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New media for increasing sporulation and germination of *Phaeoisariopsis griseola* conidia

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Abstract

The slow growth rate of the fungus *Phaeoisariopsis griseola* and the availability of a homogeneous highly concentrated inoculum is an important constraint for pathogenicity or virulence studies, where plant inoculations are needed. Therefore, the purpose of this paper was to evaluate the effect of supplementing culture media with *Amaranthus cruentus* seed meal on fungal growth and sporulation of isolates of *P. griseola* belonging to the Mesoamerican and Andean groups. The amendment of PDA or V8 media with *A. cruentus* seed meal resulted in a considerable increase in the number of conidia and also in their capacity to germinate; this depended mostly on the stage of maturity of conidia. Mesoamerican and Andean isolates produced a different number of conidia when cultured *in vitro*. Furthermore, while in Mesoamerican isolates a second degree polynomial represented the relationship between number of conidia and amount of *A. cruentus* supplementation, in Andean isolates the relationship was linear. It seems that either one or several of the nutritional factors provided by *A. cruentus* contributed to the increased production of conidia and their development, resulting in faster development of the disease and an earlier appearance of symptoms. Therefore, for cultural studies, especially for inoculum production and for pathogenicity evaluations, supplementation of the media with *A. cruentus* seed meal proved to be a good alternative.

Introduction

Phaeoisariopsis griseola, a Mitosporic fungus in the class Hyphomycetes, order Moniliales, family Stilbaceae, is the causative agent of Angular Leaf Spot (ALS), a disease of common bean that causes yield losses in many countries around the world (Jesus-Junior et al., 2001, 2003; Stenglein et al., 2003). Based on pathogenicity and molecular markers, isolates of the fungus have been divided into Mesoamerican or Andean, in corresponding with the gene pools of origin that have been defined for common bean (Boshoff et al., 1996; Correa-Victoria, 1987; Guzmán et al., 1995; Mahuku et al., 2002; Pastor-Corrales et al., 1998).

One of the most important constrains for the study of *P. griseola* is its slow growth rate on artificial media. Since mycelial growth precedes sporulation, development of conidia also occurs slowly; thus the isolation of monosporic cultures

of *P. griseola* can be a difficult task. Inglis et al. (1988) reported that colonies of the fungus, growing on different media, increased their diameter at a rate lower than 1 mm week^{-1}. Furthermore, whether the fungus was grown on media supplemented with vitamins, bean plant extracts and/or amino acids its growth rate remained unaltered (Cardona-Alvarez and Walker, 1956). Several investigators have cultured *P. griseola* on different media; light, temperature, pH and day-length have also been found to affect conidial production. *Phaeoisariopsis griseola* sporulation is induced either by continuous darkness (Santos-Filho et al., 1976; Schwartz et al., 1982) or by a 12 h light regime (Santos-Filho et al., 1976) and is more efficient at 24 °C.

Phaeoisariopsis griseola has been grown in culture on potato dextrose agar (PDA)(Cardona-Alvarez and Walker, 1956), yeast-extract agar (Cardona-Alvarez and Walker, 1956), bean leaf dextrose agar (Silvera, 1967), honey peptone agar (Santos-Filho et al., 1976), vegetable juice agar (V8) (Campos and Fucikovsky, 1980; Correa-Victoria, 1987) and tomato juice agar (Dalla Pria et al., 1997). However, *P. griseola* has been mostly grown on V8 media adjusted to pH 6.0, in the darkness at 24 °C ± 2 (Campos and Fucikovsky, 1980; Pastor-Corrales et al., 1998; Busogoro et al., 1999; Nietsche et al., 2001). Although vegetable juice agar (V8) was the best medium to induce sporulation of *P. griseola*, it grew slowly and formed few conidia.

Amaranthus cruentus is a native crop plant from Southern México and Central America that belongs to the family Amaranthaceae (Sauer, 1967). The seeds contain high levels of many minerals, amino acids and vitamins (Bressani, 1990; Pastor, 1999) and the seed meal has already been used, as a source of essential nutritional factors, to increase bacterial growth (Videira et al., 2002).

We hypothesized that the slow growth rate and low sporulation of *P. griseola* when cultured *in vitro* is due to a lack of nutritional factors. Therefore, the purpose of this work was to evaluate the effect supplementation of the culture media with a natural source rich in amino acids, vitamins and minerals, such as *A. cruentus* seed meal, has on *P. griseola* growth, sporulation and conidial germination.

Materials and methods

The number of monosporic isolates used in the experiments was six (Table 1) and were classified as representatives of the Mesoamerican or Andean groups by means of the RAPD markers as described by Guzmán et al. (1999). To avoid intra-specific variation three isolates representing each group were used in these studies.

Amaranthus cruentus seed meal was obtained by milling seeds (200 g) in a Tecator Cyclotec 1,093 at 10,000 rev min^{-1}. The seed meal was added at concentrations of 0.2, 0.5, 1, 2 and 4% w/v on both PDA and V8 medium, as required.

Single spores from 14 day-old cultures of each isolate were transferred to Petri dishes (9 cm diameter) filled either with 25 ml of PDA or V8 culture media, pH 6.0 and incubated in the darkness at 24 ± 2 °C. Two isolates of *P. griseola* characterized by Centro Internacional de Agricultura Tropical (CIAT), Pg1 and Ecu6, were used as Mesoamerican and Andean controls (Table 1). Fungal growth was estimated on the third

Table 1. Phaeoisariopsis griseola isolates and their origins

Isolates	Bean cultivar	Seed type	Seed size[1]	Department/Province	Source of isolates	Date[2]	RAPD group[3]
T1	TUC 390	Black	Small	Trancas/Tucumán	Leaf	XII/2001	M
S1	NAG 12	Black	Small	Rosario de Lerma/Salta	Legume	VI/2002	M
Pg1[4]	DOR 500	Black	Small	Trancas/Tucumán	Not available	Not available	M
S3a	Overito	Cranberry	Large	Cerrillos/Salta	Leaf	IV/2003	A
J2c	Alubia	White	Large	San Pedro/Jujuy	Leaf	IV/2003	A
Ecu6[4]	COS 16	Not available	Large	Pinhanpiro	Not available	Not available	A

[1]Seed size: small = < 20.0 g/100 seeds, large = > 30.0 g/100 seeds.
[2]Date of collection of isolates.
[3]*P.griseola* groups as defined in text: M = Mesoamrican; A = Andean.
[4]Centro International de Agricultura Tropical (CIAT).

subculture of each isolate by measuring the diameter of 10 single colony dishes per isolate after 10, 14 and 20 days of incubation. The production of conidia was estimated by dispersing and crushing a colony in an Eppendorf tube filled with 1 ml of distilled water supplemented with 70 μl of Tween 20 ®. After vortexing the tubes for 30 s, an aliquot of the suspension was placed in a haemocytometer. The number of replicates was 10 colonies per isolate. Conidial number per sample was estimated by six independent measures performed 10, 14 and 20 days after inoculating the plates. ANOVA and regression analysis were done according to Sokal and Rohlf (1995).

Germination of conidia was evaluated on microslides covered with a thin layer of unamended PDA and 1% w/v of *A. cruentus* seed meal amended PDA. Conidial suspensions (2×10^2 conidia ml^{-1}) were poured on microslides and kept in sterile Petri dishes at 24 ± 2 °C. Conidia with elongating germ tubes were considered as germinated and their number was counted in an inverted light microscope 6, 12, 24 and 48 h after inoculation.

The ability of spores to cause disease on beans was evaluated on susceptible cv. TUC 500, a representative of the Mesoamerican gene pool. Twelve seeds were surface-sterilized by immersing them for 3 min in 50 % ethanol, 3 min in sodium hypochlorite and by washing three times with sterile distilled water. Seeds were sown in pots (1 l) filled with a mixture of farm soil, organic soil and sand, in a 2:1:1 ratio. After emergence, seedlings were selected based on size uniformity, leaving only one seedling per pot. Three plants were inoculated 18 days after sowing by spraying a conidial suspension onto the upper and lower surfaces of the first trifoliate leaf. The source of inoculum was conidia from *P. griseola* isolate S1, which were obtained from 10 day-old cultures grown on both unamended and 1% w/v *A. cruentus* seed meal amended PDA. The conidial suspension was prepared by adding distilled water to the plates and agitating with a spatula, filtering and adjusting with a haematocytometer to 2×10^4 conidia ml^{-1}. After inoculation, plants were placed in a growth chamber under a 12 h day photoperiod at 24 °C and 95/100% RH; 48 h later, plants were moved back to the greenhouse. Disease severity was assessed by counting the number of angular leaf spots per leaflet 8, 12, 15 and 18 days after inoculation.

Results and discussion

Based on the morphological and agronomical traits of seed proteins and molecular markers, two pools of origin have been defined for beans, namely Mesoamerican and Andean (Gepts et al., 1986). Whether symbiotic or pathogenic, microorganisms have been shown to co-evolve with their hosts and the isolates of *P. griseola* have also been grouped after the pool of origin of beans as Mesoamerican or Andean.

The low growth rate of *P. griseola in vitro* might be a genetic characteristic of the fungus and not a response to nutrient deficiencies of the media, since its supplementation with *A. cruentus* did not alter growth rate. However, conidial production increased when the media were supplemented with *A. cruentus* seed meal. Mesoamerican and Andean isolates showed a similar behaviour on PDA and V8 media; results were therefore pooled.

Mesoamerican and Andean isolates of *P. griseola* differed in the number of conidia they produced *in vitro*. The former produced a much lower number of conidia (approximately 14, Figure 1A) than the latter (approximately 45, Figure 1B). Furthermore, Mesoamerican isolates reached a peak of conidial production after 14 days of culture, while it took longer for Andean isolates (20 days) to produce the maximum number of conidia (Figure 1).

The number of conidia produced by Mesoamerican and Andean isolates in response to *A. cruentus* supplementation was different. In Mesoamerican isolates, the number of conidia and the amount of *A. cruentus* added to the media was related by a second degree polynomial (Figure 1A). Spore number increased from the first sampling date, was at a maximum four days later after 14 days of culture and decreased thereafter. In the Andean isolates conidial production was linearly related to concentrations of *A. cruentus*, except at 1 and 2% (Figure 1B). Therefore, the highest production of conidia in Andean and Mesoamerican isolates occurred consistently after 20 and 14 days of culture, respectively, suggesting that there are physiological differences between isolates from both gene pools of origin.

Regression analysis indicated that Mesoamerican and Andean isolates responded differently to increasing amounts of *A. cruentus* seed meal (Figure 2). In Mesoamerican isolates the number

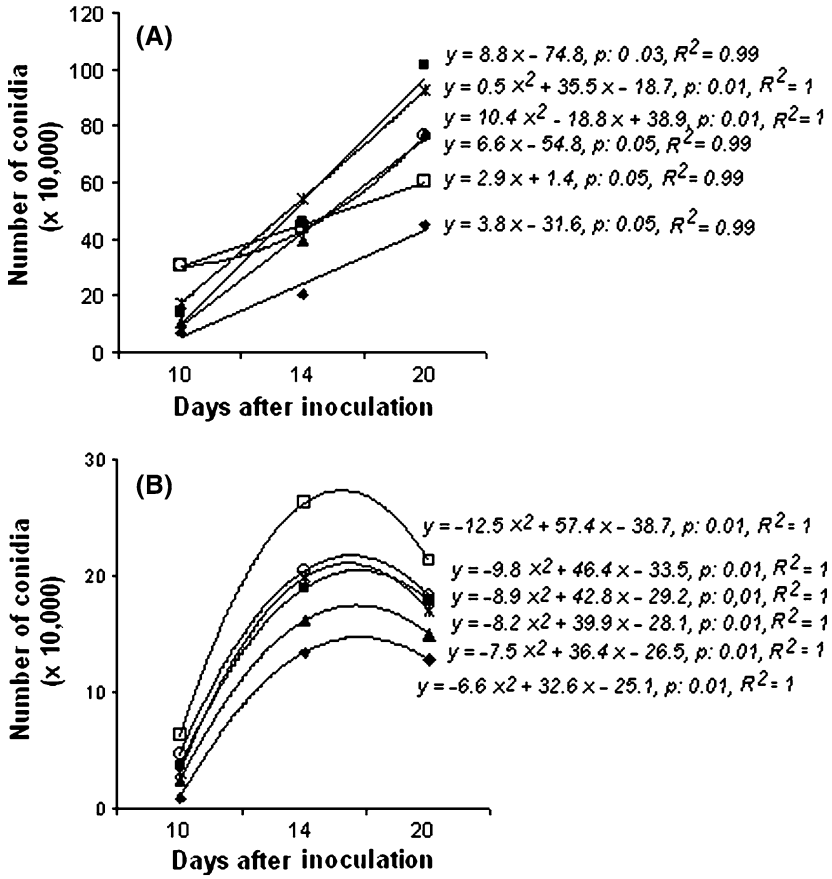

Figure 1. Conidial production of Andean (A) and Mesoamerican (B) isolates of *P. griseola* after a 20 day incubation period in response to the amendment of media with *A. cruentus* (♦0%; ▲0.2%; ■ 0.5% × 1%; ○ 2%; □ 4%). Regression analysis showed that there were statistically significant differences among sampling times and all the *A. cruentus* concentrations assayed ($P = 0.001$).

of conidia was linearly related with the amount of *A. cruentus* supplementing the media, even at high concentrations such as 2 and 4% (Figure 2A–C). The relationship of sporulation of Andean isolates with supplementation of *A. cruentus* was represented by a second degree polynomial (Figure 2D–F). Andean isolates unlike Mesoamerican isolates, developed more conidia in response to the amendment of the media with *A. cruentus* but only up to 0.5% (Figure 1D–E). Further addition of seed meal to the media was deleterious to sporulation since the number of conidia was reduced (Figure 1D–F). Therefore, a differential effect of *A. cruentus* seed meal on each group of *P. griseola* isolates was demonstrated.

Germination of conidia is indirect evidence of maturity and was assessed by counting the number of spores with developing germ tubes over a short and a long period of time. Conidia of Mesoamerican and Andean isolates germinated in culture after 6 h (Figure 3A, B). Whether or not isolates belong to the Mesoamerican or Andean group, after 12 h of culture germination was significantly higher in *A. cruentus* amended than in non-amended PDA media (Figure 3A, B). Furthermore, after an incubation period of 48 h, 97 and 77% of conidia germinated in amended and unamended media, respectively. These findings were confirmed by counting the number of germinated conidia in fungal cultures supplemented with *A. cruentus* seed meal. In this case, the effect was pronounced after 10 and 14 days of culture and almost undetectable thereafter (Figure 3C, D). Furthermore, if germination induced by *A. cruentus* seed meal is

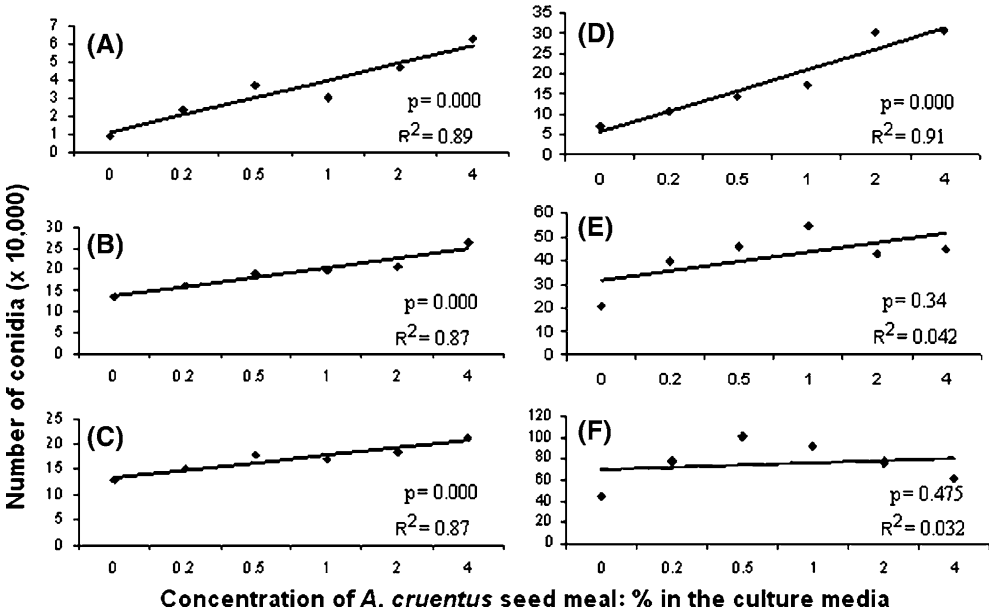

Figure 2. Regression analysis of the number of conidia (10,000×) and % of *A. cruentus* seed meal. Figures 2A, 2B and 2C represent the response of *P. griseola* isolates belonging to the Mesoamerican group incubated for 10, 14 and 20 days, respectively. Figures 2D, 2E and 2F show the response of *P. griseola* isolates belonging to the Andean group incubated for a period of 10, 14 and 20 days, respectively.

calculated as a percentage of spore germination on unamended media, a remarkable effect of *A. cruentus* can be seen after only ten days of culture. It is likely that *A. cruentus* provided nutritional factors that enhanced the maturity of conidia, an effect that was more pronounced in Andean isolates.

The availability of homogeneous highly concentrated inoculum is a requirement to validate monocyclic parameters of disease in plants. Deficiencies of the inoculation method are one of the main causes of variability among experiments under controlled conditions (Ribeiro, 1991). Because of this, the development of media that generate a large number of mature conidia is very important for obtaining high quality inoculum (Dalla Pria et al., 1997).

Phaeoisariopsis griseola inoculum preparation and symptom evaluation are potentially simple. However, it takes 12–21 days for cultures to sporulate and 14–18 days after inoculation for plants to develop symptoms of ALS disease (Bassanezi et al., 1998; Pastor-Corrales et al., 1998; Busogoro et al., 1999; Nietsche et al., 2001; Sartorato, 2002), which might be due to the slow growth rate of the fungus and/or further maturation of spores, once they reach the leaf surface. We inoculated susceptible bean plants with spores of *P. griseola* produced on cultures grown on PDA or *A. cruentus* amended media and the number of spots counted per leaflet is presented in Figure 4. Symptoms were visible eight days after inoculation and there were significant differences ($P \leq 0.05$) between plants inoculated with conidia produced on unamended and *A. cruentus* amended media. These results suggest that a higher number of mature conidia developed more angular leaf spots in a shorter period of time. Spore maturation might be affecting infection foci and not disease development since *P. griseola* is a hemibiotroph that has a biotrophic phase influenced by the prevailing environmental conditions and the bean genotype in use.

Several lines of evidence indicate that pathogenic tests can be performed in a shorter period of time by using *A. cruentus* supplemented cultures without altering the outcome of the host-pathogen interaction. Firstly, germination of *P. griseola* conidia does not require a plant inducer to germinate and fungal growth is not altered by *A. cruentus* nutritional factors. Secondly, conidial germination occurs in a shorter period of time

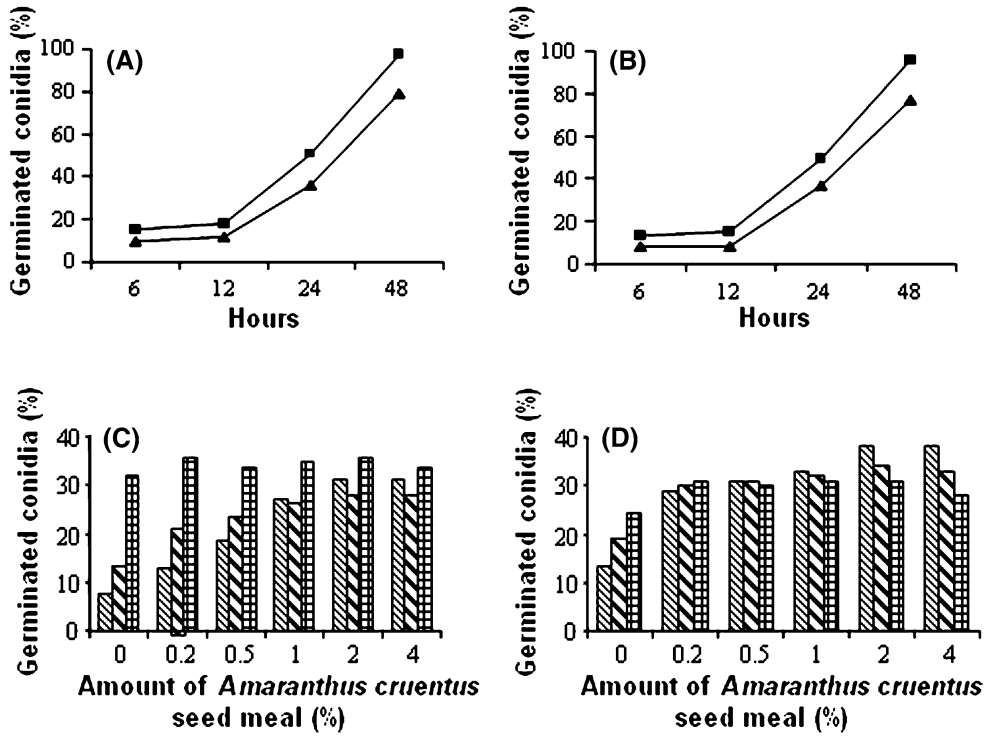

Figure 3. Germination of conidia of *Phaeoisariopsis griseola*. Percentage of germinated conidia from isolates belonging to the Andean (A) and Mesoamerican (B) groups during a 48 h period. Conidia germinated in plain media (▲) and in media supplemented with *A. cruentus* (■). Percentage of germinated conidia of isolates belonging to the Andean (C) and Mesoamerican (D) groups, after (10), ▨ (14), ▩ (20) ▦ days of incubation.

when cultures are supplemented with *A. cruentus*. Thirdly, a higher number of mature conidia produced a higher number of angular leaf spots on the plants in a shorter period of time.

To our knowledge, this is the first report comparing growth, conidial production and germination of Mesoamerican and Andean isolates of *P. griseola*. The amendment of PDA or V8 media with *A. cruentus* seed meal resulted in an increase in the number of conidia produced and also in their capacity to germinate, which seemed to depend mostly on their stage of maturity. It seems that either one or several of the nutritional factors provided by *A. cruentus* contributed to an increase in the number of conidia and their development, resulting in a faster development of disease and an earlier appearance of symptoms on beans. Therefore for cultural studies, especially for inoculum

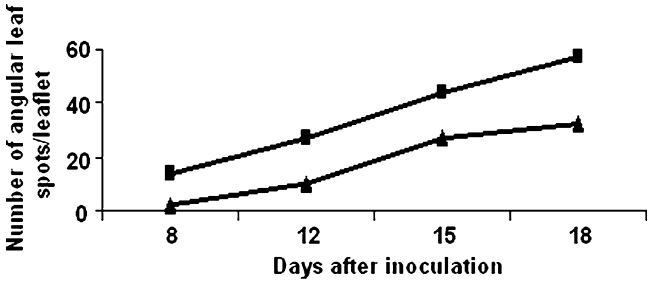

Figure 4. Number of angular leaf spots determined on common bean leaflets 18 days after inoculating bean plants with conidial suspensions obtained from unamended (▲) and *A. cruentus* seed meal supplemented PDA media (■).

production and for pathogenicity evaluations, supplementation of media with *A. cruentus* seed meal appears to offer a good method.

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