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MATING TEST AND *IN VITRO* PRODUCTION OF PERITHECIA BY THE COFFEE WILT PATHOGEN, *GIBBERELLA XYLARIOIDES* (*FUSARIUM XYLARIOIDES*)

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ABSTRACT: *Gibberella xylarioides* Heim & Saccas, the teleomorphic state of *Fusarium xylarioides* Steyaert, is a fungal pathogen causing a vascular wilt disease of coffee known as tracheomycosis. Coffee wilt disease has been one of the endemic diseases of Arabica coffee (*Coffea arabica*) with increasing outbreaks and prevalence in Ethiopia. In the present study, *in vitro* production of fertile perithecia was assessed by crossing eight randomly selected monoconidial isolates of *G. xylarioides* (*F. xylarioides*) collected from *C. arabica*, including few strains from *C. canephora* and *C. excelsa*. Five media types and two temperature levels were compared for inducing the sexual structures in the fungus. *In vivo* development of perithecia on coffee trees in the field and on inoculated young seedlings in the greenhouse was also examined. More than 30% of the crosses formed perithecia *in vitro* including those pairings of isolates recovered from the different *Coffea* spp. Isolate Gx1 (BBA 71975) showed high intra- and inter-fertility with most of the strains indicating that this isolate was most probably 'female fertile' mating type. This interfertility was successfully achieved only on carrot agar and V-8 juice medium at 20^{o}C and 12-hr photoperiod 10 – 12 wks after plating. The fungus often produced abundant perithecia containing enormous ascospores in the barks of dead coffee trees in the field and in the artificially infected young seedlings. The *in vitro* and *in vivo* perithecial developments were most likely favoured by cool and moist (wet) conditions. Abundant production of ascospores in the field is an integral part of survival and dispersal mechanisms along with genetic exchange system. The *in vitro* formation of fertile perithecia enables to study inheritance of traits such as pathogenicity (virulence) and gene flow and genetic diversity in populations of *G. xylarioides*.

Key words/phrases: Coffee wilt disease; *Coffea arabica*; Ethiopia; *Gibberella xylarioides*; Mating test; Perithecia.

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INTRODUCTION

Gibberella xylarioides Heim & Saccas, the teleomorphic state of *Fusarium xylarioides* Steyaert, is a fungal pathogen causing a vascular wilt disease of coffee frequently referred to as coffee tracheomycosis. This pathogen was reported to wipe out *Coffea excelsa* and *Coffea canephora* production in West and Central African countries between the 1950s and the 1960s (Muller, 1997) although it was rarely described in the literature. Coffee wilt disease was contained through the use of resistant host and sanitation for some three decades, although it re-emerged as a destructive disease of coffee in East and Central Africa in the 1980s (Flood, 1996; Flood and Brayford, 1997; CABI, 2003). It has been an endemic disease with increasing outbreaks and prevalence on Arabica coffee in Ethiopia (van der Graaff and Pieters, 1978; Girma Adugna, 1997; Girma Adugna *et al*., 2001; CABI, 2003; Girma Adugna, 2004). The fungus life cycle and the disease epidemiology were little understood and this hampered coffee wilt management. Studies on pathogenicity tests proved host specificity and diversity in aggressiveness of *G. xylarioides* populations obtained from *Coffea arabica*, *C. canephora* and *C. excelsa* (Girma Adugna and Mengistu Hulluka, 2000; Girma Adugna *et al*., 2005; 2007). The population structure, however, seemed to be exceptionally homogeneous within the respective subgroup by microsatellite (Janzac *et al*., 2005) and RAPD analyses (Girma Adugna *et al*., 2005).

G. xylarioides reproduces both sexually and asexually forming ascospores and conidia, respectively. The production of perithecia *in vivo* was observed by a number of researchers although there was no report on genetic analysis and contribution of the structures to population diversity (van der Graaff and Pieters, 1978; Flood, 1996; Girma Adugna and Mengistu Hulluka, 2000; Girma Adugna *et al*., 2001). *In vitro* perithecia formation by *G. xylarioides* was first noted by Heim and Saccas (1951). Booth (1971) also mentioned the possibility of perithecia development in culture only if correct mating types were brought together under suitable cultural conditions. Booth (1971) attempted to re-describe the species as heterothallic ascomycete with the presence of sex-linked “male and female strains” that can be differentiated based on colony and spore morphology. Von Blittersdorff and Kranz (1976) later identified the so called “male strain” as *G. stilboides* (*F. stilboides*), and only the “female strain” was encountered so far from large wild collections of the fungus (van der Graaff and Pieters, 1978; Nelson *et al*., 1983; Girma Adugna and Mengistu Hulluka, 2000). There has been, however, limited knowledge that substantiates development of fertile

perithecia of this fungus under controlled artificial conditions. The ability to manipulate sexual recombination in the laboratory would facilitate study of inheritance, gene mapping, and genetic exchange or gene flow between populations through the sexual cycle (Bowden and Leslie, 1999). Thus, this article presents results of studies on *in vitro* formation of fertile perithecia in comparison to *in vivo* produced sexual structure of *G. xylarioides*.

MATERIALS AND METHODS

In vitro mating tests

Mating tests were carried out by crossing eight monoconidial isolates of *G. xylarioides* randomly sampled from large culture collections in the Phytopathological laboratory at the Institute of Plant Diseases of Bonn University. The isolates Gx1 (BBA 71975), Gx3, Gx5, Gx7 and Gx11 represented recent (2001) collections from infected *C. arabica* (Arabica coffee) trees in different districts in Ethiopia; Gx12 (BBA 71980) was a recent isolate obtained from *C. canephora* (Robusta coffee) in Uganda in 1997; and Gx20 (BBA 62721) and Gx21 (DSMZ/BBA 62457) were historical strains of the 1960s collections from Robusta coffee in Guinea and *C. excelsa* (Excelsa coffee) in Central African Republic, respectively (Table 1). Five nutrient media, namely, fresh potato dextrose agar (PDA) and potato sucrose agar (PSA) prepared according to Booth (1971), water agar with a sterile coffee branch (WACB), carrot agar (CA) and V-8 juice medium (V-8) were used for the mating tests. Carrot agar was prepared according to Klittich and Leslie (1988) from fresh carrot (400 g) diced and autoclaved for 10 minutes in 400 ml distilled water. The carrot extract was mixed with additional 600 ml distilled water with 20 g agar (Merck), autoclaved for 20 minutes, and dispensed into 9 cm Petri dishes of about 15-20 ml per dish. The V-8 agar medium was made from 200 ml vegetable juice, 1.5 g $CaCO_3$ (Merck), and 20 g agar-agar (Sigma) per liter of distilled water (Hsieh *et al*., 1977). The water agar was prepared from 20 g agar (Merck) in 1 liter of tap water, after autoclaving and pouring into plates; a sterile Arabica coffee twig (7 cm long) was aseptically placed at the center of each plate and pressed into the agar.

All the isolates were first retrieved and grown on synthetic low nutrient agar (SNA) (Nirenberg, 1976) and then mated with each other adopting the mycelial plug crossing method developed by Bowden and Leslie (1999) in all possible combinations on individual medium (PDA, PSA, WACB, CA and V-8). Actively growing mycelia from cultures of each parent isolate grown on SNA (Nirenberg, 1976) were cut with a sterile cork borer (2 mm)

and placed opposite to each other. The plates were incubated in growth chambers adjusted at 20 and 25^{o}C temperature levels under 12-hr dark/12-hr fluorescent light cycles. After a week, 1 ml of sterile 2.5% Tween 60 (Merck) solution was added to each plate and the aerial mycelia were knocked down with a sterile glass rod while rotating the plate to distribute the solution (Bowden and Leslie, 1999). The cultures of all crosses were examined weekly for the presence of mature perithecia and ascospores for 12 wks, and scored as positive (present) or negative (absent). Thus, in a set of mating test with eight isolates paired on each medium and incubated at two temperature conditions, about 128 pairs (8^{2}x2) were made to identify cross-fertile isolates interactions. In total, five sets were undertaken, with each set replicated at least twice.

In vivo perithecial development study

In vivo perithecial development was studied in naturally infected Arabica coffee trees in the field at Jimma (1,750 m above sea level) and Gera (1,950 m) in Ethiopia in 2003 and 2004. At first, five coffee trees showing the characteristic partially wilting symptoms were tagged, and the main stem and primary branches in the three canopy layers (bottom, middle and top) were examined fortnightly. Similarly, five artificially inoculated and symptomatic coffee seedlings were marked in the greenhouse at Jimma Agricultural Research Center. Samples of black fruiting bodies were successively collected from these trees and seedlings by gently scrubbing with pieces of the bark for detailed microscopic examination of perithecial maturation and further characterization in the laboratory.

Characterization of *in vitro* and *in vivo* produced sexual structures

Morphological appearances of *in vitro* formed perithecia, asci and ascospores were described and compared with those field-collected ones. A single perithecium (3-5 perithecia per plate or bark pieces) was carefully removed and squashed with a needle after placing on a slide with a drop of sterile water. The color, shape and dimension measurements (width and length) of perithecia, asci and ascospores were microscopically recorded and documented. The viability of both *in vitro* and *in vivo* ascospores was tested on water agar plates.

RESULTS

Mating test and *in vitro* production of fertile perithecia

The mating test result showed that about 30% of *G. xylarioides* isolates were compatible and produced fertile perithecia. These fertile perithecia were formed only on carrot agar and V-8 juice medium at 20°C under 12-hr dark/light cycle. In this case, perithecial initials were observed between 6 and 8 wks that subsequently developed to mature perithecia 10 to 12 weeks after plating. Except some proto-perithecia, all the remaining crosses and selfed isolates, however, were infertile (perithecia without ascospores) or did not form perithecia (Table 1). Isolate Gx1 (BBA 71975) and Gx7 showed high fertility and readily mated with most strains. The crosses between isolate Gx1 (Arabica isolate) and Gx12 (Robusta isolate) (Fig.1) and with Gx21 (Excelsa strain) were fertile (Table 1). Similarly, the mating of another Arabica isolate Gx11 with a historical Robusta isolate Gx20 was also successful (Table 1).

Table 1 Crosses and *in vitro* formation of fertile perithecia by *G. xylarioides* from different *Coffea* spp. on carrot agar at 20°C under 12-hr light/dark cycles.

Isolates[a]	BBA No.[b]	Gx1	Gx3	Gx5	Gx7	Gx11	Gx12	Gx20	Gx21
Gx1	71975	NP							
Gx3	---	FP[c]	NP						
Gx5	---	FP	IP	NP					
Gx7	---	FP	FP	FP	NP				
Gx11	---	FP	IP	IP	FP	NP			
Gx12	71980	FP	NP	IP	NP	IP	NP		
Gx20	62721	IP	NP	NP	IP	FP	FP	NP	
Gx21	62457	FP	IP	IP	NP	IP	IP	FP	NP

[a]Gx1, Gx3, Gx5, Gx7 and Gx11 were *G. xylarioides* isolates collected, respectively, from infected *Coffea arabica* trees at Jimma, Gechi, Mettu, Teppi and Yirgacheffe in Ethiopia; Gx12 (recent) and Gx20 (historical) isolates from *C. canephora* trees in Uganda and Guinea, respectively, and Gx21 a historical strain from *C. excelsa* trees.
[b]BBA No. refers to culture collections number at Biologische Bundesanstalt, Berlin, Germany.
[c]FP = fertile perithecia with ascospores, IP = infertile perithecia (perithecia without ascospores), NP = no perithecia.

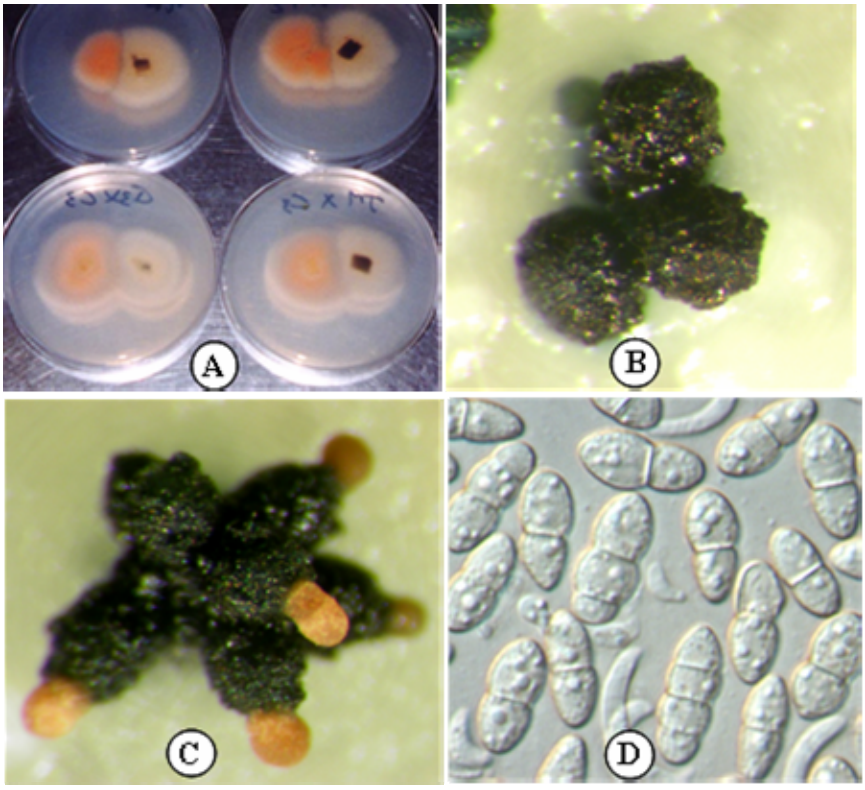

Fig. 1. Formation of *G. xylarioides* perithecia in *in vitro* mating test on carrot agar; A) crosses between strains from *Coffea canephora* (dark) and *C. Arabica* (whitish) isolates, B) fertile perithecia in group, C) perithecia oozing ascospores, and D) ascospores.

In vivo production of perithecia

G. xylarioides produced perithecia in dark stromatic fruiting bodies in the barks of stems of dead coffee trees in the field after 2-3 months. These stromatic structures were mostly observed around the crown region of the trees within 30 to 50 cm above the ground level. They were also occasionally seen higher on the stem and branches of the trees. The perithecia occurred frequently in cooler and damp places where shade and humidity were high from weeds or dense canopy stands of coffee trees. They developed often in clumps, conspicuously protruded or submerged in the bark (Fig. 2). These sexual structures also developed on some of artificially inoculated dead coffee seedlings with single spore isolate which were watered frequently under greenhouse conditions.

Description of *in vitro* and *in vivo* perithecia and ascospores

G. xylarioides perithecia are dark blue and born either singly or in group on the surface of the agar medium with extruded ascospores (Fig. 1). Most of these perithecia contained only free ascospores without asci while others had ascospores too immature to be seen microscopically in the asci. The morphological features of *in vitro* formed perithecia and ascospores are essentially similar to those produced in nature (Figs. 1 and 2). The perithecia were apparently globose and sometimes turbinate or napiform in shape with enormous ascospores oozing out upon crushing single perithecium (Fig. 2B, C and E). The dimension ranged from 98 x 123 µm (globose) to 340 x 485 µm (turbinate) (Table 2). The asci were cylindrical, and each ascus often contained 8 ascospores. The ascospores were hyaline, mostly fusoid in shape and 1 - 2 septate with a slight constriction at the septum (Fig. 2D), and measured 5.2 µm x 12.1 µm on average (Table 2). Both *in vitro* and *in vivo* developed ascospores germinated by forming germ tubes from one end (1-celled) or both ends (2-celled) and about 90–100% germination was estimated on water agar.

Table 2 Dimension of perithecia and ascospores of *G. xylarioides* collected from *C. arabica* trees in Ethiopia.

Sample group	Perithecial dimension	Ascospore dimension (µm)			
		Width		Length	
	(µm)	range	mean and SD [a]	range	mean and SD [a]
1	98 x 123	4.4 – 5.7	5.1 ± 0.5	11.5 – 14.9	12.8 ± 1.5
2	194 x 196	4.8 – 5.8	5.2 ± 0.3	11.5 – 13.2	12.2 ± 0.6
3	293 x 366	5.0 – 6.0	5.4 ± 0.4	10.2 – 13.5	11.8 ± 1.0
4	299 x 374	4.5 – 5.8	5.3 ± 0.5	10.3 – 14.0	12.3 ± 1.6
5	313 x 350	4.7 – 5.5	5.2 ± 0.3	11.9 – 13.5	12.6 ± 0.6
6	340 x 485	4.2 – 5.4	5.1 ± 0.4	11.3 – 12.9	12.0 ± 0.8
7	382 x 428	4.5 – 5.3	5.0 ± 0.3	9.7 – 11.8	11.1 ± 0.6
	Mean	4.2 – 6.0	5.2 ± 0.1	9.7 – 14.9	12.1 ± 0.6

[a] SD = standard deviations of the mean

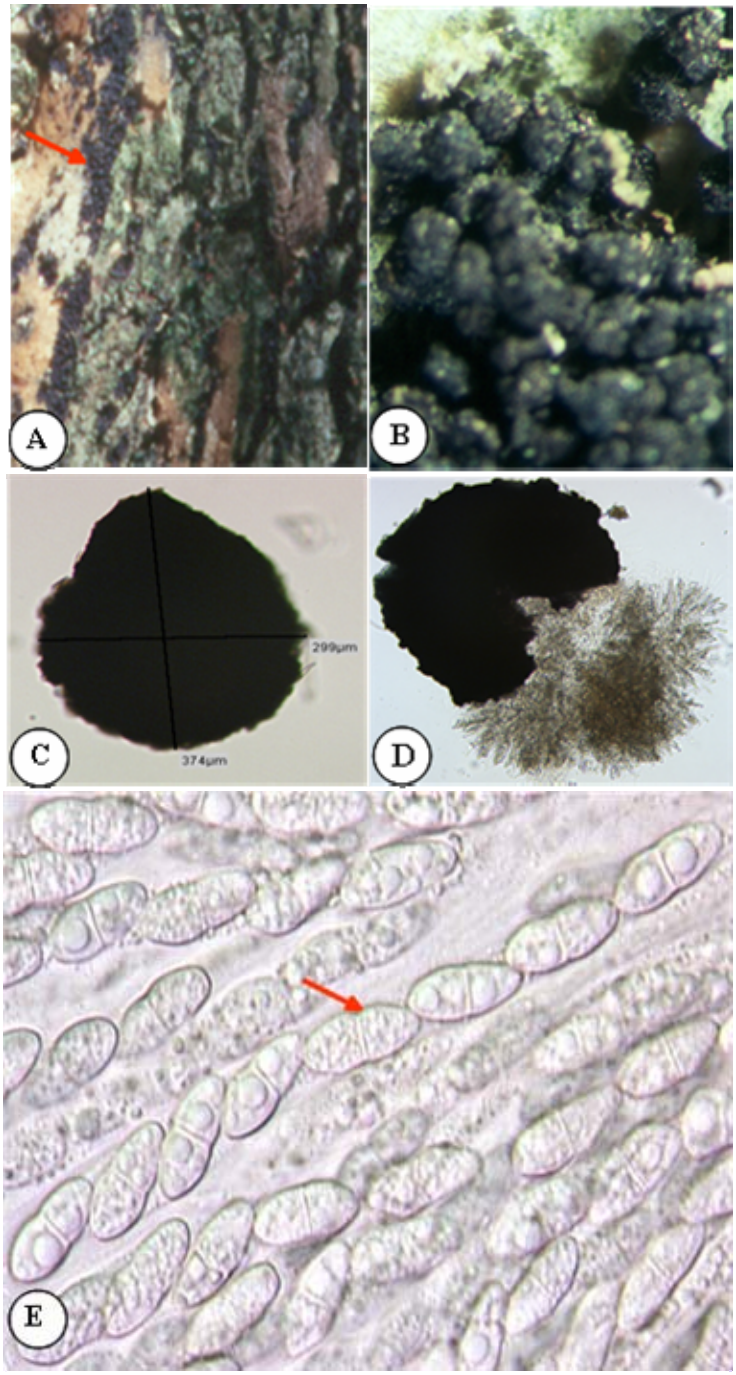

Fig. 2. Sexual (teleomorphic) structures of *Gibberella xylarioides*; A) protruded perithecia in coffee bark (arrow), B) perithecial aggregates, C) mature perithecium, D) squashed perithecium with asci and ascospores, E) a typical 8-ascospores in ascus.

DISCUSSION

The study, destined to produce fertile perithecia *in vitro* by crossing strains of *G. xylarioides* under artificial conditions, was successful at the end. Sexual crosses of some isolates (30%) were compatible and yielded mature perithecia with typical ascospores of the species. Among the isolates, Gx1 (BBA 71975) showed high intra- and inter-fertility with most of the strains implying that this isolate was most probably 'female fertile' mating type. Those crosses that resulted into either infertile perithecia (perithecia without ascospores) or no perithecia might be grouped as 'self-sterile hermaphrodites' that could function as either male or female parents in a pair. This interpretation corresponds with the hypothesis of Leslie and Klein (1996) that sexual reproduction increases the relative number of hermaphrodites. In field populations, polymorphism occurs for female-sterile/ hermaphrodite status and female-sterile mutants that function only as males during sexual reproduction may comprise > 50% of the population (Leslie and Klein, 1996).

The pairings of isolate Gx12 (BBA 71980) and Gx20 (BBA 62721), both from *C. canephora* of the recent and historical collections, were sexually intra-fertile. The interfertility between the two historical strains from *C. canephora* Gx20 (BBA 62721) and *C. excelsa* Gx21 (DSMZ/ BBA62721) agrees with our earlier conclusion that the recent strains attacking *C. canephora* in East and Central Africa might have originated from *C. excelsa*. In addition, successful interfertility among strains from *Coffea arabica*, *C. canephora*, and *C. excelsa* demonstrated that these strains belonged to the same biological species (mating population) of *G. xylarioides* (*F. xylarioides*), although host specialization and variation in aggressiveness was well documented from a number of host-pathogen interaction studies (Girma Adugna and Mengistu Hulluka, 2000; Girma Adugna *et al*., 2005; 2007). In this case those strains attacking only *C. arabica* in Ethiopia were named as *Gibberella xylarioides* f. sp. *abyssiniae* (anamorph: *Fusarium xylarioides* f. sp. *abyssiniae*) while isolates pathogenic to *C. canephora* and *C. excelsa* were termed as *G. xylarioides* f. sp. *canephorae* (anamorph: *F. xylarioides* f. sp. *canephorae*) (Girma Adugna, 2004; Girma Adugna *et al*., 2005; 2007). Rutherford (2006), based on various molecular analyses, reported that two clonal populations were responsible for the current CWD outbreaks in Africa, one comprising isolates obtained from affected *Coffea arabica* in Ethiopia (variant 'A'), and the other isolates from affected *C. canephora* in DR Congo, Uganda, and Tanzania (variant 'C').

Among the five culture media and two temperature regimes tested in *G. xylarioides*, fertility of the crosses was achieved on carrot agar and V-8 juice medium at 20°C under 12-hr light and dark cycling conditions. Lawrence *et al*. (1985) reported that isolates of *G. baccata* (*Fusarium lateritium*) produced perithecia on carrot agar at 22°C under mixed cool-white and black fluorescent light on a 12-hr alternating light/dark schedule. Desjardins and Beremand (1987) obtained proto-perithecia developed in some strains of *G. pulicaris* (*F. sambucinum*) on V-8 agar, potato dextrose agar, potato-carrot agar and water agar containing mulberry twigs or wheat straw. However, mature perithecia with extruded ascospores were produced only in crosses on mulberry twigs or rarely on wheat straw and only after incubation at 15°C. On the other hand, Hsieh *et al*. (1977) indicated that the V-8 juice agar was the best medium for formation of the *Gibberella* stage in mating groups A and B of *F. moniliforme*, while dried banana leaves, carrot leaves or rice straw added to PDA were excellent for mating group C. These perithecia were formed most abundantly at 20°C under 12-hr alternating light and dark than at higher or lower temperatures, constant light or constant darkness. Carrot agar was the most effective media used in making crosses of *G. zeae* to obtain much more fertile perithecia as compared to V-8 agar or carnation agar (Leslie, 1991).

G. xylarioides perithecia developed *in vivo* in the stem bark of dead trees and stumps of coffee, after completely killing the plant. They were frequently encountered in most surveyed fields in various habitats ranging from the very low altitude of Bebeka (1,000 m) and Teppi (1,200 m) with hot and wet climate to as high as Gera and Gechi (2,000 m) districts having wet and cool weather conditions in Ethiopia (Girma Adugna *et al*., 2001; Girma Adugna, 2004). They could also be seen in dead coffee seedlings after artificial inoculation in the greenhouse. The *in vitro* study showed that the low temperature of around 20°C favored maturation of perithecia, their development in the field and greenhouse also required cool and damp places resulting from dense shade and relatively humid conditions. Van der Graaff and Pieters (1978) also noted that *G. xylarioides* ascospores could only be found in the field in August and September at the end of the rainy season. Paulitz (1996) suggested that rainfall might be needed for formation and maturity of *G. zeae* (*F. graminearum*) perithecia and ascospores on crop residues. *G. xylarioides* rarely forms chlamydospores, and thus the production of persistent perithecia with abundant ascospores in the field is an important source of inoculum for coffee wilt disease epidemics (Flood and Brayford, 1997; Girma Adugna *et al*., 2001; Girma Adugna, 2004).

Desjardins and Platter (2003) also reported that production of sexual spores enhanced head blight in wheat under field conditions. Besides the degree of sexual *vs*. asexual reproductions in natural populations of organisms, it can have profound effects on their genetic variability and population structure (Chen and McDonald, 1996).

In conclusion, the coffee wilt pathogen *G. xylarioides* formed mature perithecia with ascospores *in vitro* on carrot agar and V-8 medium that were similar in typical appearances to those developed *in vivo*. The development of fertile perithecia by crossing different strains under controlled laboratory conditions helps to understand the genetic structure of the fungus populations such as gene flow and inheritance of traits like virulence/ aggressiveness.

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