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Tuber borchii mycelial protoplasts isolation, characterization and functional delivery of liposome content, a new step towards truffles biotechnology

Anna Poma [a,*], Sabrina Colafarina [a], Tania Limongi [a], Giovanni Pacioni [b]

[a] *Department of Basic and Applied Biology, Faculty of Biotechnology and Faculty of Sciences, University of L'Aquila, Via Vetoio 1, Località Coppito, I-67010 L'Aquila, Italy*

[b] *Department of Environmental Sciences, University of L'Aquila, Via Vetoio, Coppito, I-67010 L'Aquila, Italy*

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Abstract

The filametous ascomycete *Tuber borchii* is a plant-symbiotic ectomycorrhizal microrganism with an high value due to the production of hypogeous fruitbodies (truffles). The present work was undertaken to develop a procedure for the release of *T. borchii* viable protoplasts from *Tuber* mycelium, isolate ATTC 96540; several factors which affect the isolation, morphology and viability were examined and developed in order to improve applications of *T. borchii* protoplasts in morphological, biochemical and genetic investigations (protoplast fusion or transformation). Functional delivery of liposome content into *T. borchii* protoplasts has also been examined with a cytotoxic ribosome inactivator as saporin. *T. borchii* protoplasts incubation/fusion with saporin containing liposomes were made to demonstrate the absence of cell wall of 16 days cultured protoplasts.

Keywords: Mycorrhizal fungi; *Tuber borchii*; Protoplasts; Liposomes

1. Introduction

The filametous ascomycete *Tuber borchii* is a plant-symbiotic microorganism that colonizes trees by the formation of a specialized structure, the ectomycorrhiza. The *Tuber* mycelium of mycorrhiza has cells containing two nuclei likely originated by a secondary homothallism or a self-reproduction process. The absence of heterozigosity supports the hypothesis that the two nuclei of dikaryotic hyphae of *Tuber* are the product of a self-fertilization process [1–4,1,5]. These knowledge about *Tuber* mycelium may be useful to have an understanding of the cell biology and genetics of the strain (*T. borchii* Vittad., isolate ATTC 96540) being used for protoplasts isolation. Initially, the isolation of protoplasts was a very useful procedure in preparing cell free extracts and organelles; after that the role of fungal protoplasts in a variety of morphological, biochemical and genetic investigations has gained considerable importance. Protoplast reversion or regeneration technique has been applied to the elucidation of the details of cell wall polymer biogenesis and disposition [6,7]. Interspecies protoplasts fusions have been reported such as fusants, i.e., between *Aspergillus*, *Penicillium*, *Ganoderma* [8]. Removing the wall and exposing the protoplast membrane allow for manipulations involving fusion or uptake of nucleic acids. The fungal protoplast

* Corresponding author. Tel.: +39 862 433268; fax: +39 862433273.
E-mail address: poma@univaq.it (A. Poma).

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fusion and transformation systems have been developed as an aid to further understanding of some phenomenon such as genetic incompatibility between strains and species. *T. borchii* protoplasts also may be used as an effective experimental tool for studying wall-free cells. Generation of fungal protoplasts is essential for fusion and transformation systems so transformation of *Tuber borchii* mycelium protoplasts could also offer great potential for the improvement of ectomycorrhizal fungi of economic value.

The present work was undertaken to develop a procedure for the release of *T. borchii* viable protoplasts; several factors which affect the isolation, morphology and viability were examined in order to develope future application of protoplast fusion or transformation. Functional delivery of liposome content into *T. borchii* protoplasts has also been examined with a cytotoxic ribosome inactivator as saporin, an enzyme purified from *Saponaria officinalis* seeds. This molecule allows assessment of liposomes contents delivery because it requires a small number of molecules to inhibit protein synthesis. This work is the first report about *T. borchii* mycelial protoplasts isolation and fusion with liposomes, suggesting that the ascomycete *T. borchii* (truffle) could be transformed through liposome-mediated delivery of nucleic acid, considering the here reported delivery of the protein saporin.

2. Materials and methods

2.1. Organism and growth conditions

T. borchii Vittad. mycelia (isolate ATCC 96540) to be used for the preparation of protoplasts were grown and propagated on Potato Dextrose Agar solid medium (PDA; BD-Difco BBL, Sparks,MD) 5–10 cm petri dishes at 24 °C in the dark for 35 days.

2.2. Preparation of protoplasts

The mycelium (0.5 g) was mechanically harvested by a scraper from culture dishes and resuspended in 5 ml of the following digestion mixture: bovine serum albumin (BSA, Sigma, Milan, Italy) 1,25 mg/ml; "Lysing Enzymes"(from *Trichoderma harzianum*, Sigma), containing cellulase, protease and chitinase, 20 mg/ml. The suspension was gently shaken in a water bath at 30 °C for 180 min. Protoplasts were harvested by filtration through a 20, 40 and 60 μm nylon net to remove hyphal debris. The protoplast suspension was then centrifuged for 15 min at 4 °C and 500 g ($r_{av} = 7.78$ cm) in a SH80 Sorvall rotor (DuPont, Wilmington, Delaware) with swinging buckets. The pellet was centrifuged for 15 min at 4 °C and 1000 g ($r_{av} = 7.78$ cm), suspended in the isoosmotic medium 1.2 M sorbitol, 10 mM Tris–HCl pH 7,5, 50 mM $CaCl_2$, 25 mM NaCl and centrifuged a second time for 15 min at 4 °C and 1000 g ($r_{av} = 7.78$ cm) in order to remove small cell wall and hyphae debris. The protoplasts pellets finally were resuspended in 100/200 μl of the isoosmotic medium and the yield determined by a Nageotte counter device. Integrity of the isolated protoplasts suspended in the isoosmotic medium was checked by counting protoplasts under a phase-contrast microscope (Amplival, Zeiss Jena; magnification: 400×). The protoplasts were allowed to duplicate by incubating in the osmotically stabilized liquid medium (modified Melin-Norkrans nutrient solution MMN containing 1.2 M sorbitol), at 20 and 30 °C and were harvested for counts at appropriate times. Protoplasts were also examined by phase-contrast microscopy (Zeiss; magnification: 100×).

2.3. Fluorescence staining and microscopy

Protoplasts nuclei were observed using a staining method with propidium iodide (PI) (Sigma, Milan, Italy). 5% (w/v) stock solution of PI was made in distilled water. Protoplasts were fixed with 0.1 M phosphate-buffered 2% gluraraldehyde (pH 6.8) for 1 h at room temperature, washed three times with buffer containing 1.2 M sorbitol, stained with PI 1 μg/ml (15 min, in the dark at room temperature). Slides were mounted with Vectashield™ (Vector Laboratories, Burlingame, California) to prevent photobleaching and were stored in the dark at 4 °C before observation with a Leitz fluorescence light microscope.

The percentage of viable nuclei of protoplasts and mycelium was measured under the fluorescence microscope by staining the slides with acridine orange and ethidium bromide. Acridine orange (100 μg/ml) was mixed to 100 μg/ml ethidium bromide (Molecular Probes, Eugene, OR) in phosphate buffer solution (PBS). Dye (1 μl) was mixed with 25 μl of protoplasts (5×10^4). Live cells were determined by the uptake of acridine orange (green fluorescence) and exclusion of ethidium bromide (red fluorescence) stain. Live and dead apoptotic cells were identified by the perinuclear condensation of chromatin stained by acridine orange or ethidium bromide, respectively. Necrotic cells were identified by uniform labelling of the cells with ethidium bromide. Slides were observed immediately with a fluorescence light microscope.

The method for staining the mycelium and protoplasts cell wall with calcofluor white (Merck) was adopted from Butt et al. [9].

To make the F-actin visible, the fixed protoplasts (3% paraformaldehyde PFA in buffer containing 1.2 M sorbitol for 1 h) were washed three times with buffer containing 1.2 M sorbitol, permeabilized with 50 mM β-mercaptoethanol in phosphate-buffered saline (PBS; 50 mM potassium phosphate buffer, pH 7.3, containing

150 mM NaCl) for 30 min and 1% Triton-X 100 in PBS for 30 min. Then protoplasts were stained with rhodamine-conjugated FITC (Sigma) for 1 h. Slides were viewed with an Olympus FMBX60 equipped with appropriate filters for rhodamine and were photographed with a Kodak Tmax 400.

2.4. Scanning electron microscopy

Protoplasts (incubated were fixed in 2% glutaraldehyde in 0.2 M Na cacodylate buffer (pH 7) for 2 h, followed by fixation with 1% osmium tetroxide in 0.2 M Na cacodylate buffer (pH 7) for 90 min. Samples were rinsed with distilled water and dehydrated with a graded ethyl alcohol series and critical point-dried. Samples were mounted on aluminum stubs, sputter coated with gold and observed using a Philips SEM 505.

2.5. Protoplasts viability assay/succinate dehydrogenase activity measurement (MTT assay)

Protoplasts viability was determined by the Mosmann assay that employed the mitochondrial-dependent reduction of MTT (3-[4-5dimethyl-thiazol-2-yl]-2-5diphenyl tetrazolium bromide) to formazan. The cells (1600/ml) were incubated with 100 μl of 0.2 mg/ml MTT for 2 h at 30 °C, followed by a 15 min incubation at 30 °C with 100 μl DMSO. Microtiter plates were read at 595 nm in an ELISA plate reader. The results are expressed as absolute optical density (OD) readings.

2.6. Liposome-mediated delivery of saporin (a ribosome inactivating protein) into T. borchii protoplasts

Saporin was purified to homogeneity according to the method in [10] from seeds of *S. officinalis*. Lecithin and cholesterol were obtained from Sigma–Aldrich (Milan, Italy). All other chemicals used were of analytical grade (Sigma–Aldrich, Milan, Italy).

L-α-lecithin/cholesterol liposomes were prepared according to the method of Szoka and Papahadjopoulos [11] as specified in [12]. The lipid mixture (cholesterol 6.5 μmol, corresponding to 4% with respect to L-α-lecithin) was dissolved in 5 ml chloroform, the solvent was subsequently removed by evaporation at 30 °C in a rotavapor and the dried lipids were dispersed in 50 mM phosphate buffer (pH 6.5) containing 0.1 M NaCl. Saporin was added at the final concentration 3.4 mg/ml to the vesicles and the dispersion maintained for 90 min at 4 °C, then sonicated for 1 min with a probe sonicator (10 W, duty cycle 80%). The vesicles were washed using 3 ml of buffer and the non-entrapped enzyme was separated from vesicles by centrifugation at 110,000*g* for 60 min (super speed centrifuge Beckman Spinco L2 65 B). This step was repeated at least three times. After Liposomat extrusion (pore size 400 nm), unilamellar vesicles were examined by contract phase microscopy to check their size. Vesicles were then incubated with the protoplasts suspension (ratio v/v 1:1) for 4, 6, 10 and 16 h; protoplasts viability was determined by the MTT assay.

3. Results

Protoplast formation from hyphae of *T. borchii* started 60 min after the cell wall degrading enzymes had been added (Fig. 1A). Hyphae, coming from 20 days old cultures and subcultured for 48 h were the most susceptible to enzyme cocktail. After 3 h of incubation with enzyme cocktail and using 1.2 M sorbitol as osmotic stabilizer, the average diameter of protoplasts was 8–10 μm (approximately 0.5 pL per protoplast as internal volume, Fig. 1B). These sizes agree with the internal volume of hyphae single cells. The protoplasts were spherical (Fig. 1C) and burst upon a hypoosmotic shock (Fig. 1D). A typical experiment when followed as a function of time (growth curves, at 20 °C Fig. 2A and 30 °C Fig. 2B), gave the maximum average yield ($28–30 \times 10^3$ ml^{-1}) of protoplasts at the end of 70 culture days. However, there was not a significative increase in the yield of protoplasts with increase in the growth temperature.

Protoplasts viability was determined by the Mosmann MTT (3-[4-5dimethyl-thiazol-2-yl]-2-5-diphenyl tetrazolium bromide) test; this colorimetric assay determines the ability of viable cells to convert soluble tetrazolium salt (MTT) into a insoluble precipitate (blue crystals that are dissolved and read spectrophotometrically). MTT is reduced at sites in the mitochondrial electron transport system and we have here demonstrated that is a test for succinate dehydrogenase activity also suitable for protoplasts. As shown in Fig. 3, the mitochondrial activity is 25% of the starting at 35 culture days; this is correlated to the growth curve plateau because usually MTT reduction correlates to indices of viable cell number.

The percentage of viable protoplasts was determined by: (i) staining with propidium iodide (Fig. 4A) and (ii) simultaneous staining with acridine orange and ethidium bromide (Fig. 4B–D), mycelium stained with acridine orange and ethidium bromide. Protoplasts with an intact plasma membrane appeared yellow whereas protoplasts with a damaged plasma membrane appeared orange. The portion of viable protoplasts determined by fluorescence staining was between 50% and 65%.

Between 5% of the 16 days cultured (20 °C) protoplasts, stained with calcofluor white (Fig. 4E), started to regenerate a new cell wall. The cell wall regeneration process was very slow, it was more detectable after 30 culture days (50% of protoplasts fluoresce). On the contrary, calcofluor white stained mycelium (16 days cultured) showed (Fig. 4F) hyphae with cell wall appearing fluorescent under illumination.

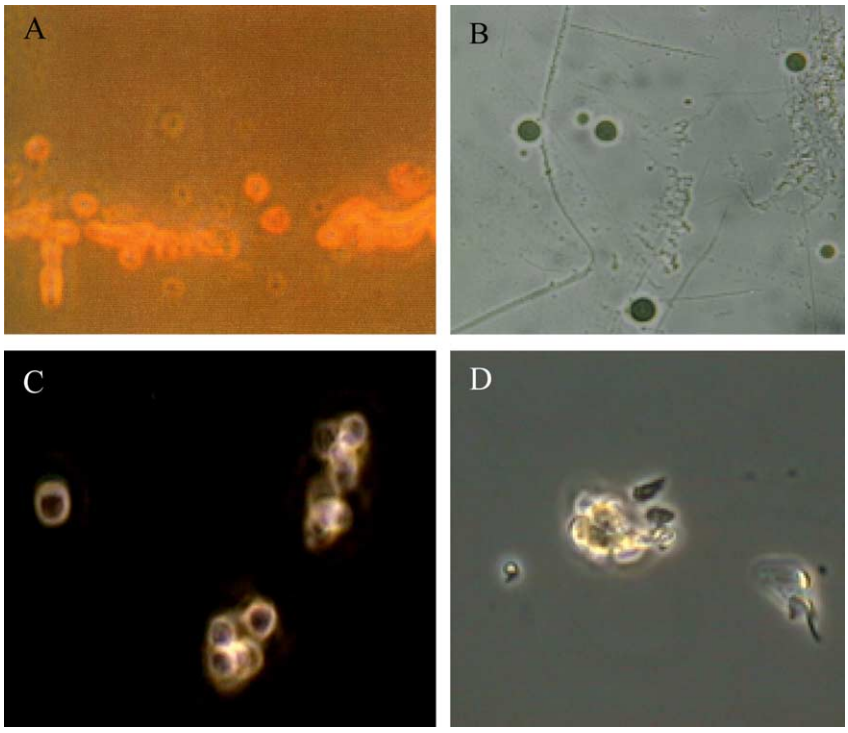
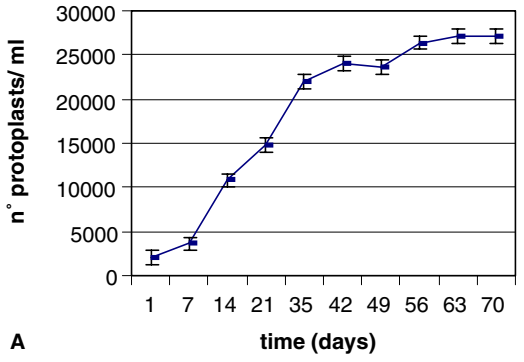


Fig. 1. Images of protoplasts from *T. borchii* hyphae. (A) Plasmolysis observed with phase contrast microscope (600×) and (B) protoplasts perfectly spherical can be observed (400×). (C) Protoplasts distilled water broken observed under a phase contrast microscope (400×). (D) *T. borchii* protoplasts observed by inverted microscope (400×).

The behaviour of the protoplasts (16 days cultures) actin cytoskeleton was analysed (Fig. 4G and H) with the rhodamine conjugated FITC staining: a continuous actin ring is visualized under the protoplasts surface; this is according to *S. pombe* protoplasts [13].



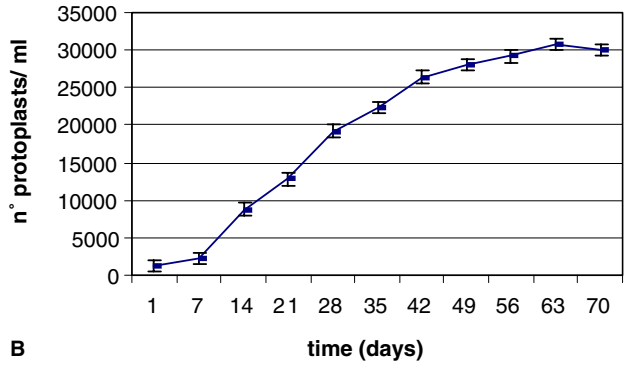


Fig. 2. Growth curve of *T. borchii* protoplasts. (A) At 20 °C and (B) at 30 °C.

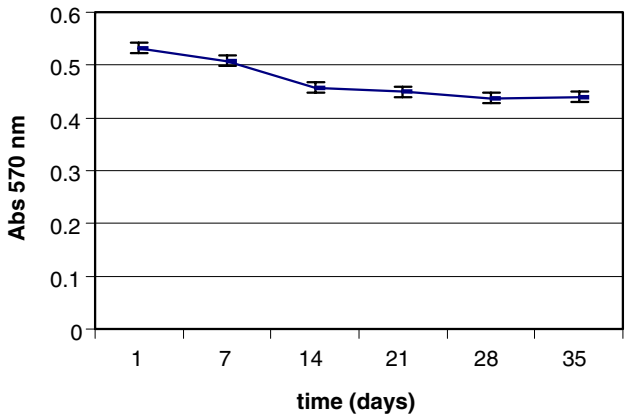


Fig. 3. *T. borchii* protoplasts viability as determined by the MTT test.

T. borchii protoplasts incubation/fusion with saporin containing liposomes were made to demonstrate the absence of cell wall of 16 days cultured protoplasts. A great variety of plant species contains toxins, which inhibit protein synthesis through the catalytic inactivation of eukaryotic ribosomes. These ribosome-inactivating proteins (RIPs) are classified in two groups based on their subunit composition: type 1 RIPs are single chain polypeptides while in type 2 RIPs the enzimatically active A chain is covalently (disulfide) bonded to a B (binding) chain with lectin properties [12]. Saporins are

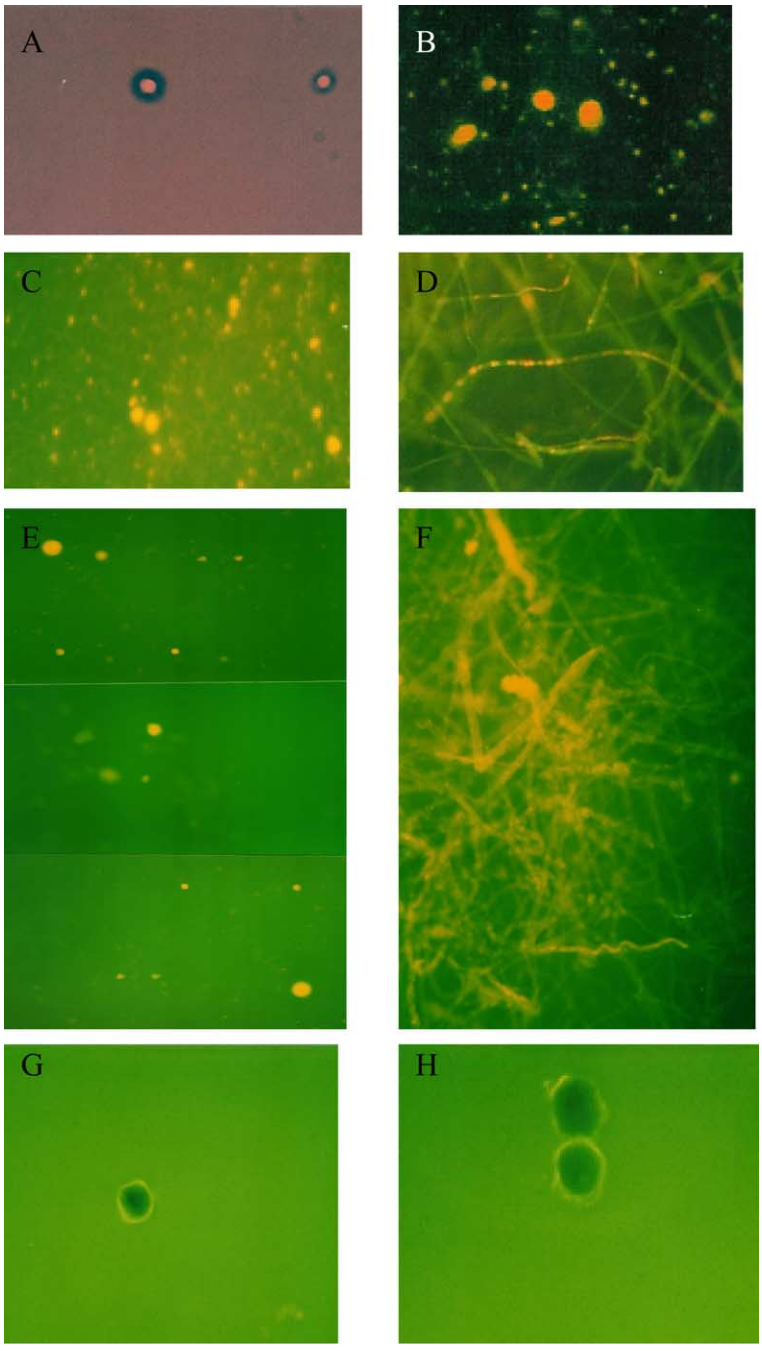


Fig. 4. *T. borchii* protoplasts observed under a fluorescence microscope. (A) Protoplasts stained with propidium iodide (400×); (B) non-vital protoplasts (acridine orange stained 400×); (C) vital protoplasts (ethidium bromide stained, 400×); (D) acridine orange and ethidium bromide stained *T. borchii* mycelium (400×); (E) calcofluor white stained *T. borchii* protoplasts (400×) after 16 days in culture; (F) calcofluor white stained *T. borchii* mycelium, subcultured for 16 days (400×); and (G and H) rodamine-FITC stained *T. borchii* protoplasts from 16 days cultures (400×) microscope.

higly basic type 1 RIPs present in different organs of the plant *S. officinalis* L.

In this work, saporine containing liposomes were prepared and incubated with protoplasts for 4, 6, 10 and 16 h. Control protoplasts number/ml was determined and MTT test was performed. Before incubation, the number/ml is 20.000; MTT test 595 nm absorbance is 0.6512. After 16 h incubation the protoplasts intact number/ml and MTT test 595 nm absorbance in both are 0.000 (Table 1). By following, it appears that the saporine containing liposomes, fusing with the protoplasts, just after 6 h contact time, cause their death at first by protein synthesis inhibition and consequently by mitochondrial metabolism block as assessed by MTT test.

This results are agree with the calcofluor white test, the cell wall is not regenerated until 18 culture days in a few number of protoplasts.

Table 1
Effects of saporin containing liposomes after 4, 6, 10 and 16 h incubation time on the viability of *Tuber borchii* protoplasts as evaluated by MTT reduction assay (ratio liposomes/protoplasts v/v 1:1; the results are expressed as absolute optical density readings at 595 nm)

Incubation time (h)	Absorbance (595 nm)	Cells/ml number
0	0.6512	20,000 ± 450
4	0.5500	15,000 ± 350
6	0.0030	80 ± 12
10	0.0016	30 ± 05
16	0.0000	0.0000

Protoplasts were also observed by scanning electron microscopy (SEM). As shown in Fig. 5A, protoplasts (from 16 days culture) have an average diameter of 10 μm and maintain a spherical shape; the shape appears dramatically modified and diameter reduced as shown in Fig. 5B after saporin/liposome treatment.

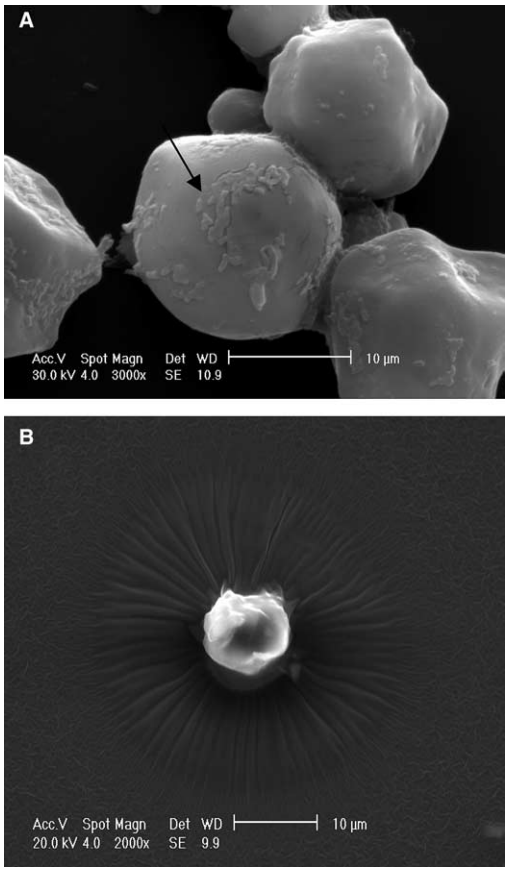


Fig. 5. (A) *T. borchii* protoplasts (16 days in culture, incubated with the liposomes suspension (black arrows, ratio v/v 1:1) for 2 h) observed under a scanning electron microscope (SEM). (B) *T. borchii* protoplasts incubated 6h in culture medium supplemented with saporin containing liposomes.

4. Discussion

The aim of this work was to isolate for the first time protoplasts from *T. borchii* mycelium and to obtain liposomes delivery content into protoplasts. The protoplast yield obtained is lower in respect to the yields reported in the literature for other fungi [6,8] but the digestion with higher concentrations of the lysing enzymes had no positive effect (data not shown); therefore a compromise between a not-high yield of protoplasts and a low degree of damage of the plasma membrane was sought. We used the double fluorescence method for the estimation of the percentage of nonviable protoplasts and for the first time in *T. borchii* an assay of the protoplasts viability and respiratory activity was determined by the mitochondrial-dependent reduction of MTT to formazan. It has also been shown that *T. borchii* protoplasts, under saporin containing liposomes treatment, undergo metabolic changes that affect mitochondrial functions as assessed by the MTT test.

Protoplasts from *T. borchii* show a cytoskeleton composed by a F-actin ring connected to the plasmatic membrane so could be proposed a role for actin in the activation of the synthesis of the new cell wall as yet suggested for *Schizosaccharomyces cerevisiae* protoplasts [13,14]. The exposure of *Tuber borchii* protoplast membrane will allow manipulations involving fusion or uptake of nucleic acids while protoplast fusion and transformation systems could be developed. *T. borchii* protoplasts also may be used as an effective experimental tool for studying wall-free cells. It is an open question for *T. borchii* if binucleated hyphal cells are derived from the fusion of mononucleated cells belonging to mycelia characterized by different mating type or from automittic processes. Protoplasts fusion could be an useful system to establish if binucleated hyphal cells are derived from the fusion of mononucleated cells belonging to mycelia characterized by different mating type or from automittic processes as above indicated [15]. About a possible *Tuber* genetic transformation, we must consider that the electroporation method is employed for gene transfer into otherwise untransformable filamentous fungi [16] and applied to conidia that are not available under laboratory conditions for *Tuber*. Alternatively, the PEG method [16] also could be prevented by the here demonstrated low cell wall regeneration ability. An *Agrobacterium tumefaciens* strain has been used for *Tuber* mycelia (but not protoplasts) transformation [17]. From our first results about liposomes delivery content into protoplasts, we are working out basic conditions for a possible *T. borchii* protoplasts transformation by liposome-mediated transfer of genetic material for the improvement of ectomycorrhizal ascomycetes of high economic value. On the other hand, our results show that the L-α-lecithin/cholesterol liposomes represent also a promising vehicle system for introducing cytotoxic ribosome-inactivators (i.e.,

saporin) in Ascomycetes protoplasts and for better understanding protoplast cell physiology.

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