Reduction of the number of nuclei per cell of *Helminthosporium euphorbiae* by protoplast isolation

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Helminthosporium euphorbiae is a pathogen of the weed *Euphorbia heterophylla*, which causes severe losses in soybean (*Glycine max*) crops. The fungus causes leaf loss and affects germination, making it a promising biocontrol agent for this weed. In order to start a breeding program for this species, four isolates were examined for number of nuclei in the conidia and hyphae and nuclear behavior at different cultivation stages. The conidia were multinucleated with about 20 nuclei per conidium, and 5 to 7 nuclei were observed in the hyphae compartments. The high number of nuclei makes the genetic manipulation of this species difficult, so the protoplast formation is an alternative for obtaining cells with a reduced number of nuclei. Thus the experimental conditions for the production and regeneration of protoplasts in *H. euphorbiae* were determined by assessing three enzymatic complexes and seven osmotic stabilizers. The efficiency of formation and regeneration frequency of the protoplasts varied depending on isolates, stabilizers and enzyme mixture used. The number of nuclei estimated per protoplast was reduced to 1 to 6, depending on the stage of mycelial growth during the protoplast formation process.

Key Words——cytology; genetics; *Helminthosporium euphorbiae*; protoplasts.

Helminthosporium euphorbiae Hansford (Muchovej and Carvalho, 1989) affects germination and causes leaf loss in *Euphorbia heterophylla* L. (Euphorbiaceae), known popularly as milk weed in soybean (*Glycine max* (L.) Merril) crops, which is common in the south of Brazil (Cerdeira and Voll, 1981; Yorinori, 1985; Gazziero, 1985). When this weed is not controlled it causes severe crop losses because it interferes with the yield and reduces the quality of grains. According to Yorinori (1987), the weed infests about 200,000 ha of cultivated land in Brazil, and the frequently inadequate control increases annual costs for chemical herbicide, thus increasing production costs. The use of *H. euphorbiae* as a bioherbicide in the *E. heterophylla* control has become important because it is highly specific and virulent (Yorinori, 1985; Yorinori and Gazziero, 1987) and does not interfere with the development of soybean crops (Souza, 1992). Its application does not depend on the age of the weed and may be associated with chemical herbicides or insecticides in an integrated management (Yorinori et al., 1985a, b; Gazziero and Yorinori, 1987, 1988; Yorinori

and Gazziero, 1987; Gazziero et al., 1989, 1993). In spite of its potential as a biological herbicide, little is known about the biology of the fungus species. It is known that most representatives of the genus *Helminthosporium* have multinucleated conidia (Knox-Davies and Dickson, 1960), which hinder a breeding program using mutant induction and selection.

Protoplast formation is a possible alternative to obtain cells with a reduced number of nuclei, which would ease mutant acquisition. This study was carried out to cytologically characterize the *H. euphorbiae* species and standardize the methodology for protoplast formation and regeneration. The cultural conditions of mycelia for obtaining protoplasts with reduced number of nuclei were mainly investigated.

Materials and Methods

Microorganisms The *H. euphorbiae* isolates were kindly given by Dr. José Tadashi Yorinori (EMBRAPA-Soja-Londrina PR) and are listed in Table 1. Cultures of these isolates were maintained on potato dextrose agar (PDA) medium at 28°C.

Cytological analysis The isolate 1 was used for the cytological analyses. Conidia were produced in PDA medium at 28°C after 7 d of cultivation. For light microscopic observation of conidia, slides containing a thin layer of 50% albumin were pressed on the 7 d culture with abundant conidia. The slides with attached conidia were placed in sodium hypochlorite solution for 1 min,

Table 1. *Helminthosporium euphorbiae* isolates.

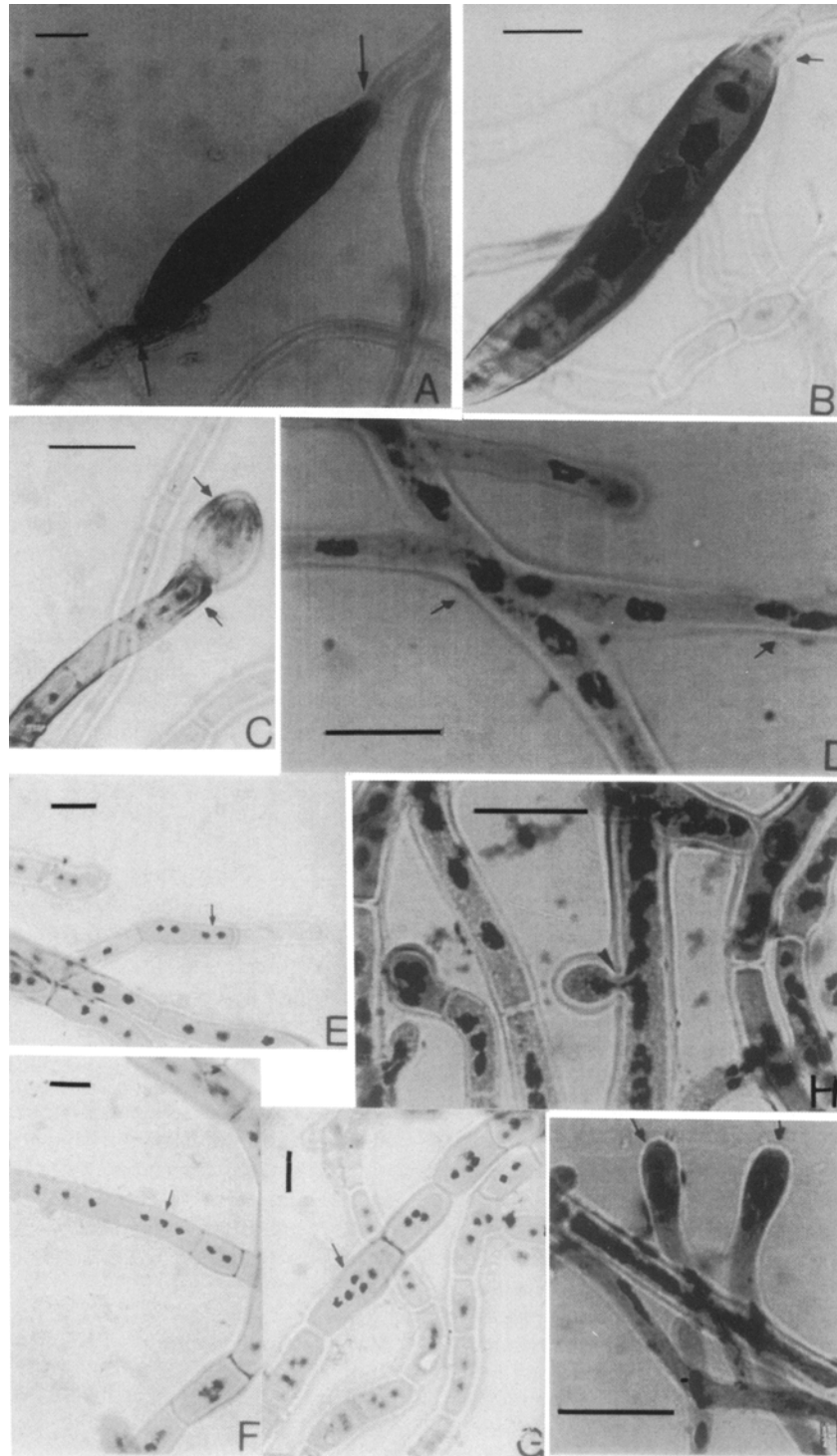| Isolate	Locality
1	Paraná-Brazil
2	Mato Grosso-Brazil
3	Mato Grosso do Sul-Brazil
4	Rio Grande do Sul- Brazil

Fig. 1.   A. Bipolar germination at 8 h growth.   B. Nuclear migration during conidiogenesis; conidium with more than 20 nuclei.   C. Multinucleated conidiophore (arrow a) and conidia in formation (arrow b) at 18 h growth.   D. Hyphal anastomosis.   E. Mycelia at 18 h development (predominance of 2 nuclei/cell) (arrow).   F. Mycelia at 30 h development (predominance of 3 nuclei/cell) (arrow).   G. Mycelia at 48 h development (predominance of 5 to 6 nuclei/cell) (arrow).   H. Nuclear migration during hyphal ramification (arrow).   I. Apex region of hyphae during growth; the arrows indicate the accumulation of nuclei in this region.   Scale bars: A–C = 10 μm; D–I = 5 μm.

then stained with Giemsa's solution following the technique described by Tanaka et al. (1979). For light microscopic observation of hyphae, agar discs of 5 mm in diam from Petri plate cultures of four isolates were inoculated on sterile dialysis membrane on PDA medium, incubated at 28°C to assess germination and mycelia growth. The membranes were removed from the culture media at predetermined periods and stained with Giemsa's solution (Tanaka et al., 1979). The membranes were placed on slides for microscopy, covered with slips, and sealed with nail enamel. The slides were observed under a light microscope.

Protoplast production and regeneration A suspension containing 10^6 conidia ml^{-1} was inoculated in 50 ml of potato broth. After 45 h of incubation at 28°C, the mycelia were collected and washed three times in a centrifuge with osmotic stabilizer solution and then preincubated at 28°C for 1h in the same stabilizer (50 mg mycelia/ml of osmotic stabilizer). Seven osmotic stabilizers were assessed: 0.6 M KCl; 0.7 M NaCl; 0.7 M CaCl$_2$; 0.7 M MgCl$_2$; 0.7 M MgSO$_4$; 0.05 M KH$_2$PO$_4$ (added to 5 mM MgSO$_4$+0.7 M KCl+3 mM cysteine); and 0.7 M sucrose. The enzymatic mixture was added and the mixture was incubated at 28°C with shaking (80 rpm/min) for various incubation times. The following enzymatic complexes were assessed: N+CP [0.25% w/v Novozym 234 (Novo Enzyme Products Ltd, Windsor) +0.25% w/v Cellulase CP (Sigma)]; N+O [0.25% w/v Novozym 234+0.25% w/v Cellulase R10 (Onozuka)] and N+B [0.25% w/v Novozym 234+0.25% w/v Cellulase (Biobrás)]. After enzymatic digestion, the protoplasts were separated by filtration on glass wool. The protoplasts obtained were washed three times in

osmotic stabilizer by centrifuge at 3000 rpm for 15 min. The number of protoplasts was estimated using a Neubauer haemocytometer counting chamber. For regeneration, protoplasts were plated onto the culture media, PDA and PDA+osmotic stabilizers, by the pour-plate technique. After 7d of incubation at 28°C, the colonies were counted and the regeneration frequency was calculated using the following ratio: (number of colonies obtained from protoplasts/number of protoplasts plated) × 100 (Peberdy, 1978).

Number of nuclei in protoplasts The number of nuclei in the protoplasts was determined using the Giemsa staining technique. Protoplasts were adhered on slides containing a thin layer of 50% albumin, fixed in methanol for 5 min, hydrolyzed in 1 N HCl for 3 min (60°C) and washed in 0.02 M phosphate buffer, pH 6.9. They were then stained with the Giemsa's solution and 0.02 M phosphate buffer, pH 6.9 (1 : 10) for 30 min. The slides were covered with slips, sealed with nail enamel, and the nuclei of protoplasts were observed under a light microscope.

Results and Discussion

Cytological analysis Cytological analysis was performed on isolate 1. The morphology and coloration of these conidia agreed with the description of Muchovej and Carvalho (1989): brown conidia, almost cylindrical to fusiform, bent, hyaline, barrel-shaped, pseudoseptate, with 63–96 × 14.7–17.5 μm with 7–8 pseudosepta.

Conidiophores and conidia of *H. euphorbiae* were observed to be multinucleated (Fig. 1B, C). The conidia had up to 20 nuclei/cell with bipolar germination after 8 h
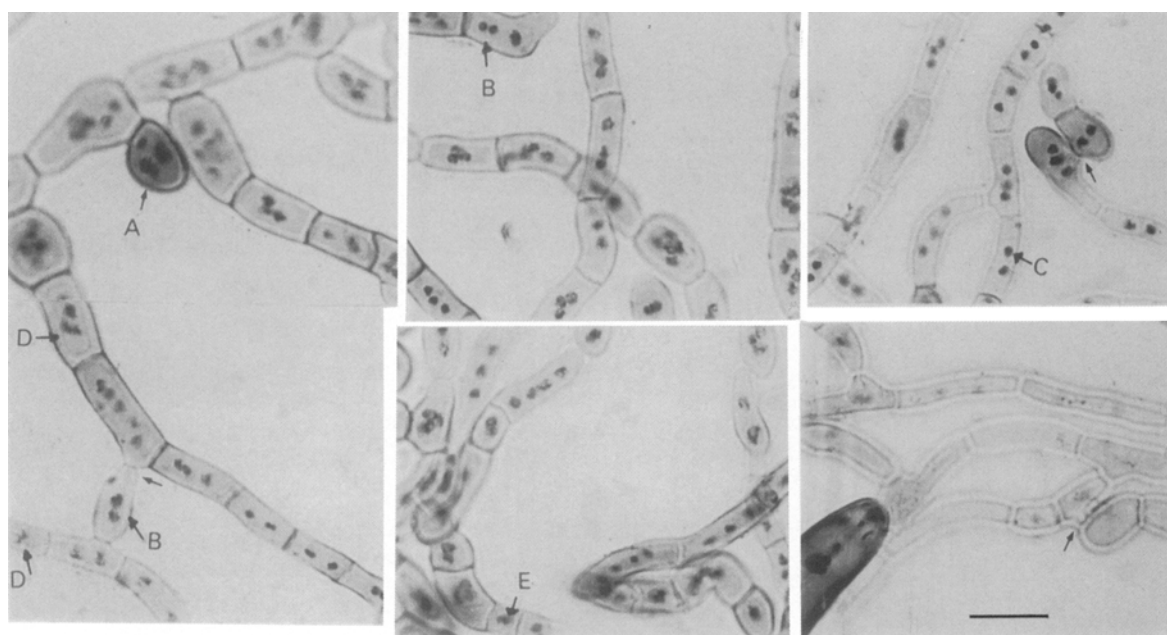

Fig. 2. Cytological aspects of hyphae from Isolate 1 of *Helminthosporium euphorbiae* at 18 h of culture in artificial medium. The arrows indicate hyphal anastomosis (arrows without letters) and different nuclear division stages: A. anaphase; B. telophase; C. interphase; D. metaphase; and E. prophase. Scale bars: 10 μm.

Table 2. Protoplasts ($\times 10^6$ protoplasts ml^{-1}) obtained from *Helminthosporium euphorbiae* isolates by incubation for 4 h with different enzimatic complexes and osmotic stabilizers.

Isolate	Enzimatic complex	NaCl 0.7 M	MgCl$_2$ 0.7 M	MgSO$_4$ 0.7 M	CaCl$_2$ 0.7 M	KCl/MgSO$_4$*	Sucrose 0.7 M	KCl 0.6 M
1	N+CP	0.11[a]	0.14[a]	0.00[b]	0.44[a]	0.75[a]	0.05[a]	0.44[a]
1	N+O	0.20[a]	0.06[a]	0.04[a]	0.02[a]	0.06[a]	0.00[b]	0.03[a]
1	N+B	0.06[ab]	0.10[a]	0.05[ab]	0.02[b]	0.04[ab]	0.00[b]	0.02[b]
2	N+CP	0.51[ab]	0.16[bc]	0.00[c]	0.16[bc]	0.71[a]	0.01[c]	0.21[bc]
2	N+O	0.52[a]	0.25[b]	0.00[c]	0.12[bc]	0.63[a]	0.00[c]	0.03[c]
2	N+B	0.64[a]	0.30[b]	0.00[c]	0.45[ab]	0.22[bc]	0.00	0.03[c]
3	N+CP	0.50[c]	0.65[b]	0.00[e]	0.77[a]	0.08[e]	0.00[e]	0.20[d]
3	N+O	0.09[cd]	0.73[a]	0.00[d]	0.12[c]	0.43[b]	0.02[dc]	0.02[dc]
3	N+B	0.11[b]	0.13[a]	0.02[b]	0.08[b]	0.47[b]	0.02[b]	0.02[b]
4	N+CP	0.47[a]	0.24[abc]	0.00[d]	0.03[bc]	0.27[ab]	0.00[d]	0.15[bc]
4	N+O	0.11[b]	0.11[b]	0.00[c]	0.15[b]	0.77[a]	0.00[c]	0.02[c]
4	N+B	0.10[a]	0.20[a]	0.00[b]	0.07[a]	0.50[a]	0.00[b]	0.01[a]

Means followed by the same letter did not differ by the Tukey test ($p \leq 0.05$).
N = Novozym 234 (0.25% w/v).
CP = Cellulase CP (0.25% w/v)
O = Cellulase Onozuka (0.25% w/v)
B = Cellulase Biobrás (0.25% w/v)
* KCl/MgSO$_4$ (5 mM MgSO$_4 \cdot$7H$_2$O + 0.7 M KCl + 3 mM cysteine)

of incubation in artificial culture medium (Fig. 1A, B). The young conidia formed septa at the beginning of their formation. The conidiophores were in the shape of a differentiated hyphae, and the conidium began to develop at the apex (Fig. 1C). Nuclear migration (Fig. 1B) was observed during the conidial formation but no nuclear division was observed in conidiophores or conidia. Hyphae were found to contain 1–6 nuclei per compart-

ment, depending on the growth stage (Fig. 1D–H). Nuclear migration was observed during hyphal branching, and hyphal anastomosis occurred frequently (Fig. 1D–I). Leonard (1988) observed similar phenomena in the conidial states of *Setosphaeria turcica* (Luttrell) Leonard & Suggs, of which hyphae were also multinucleated, containing up to 20 nuclei, and he estimated the haploid chromosome number to be 8–9 for this species. The
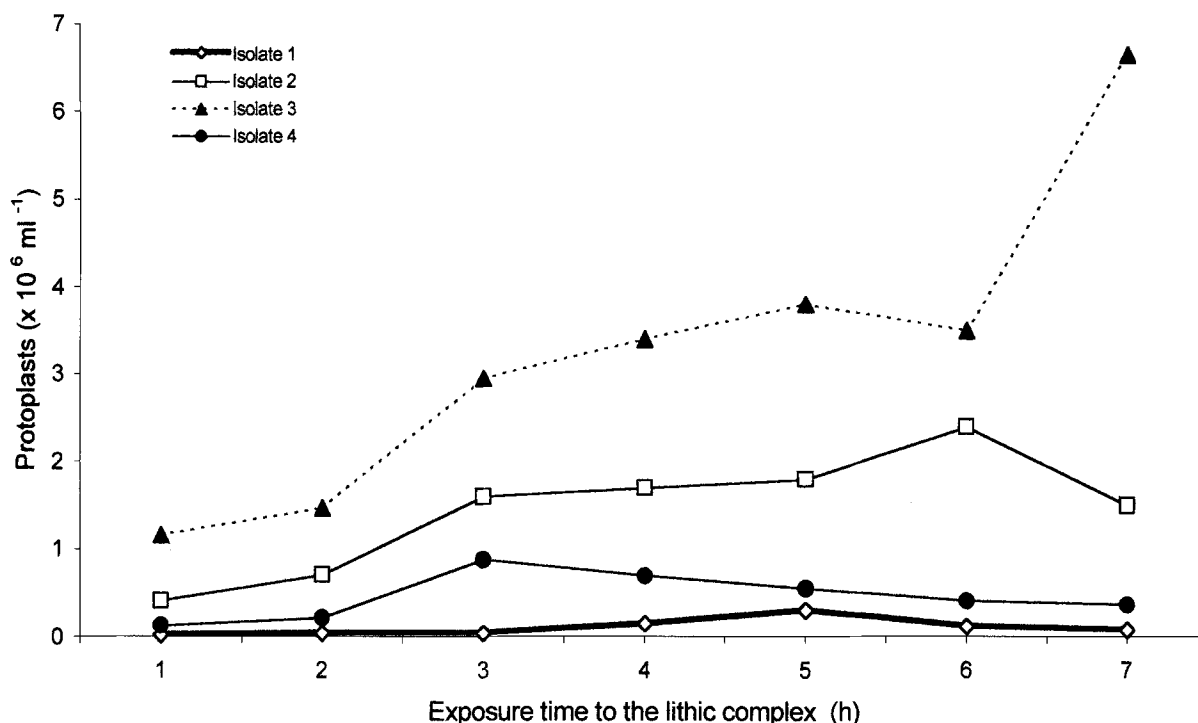

Fig. 3. Protoplast production of four *Helminthosporium euphorbiae* isolates at different incubation times in the N+CO enzymatic complex and 0.05 M KH$_2$PO$_4$ (added to 5 mM MgSO$_4 \cdot$7H$_2$O + 0.7 M KCl + 3 mM cysteine) osmotic stabilizer.
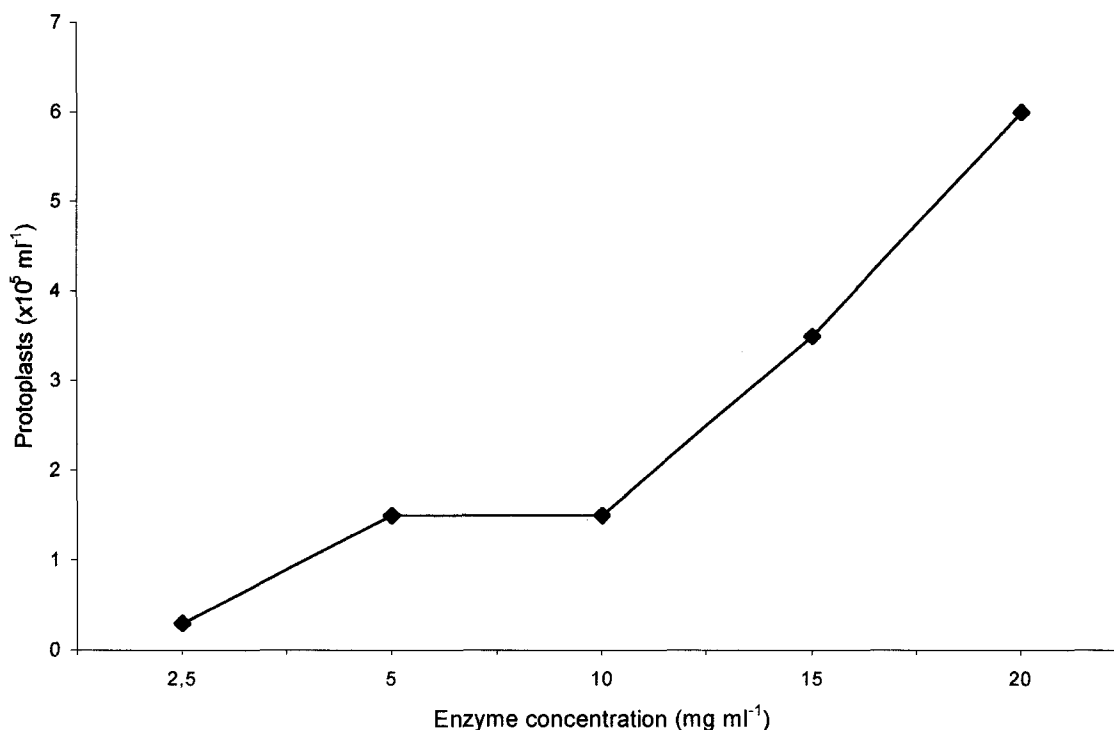
Fig. 4. *Helminthosporium euphorbiae* isolate 1 protoplast production after 4 h incubation with different concentrations of the N+CP enzymatic complex.

conidiophores were multinucleated, 3 to 18 μm long, and showed no nuclear division in their interior. Leonard (1988) also observed hyphal anastomosis and nuclear migration in certain isolates, suggesting that the hetero-karyon could occur naturally, but there was no cytological evidence that this happens in the conidium.

Figure 2 shows some mitotic phases of the fungus.

Table 3. Protoplast regeneration frequency (%) from *Helminthosporium euphorbiae* isolates incubated at 28°C for 7d with two osmotic stabilizers.

Isolate	Enzymatic complex	0.6 M KCl	KCl/MgSO$_4$*
1	N+CP	0.67	18.10
1	N+O	0.44	9.25
1	N+B	2.22	25.37
2	N+CP	0.72	1.62
2	N+O	1.96	5.93
2	N+B	2.21	11.16
3	N+CP	0.70	12.96
3	N+O	0.75	6.03
3	N+B	0.98	3.07
4	N+CP	0.80	10.31
4	N+O	0.52	1.67
4	N+B	0.81	1.66

N =Novozym 234 (0.25% w/v)
CP=Cellulase CP (0.25% w/v)
O =Cellulase Onozuka (0.25% w/v)
B =Cellulase Biobrás (0.25% w/v)
* KCl/MgSO$_4$ (5 mM MgSO$_4$·7H$_2$O + 0.7 M KCl + 3 mM cysteine).

The nuclear division is asynchronous, with several division phases at a single hyphal development stage. In *Helminthosporium turcicum* Pass., Knox-Davies and Dickson (1960) observed that the nuclei in hyphae divide simultaneously and quickly, so the prophase, prometaphase, metaphase and anaphase stages cannot be distinguished. The hyphae, conidiophores and conidia are multinuclear.

Protoplast production and regeneration Seven osmotic stabilizers were assessed together with three enzymatic complexes, incubated for 4 h, to obtain protoplasts of the *H. euphorbiae* isolates. An ideal osmotic stabilizer for all the isolates was not found. According to Davis (1985), the production and stability of protoplasts is highly influenced by the osmotic stabilizer type and concentration, depending on the organism, so there is no 'universal' stabilizer useful for protoplast formation of many fungi. The protoplast formation varied depending on isolates, stabilizers and enzyme mixture (Table 2). The 0.7 M MgSO$_4$ and sucrose stabilizers produced fewer protoplasts.

Among the enzyme mixtures used, the most suitable were 0.25% Novozym 234+0.25% Cellulase CP and 0.25% Novozym 234+0.25% Cellulase Onozuka R10 (Table 2). Hamlyn et al. (1981) tested the efficiency of several enzymes for protoplast production in various fungal species, including: *Aspergillus nidulans* (Eidam) Winter, *Aspergillus niger* van Tieghem, *Aspergillus ochraceous* Wilhelm, *Aspergillus rugulosus* Thom & Raper, *Penicillium chrysogenum* Thom, *Saccharomyces cerevisiae* Meyen and *Volvariella volvacea* (Bull. : Fr) Singer. Twelve commercial enzymes were tested, in-

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Table 4. Number of nuclei/protoplast of *Helminthosporium euphorbiae* 1 isolate at four mycelial growth times (4 h in
Cellulase CP+Novozym 234 enzymatic complex).

Mycelial growth (h)	Number of nuclei/protoplast (%)							
	0	1	2	3	4	5	6	total
8	27 (29.6)	10 (10.9)	21 (23.0)	20 (21.9)	10 (10.9)	2 (2.1)	1 (1.05)	91
12	8 (5.7)	18 (12.9)	45 (32.3)	32 (23.0)	21 (15.1)	7 (5.0)	6 (4.3)	139
18	8 (3.9)	27 (13.1)	49 (24.3)	51 (25.3)	45 (22.3)	15 (7.4)	6 (2.9)	201
24	17 (10.6)	26 (16.2)	36 (22.5)	37 (23.7)	26 (16.2)	14 (8.7)	4 (2.5)	160

cluding cellulases and chiitinases. Novozym 234 performed best for all the species analyzed, but the protoplast production increased when the Novozym 234+Cellulase CP system was used.

The N+CP and the osmotic stabilizer 0.05 M KH_2PO_4 (added to 5 mM $MgSO_4 \cdot 7H_2O$ +0.7 M KCl+3 mM cysteine) was used to assess the exposure time to the enzymatic system. Figure 3 shows that protoplast production varied with exposure time to the enzymatic

complex among the *H. euphorbiae* isolates. The greatest rate of protoplast production was obtained at 5, 6, 7 and 3 h of enzymatic treatment for the isolates 1, 2, 3 and 4, respectively. The N+CP enzymatic concentration was assessed (incubated for 4 h), and higher concentrations of this complex yielded higher protoplast production (Fig. 4).

The *H. euphorbiae* protoplast regeneration frequency varied among the isolates (Table 3). It was generally
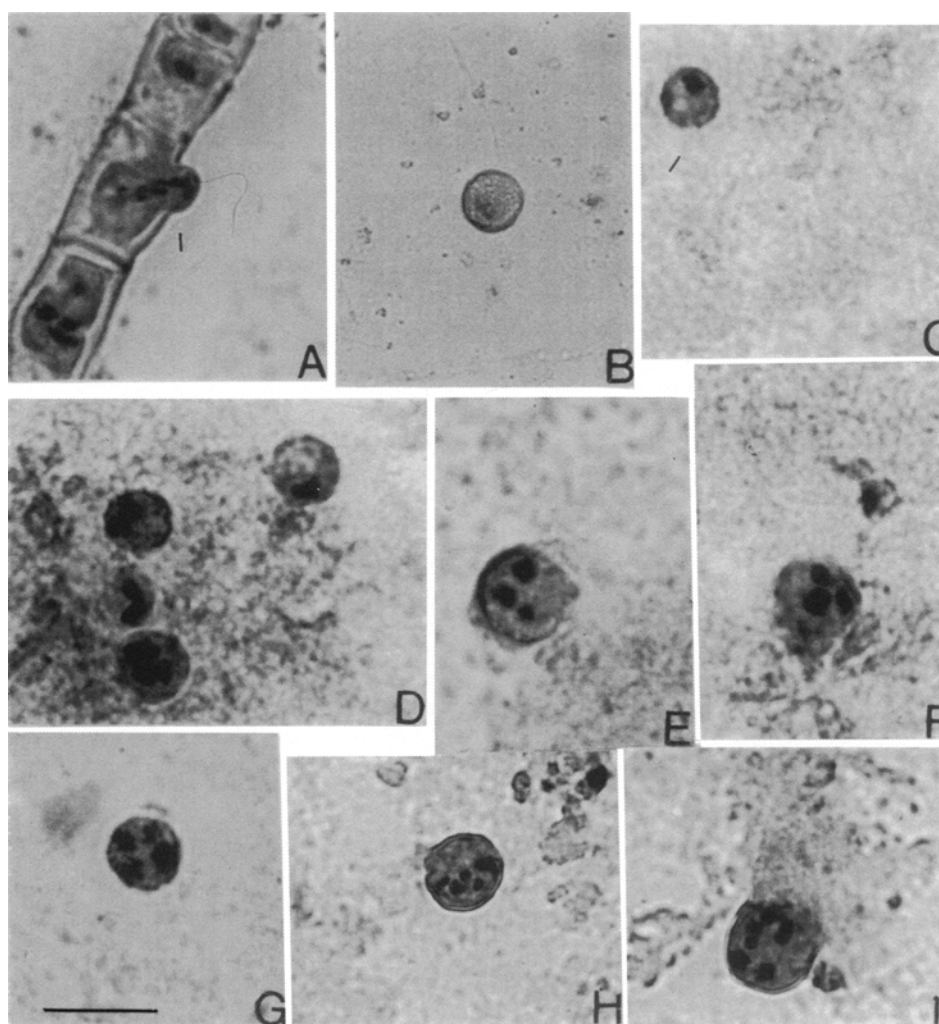

Fig. 5. Distribution of the number of nuclei in the protoplasts of *H. euphorbiae*. A. Protoplasts formation; B. Anucleate protoplasts; C. Uninucleate protoplasts: D. Protoplasts with 1, 2 and 3 nuclei. E, F, G, H, I. Protoplasts with 3, 4, 5, and 6 nuclei. Scale bars: 10 µm.

highest when 0.05 M KH_2PO_4 (added to 5 mM $MgSO_4$·$7H_2O$ +0.7 M KCl+3 mM cysteine) was used as osmotic stabilizer, but it varied with the enzymatic mixture. The N+B complex was more effective for the isolates 1 and 2, while the N+CP complex was more effective for isolates 3 and 4. Bagagli et al. (1991) obtained a relatively low protoplast regeneration frequency in *Metarhizium anisopliae* (Metsch.) Sorokin that varied from 0.05 to 0.44% depending on the osmotic stabilizer used. Barcellos (1993) used several *M. anisopliae* lines and obtained a regeneration frequency with 0.7 M KCl that varied from 3 to 19% depending on the line. Differences in protoplast regeneration among the isolates may reflect differences in the cell wall constitution. The enzyme combination and concentration are dependent upon the specificity of the chemical composition and the structure of the cell wall (Iijima and Yanagi, 1986).

Protoplast cytological analysis The protoplast cytological analysis showed a distribution of the frequencies of the number of nuclei per protoplast (Table 4), which varied from 0 to 6 (Fig. 5). The majority of protoplasts in mycelia at 8 h of culture are anucleate. In mycelia of 12 to 24 h of age, there was a greater percentage of protoplasts with 1–3 nuclei/cell (Table 4). Bagagli et al. (1991) used the same technique for protoplasts obtained from a *M. anisopliae* monokaryotic line and observed the following distribution of nuclei/protoplast: 10 to 20% anucleate, 71.4% uninucleate, 23.4% binucleate, up to 5% trinucleate, and 0.2% with 5 nuclei.

Since the high number of nuclei in the *H. euphorbiae* conidia (about 20 nuclei/conidium) makes the genetic studies very difficult, the technique of protoplast production described here offers an alternative for obtaining cells with a reduced number of nuclei, thereby facilitating the production of mutants for genetics and breeding studies in this important species.

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