Lewia chlamidosporiformans, a mycoherbicide for control of *Euphorbia heterophylla*: isolate selection and mass production

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Summary

The potential of the fungus *Lewia chlamidosporiformans* Vieira and Barreto as a biological control agent for wild poinsettia, *Euphorbia heterophylla* L., a noxious invader of soybean fields in Brazil, is being assessed. One isolate was selected from nine that were tested as being the most aggressive to a series of wild poinsettia populations (including one known to be herbicide-resistant). The biphasic technique was investigated as an option for mass production of conidia of *L. chlamidosporiformans*. This method involves the production of mycelia in liquid culture that are later blended and poured into trays containing a solid medium and incubated under a specific light regime until conidia form. After 3 days, conidia are harvested once per day by pouring sterile water over the surface of the colonized medium and scraping the surface with a rubber spatula. A semi-synthetic liquid medium (with a sucrose and asparagin base) was selected as the best for the first phase of growth. A vegetable broth-agar medium supplemented with $CaCO_3$ was the best solid medium for fungal growth and sporulation in the second phase.

Keywords: bioherbicides, pathogenicity, biphasic.

Introduction

Losses caused by weeds represent one of the main limiting factors in agriculture production worldwide. Chemical herbicide applications are gradually becoming the dominant method of control of weeds in both developed and developing countries (Wyse, 1992; Abernathy and Bridges 1994). However, parallel to this, problems with contamination of water resources, accumulation of chemical residues in the soil, emergence of herbicide resistence in weed species and threats to biodiversity are also on the rise. This justifies the search for alternatives that might allow the reduction or replacement of chemical herbicide applications such as through biological control of weeds with plant pathogens (Rosskopf *et al*., 1999).

Wild poinsettia (*Euphorbia heterophylla* L.), known in Brazil as 'amendoim-bravo' or 'leiteiro', is a native euphorb of tropical and subtropical America (Lorenzi, 2000). In Brazil, it is regarded as one of the worst weeds in important crops such as corn, sugarcane, common bean and soybean (Guedes and Wiles, 1976; Arevalo and Rozanski, 1991). The reduction in the soybean harvest caused by competition with *E. heterophylla* varies depending on the weed density in an invaded area and the soybean cultivar, but it is estimated that losses range from 35% to 62% (Constantin *et al*., 1997; Voll *et al*., 2002). Acetolactate synthase (ALS) inhibiting herbicides have been the favorite product used in the control of *E. heterophylla* in soybean. However, the repetitive use of these products and their residual effect in the soil led to a continuos selection of populations of *E. heterophylla* that are now resistant to these products (Gazziero *et al*.1998; Melhorança and Pereira, 1999). Such a situation offers an ideal opportunity for the use of a fungus formulated as a mycoherbicide (Charudattan, 2001).

Lewia chlamidosporiformans Vieira and Barreto is a newly described fungus capable of causing severe inflorescence necrosis, foliar blight and stem canker on *E. heterophylla* under natural conditions. Since its discovery, it has been intensively evaluated as a potential mycoherbicide. This paper reports some of the results obtained during these studies, namely isolate selection

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and development of a method for mass production of *L. chlamidosporiformans* inoculum.

Materials and methods

Fungal isolates

Samples of *E. heterophylla* with symptoms of attack by *Lewia* were collected in the Brazilian states of Minas Gerais, Rio de Janeiro, and Rio Grande do Sul.

Direct isolation from sporulating lesions as well as indirect isolations through surface sterilization and plating of diseased tissues was performed in vegetable-broth agar (VBA; Pereira *et al*., 2003). Cultures were preserved in silica gel as described in Dhingra and Sinclair (1995).

Inoculum production

Conidia of *L. chlamidosporiformans* of all fungal isolates were produced for the first isolate-selection experiment using the methodology described by Walker (1980) with the following modification: ten culture disks obtained from the margin of 7-day-old cultures grown in VBA were transferred to a series of Erlenmeyer flasks containing 100 ml of VB, i.e., the same as described in Pereira *et al.* (2003) but without agar. The erlenmeyers were left on a shaker at 140 rpm for 7 days at room temperature. After this period, the mycelial mass was blended within the remaining liquid medium within each flask and poured onto 20 × 28 cm aluminum trays, each containing 100 ml of solidified VBA. Trays were kept in a controlled temperature room at 26 ± 2°C under a 12-h photoperiod (light from two 40-W daylight fluorescent lamps and two 40-W fluorescent, near-ultra-violet light lamps). After 2 days, conidia were collected by pouring 50 ml of sterile water on the culture surface and scraping it with a rubber spatula. The resulting suspension was then filtered through two layers of cheese cloth, and the final concentration of the suspension was evaluated and adjusted to the adequate concentration for use in the experiment.

E. heterophylla plants for the experiments

The populations represented in the experiment were produced from seeds obtained from different locations and included plants with the following characteristics: resistance to the herbicide imazetaphyr and resistance to *Bipolaris euphorbiae* (Hansford) Muchovej, a fungus previously evaluated as a mycoherbicide for wild poinsettia (Yorinori and Gazziero, 1989; Marchiori *et al*., 2001, Nechet *et al*., 2006). Seeds to be used in the experiments (Table 1) were harvested from healthy plants grown in a greenhouse and stored at 5°C until use. Plants used in the experiments were produced from pre-germinated seeds that were planted in 500-ml pots containing sterile soil. The plants were maintained in a greenhouse (26 ± 2°C) and watered daily. Plants were inoculated at the three- to four-leaf stage.

Screening of fungal isolates

Groups of plants of nine populations listed in Table 1 were inoculated with conidial suspensions representing each isolate obtained in the survey. Inoculum consisted of suspensions of 1.0×10^4 conidia/ml + 0.05% Tween 20 (polyoxyethylene monolauratic) + 0.05% Breakthru® (polyether-polymethyl siloxane copolymer + polyether; T.H. Goldschmidt, Guarulhos, São Paulo). After inoculation, plants were kept in a mist room at 25°C for 24 h and then moved to a greenhouse (26 ± 2°C). Plants inoculated with a suspension with the same components as described above but without *L. chlamidosporiformans* conidia served as the control. The number of dead plants and percentage of diseased leaves (proportion of number of diseased leaves per total number of leaves) were evaluated at 5-day intervals for 30 days, and the area under the disease progress curve (AUDPC) was estimated (Campbell and Madden, 1990).

The experiment was carried out in a completely randomized design with a factorial of nine isolates, nine plant populations and three replications per treatment. Each replicate consisted of one pot containing two plants.

Table 1. *Euphorbia heterophylla* populations included in the study.

Code	Origin
EKLN16	Niterói-RJ
EKLN19	Viçosa-MG
EKLN247	Itabuna-BA
ERWB274	Nova Laranjeira-PR
ERWB280	Nova Petrópolis-RS
ETSB	Londrina-PR
ETRB	Londrina (resistant to *B. euphorbiae*)
ESH	Viçosa-MG
ERH	Viçosa-MG

Evaluation of liquid-culture media on the mycelial growth of *L. chlamidosporiformans*

This experiment aimed to evaluate the growth of *L. chlamidosporiformans* (isolate KLN-06) in a series of five common liquid-culture media of different compositions (see below for details) at either standard or double concentration. This test aimed to determine the composition of a liquid medium, from among the following, that might be adequate for mass production of mycelium for the first stage of a biphasic-technique (Walker, 1980):

1. PS (Dhingra and Sinclair 1995, standard concentration): decoction of 200 g potato; 20 g sucrose; 1 l distilled water;
2. PS × 2 (doubled concentration): decoction of 400 g potato; 40 g sucrose; 1 l distilled water;
3. VBS (VB as mentioned above supplemented with sucrose)-100 ml vegetable broth; 20 g sucrose; 450 ml distilled water;
4. VBS × 2 (doubled concentration)- 200 ml vegetable broth; 40 g sucrose; 450 ml distilled water;
5. Marine ammonium mineral salt (MAMS; standard concentration): decoction of 200 g castor-bean plant leaves; 20 g sucrose; 1 l distilled water;
6. MAMS × 2 (doubled concentration): decoction of 400 g castor-bean plant leaves; 40 g sucrose; 1 l distilled water;
7. MANDS (standard concentration): decoction of 200 g cassava leaves; 20 g sucrose; 1 l distilled water;
8. MANDS × 2 (doubled concentration): decoction of 400 g cassava leaves; 40 g sucrose; 1 l distilled water;
9. MSSA: semi-synthetic sucrose-asparagin medium (Alfenas, 1998; normal concentration): 10 g sucrose, 2 g L-asparagin, 2 g yeast extract; 1 g KH_2PO_4; 0.1 g $MgSO_4 \cdot 7H_2O$; 0.44 mg $ZnSO_4 \cdot 7H_2O$; 0.48 mg $FeCl_3 \cdot 6H_2O$; 0.36 mg $MnCl_2 \cdot H_2O$; 1 l distilled water.
10. MSSA · 2 (doubled concentration): 20 g sucrose, 4 g L-asparagin, 4 g yeast extract; 2 g KH_2PO_4; 0.2g $MgSO_4 \cdot 7H_2O$; 0.88 mg $ZnSO_4 \cdot 7H_2O$; 0.96 mg $FeCl_3 \cdot 6H_2O$; 0.72 mg $MnCl_2 \cdot H_2O$; 1 l distilled water.

Three mycelial plugs from 7-day-old cultures grown on PDA were transferred to 125-ml Erlenmeyer flasks, each containing 50 ml of one of the liquid-culture media described above. The flasks with plugs were placed on a shaker at 100 rpm at room temperature (25°C). Dry weight of mycelia produced was evaluated after 7 days of incubation. The contents from each flask were vacuum filtered through filter paper until the mycelium was dry. The mycelial mass was then scraped and weighed after drying in an electric oven at 70°C for 24 h. The experiment had a completely randomized design with four replications.

Effect of different solid-culture media on sporulation of *L. chlamidosporiformans*

This experiment aimed to determine the influence three different solid-culture media (supplemented or not with $CaCO_3$ at 3 g/l), on the sporulation of *L. chlamidosporiformans* during the second growth phase of the biphasic system of mass production. The following culture media were tested:

1. Concentrated PCA: decoction of 200 g potato; 200 g carrot; 1 l distilled water; 20 g agar;
2. Concentrated PCA + $CaCO_3$
3. VBA (Pereira *et al.* 2003)
4. VBA + $CaCO_3$
5. FLA: decoction of 200 g 'wild poinsettia leaves', 20 g sucrose, 1 l distilled water.
6. FLA + $CaCO_3$

The medium utilized during the first phase (mass production of mycelium in liquid culture) was MSSA (described above), and the procedure was also as described above. The mycelium was blended inside of the erlenmeyers, and 100 ml of the resulting suspension was poured into each of 24 aluminum trays (35 × 20 cm), containing 100 ml of the solid media that were being tested. Trays were kept in a controlled temperature room at 26 ± 2°C under a 12-h photoperiod. After 3 days, conidia were harvested once per day by pouring 50 ml of sterile water over the surface of the colonized medium and scraping the surface with a rubber spatula, with a total of four harvests per tray. The obtained suspension was filtered through three layers of cheesecloth. An aliquot of 20 μL of the conidial suspension obtained from each tray was removed and mounted on a microscope slide, and the number of conidia produced was counted, with results converted into conidia/ml from each treatment. The experiment had a completely randomized design with four replications and each tray represented a replicate. Statistical analysis was made of the sum of conidial concentrations obtained for the four harvests.

Results

Selection of a fungal isolate

Nine isolates were obtained from several locations (Table 2). Among these isolates, only three were pathogenic to all *E. heterophylla* accessions that were screened. Isolate KLN06 caused the highest disease-intensity levels resulting in larger values of AUDPC for five of the wild poinsettia populations involved in the test, including population ERH (resistant to the herbicide imazethaphyr) and was equivalent to other isolates in disease severity caused to the remaining weed

Table 2. *Lewia chlamidosporiformans* isolates included in the study.

Code	Origin
KLN06	Viçosa-MG
KLN09	Araruama-RJ
KLN14	Italva-RJ
KLN15	Niterói-RJ
KLN17	São Miguel do Anta-MG
KLN18	Viçosa-MG
KLN19	Viçosa-MG
KLN20	Viçosa-MG
RWB280	Nova Petrópolis-RS

populations (Figure 1). The first symptoms appeared 5 days after inoculation with KLN06, and plant death started appearing 7 days after inoculation in populations EKLN19, ERWB247, ETSB, ERH and ESH. KLN06 was selected as the most promising isolate for further studies.

Evaluation of liquid-culture media on the mycelial growth of *L. chlamidosporiformans*

The liquid-culture media that resulted in the hightest levels of *L. chlamidosporiformans* mycelial production was MSSA × 2 (Figure 2). Other media yielded inferior results for the production of *L. chlamidosporiformans* mycelia, reaching values that varied from 1/3 to 1/2 that obtained with MSSA × 2. A smaller production of mycelial mass was obtained for: PS, MAMS and MAMS × 2. The doubled concentration of the ingredients in the liquid-culture media only resulted in significant increase in the yield of mycelial biomass for MSSA × 2 and PS. Mycelial production for MSSA × 2 was triple that obtained for standard MSSA (Figure 2). MSSA × 2 was hence selected for use as medium in the first phase of biphasic mass production of *L. chlamidosporiformans*.

Effect of different solid-culture media on sporulation of *L. chlamidosporiformans*

Results obtained in this experiment are presented in Figure 3. Among the solid-culture media that were tested for sporulation in the second phase of the biphasic mass production of *L. chlamidosporiformans*, VBA + $CaCO_3$ had the best performance. Its use resulted in a production of 8.1×10^5 conidia/ml and was followed by PCA + $CaCO_3$ (7.03×10^5 conidia/ml) and FLA + $CaCO_3$ ($5{,}6 \times 10^5$ conidia/ml). There were significant statistical differences among most culture media being tested (Figure 3). The supplementation of $CaCO_3$ (3g/l) significantly increased the production of spores of the fungus for all solid-culture media being tested, and this

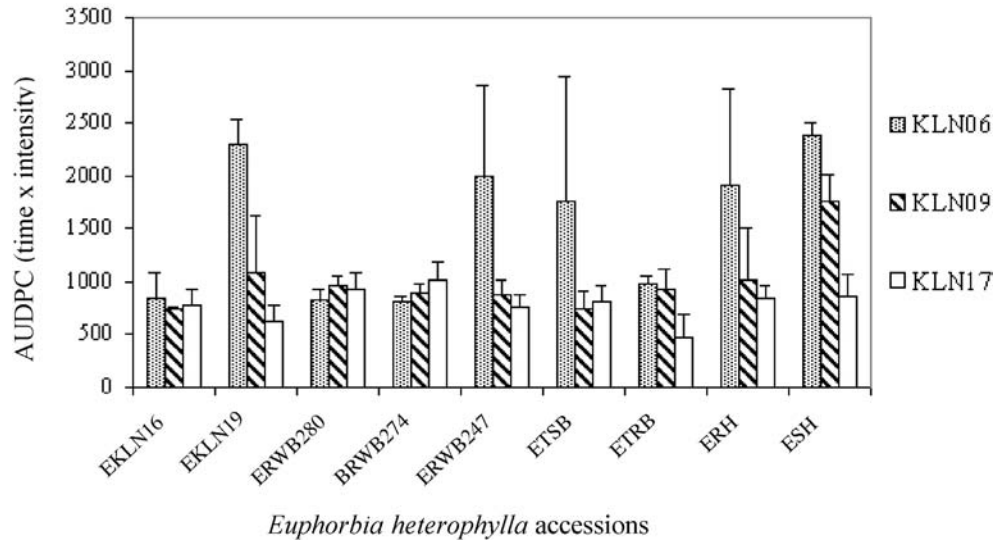

Figure 1. Area under the disease progress curve for the isolates KLN06, KLN09, and KLN17 of *Lewia chlamidosporiformans* based on disease severity (means of three repetitions; *bar* standard deviation).

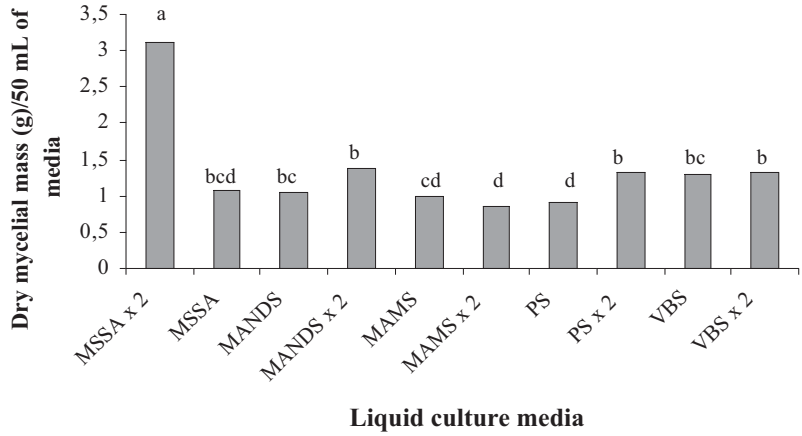

Figure 2. Production of mycelial mass of *Lewia chlamidosporiformans* in different liquid-culture media (means of four repetitions, *bars* standard deviation, *means followed by the same letter* did not differ under Tukey test at the level of 5% of probability).

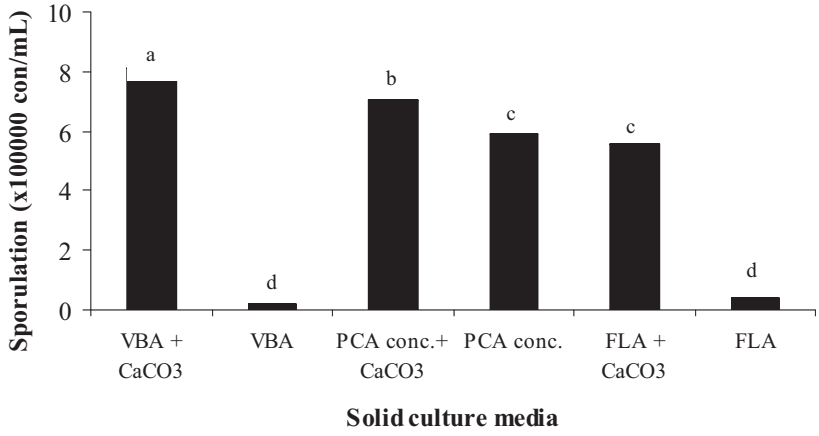

Figure 3. *Lewia chlamidosporiformans* conidial production on different solid-culture media (means of four repetitions, *bars* standard deviation, *means followed by the same letter* did not differ under Tukey test at the level of 5% of probability).

was particularly significant for VBA. The addition of $CaCO_3$ (3g/l) increased conidial production in VBA 16-fold. It also increased conidial production for FLA by a factor of 9.3 and by a factor of 1.24 for PCA.

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