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Use of Oval Conidia as a Tool to Assess the Genetic Transfer Among *Colletotrichum sublineolum* Mutants

Maria Paula Nunes, Ednéia Aparecida de Souza-Paccola, Gisele Maria Andrade-Nóbrega and Luzia Doretto Paccola-Meirelles*

Departamento de Biologia Geral; Laboratório de Genética de Fungos; Universidade Estadual de Londrina; C.. P.: 600; 86051- 990; Londrina - PR - Brasil

ABSTRACT

The aim of this study was to assess oval conidia production in mutants for the reductase nitrate gene in C. sublineolum and verify the possibility of using them as a tool to transfer the genetic material in this species. The mutants used in the present study lost the ability to form falcate conidia but all produced oval conidia. The number of nuclei by conidium was evaluated. Oval conidia were efficient in the heterokaryon formation and these heterokaryons in liquid culture medium they also produced oval conidia in abundance. Recombinants were obtained and the genetic exchanges were confirmed by the RAPD analyses.

Key words: genetic recombination, nit mutants, parameiosis

INTRODUCTION

Anthracnose, caused by *Colletotrichum sublineolum*, is one of the most important diseases of sorghum crops [Sorghum bicolor (L.) Moench] in Brazil. This disease can cause losses of up to 50% in grain production. The use of resistant cultivars has been the most efficient strategy to control anthracnose, but it can be limited by the high genetic variability presented and pathogenic specialization of the phytopathogen, which is associated with frequent breaks in sorghum resistant cultivars (Casela and Ferreira, 1998). Heterokaryosis, aneuploidy, recombination and mutations caused by the transposable genetic elements have been indicated as possible generating mechanisms of this high pathogenic variability and instability in *C. sublineolum* (Casela and Frederiksen, 1994). Little is known regarding the biology and the mechanisms of genetics exchange used by *C. sublineolum*. Souza-Paccola et al. (2003a) demonstrated the existence of conidial dimorphism in this species. The falcate conidia, with a falciform shape, are produced blastically from the morphologically different conidiogenous cells. The second type, a secondary conidium, is oval to elliptic in shape, variable in size, smaller than the falcate conidia and produced blastically from hyphae. According to these authors, the production of these oval conidia represents a promising alternative for the studies that require conidial use, such as genetic and pathogenicity studies, especially for the strains with a few or no spores in agar media.

Falcate conidia obtained from mutant strains, carriers of nutritional, morphological and resistance markers were used by Souza-Paccola et al. (2003b) in complementary crosses that resulted

*Author for correspondence: paccola@uel.br

in the production of heterokaryon in *C. sublineolum*. The genetic recombination without typical parasexuality was shown in this species. Recombinant haploid segregants were recovered directly from the heterokaryon (Souza-Paccola et al., 2003b). The term parameiosis was used by Bonatelli Jr. et al. (1983) in *Aspergillus niger* to explain this type of instability in the diploid heterozygote nuclei in which the process of mitotic exchange and haploidization occurred still in the interior of the heterokaryotic hyphae, retaining similarities with the meiotic process. Thus, the conidia recovered directly from the heterokaryon can be haploids, aneuploids or even diploids. Fávaro et al. (2003) using the mutants unable to use nitrate, the Nit mutants, suggested that the instability in *C. sublineolum* could be the result of the activity of transposable elements. The Nit mutants have also been used in different fungal species to investigate the genetic instability, the genetic structure and population dynamics and to assess the diversity of compatibility reactions among strains (Leslie 1993). These mutants contain changes in structural loci or nitrate catabolism regulators or related pathways. The Nit mutants are resistant to chlorate, a toxic analogue of nitrate, and may be isolated as fast-growing sectors on medium containing chlorate. The enzyme reductase nitrate converts nitrate to nitrite, but can also convert chlorate to chloride, which is toxic to the cell. Therefore, if there is some alteration in the gene that codifies the reductase nitrate enzyme, the cell will not be able to use nitrate but will be resistant to chlorate (Cove, 1976, 1979; Tomsett and Garrett, 1980; Marzluf, 1981; Marzluf et al., 1985; Crawford and Arst, 1993).

Nit mutants of *C. sublineolum* were classified by Fávaro et al. (2007) in six phenotypic classes: mutations in the structural nitrate reductase locus (*nit*1); in the structural nitrite reductase locus (*nii*A); in the specific regulator locus (*nit*3); in the main regulator locus (*nit*2); in loci that codified the cofactor containing molybdenum necessary for nitrate reductase activity (*nit*M) and one or more genes responsible for nitrate intake (*crn*). The genetic control of the *C. sublineolum* metabolism seems to be similar to other fungi such as *Aspergillus*, *Neurospora* and *Fusarium* (Fávaro et al., 2007). Vaillancourt and Hanau (1994), using this type of mutant described the heterokaryosis in *Colletotrichum graminicola* and found that the vegetative compatibility in the species was controlled by five non-linked loci.

The objective of this study was to assess the oval conidia production in mutants for the reductase nitrate gene in *C. sublineolum* and evaluate the possibility of using them as a tool to transfer the genetic material in this species.

MATERIALS AND METHODS

Strains and culture media

Mutant strains for the reductase nitrate gene, mutants resistance to benomyl and cyclohexamide resistant were used along with morphological mutants obtained by Fávaro et al. (2003) (Table 1). These selected mutant strains could not produce falcate conidia in culture media. They were cultured at 25°C under continuous light in basal culture media containing KH_2PO4 (1.5g), KCl (0.5g), $MgSO_4.7H_2O$ (0.5g), $FeSO_4$ (0.01g), $ZnSO_4$ (0.01g), glucose (10g), 5mL biotin solution (0.004g/100mL distilled water), 10mM sodium glutamate and agar (15g) per liter distilled water. The nutrient was added to this culture medium for which the strain presented the deficiency.

Oval conidia production and assessment of the nuclear condition

Mycelial discs (65 mm diameter) of the mutant strains from 7-day-old, cultured on oat-agar culture medium at 25°C under continuous light, were inoculated in Erlenmeyer flasks containing 50 mL liquid basal culture medium. The flasks were kept under constant agitation (90 rpm) for three days at 25°C under continuous light. Later, the contents of the flasks were filtered through eight layers of sterilized gauze and centrifuged at 3000 rpm for 20 minutes. The conidia were re-suspended again in 0.1% Tween (v/v) (aqueous solution) and centrifuged twice in saline solution (NaCl, 0.8%). Oval conidia production was quantified in a Neubauer chamber.

To assess the nuclear condition, oval conidia from each mutant strain were stained by the HCL-Giemsa technique described by Tanaka et al. (1979). An oval conidia suspension was spread on slides containing a thin film of 50% albumin. The

material was left to dry at room temperature and later fixed in ethanol: acetic acid (3: 1) for 30 minutes. Dehydration was performed in 95% alcohol (5 minutes) and in 70% alcohol (20 minutes). The material was then washed three times in distilled water and hydrolyzed in HCl (1N) at 60ºC for 3 to 7 minutes and then stained in Giemsa solution according to the methodology reported by Tanaka et al. (1979).

Table 1 - Genetic markers of the *Colletotrichum sublineolum* mutants used in this study

Mutants*	Genetic markers**
243.1/2 3	*ben*R; *bio*-; *cic*R; *nit*M;
243.1/2 30	*ben*R; *bio*-; *cic*R; *nit*1;
15B 17	*nit*M
15E 16	*bio*-; *cic*R; *crn*
15E 17	*bio*-; *cic*R; *nit*3
15E 24	*bio*-; *cic*R; *nit*2
30C 17	*nit*M
30C 20	*bio*-; *cic*R; *nit*1
30C 33	*nit*1
30C 37	*nit*M
30C 75	*nit*1
30C 104	*nit*M
30C 118	*nit*M
30C 121	*nit*1
30399	*bio*-; *cic*R; *nii*A

*All strains have the normal wild type colour, except the strains 243.1/2 3 and 243.1/2 30 that present orange color.
** Mutations in the structural locus of the reductase nitrate gene (*nit1*); in the structural locus of nitrite reductase (*nii*A); in the specific regulator locus (*nit*3); in the main regulator locus (*nit*2); in loci that codified the cofactor containing molybdenum necessary for nitrate reductase activity (*nit*M); in one or more genes responsible for nitrate intake (*crn*); deficiency for biotin (*bio*); resistant to the benomyl fungicide (benR); resistant to cyclohexamide (*cic*R).

Obtaining recombinants and their characterization

Solutions containing 10^6 oval conidia/mL, derived from two complimentary mutants strains, were mixed and incubated in tubes containing 2mL of Pontecorvo culture medium (Pontecorvo et al., 1953) with the addition of 2% complete culture medium (Pontecorvo et al., 1953). After 20 to 25 days incubation at 25°C, under continuous light, a dense mycelia film was visible on the surface of the culture medium. This was transferred to Pontecorvo minimum agar culture medium and incubated until heterokaryon formed.

The heterokaryon formed was fragmented, inoculated in 50 mL Pontecorvo minimum liquid medium and incubated at 25°C under constant agitation (90 rpm) for seven days for the production of the oval conidia. The content of the flasks was filtered through sterile cheesecloth and oval conidia were recovered by centrifugation at 3000 rpm for 20 minutes. The pellets were washed three times by centrifugation (3000 rpm) in 0.8% saline solution and resuspended in 2 mL of the same solution.

An aliquot of approximately 100 µl of the oval conidia suspension was plated in Pontecorvo minimum medium and in minimum culture medium containing glutamate as nitrogen source. After three days growth at 25°C, under continuous light, the colonies capable of using nitrate as nitrogen source were selected and inoculated in selective culture medium for the marks used: glutamate, ammonia, nitrite, nitrate and urea to confirm the presence of recombinants.

Genomic DNA extraction

Mycelia discs taken from the 10-day-old recombinant colonies were cultured in Pontecorvo liquid medium. After seven to ten days growth, under agitation (90 rpm) at 25°C, the mycelia were collected by filtering and washed in sterilized distilled water. The genomic DNA was extracted according to methodology reported by Reader and Broda (1985). The DNA of the samples was quantified in a fluorimeter.

RAPD reactions

RAPD reactions were performed with mutant

parental strains and recombinants to confirm the recombinant identity. The following primers were used: OPAD17, OPAX17, OPAP06, OPAW06, OPAM18, OPAR16, OPAA04 and OPAX10. The reactions were prepared in a final 25 µl volume, containing 0.25 mM of each nucleotide; 0.25 mM primer; 0.4U Taq DNA polymerase enzyme (Invitrogen, Brasil); 3.0mM $MgCl_2$ and 20ng genomic DNA. The control reactions consisted of adding all the reaction components, except the genomic DNA. Amplification was made in a PTC – 200 thermocycler (MJ Research) programmed for initial denaturation at 94°C for 4 minutes, followed by 40 amplification cycles. Each cycle consisted of one minute at 92°C, one minute at 35°C, two minutes at 72°C and a final extension of five minutes at 72°C. The products generated in this reaction were separated in 1.4% agarose gel, stained with ethydium bromide and photodocumented under UV light.

RESULTS AND DISCUSSION

The type of conidium normally produced by *C. sublineolum* is the falcate conidium which represents the primary inoculum source of this pathogen, and is also the propagule frequently used in genetic studies and pathogenecicity tests. Some *C. sublineolum* strains have lost the capacity to produce this type of asexual spore, or produce insufficient quantities for carring out inoculations. Souza-Paccola et al. (2003a) reported a second type of conidium in this species, the oval conidium. The production of this type of secondary conidia may have epidemiological consequences not only because it represents an additional inoculum source (Souza-Paccola et al. 2003a) but also because it represents an alternative propagation tool when there are no primary conidia.

The mutants strains used in the present study lost the ability to form falcate conidia but all produced oval conidia when cultured in liquid culture medium. The quantity of oval conidia ranged from $0.01x10^6$ to 73.6×10^6 oval conidia/mL, depending on the strain used (Table 2). This high variability in production of the oval conidia was reported in wild *C. sublineolum* strains by Souza-Paccola et al. (2003a) when cultured in Fries liquid culture medium both in shaking and static culture.

Table 2 - Oval conidia production by *Colletotrichum sublineolum* mutants in liquid basal culture medium.

Mutants	**Oval conidia (x 10^6/mL)***								
30399	73.60	a							
15B 17	41.60	a							
243.1/2 3	39.47	a							
15E 24	36.53	a							
15E 17	27.13	a	b						
15E 16	17.17		b						
243.1/2 30	15.07		b						
30C 17	14.30		b						
30C 33	4.00				c		d		
30C 75	0.38						d		
30C 118	0.37						d		
30C 20	0.29						d		
30C 104	0.15						d		
30C 37	0.03								e
30C 121	0.01								e

*Means followed by the same letter did not differ by the Tukey test ($p \leq 5$) (Mean of 4 replications).

The nuclear condition of the conidia was assessed in eight of the mutant strains. A variable number of nuclei (one to five/spore) was observed, but the uninucleate and binucleate condition was predominant (Table 3). Souza-Paccola et al. (2003a) observed a greater frequency of uninucleate conidia in wild *C.sublineolum* strains. In *C.graminicola,* Panaccione et al. (1989) detected about 60% uninucleate oval conidia, 30% binucleate and 10% tri and tetranucleate. The distribution of the number of nuclei in the conidia produced by any one of the strains was relatively stable, even after repeated subcultures. The oval conidia were used in complementary crosses for heterokaryon production. All the heterokaryon when inoculated in the liquid culture medium also produced oval conidia in abundance.

Table 3 - Nuclear condition of the oval conidia produced by mutant strains for the reductase nitrate gene in *Colletotrichum sublineolum*.

	Number of nuclei /cell (%)			
Strains	**1**	**2**	**3**	**≥4**
15E 24	46	44	6	4
30C 17	32	48	16	4
30C 20	16	34	40	10
30C 33	38	54	8	0
30C 37	86	14	0	0
30C 75	42	54	2	2
30C 104	42	54	4	0
30399	38	44	10	8

A high reversion rate was observed in most of the crosses unlike that observed by Souza-Paccola et al. (2003b) in crosses using other types of genetic markers. According to Fávaro et al. (2003), the reversion rate of *nit* mutant in *C. sublineolum* was high and ranged from $1x10^{-5}$ to 10^{-4}. This instability has been reported frequently in this pathogen (Casela and Frederiksen, 1994) and can be explained by the presence of transposable genetic elements (Fávaro et al., 2007). Thus, of the 83 crosses carried out, only five remained stable, allowing recombinant recovery nevertheless at relatively low frequencies (Table 4). Colonies were considered recombinant when grew in culture medium containing nitrate as a single nitrogen source, an indication of the occurrence of genetic exchanges among the mutants.

Table 4 - Crosses among *Colletotrichum sublineolum* mutants carriers of complementary marks.

	Segregants	
Crosses	**Nit $^+$**	**Nit $^-$**
15B 17 *nit* M x 30C 33 *nit*1	2	19
15E 24 *nit* 2 x 30C 17 *nit* M	1	105
15E 24 *nit* 2 x 30C 33 *nit*1	9	4
30C 17 *nit* M x 30C 33 *nit*1	21	22
30C 33 *nit*1 x 30C 118 *nit* M	22	30

The confirmation of the occurrence of genetic material exchange was also obtained by the RAPD analyses. All the primers tested amplified the samples. Amplifications with the primers OPAX 17 and OPAD17 are shown in Figures 1 and 2, respectively. The segregant bands were identified from both parents, proving the occurrence of recombination. Souza-Paccola et al. (2003b) demonstrated the genetic transference among the carrier strains of auxotrophic, resistance and morphological marks by the electrophoresis profile for esterases. Wang et al. (2002) characterized heterokaryons in *B. bassiana* by an intronic sequence of the gene of the 28S subunit of the rRNA, confirming previous reports of the occurrence of recombination in this species (Paccola-Meirelles and Azevedo, 1991; Bello and Paccola-Meirelles, 1998).

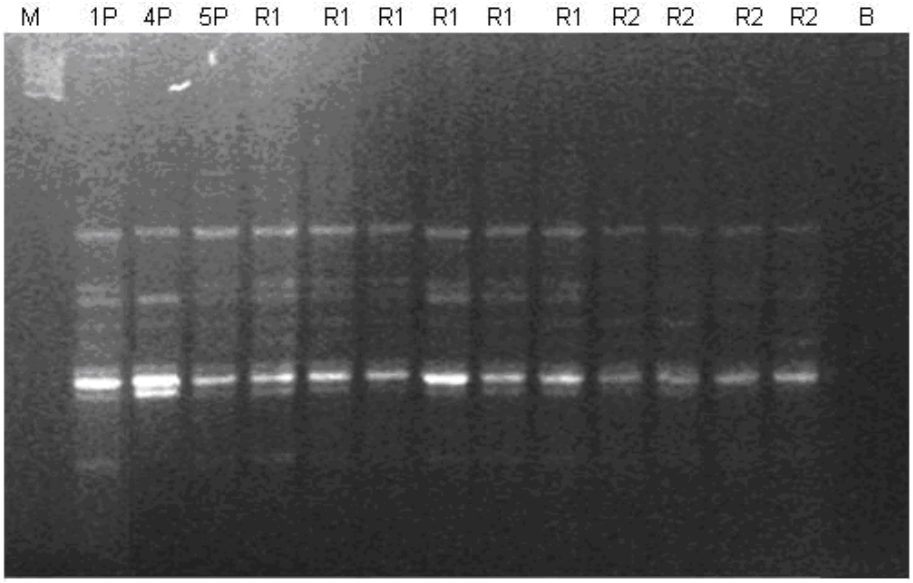

Figure 1 - Profile of RAPD bands of *C. sublineolum* parents and recombinants obtained by amplification with the OPAD17 primer, M = molecular marker (1Kb); 1P = parent 30C 17; 4P = parent 15E 24; 5P = parent 30C 33; R1 = recombinant of the cross between parents 4P and 5P; R2= recombinant of the cross between the 1P and 5P parents; B = control without DNA.

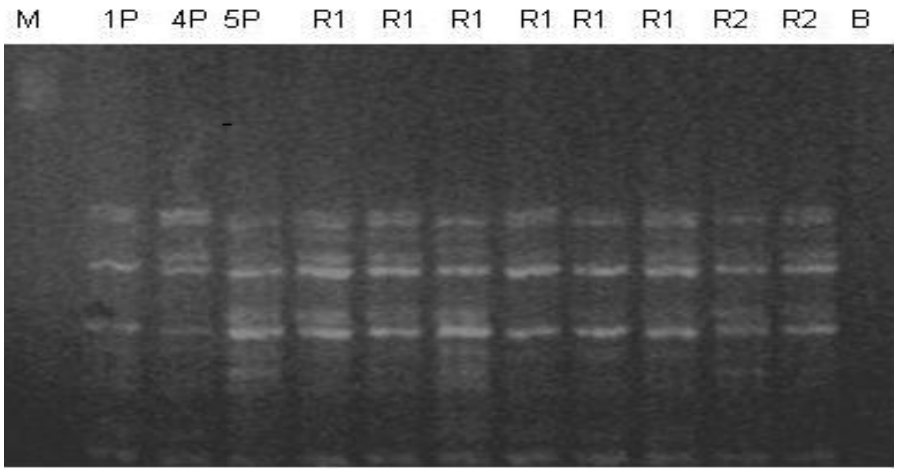

Figure 2 - Profile of RAPD bands of *C. sublineolum* parents and recombinants obtained by amplification with the OPAX 17 primer; M= molecular marker 1Kb; 1P= parent 30C 17; 4P= parent 15E 24; 5P= parent 30C 33; R1 = recombinant of the cross between parents 4P and 5P; R2= recombinant of the cross between the 1P and 5P parents; B= control without DNA.

The parasexual cycle was originally reported in *A. nidulans* but can be observed in many Ascomycetes, Deuteromycetes and Basidiomycetes. The parasexual cycle has also been useful in genetic improvement of fungal strains (Loera and Córdova, 2003) However, in various fungal species, the processes of parasexual recombination take place differently from the pattern described originally by Pontecorvo et al. (1953) (Debets, 1998). In some species, the

diploid phase is considered transient, usually not identified, and the recombinants can be obtained directly from the heterokaryon. This process was called parameiosis (Bonatelli Jr et al., 1983; Bagagli et al., 1991) and have been observed in many fungal species, including *Fusarium oxysporum* (Molnar et al., 1990), *Metarhizium anisopliae* (Bagagli et al., 1991; Kava-Cordeiro et al., 2005), *Cladosporium fulvum* (Arnau and Oliver, 1993), *Colletotrichum gloesporioides* (Chacko et al., 1994), *Beauveria bassiana* (Paccola-Meirelles and Azevedo, 1991; Bello and Paccola- Meirelles, 1998).

The results presented here showed that the oval conidia were efficient in obtaining the heterokaryon in *C. sublineolum*, representing an additional tool for genetic studies, mainly in strains incapable of producing falcate conidia. All of the heterokaryon analyzed also produced oval conidia when placed in the liquid culture medium, enabling the recovery of segregants directly from the heterokaryon, thus confirming the occurrence of parameiosis in this species.

RESUMO

O fungo *Colletotrichum sublineolum* agente causal da antracnose em sorgo mostra dimorfismo conidial, produzindo conídios falcados ou ovais dependendo das condições de cultivo. Conídios falcados têm sido usados para obter heterocários entre mutantes complementares. Muitas linhagens de *C. sublineolum* não produzem conídios falcados ou produzem em quantidades insuficientes para analises. O objetivo do presente estudo foi avaliar a produção de conídios ovais em mutantes para o gene da nitrato redutase e verificar a possibilidade de sua utilização como ferramentas na transferência de material genético nesta espécie. Os mutantes usados neste estudo perderam a capacidade de produzir conídios falcados, porém todos produziram conídios ovais. O número de núcleos por conídio foi determinado. Conídios ovais foram eficientes na formação de heterocários e os heterocários quando cultivados em meio líquido também produziram conídios ovais em abundância. Recombinantes foram obtidos e as trocas genéticas foram confirmadas pelas análises de RAPD.

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