (2006).

Result and Discussion

Inoculum production: The rate of conidia formation was affected by production method, media and *Fusarium* species. With the improved method, all isolates of the tested *Fusarium* species produced a high amount of conidia in a very short period of time (Table 2). For example, after three days in PDB and two days on LSPDA, SNA and WA the number of conidia produced by *F. graminearum* was 70, 55, and 24 times, respectively, higher than using conventional method ($P \leq 0.05$). The respective multiplication factors were 12, 5.4, and 3.4 for *F. culmorum*, 3.9, 2.9, and 1.4 for

F. avenaceum, 6.5, 3.4, and 1.6 for *F. poae*; 3.9, 3, and 1.5 for *F. tricinctum*. For *F. culmorum* and *F. graminearum* Pearson's Chi-squared test showed significant differences between the conventional and improved method to produce conidia ($P < 0.05$), while there were no statistical significant differences for the other species to produce conidia through both methods. There were significant differences among the species to produce conidia in the conventional method ($P < 0.05$), which were not significant for the improved method.

The average number of conidia produced by *F. graminearum* was 0.8 * 106 (per plate) using the conventional method, and ranged from 18.8 *106 to 56.2*106 for the improved method depending on the medium used (Table 2). Compared to the other *Fusarium* species, *F. graminearum* produced low amounts of conidia in both procedures of spore production on all media. With the procedure introduced here, the number of conidia produced was 31 to 90 % of the other *Fusarium* species, compared to 2 to 8 % with the conventional method. With the improved method of spore production, the ratios of conidia produced on LSPDA were 1.3 to 3.9 times higher than on the other media. The number of spores only slightly increased until day 5 with the improved procedure, and further incubation resulted in the production of abundant mycelium (Fig 1D). The conventional methods required considerably more time and still resulted in lower conidiation, especially for *F. graminearum.*

Table 2. Conidium production of *Fusarium* species using a conventional and improved procedure of inoculum production on different media. Plates were incubated for 21 days (conventional) and 2 days (improved method), respectively

	Number of conidia per plate × 10^6					Yates' *	
	Conv. on LSPDA[a]		Improved procedure on			*P*-value	Chi-square
***Fusarium* species (isolate)**	**After 5 days**	**After 21 days**	**LSPDA**	**SNA[b]**	**WA[c]**		
F. graminearum (5.1)	Rarely	0.8 C[d]	56.2 A	44.0 A	18.8 B	0.037	8.465
F. culmorum(3.11)	-	10.6 D	128.3 A	57.3 B	35.6 C	0.041	8.236
F. avenaceum (1.7)	-	21.0 B	83.3 A	61.7 A	30.4 B	0.463	2.568
F. poae (7.8)	-	14.6 B	94.7 A	49.0 B	24.3 B	0.585	1.937
F. tricinctum(10.11)	-	38.4 B	150.0 A	118.7 A	59.3 B	0.101	6.208
Yates' p-value		0.006	0.090	0.300	0.970		
Yates' chi-square		14.216	7.790	4.832	0.570		

a. Low-strength PDA; b. Synthetic nutrient-poor agar; c. Water agar; d Means separated by independent multivariate analyses for *Fusarium* species (Student Newman-Keuls test, $p \leq 0.05$) ;*. Pearson's Chi-squared test with Yates' continuity correction was applied to compare the inoculum production between *Fusarium* spp. as well as conventional and improved procedures.

Microscopy of conidia production: Microscopic observations of the mechanisms of *Fusarium* species conidiogenesis using the improved method revealed the production of sporodochial conidial masses on the surface of the media in orange, red purple, violet, blue violet and red (Figs 1C, E-H, M-P). Conidia were budded on monophialides formed on hyphae, branched conidiophores or conidiogenous cells. After adding the mycelia from liquid to solid media, mycelia predominantly shifted to the production of sporodochia within the first 2 days, while the mycelia, conidia or visible sporodochia were the predominant stages in the conventional method even though mycelia were the main biomass (Figs 1 A-B, I-K).

After five days of incubation with the improved method, the produced conidia had germinated and formed mycelium covering the plate (Fig 1D). No morphological

differences were observed in *Fusarium* spp. neither on conidiogenesis nor on the morphology and germination behaviour of the produced micro- and macroconidia (Figs 1Q-T). Similar to the conventional method, with the improved method *F. graminearum* and *F. culmorum* produced only macroconidia while all types of conidia were observed in cultures of *F. avenaceum*, *F. poae* and *F. tricinctum*.

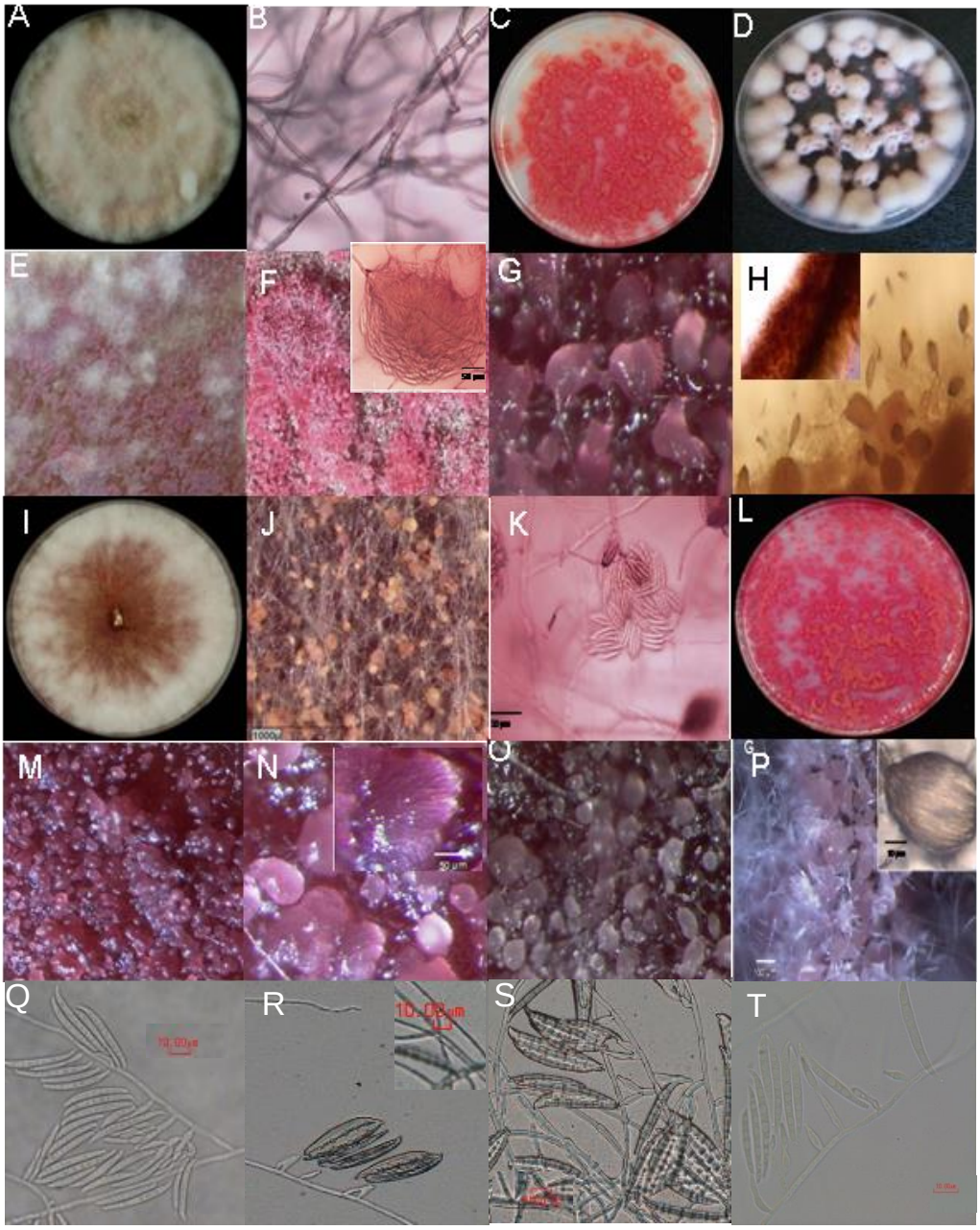

Fig. 1. Morphology of conidia produced by *Fusarium* species with a conventional and the improved inoculum production method on low-strength PDA for 10 and 2 days of incubations, respectively. A and B, *F. graminearum* (conventional method); C to H, *F. graminearum* (improved method); I to K, *F. culmorum* (conventional method); L to N, *F. culmorum* (improved method); O, *F. avenaceum* (improved method); and P, *F. tricinctum* (improved method); Q to T, morphology of conidiophores of *F. graminearum* on LSPDA which produced abundant macroconidia on branched and unbranched monophialides. Inserts (F, H, N, P) display higher magnifications of conidia produced in mucilaginous droplets.

Aggressiveness of Fusarium inoculum on wheat ears: The quality of *Fusarium* inoculum produced with both procedures was compared in inoculation experiments. For both inocula, the ability to produce disease was evaluated using disease severity, frequency of infected kernel and fungal biomass (Table 3). All *Fusarium* species tested were able to produce FHB either in greenhouse or field experiments as shown for *F. graminearum* in Fig 2. For all species and estimated parameters the aggressiveness decreased in the order *F. graminearum*, *F. culmorum*, *F. avenaceum* and *F. poae* independent of the method of conidia production. In both methods, *F. graminearum* also gave by far the highest amount of fungal DNA, followed by *F. culmorum*, *F. avenaceum* and *F. poae* with 60%, 5%, and 0.3%, respectively, of the fungal DNA content in kernels as compared to *F. graminearum*.

A t-test was used to compare differences between means of the conventional and improved method for disease severity, frequency of infected kernel and fungal biomass. The results revealed no significant differences between the two methods. Although the frequency of *F. poae* infected kernels was significantly higher with the improved (7 %) method than the conventional method (2 %), no significant differences were observed for fungal biomass (0.05-0.06 ng fungal DNA per mg kernel dry weight) and disease severity rating (1) ($p \leq 0.05$).

Table 3. Aggressiveness of *Fusarium* conidia produced with conventional and improved procedure of inoculum production on FHB severity of wheat (cv. Munk), frequency of kernel colonization, and fungal biomass after inoculation with *Fusarium* spp. at GS 65. Greenhouse study

Fusarium species (isolate)	Disease severity (rating, 1 – 9)*		Frequency of infected kernel [%]		Fungal biomass [ng DNA/mg DW[a]]	
	Conv. [b]	Improved [c]	Conv.	Improved	Conv.	Improved
Non-inoculated	1.0	1.0	0	0	0	0
F. graminearum (5.1)	6.5	7.1	65.2	63.5	15.9	17.4
F. culmorum(3.11)	4.5	5.2	49.6	47.5	9.4	10.5
F. avenaceum (1.7)	3.4	3.5	35.2	28.5	1.0	0.6
F. poae (7.8)	1.0	1.0	2.0	7.0**	0.05	0.06

a. ng fungal DNA per mg kernel dry weight; b. Conventional method of inoculum production; c. Improved method of inoculum production; *. 1 = no infection, 2 = <5; 3 = 5-15; 4 = 16 to 25%; 5 = 26 -45%; 6 = 46-65%; 7 = 66 - 85%; 8 = 86 - 95%, 9 = 96 - 100% of bleached spikelets; **. Significantly different from conventional method (t-test, $p \leq 0.05$).

Fig. 2. Development of Fusarium head blight on wheat ears after inoculation with macroconidia of *F. graminearum* produced with the improved method. A: greenhouse; B: field experiments

A simple and rapid method for the production of spores of *Fusarium* species has been developed. All species produced large amounts of mycelium in PDB. Mucilaginous colonies characterized by great reduction of mycelia formation and the production of large quantities of conidia developed within 2 days after transferring the mycelia onto solid media. As the liquid medium prevented *Fusarium* species from producing conidia, it was important to dry the culture plates before incubation. In the conventional method, all species produced mycelia, conidia or visible sporodochia, even though mycelia were the main source of biomass.

In the improved method, the quantity of produced conidia in the *Fusarium* species decreased in the order of *F. tricinctum, F. culmorum, F. poae, F. avenaceum* and *F. graminearum*, respectively. Similar to conventional method, both macro- and microconidia were produced in *F. avenaceum, F. poae* and *F. tricinctum* with the improved method.

Although different isolates of *F. graminearum* are able to produce conidia in MBB or MBA, However the current improved method takes shorter time with higher concentrations of conidia and its simplicity is superior. For example, the overall required time to produce large quantity of conidia using the MBB or MBA is between one and four weeks in *F. graminearum* isolates ((De Villiers, 2009; Gale *et al.*, 2002 and 2005; Zhang *et al.*, 2012). The present method may also be useful in production of secondary metabolites by *Fusarium* species, such as gibberellic acid in solid state and submerged fermentation in which a large amount of conidia in a short time is needed.

In the conventional procedure, isolates of *F. graminearum* did not produce high numbers of conidia and the variability in spore production even among plates within a single isolate was high. Pearson's Chi-squared test showed high variability among the species to produce conidia using the conventional method for *F. culmorum* and *F. graminearum* between the conventional and the improved methods.

The extreme change from liquid to solid media is likely to trigger response mechanisms by the *Fusarium* species/isolates to form mucilaginous colonies with high numbers of conidia. This may be explained by the fact that *Fusarium* species produce high amounts of mycelium in PDB. Therefore, a high biomass in liquid medium depleted of nutrients and extreme changes in environmental conditions – dryness and aeration - may shift the metabolism and ontogenesis of *Fusarium* species to exponential production of phialides and conidia in mucilaginous colonies. Slade *et al.* (1987) reported the occurrence of *Colletotrichum gloeosporioides* microcycle conidiation on commonly used microbial media, however, only at high inoculum densities. They also mentioned that microcycle induction is a function of diffusion restricted nutrient availability to the fungal colony and/or accumulation of microcycle-inducing fungal metabolites. Conidiation in *Cercospora zeae-maydis* was sensitive to α-amanitin, an inhibitor of mRNA synthesis, and cycloheximide, an inhibitor of protein synthesis, suggesting that new RNA and proteins must be synthesized (Lapaire and Dunkle, 2003). Kølmark (1984) mentioned that many filamentous fungi conidiate poorly or not at all in submerged culture even though such proliferation may be abundant during growth on open surfaces. Son *et al.* (2013) showed that microcycle conidiation induced in *wetA* deletion mutant of *F. graminearum* by vigorous generation of single-celled conidia through autophagy-dependent MC, while in the wild type conidia budded from phialides originating from hyphae.

In tests on the aggressiveness of conidia produced with both methods, the frequency of kernel infection, FHB severity and *Fusarium* biomass of kernels measured as *Fusarium* DNA content were not significantly different. For example, the means of frequency of kernel infection, FHB severity and *Fusarium* biomass for *F. graminearum* with the both methods were ranged 63.5- 65.2%, 6.5-7.1% and 15.9-17.4%, respectively. This indicated that the pathogenic potential of conidia produced with the improved method was similar to those conidia produced with the conventional method. For highly aggressive species, symptoms were observed within 1 to 2 days after inoculation which indicated the efficiency of conidia to cause disease. For both methods, *F. graminearum* resulted in the highest rates of infection and colonization as well as FHB severity, followed by *F. culmorum*, *F. avenaceum* and *F. poae*, respectively.

Pathogenicity of *Fusarium graminearum* mutants with *FgStuA* gene deletion on wheat heads and production of

secondary metabolites was greatly reduced (Lysøe *et al.*, 2011). They also mentioned that the wild type produced macroconidia on solitary or multiple phialides on conidiophores, while mutants lacked conidiophores and phialides, leading to delayed production of aberrant macroconidia. Son *et al*. (2013) showed that in *F. graminearum* the *wetA* gene is involved in conidiogenesis and conidium maturation via maintenance conidia dormancy by suppressing MC.

The method has been successfully applied to produce inoculum for several *Fusarium* isolates on maize and wheat including *F. avenaceum*, *F. culmorum*, *F. graminearum*, *F. poae, F. tricinctum, F. verticillioides* and *F. proliferatum* for different aims (Görtz, 2010; Moradi, 2008). The method described here facilitates studies on different aspects of *Fusarium* species and their conidia where large amounts of conidia are required.

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