Ideal culture conditions for *Dicyma pulvinata* conidia mass production

Débora Ferreira Melo[1] and Sueli Corrêa Marques de Mello[2]

[1]Universidade de Brasília, Departamento de Fitopatologia, Caixa Postal 4457, CEP 70910-900 Brasília, DF. E-mail: deboramelo@pop.com.br
[2]Embrapa Recursos Genéticos e Biotecnologia, Caixa Postal 02372, CEP 70770-900 Brasília, DF. E-mail: smello@cenargen.embrapa.br

Abstract – The objective of this work was to set up ideal conditions for conidia mass production of *Dicyma pulvinata*. Four isolates were compared in terms of their growth and conidia production on various substrates (grains of parboiled rice, common rice, maize and wheat, besides chipped maize and rice husk), temperatures (19, 22, 25, 28 and 31°C), growth containers (aluminum trays, polypropylene bags and Erlenmeyers) and light regimes (continuous darkness, 6 and 12 hours of light/darkness, and continuous light). Temperature effects on conidia germination capacity were also evaluated. The experiments were done in randomized complete block designs, in factorial arrangements (isolates x treatments – substrates, containers, temperatures and light regimes), with four replicates. In general, parboiled rice and polypropylene bags provided the best development of the fungus. Complete darkness and 6 hours of light increased mycelial growth, whereas continuous light favored sporulation. All tested temperatures favored the cultures of the fungus, except 31°C. Temperatures between 19 and 25°C ensure spore germination of more than 76%.

Index terms: *Microcyclus ulei*, biocontrol, biofungicide, leaf blight, rubber tree.

Condições ideais de cultivo para produção massal de conídios de *Dicyma pulvinata*

Resumo – Este trabalho teve como objetivo estabelecer condições ideais para a produção massal de conídios do fungo *Dicyma pulvinata*. Quatro isolados do fungo foram comparados em termos de seu crescimento e produção de conídios em diferentes substratos (grãos de arroz parboilizado, arroz comum, milho e trigo, além de flocos de milho e casca de arroz), temperaturas (19, 22, 25, 28 e 31°C), recipientes de cultivo (bandejas de alumínio, sacos de polipropileno e Erlenmeyers) e regimes de luz (escuridão contínua, fotoperíodos de 6 e de 12 horas de luz/escuro, e luz contínua). Os efeitos da temperatura sobre a capacidade germinativa dos conídios também foram avaliados. Os experimentos foram conduzidos em delineamento de blocos ao acaso, em arranjos fatoriais (isolados x tratamentos – substratos, recipientes, temperaturas e regimes de luz), com quatro repetições. De modo geral, sacos de polipropileno e arroz parboilizado proporcionaram o melhor desenvolvimento do fungo. Fotoperíodos de 0 e 6 horas de luz favoreceram o crescimento micelial, enquanto que a luz contínua favoreceu a esporulação dos isolados. Todas as temperaturas testadas propiciaram o cultivo do fungo, exceto a de 31°C. Temperaturas entre 19 e 25°C asseguram germinação superior a 76% dos conídios do fungo.

Termos para indexação: *Microcyclus ulei*, controle biológico, biofungicida, mal-das-folhas, seringueira.

Introduction

South American leaf blight (SALB) poses a serious problem when establishing rubber plantations in humid regions of Central and South America. This disease, caused by the fungus *Microcyclus ulei* (P. Henn.) Arx. (ascogenic phase) or *Fusicladium macrosporum* Kuijper (conidial phase), is widespread in almost all areas of rubber tree cultivation in Brazil (Mello et al., 2006). Although it is currently restricted to the Americas (International Rubber Research and Development Board, 2006; Lieberei, 2007), the pathogen represents a considerable threat, especially to Southeast Asia, where the main rubber-producing countries are concentrated.

The fungus has not yet spread to Southeast Asia and the Pacific, at least partly because of the intense monitoring of air and sea cargo coming from South America (Lieberei, 2007). In the 1950's, the Asia and Pacific Plant Protection Commission (APPPC) was created, under the auspices of FAO. Since then, Asian and Pacific countries have adapted a number of quarantine-related measures and bans to prevent

the entrance of any material contaminated with the pathogen (International Rubber Research and Development Board, 2006; Food and Agriculture Organization of the United Nations, 2009). However, it has been postulated that the likely increase in direct flights from Brazil to other tropical humid countries may make it more difficult to apply these measures (Lieberei, 2007). This problem is so serious that it has recently led to the inclusion of *M. ulei* on the list of biological weapons (Croddy & Newhouse, 2009).

Among the main measures adopted to contain epidemics under control in countries where the pathogen occurs, the most notable are the use of chemical fungicides, the selection of resistant clones and crown budding (Mattos et al., 2003). Over the years, however, these measures have not yielded satisfactory results in permanently humid regions of Brazil, such as Amazonia and the southeast of Bahia state. This has led to rubber plantations moving to less humid areas, such as the Midwest, Southeast and South of Brazil, where climatic conditions are mainly unfavorable to the disease during the period of leaf renewal, from July to September (Furtado & Trindade, 2005).

Despite the evidence of good performance from the rubber plantations, cultivated in adverse conditions to the disease, *M. ulei* is a fungus that has a great capacity for mutation and could, also, become a serious problem in the escape zones, such as the Brazilian Cerrado (Gasparotto & Junqueira, 1994).

Biological control represents an additional strategy to be used as part of an integrated management of SALB. In natural conditions, the fungus *Dicyma pulvinata* (Berk. and M.A. Curtis) Arx [syn. *Hansfordia pulvinata* (Berk. and M.A. Curtis)] is capable of colonizing the structures of the pathogen, both in the ascogenic and the conidial phases (Junqueira & Gasparotto, 1991; Mello et al., 2006). Greenhouse and field trials showed that some isolates of this fungus species were able to reduce leaf fall and the pathogen inoculum rate for re-infections (Melo et al., 2008). *D. pulvinata* also shows biocontrol against other crop diseases (Peresse & Picard, 1980; Mitchell et al., 1987; Tirilly, 1991). Commercial application of this biocontrol agent depends on the production of large quantities of conidia. Thus, an efficient conidia production process should be developed.

The production of fungal biocontrol agents in Brazil, as well as in other countries, has been based on the use of cereal grains or other organic materials as substrates (Faria & Magalhães, 2001; Grimm, 2001; Thangavelu et al., 2004; Prakash et al., 2008). This is partly due to the fact that organic matter is readily biodegradable, making field application easier (Thangavelu et al., 2004). Solid-state fermentation, in turn, mimics the natural environmental conditions and habitat for some microorganisms (Spadaro & Gullino, 2005).

The objective of this work was to determine ideal conditions for conidia mass production of the fungus *D. pulvinata*, by evaluating the effects of substrate, container, temperature and light regime on growth and sporulation of four isolates of the fungus.

Materials and Methods

The four *D. pulvinata* isolates used in this study, CEN 58, CEN 62, CEN 91 and CEN 93, belong to the culture collection of Embrapa Recursos Genéticos e Biotecnologia, and were originally obtained from stomatic lesions of *Hevea* rubber tree (Mello et al., 2006). The selection of the isolates was based on the previous evaluation of their biological control potential (Melo et al., 2008).

Long-term cultures stored in liquid nitrogen (-193°C) were revived in PDA (potato-dextrose-agar) medium, and the working cultures were maintained at 4°C. When tests were performed, the isolates were subcultured on the same medium. Except for the specified cases, in all assays the colonies were incubated at 25ºC, under a 12-hour photoperiod. Photoperiods were simulated using four daylight fluorescent 20-watt bulbs, installed in the door of the BOD (Model NT 708-AT; Nova Técnica, Piracicaba, SP). In the cases where there was mycelial production in a liquid medium, the incubation took place in a rotary shaker (Model NT 711; Lab-line Instruments, Inc., Melrose Park, IL, USA) at 150 rpm and 25ºC, for seven days. All the experiments were done twice.

Grains of parboiled rice, common rice, maize, and wheat, as well as chipped maize and rice husk, were evaluated as substrates. Except for rice husks, 25 g of each substrate with 15 mL of distilled water were placed in 250 mL Erlenmeyer flasks, sealed, left to hydrate for about two hours, and then sterilized

by autoclaving at 98 kPa and 120ºC for 25 min. For the substrate based on rice husks, 8 g of material was hydrated with 15 mL of the liquid from cooking potatoes in water (200 g L^{-1}) and submitted to the same autoclaving process. Under aseptic conditions, mycelial discs (9-mm diameter) were cut from the edges of 10-day-old *Dicyma pulvinata* colonies and introduce on the substrate contained in the Erlenmeyers (four discs per flask) that were sealed again. After 17 days of incubation, the number of conidia per gram of substrate was estimated, using the suspension obtained by adding 100 mL of water containing Tween 80 (polyoxyethylene sorbitan monolaurate) at 0.05% (v/v) to the colonized substrate. The suspensions were vigorously shaken in the rotary shaker for two minutes, and filtered through four layers of cheesecloth. From each flask, samples were taken and appropriate dilutions were made, in order to count conidia in a Neubauer chamber at 400x magnification. Three readings of each replicate were carried out.

Aiming to compare containers usually employed for production of the fungus, as for spore count, an experiment was set up with trays, polypropylene bags and Erlenmeyers. Rectangular aluminum trays (30x44x4.5 cm) were covered with autoclavable plastic bags, whose openings were folded and stapled after 500 g of parboiled rice had been introduced and spread around. In the same way, the polypropylene bags (28x42 cm) were closed with a stapler, after receiving 200 g of parboiled rice, then placed vertically; and the Erlenmeyer flasks, with 2-L capacity, were sealed with cotton plugs after receiving 250 g of parboiled rice. Rice had been previously moistened with 60% (p/v) of distilled water, left to soak for about two hours and sterilized by autoclaving at 98 kPa, at 120ºC for 25 min. The inoculation of mycelia in the substrates, of the four previously mentioned isolates, took place in a laminar flow chamber. The mycelial mass for inoculation was produced in liquid SDY medium (peptone 10 g L^{-1}; dextrose g L^{-1}; and yeast extract 10 g L^{-1}). For this, five mycelia discs (9-mm diameter) from colonies produced as in the previous item, were transferred to the SDY medium contained in 500 mL Erlenmeyer flasks (150 mL per flask). Incubation took place while shaking for seven days. The concentration used for inoculation was of 10% (w/v) of mycelium suspension, independently of the container that held the substrate. After 17 days of incubation, the estimation of conidial production was made. Conidia were obtained from each replicate container by taking 3 g samples of colonized rice and by suspending each sample in 30 mL of sterile water. The suspensions of conidia were vigorously shaken in a Laboratory mixer (MS 2 Minishaker IDA-Works, Inc., Wilmington, NC) operated at full speed for 2 min, filtered, and used to determine conidia concentrations, as described above.

To check the effect of light regime on the fungus growth and conidia production, two experiments were run with the four isolates of *D. pulvinata*, using two types of cultivation: on PDA medium and on parboiled rice. In the first experiment, mycelial discs (9-mm diameter) taken from the colonies, developed in the previously described conditions, were placed in the center of Petri dishes containing PDA medium, with the mycelia facing downwards. In the second experiment, 250-mL Erlenmeyer flasks containing 25 g of parboiled rice, duly moistened and sterilized, were used. Each flask received four mycelial discs from the same colonies. The plates and flasks were submitted, after inoculation, to the following photoperiods: continuous darkness, 6 hours of light/darkness, 12 hours of light/darkness and continuous light. The evaluation of mycelial growth was carried out by taking measurements of the diameter of colonies developed on PDA medium at 4, 8, 12 and 16 days of incubation. The conidial production, both on PDA and on parboiled rice, was quantified at 17 days after inoculation by taking samples from the suspension in distilled water with addition of Tween 80 at 0.05% (v/v), to count conidia, as described earlier.

To evaluate the effects of temperature on growth and conidia production, the fungus was cultivated on PDA and on parboiled rice, following the same procedures for inoculation and evaluation, as described in the previous experiment. The temperatures tested were 19, 22, 25, 28 and 31ºC, under a regime of 12 hours of light/darkness. The period of incubation for determination of mycelial growth and for evaluation of conidia production was 16 days.

The germinative capacity of the isolates CEN 58, CEN 62, CEN 91 and CEN 93 of *D. pulvinata* were evaluated at the temperatures 19, 22, 25, 28 and 31ºC. Conidial suspensions, whose incubation took place at 25ºC with 12 hours of light/darkness photoperiod, were sown on Petri dishes containing agar-water medium and incubated for 17 hours. For each isolate, four dishes were prepared for each of the temperatures to be

tested, and each dish received 200 UL of suspension at a concentration of 1 x 10^5 spores mL^{-1}. The germination percentage was determined by means of a test with 100 conidia per dish, visualized in randomly chosen microscopic fields, at 400x magnification. Conidia with a germinative tube of equal size or larger than their diameter were considered to have germinated.

The experiments were done in a randomized complete block design, in a factorial arrangement – isolates x treatments (substrate, container, temperature or light regime) –, with four replicates. Data were submitted to analysis of variance, using the Estat System for statistical analysis v. 2.0, followed by mean comparison using Tukey test, at 5% probability.

Results and Discussion

In general, all the substrates were conducive to cultivation of the fungus, independently of the isolate used (Table 1). However, spore production was highest on parboiled rice. The lowest sporulation rate was observed on rice husk substrate, even with the addition of potato-water.

Based on the above, parboiled rice is still the best substrate for production of *D. pulvinata* conidia and can be used as a starting point for research in the development of an efficient production technology. However, other substrates, such as industrial and agricultural residues, should be tested to avoid the use of an important staple food for humans as a substrate for fungal growth.

Table 1. Sporulation of *Dicyma pulvinata* isolates on different solid substrates (10^6 spores g^{-1} of substrate) and containers (10^7 spores g^{-1} of substrate)[(1)].

Treatments	Isolates			
	CEN 58	CEN 62	CEN 91	CEN 93
Substrate				
Parboiled rice	5.153Ab	4.795Ab	3.598Ac	7.774Aa
Common rice	3.844Bb	3.091Bc	2.593Bc	6.113Ba
Maize	3.112BCb	2.583BCb	2.509Bb	5.081Ca
Chipped maize	2.394CDb	2.034Cb	2.048Bb	3.137Da
Wheat	2.212DEb	1.868Cb	1.816Bb	2.915Da
Rice husk	1.507Ea	0.993Da	0.893Ca	1.301Ea
Containers				
Poly-Propylene bags	1.861A	1.205Abc	0.790Ac	1.306Bb
Erlenmeyer flasks	1.790Aa	1.197Ab	0.840Ab	1.891Aa
Trays	2.195Aa	1.464Ab	0.741Ac	1.021Bbc

[(1)]Means of each treatment followed by equal letters, uppercase in the columns and lowercase in the lines, do not differ by Tukey's test, at 5% probability. Data transformed by SQRT (x + 0.5).

The containers used for propagules production of *D. pulvinata* isolates, compared in terms of sporulation, did not differ statistically from one another, except for CEN 93, in which the production in Erlenmeyers was greater than the obtained in the other two containers (Table 1). The use of polypropylene bags offered easy handling, which is related to the fact that they are widely used in the production of various fungi, such as *Metarhizium* spp. and *Beauveria bassiana* (Leite et al., 2003).

There was no significant interaction between the photoperiods and isolates, for the diameter size of colonies. Continuous darkness and six hours of light/darkness were the regimes that most favored the growth of the fungus. These data agree with those of Rodrigues (2002) who, in evaluations of the influence of light on mycelial growth of a *D. pulvinata* isolate, stated that the absence of light promoted the growth of the fungus.

After four and eight days of incubation, no difference was observed on the growth of the isolates (Table 2). However, at 12 and 16 days, isolates CEN 58 and CEN 91 showed larger mean size of colony diameter.

In general, continuous light favored sporulation of the isolates of *D. pulvinata* (Table 3). In the experiment carried out on PDA culture medium, only CEN 93 was not influenced by the tested photoperiods. As for the experiment with parboiled rice, sporulation was similar with photoperiods of 12 hours of light/darkness and of 24 hours of continuous light, for the isolate CEN 58. However, the isolate CEN 93 responded better to 24 hours of continuous light, while CEN 62 and CEN 91 showed no significant difference for the light regimes studied.

According to Rodrigues (2002), *D. pulvinata* presents a variety of responses to different wavelengths, but sporulation was greatest with white and blue light and with the absence of light.

Table 2. Mean diameter of colonies (cm) of *Dicyma pulvinata* isolates, after different cultivation periods[(1)].

Days of cultivation	Isolates			
	CEN 58	CEN 62	CEN 91	CEN 93
4	0.20Da	0.20Da	0.15Da	0.20Da
8	1.01Ca	0.99Ca	0.93Ca	0.94Ca
12	1.86Ba	1.66Bbc	1.78Bab	1.48Bc
16	2.26Ab	2.51Aa	2.02Ac	2.55Aa

[(1)]Means followed by equal letters, upper-case in the columns and lowercase in the lines, do not differ by Tukey's test, at 5% probability.

The data obtained here corroborated that during their development, fungi respond differently to light stimulation (Cochrane, 1958). As such differences have shown to also be dependent on the type of culture medium or substrate, it is important to consider the means of production and the isolate chosen in terms of their biocontrol efficiency. In the case of isolates that do not present demands regarding photoperiod, the inoculum production process may become simpler and less demanding.

Loureiro et al. (2002) have suggested that conidial production is generally favored by the presence of light; they stress, however, that to obtain maximum numbers of conidia, the right light type and regime should first be determined, since both are parameters that vary considerably between isolates of the same species.

The temperatures tested enabled the cultivation of the four *D. pulvinata* isolates (Figure 1), except for 31ºC. At 28ºC, less mycelial growth of the fungus was observed. For isolates CEN 58 and CEN 91, the best temperature was 25ºC, but for isolates CEN 62 and CEN 93, temperatures of 19, 22 and 25ºC did not have any significant effect on the growth of *D. pulvinata*.

As for sporulation, differences were observed in isolate responses to temperature, both on PDA medium and on parboiled rice (Table 3). On PDA medium, temperatures of 19, 22 and 25ºC did not exert a significant effect on isolates CEN 58 and CEN 93. However, isolates CEN 62 and CEN 91 sporulated better at 22ºC. For the culture on parboiled rice, isolate CEN 58 sporulated better when maintained at 19ºC, while the other isolates showed no statistical differences in terms of conidia per gram of substrate. CEN 62 and CEN 91 were favored by temperatures of 19 and 22ºC, with greater conidia production. Isolate CEN 93 showed no difference in terms of conidia production at 19, 22 and 25ºC, but rate and quantity of conidia per gram of substrate were significantly higher at these temperatures than at 28ºC.

For conidium germination, there was no interaction between treatments and isolates; the general means for temperatures and isolates are presented in Figure 2. The mean percentage of germinated spores was higher at 22ºC, but the rate varied little from those obtained at 19 and 25ºC. Among the isolates, the highest mean rates of germination were for CEN 62 and CEN 93, which had no difference in rates. These results matched information from Leite et al. (2003), who stated that the most hifomycete fungi, during growth and conidiogenesis, are favored by temperatures close to 25ºC.

Mitchell & Taber (1986) studied an isolate of *D. pulvinata* from Texas and reported a maximum growth point in liquid medium at between 23 and 25ºC, with a 22% fall in growth at 28ºC. This effect of temperature was also observed in the colonization and sporulation on lesions of *Cercosporidium personatum*, in which temperatures between 23 and 25ºC were determined as the ideal range for fungal activity.

The four isolates of *D. pulvinata* in the present study showed quite similar behaviors for favorable temperature range to growth and sporulation. This is relevant not only for the production of the fungus, but also for inferences about the environmental conditions and ideal periods in which to apply the fungus.

No development of the colonies of any isolate was observed at 31ºC, after 17 days of incubation. When

Table 3. Mean sporulation (10^5 spores ml^{-1}) of *Dicyma pulvinata* isolates, under different light regimes (continuous darkness, 6 and 12 hours of light/darkness, and continuous light) and temperatures, after 17 days of growth on PDA culture medium or on parboiled rice[(1)].

Treatments	PDA culture medium				Parboiled rice			
	CEN 58	CEN 62	CEN 91	CEN 93	CEN 58	CEN 62	CEN 91	CEN 93
Photoperiod								
0h	2.57Ca	0.85Ba	1.17Ba	0.32Aa	3.05Bb	5.11Aab	0.49Ac	6.38Ba
6h	7.02Ba	3.57Bab	3.62Bab	0.45Ab	2.90Bbc	4.43Ab	1.49Ac	7.44Ba
12h	3.82BCa	0.77Ba	1.97Ba	0.47Aa	4.21ABb	3.92Ab	0.39Ac	7.13Ba
24h	17.45Aa	9.77Ab	11.67Ab	0.85Ac	6.35Ab	5.82Ab	1.62Ac	11.32Aa
Temperature								
19ºC	8.20Ab	10.45Bb	15.15Ba	3.95Ac	8.40Aa	6.06ABb	9.05Aa	5.32Ab
22ºC	9.80Ac	15.80Ab	23.90Aa	4.20Ad	5.43Bb	7.34Ab	9.65Aa	6.34Ab
25ºC	7.15Aba	2.42Cb	4.77Cab	1.40Ab	4.61Bab	4.03Bab	2.51Bb	6.04Aa
28ºC	3.47Ba	0.37Ca	2.20Ca	0.75Aa	3.86Ba	0.81Cb	0.29Cb	0.23Bb

[(1)]Means of each treatment followed by equal letters, uppercase in the columns and lowercase in the lines, do not differ by Tukey test, at 5% probability.

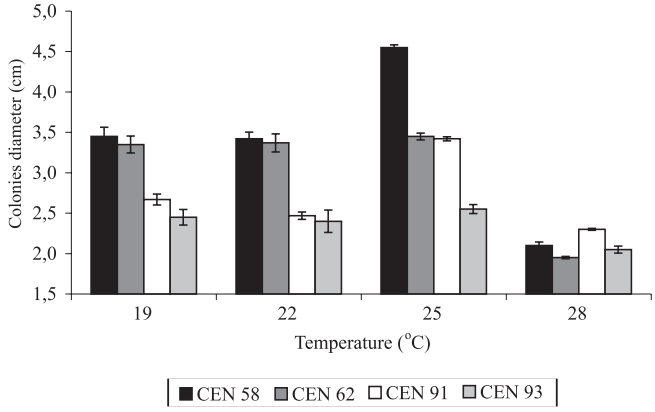

Figure 1. Mean diameter of the colonies of *Dicyma pulvinata* grown at different temperatures, at 16 days. Error bars represent 95% confidence intervals.

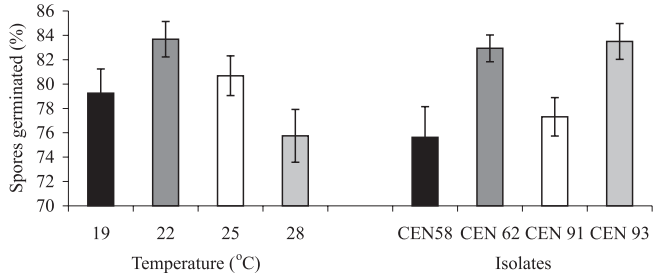

Figure 2. Germination of spores of four isolates of *Dicyma pulvinata* at different temperatures. Error bars represent 95% confidence intervals.

transferred to BOD at 25ºC, these colonies grew and sporulated, both on PDA medium and on parboiled rice. The spores obtained in this way were seen to be viable. Similar data have been noted for other fungi, such as *Cercospora piaropi*, which did not develop at 35ºC, but when transferred to 25ºC, had radial growth (Ávila & Pitelli, 2004).

Conclusions

1. The most efficient method of producing *Dicyma pulvinata*, on a large scale, is the use of parboiled rice substrate in polypropylene bags, under continuous light.

2. Temperatures between 19 and 25ºC ensure conidia germination of more than 76%.

Acknowledgements

To Conselho Nacional de Desenvolvimento Científico e Tecnológico, for scholarship.

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Received on April 7, 2009 and accepted on September 10, 2009