Reliability in Transformation of the Basidiomycete 
*Coprinopsis cinerea*

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Abstract
Transformation of the basidiomycete *Coprinopsis cinerea* makes use of unicellular haploid asexual spores called oidia. Protoplasts of oidia are generated by a cellulase/chitinase enzyme mix. Protoplasts and DNAs are incubated together in 25 mM Ca\(^{2+}\) and 5% PEG (polyethylene glycol) 4000 on ice and more PEG is added (23% final concentration) after a ‘heat shock’ step at RT (room temperature). Upon regeneration on selective media, transformation rates of several hundreds of clones might be obtained per 1 µg DNA and 10\(^7\) protoplasts. Although the technique has been invented 25 years ago by Binninger *et al.* (1), there are reoccurring pitfalls in the method that can cause failure. Successful transformation needs a good amount of skillful knowhow about the fungus and the method. Here we present our experiences with *C. cinerea* transformations, call attention to potential flaws and to optimal handlings in fungal cultivation, harvesting, protoplasting, transformation, and subsequent regeneration of the fungus.

Keywords: Transformation, protoplasts, *C. cinerea* vectors, oidia, basidiomycete

Introduction
Transformation in *Coprinopsis cinerea* has first been described by Binninger *et al.* in 1987 (1). *C. cinerea* produces unicellular aerial spores (oidia) with one haploid nucleus ([2,3]; Fig. 1) and Binninger *et al.* (1,4) used these to generate protoplasts for Ca\(^{2+}\), in-ice-incubation-, heat shock-, PEG-mediated vector transformation. Around 100 transformants per experiment (about 33 per µg vector DNA and per 10\(^7\) total and 10\(^8\)

Fig. 1. An unicellular oidium of *C. cinerea* strain AmutBmut with a haploid nucleus (N), a bilayered outer cell wall with hair-like structures contributing to a gelatinous layer surrounding the spore, and a single-layered ruptured septum cell wall found at the side of former attachment to another spore (2,3). A. Transmission electronic photograph of the complete spore (size bar = 1 µm), and enlarged views B. of the outer cell wall of the oidium and C. of the septal cell wall (size bar = 0.2 µm). Figure modified from (3).
viable protoplasts) were obtained by these authors (1,4), rates comparable to those of protoplasts made from fungal mycelium (1,5,6). Similar high rates in oidia transformation were reported in studies by other groups (7-13). Circular or linearized DNA or also single-stranded DNA can be used for the transformation of C. cinerea (4,10) and in all cases ectopic integration of the foreign DNA into the host chromosomes takes place (1,6-12). Importantly, two or more plasmids may be transformed at the same time into a same protoplast allowing through complementation of auxotrophies or through conferring antibiotic resistances indirect selection for transformed genes having no easily selectable phenotypes and, in the following, studies of different genes at the same time (7-9,11,13-16).

Many a time in C. cinerea laboratories, transformations are however also unsuccessful. Such failures are not published but negative experiences are told among the international Coprinopsis community. In over 20 years of research with C. cinerea transformation by the senior author, five longer periods of failure in transformation were experienced by members of the own research teams, distributed over three different labs in three different countries (Oxford, UK; Zurich, Switzerland; Göttingen, Germany). Accordingly, C. cinerea transformation has among some researchers a reputation to be a difficult technique. Commonly in situations of longer failure, the water used was made responsible or the enzyme used, or strains for transformation were suspected to have changed properties or the DNAs being of not good enough quality. Ultra-pure water had been bought and new enzyme batches, transformation strains newly acquired from other labs or replaced by other ones, and DNAs isolated by different methods. These measures not necessarily helped. Observations suggest that a personal factor has an important position in these negative results. Changing a person in charge of transformation to a fresh investigator rendered the outcome positive, also with the same water, enzymes, strains and DNAs. New laboratories were established at two occasions and new people were kept unladen from the idea that transformation can be difficult. Persons so introduced to transformation were excellent in performance, with no complaints and no experiences of technical difficulties. Periods of about 5 years were without major troubles. However, some newcomers to C. cinerea transformation were subsequently unable to perform the technique. In some instances, wrongly made up buffers, media and solutions were proven to be the cause and this could quickly be solved. In other instances, persons failed by changing details in the method (‘for optimizing the protocol’) and wanting to adapt to what they felt to be better in handling or to be more accurate in procedure. Otherwise well practiced researchers so never made transformation work, also not when keeping to the established procedure. The resulting idea of C. cinerea transformation being a ‘bad technique that needs to be reformulated’ turned out to be infectious. Lab colleagues could then also not transform C. cinerea or when, only with very low success of obtaining only few transformants. Consequently, there was need for the senior author to show in own action that the method perfectly works, even after having self not been active for times in the laboratory. Upon such demonstration, the transformation technique kept on going until some chance of personnel. A number of times, the C. cinerea transformation procedure had thus been taught from scratch (U. Kües, pers. observations).

With this paper, we intend to describe and explain the detailed steps and actions in the C. cinerea transformation procedure, to call attention to sensitive phases in handling, and to point out potential pitfalls that can cause failure.

Material and Methods

Coprinopsis cinerea strains and plasmids: Monokaryotic C. cinerea strains FA2222 (A5, B6, acu1, trp1.1,1.6) and PG78 (A6, B42, pabl, trp1.1,1.6) (16) were used in this study and

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homokaryotic strains EN117.9 (A6m, B5, ade8) (17) and the UV-mutant 7K17 of strain AmutBmut (A43mut, B43mut, pab1) having mutations in the mating type loci (10,16; Granado et al. unpublished). The following plasmids were used: the pUC9 derivative pCc1001 with the C. cinerea wild type trp1 gene (1), the pUC13 derivative pST17 (18,19) and the pTZ18R derivative pPAB1-2 (10) with the C. cinerea wild type pab1 gene, and cosmid pCRS1 from the C. cinerea JV6 Lorist 2 cosm id library with the ade8 wild type gene (18,20). pYSK7 as a derivative of the yeast-shuttle vector pRS426 with the C. cinerea wild type genes pab1 and lcc1 (13) and construct pYPH3 (Hoegger et al. unpublished) with gene lcc1 in pYSK7 replaced by a Pleurotus sapidus putative versatile peroxidase gene (GenBank accession number AM039632) were used in cotransformation of strain FA2222 together with pCc1001.

**DNA preparation:** Plasmid isolation from Escherichia coli performed in mini-prep form is a modification of the method of Birnboim and Doly (21). E. coli strains with the respective plasmids are cultivated overnight (ca. 14 h) at 37°C in 3 ml LB-medium (22) supplemented with the appropriate antibiotics on a shaker at 180 rpm. Cells are harvested in two portions in a 1.5 ml Eppendorf tube by centrifugation (1 min; 16,000 x g). The resulting cell pellet is resuspended in 75 µl TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0). 150 µl alkaline lysis solution (always freshly prepared by mixing 1:1 0.4 M NaOH and 2% SDS) is added and mixed with the cell suspension. After incubation for up to 20-30 min at RT (room temperature; the solution needs to become viscous and clear), 500 µl of renaturation solution (always freshly made by mixing in 1:3:6 portions 5 M NaCl, non-autoclaved 3 M Na acetate of pH 4.8 and sterile H2O) is added and mixed (white flocks form from cell debris). The mixture is kept at -20°C for 10-20 min to improve precipitation of unwanted cell debris. Afterwards, the cell debris together with the chromosomal DNA is removed by centrifugation (20 min; 16,000 x g). The supernatant with the plasmid DNA is poured into a new 1.5 ml tube and the DNA is precipitated by addition of 750 µl isopropanol, mixing the solutions at RT (multiple small gas bubbles will form to sparkle to the surface) and centrifugation (15-20 min; 16,000 x g; the junction between tube and cap should be turned to the outside of the rotor in order to mark the side of DNA pelleting during centrifugation). The supernatant is poured off and the remaining liquid sucked off by turning the opened tube upside down onto clean towelling paper. The resulting pellet is then washed by rinseing the tube with 500 µl 70% ethanol and subsequent short centrifugation (5 min; 16,000 x g). The supernatant is poured off, the remaining liquid sucked off by turning the opened tube upside down onto clean towelling paper, and the visible pearly pellet of DNA and RNA at the bottom of the tube air-dried at RT. Note that more clean DNA might be distributed as a transparent film over the wall of the tube at the side of the junction with the cap (depends on the brand of tubes used). DNA and RNA are resuspended in 50 µl sterile H2O under repeatedly rinsing the wall of the tube with the liquid (until the liquid easily runs in droplets down). The DNA concentration is determined by agarose gel electrophoresis (22).

In addition, purified maxi-prep DNA of cosmid pCRS1 for this study was prepared by CsCl-ethidium bromide gradient centrifugation (22) and purified pST17 DNA by a Qiagen Small Scale Plasmid Purification Kit (Qiagen GmbH, Hilden, Germany).

**Fungal transformation as modified by Granado et al. (10):** C. cinerea strains are grown at 37°C on fresh YMG or YMG/TR (trp auxotrophs) agar medium until the whole plate is covered by mycelium. Plates are either inoculated by a small piece of agar with mycelium or by four mycelial pieces placed onto the agar at equal distances to each other and about 2-2.5 cm apart from the edges of the Petri dish (9 cm in Ø) (Fig. 2). For optimal growth, plates are transferred into aerated dark boxes whose bottoms are covered by wet towelling paper to

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keep a high humidity in the air. Petri dishes with cultures for transformations should not be set directly on the towels but onto a spacer (e.g. another Petri dish) in between to avoid any contaminations on the outside of the plates by transfer from the wet tissues. Boxes with plates should not be set directly onto the warm bottom of an incubator to prevent heat accumulation in the box. Monokaryons of *C. cinerea* can be used as such but for transformation of strains with defects in the A mating type genes, fully grown plates should be transferred either for 2 days at 37°C or for 4 days at 25°C and high humidity into white light (light intensity 20-25 µE m⁻² s⁻¹; light source e.g. Osram L40/25; Osram AG, Munich, Germany) for induction of oidia production (10,6,23).

For harvest of spores, ca. 10-15 ml sterile H₂O are poured (from a bottle with sterile water) onto the aerial mycelium of a fully grown plate. Spores are released from the aerial mycelium by scraping with a sterile blunt spatula. The edge of the dry plastic Petri dish is sterilized by brief flaming with a Bunsen burner and the spore suspension is directly poured over the sterilized edge of the plate into a sterile thistle funnel (3-4 cm in Ø), which contains a layer of glass wool (Fig. 3A,B). The spores are filtered through the glass wool into a sterile test tube, thereby leaving agar pieces and mycelial debris behind. The filtered spore solution is then transferred into a 30 ml Sterilin polystyrene tube (item code 128A, Sterilin Ltd, Newport, UK; Fig. 3C,D) and centrifuged (5 min; 2.600 x g) against another Sterilin tube filled up from a spray bottle with water to a same volume as the spore suspension. The pelleted spores are resuspended in 5 ml freshly prepared sterile MM buffer and centrifuged (5 min; 2.600 x g). The supernatant is discarded and the enzymatic digest of the fungal cell walls started with resuspension of the spore pellet in 2 ml osmotically stabilized cellulase/chitinase solution. The mixture is incubated at 37°C with the caps of the tubes loosely closed to allow aeration as well as protection. The tubes are either gently shaken (110 rpm) in slightly tilted

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**Fig. 2.** YMG/T agar plate inoculated with four mycelial agar pieces of *C. cinerea* FA2222 after six days growth at 37°C in the dark.

**Fig. 3.** Selected materials required for transformation. A. and B. Thistle funnel with glass wool: the funnel is fixed in the test tube with a layer of cotton wool and for autoclaving the opening of the funnel and the cotton wool should be covered with aluminium foil. C. and D. Sterilin 30 ml tube after harvest of oidia from strain FA2222 grown for 8 days at 37°C: the arrows point to the pellet of spores. E. Ethidium bromide-stained agarose gel with 200 ng marker λ-DNA-HindIII (lane 1) and 1 µl pCc1001 mini-prep DNA (lane 2) and 1 µl pYSK7 mini-prep DNA (lane 3) with RNA seen as a large spot at the bottom of the gel.
position (30° angle) or laid raked down onto a stand to produce as large surface as possible without coming too close to the edge of the tube for spilling solution. After 3-4 h incubation, a first sample is taken (ca. 3 µl) and the grade of protoplasting formation is determined by examination under the microscope. The level of protoplasting defines whether samples are further incubated or whether further enzyme mix (in 1 or 2 ml portion) might be added. At a good level of protoplasting (usually ca. 50-70%), the enzymatic digest is stopped by addition of 5 ml sterile MMC buffer and gentle centrifugation (10 min; 640 x g). The supernatant is slowly poured off with caution that cells and protoplasts are minimally dispersed and that the slippery pellet does not get lost. The pellet is washed another time by 5 ml MMC buffer, with centrifugation (10 min; 640 x g) and carefully discharging the supernatant. The pellet is carefully resuspended in 300-500 µl MMC buffer to densities of about 0.2-2 x 10^8 cells/ml, depending on the strain, the quality of protoplasting and eventual losses of cells during the handling in the protoplasting procedure. Aliquots of each 50 µl are transferred to sterile 1.5 ml tubes. For transformation, plasmid DNAs (usually 1 µg per plasmid in single transformation or each 1 µg per different plasmid in cotransformation) and 12.5 µl PEG/CaCl_2 solution are added and gently mixed with the protoplasts. The tube(s) are placed for 20 min on ice. Then, 500 µl PEG/CaCl_2 solution are added to the protoplasts and gently mixed to further incubate for 5 min at RT (‘heat shock treatment’). Finally, 1 ml of sterile STC buffer is added and mixed. The transformation mix is spread in usually four portions (ca. 390 µl per plate) onto freshly prepared solid regeneration medium (if required with appropriate supplements). The plates are incubated at 37°C in aerated dark boxes on wet towelling paper. After ca. 3 to 4 days, first transformants might arise and hyphal growth should be monitored using a binocular. When the first mycelium arises, remaining free liquid should be removed from the surface of the plates by drying them shortly under a laminar flow cabinet. Growing colonies should be picked by a sterile tungsten needle and transferred onto fresh medium under appropriate selection (e.g. minimal medium for growth of prototrophs). Care must be taken that all mycelium of grown transformants is harvested from the plates prior to their further incubation at 37°C for growth of additional transformants. Every day, plates should be checked for harvest of new transformants. Usually, further transformants will appear for the next 4 to 6 following days. If too many transformants appear at a time or if transformants grow too fast, the process might be slowed down by incubating the plates at lower temperature (RT).

**Media (1,10,24,25):**
- YMG or YMG/T medium (per l: autoclave separately 4 g yeast extract, 10 g malt extract and, for YMG/T only 100 mg tryptophan in 900 ml bidest. H_2O and 4 g glucose in 100 ml bidest. H_2O to mix the two solutions after autoclaving; if required add 1% agar to the first solution for solidification); regeneration medium (per l final volume: autoclave separately 25 ml stock solution A, 1 ml stock solution B, 10 ml stock solution C, optional 50 mg adenine sulphate, 2 g L-asparagine, 172 g sucrose, 5 g soluble starch, filled up with bidest. H_2O to exactly 900 ml to then add 1.2% or 1% agar and 5 g glucose filled up with bidest. H_2O to exactly 100 ml to mix the two solutions after autoclaving; if required add 100 mg of a respective amino acid per l, and 5 mg para-aminobenzoic acid per l); minimal medium (as regeneration medium but without the optional 50 mg adenine sulphate, 172 g sucrose, and 5 g soluble starch and with 10 g instead of 5 g glucose per l; if required add 100 mg of a respective amino acid per l, and 5 mg para-aminobenzoic acid per l); stock solution A (per l final volume: 40 g KH_2PO_4, 90 g Na_2HPO_4, 11.6 g Na_2SO_4, and 20 g di-ammonium tartrate, fill up to 1 l with bidest. H_2O; store in fridge over some droplets of chloroform); stock solution B (per l: 40 mg thiamine in bidest. H_2O; autoclave and store in fridge); stock solution C (per l: 25 g MgSO_4 x 7 H_2O in bidest. H_2O; store in fridge over some droplets of chloroform). Agar

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concentrations in the media refer here to Serva agar (cat. No. 11396, Serva Electrophoresis GmbH, Heidelberg, Germany) and its specific gelling properties.

Solutions (1,10,23): MM buffer [always prepare freshly in 2:1:1 portions from sterile stocks of 1 M mannitol, 0.2 M Na maleate (pH 5.5), and bidest. H₂O]; MMC buffer [always prepare freshly in 2:1:0.1:0.9 portions from sterile stocks of 1 M mannitol, 0.2 M Na maleate (pH 5.5), 1 M CaCl₂, and bidest. H₂O]; cellulase/chitinase solution [per 20 ml MM buffer: 800 mg cellulase “Onozuka” R-10 from **Trichoderma viride** (1 U/mg, cat. No. 16419, Serva Electrophoresis GmbH, Heidelberg, Germany); 20 mg (standard) chitinase from **Streptomyces griseus** (product number C6137 with guaranteed > 200 units/g, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany); filter sterilize and store in 2 ml aliquots at -20°C]; PEG/CaCl₂ [10 g PEG (polyethylene glycol) 4000 (product number 807490, Merck KGaA, Darmstadt, Germany), 400 µl 1 M Tris-HCl (pH 7.5), and 1 ml 1 M CaCl₂, fill up to 40 ml with bidest. H₂O; filter sterilize and store in 5-10 ml portions at -20°C]; STC buffer (1 M sorbitol, 25 mM CaCl₂, 10 mM Tris-HCl (pH 7.5); autoclave). Note that the stock solutions for MM and MMC buffers as well as the STC buffer and any bottle with bidest. H₂O might be autoclaved again after usage to avoid that any accidental contamination will grow in these in between two experiments.

Results and Discussion

**DNA for transformation:** The quality of DNA is very important for transformation. We use in transformation plasmid DNA prepared by the mini-prep method modified after Birnboim and Doly (21) as described in the materials and methods. Such prepared DNA contains also large amounts of RNA (Fig. 3E). RNA in DNA samples helps to precipitate the DNA (26). However, this RNA can be of further benefit for the success of transformation as indicated by comparative transformation experiments. Transformation of strain EN117.9 for example with 1 µg pCRS1 isolated by the mini-prep method resulted in 171 ade8+ transformants whereas no transformant was obtained with 1 µg of pCRS1 purified by CsCl-ethidium bromide gradient centrifugation from RNA. Similarly in two different transformation experiments of strain PG78, 91, respectively 164 different pab1+ transforms were obtained with 1 µg pST17 prepared by the mini-prep method and only one, respectively 11 pab1+ transforms with 1 µg pST17 prepared by a Qiagen Plasmid Purification Kit including the usual RNAse treatment. Costa et al. (15) reported for the same strain similar low transformation rates from cotransformation experiments: 61 transformants were obtained from three experiments with 2 µg pST17 and 5 µg of a plasmid pSUPER-GFP of Qiagen-Kit-purified DNA.

Notably, in other experiments with PG78 protoplasts and pST17-RNA-mixtures from mini-preps we reached transformation rates of 300-700 clones/µg vector DNA, and with pCc1001-RNA-mixtures and pPAB1-2-RNA-mixtures transformation rates of each up to 300 clones/µg vector DNA. Similar transformation rates of up to 450 clones/experiment for strain PG78 and plasmid pPAB1-2 were reported by Granado et al. (10) who, although not mentioned at the time in the paper, also used mini-prep DNA. Single-stranded nucleic acids (either RNA or single-stranded DNA) applied as carrier RNA or carrier DNA in transformation of other fungi including the basidiomycete **Pleurotus ostreatus** has been shown to well enhance transformation frequencies (27-32). The mechanism(s) by which carrier RNA and DNA increase transformation rates remain(s) elusive. These extra nucleic acids may protect the plasmid DNA against nucleases by distracting them from the plasmids (27), which might happen outside of the cells or after transfer in the cells. Other papers on fungal transformation speculate on a cell-wall-binding function that helps either to cover all available DNA binding sites so that the plasmid DNA remains in solution for uptake into the cells (29,30) or to make the cell wall loose and better porous for transfer of plasmid DNA (33). If so,
this might affect transformation with spheroplasts.

An important factor is surely the overall purity of the DNA. DNA prepared by a traditional mini-prep method might be considered as only coarsely purified. Protein and other cell debris might not be well separated, especially when using a renaturation buffer made up of either only K acetate or only Na acetate (22). However, if 0.5 M NaCl end-concentration is added to the renaturation buffer, if the volume of renaturation buffer (500 µl) is large enough to allow easy mixing with the slimy-viscous sample with the denaturated DNA, protein and other cell debris, and if in addition the mixture is cooled down at -20°C (up to freezing to ice), protein and cell debris well precipitate in granular flocks that form to a compact pellet upon centrifugation. Pouring the supernatant after centrifugation into a new tube ensures that all cell debris are left behind and no attempts should be done to rescue also the small volume of liquid remaining in the tube (such as by pipetting). In the next step after isopropanol precipitation of DNA and RNA (done at RT to not favour precipitation of any left other substance), centrifugation, pouring off the supernatant, and sucking off all remaining liquid by towelling paper will remove any excessive NaCl and any other soluble compound possibly left. Repeating this step upon the 70% ethanol wash step will further contribute to obtain highest DNA (with RNA) purity.

Growth of *C. cinerea*: *C. cinerea* strains differ in speed of growth and in number of oidia produced in the aerial mycelium (16,34). Strain FA2222 for example takes 10-12 days and strain PG78 6-7 days at 37°C to fully cover with mycelium the surface of YMG/T medium in a 9 cm Ø Petri dish upon inoculation with a single mycelial piece in the middle of the plate. The freshly grown mycelium needs about 12-24 h to produce aerial spores (2,35), why a fully grown plate might be better stored another 1 or 2 days at 37°C prior to use which will increase absolute spore numbers by 2.5 fold. Two days after a plate is fully grown, strain FA2222 gives rise to $5 \times 10^9$ oidia/plate and strain PG78 $8 \times 10^8$ oidia/plate (16). Oidia are only short lived and may lose germination ability already after two further days under temperature stress. A long period needed for a strain to fully grow over a plate can thus result in high portions of non-germinable oidia (36). Survival rates will be better with shorter incubation rates at 37°C. This can be achieved by placing four pieces of mycelium onto a YMG/T agar plate for cultivation (Fig. 2). Cultivation times for mycelium of strain FA2222 to fully cover the agar are thus reduced to 7-8 days and for strain PG78 to 4-5 days.

Stress for the spores should be best avoided. High water activity ($a_w$ value) favoured by mycelium and spores is ensured by using fresh agar plates for fungal growth with only 1% agar (if employing as we agar from Serva; note that agars from different suppliers might have different gelling properties that can differentially influence the $a_w$ value of the medium). Keeping the humidity in the air high by incubating plates in dark boxes on wet tissues helps further the well-being of mycelium and spores.

Best is to use the freshly grown plates directly for transformation. To have always fresh plates at hand for transformation can be ensured by regularly inoculating plates on a daily basis. If transformation does not need to be as perfect (in high numbers of transformants), it is possible to use plates that after completing growth at 37°C were stored at lower temperature, so at RT for 1 or 2 days or at 4°C for several days (10). For own comfort, plates ready for use should be available on Thursdays and Fridays as transformation is best done on these days to then start picking first regenerated clones on Monday or Tuesday.

**Harvest of spores**: Important during harvests of spores is to avoid any contamination. Plates should be dry from the outside. During growth, this can be warranted by not placing plates directly onto the wet tissues in the dark box.

Further to avoid contamination, handling in harvest should be fast (but not hectic). An aliquot

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of sterile water can quickly be poured onto the mycelium from a flask so that the hydrophobic aerial mycelium is partially covered (needs about 10-15 ml). Scraping quickly the mycelium with the sterile blunt spatula (a sharp edge will injure the agar) distributes the liquid over the whole surface and brings spores and mycelial debris into solution. Prior to pouring off the spore solution into a funnel, short flaming the outer plastic helps to kill any attached microbes that possibly could contaminate the spores. The bell-shaped thistle funnel (Fig. 3A,B) used for filtration ensures that the glass wool nestles well to the glass surface of the funnel’s bottom and that also during spore suspension filtering, unlike in the case of a conoid funnel where pouring off the oidia suspension can easily displace the glass wool. When pouring off the spore solution, no attempt should be made to harvest the last droplet of spores from the plate as this extra handling might increase the risk of unwanted contamination and the spores lost in this left-over will not majorly change the absolute number of spores harvested.

The filtered spore suspension is sterile transferred into a Sterilin 30 ml tube (Fig. 3C,D). This translucent tube has several advantages also for the following protoplasting procedure. The conical form of the bottom allows compact pelleting of spores and protoplasts and the sizes, colours and behaviour of pellets can easily be observed through the plastic and, with experience, spore and protoplast amounts be judged from pellet size (Fig. 3C). A pellet size as shown in Fig. 3C (with ca. 4 mm height along the slant of the tube) corresponds to about 10⁸ spores that might last for 4 to 8 parallel transformation samples. Fewer spores should not be taken for experiments since pelleting works less well for lower amounts of spores and protoplasts and there is always some loss of cells. If more spores are needed for more transformations to be done in parallel, oidia from two (or more) plates might be combined. It is however also not advisable to harvest too many spores in one tube. Above 10¹⁰ cells, relations between cell numbers and the volume and total activity of the enzyme mix might become negative, for example also with regards to good aeration.

After protoplasting, in best case the pellet is only slightly reduced in size and the colour will have changed from compact whitish of the undigested spores to gleaming white of the protoplasts. Furthermore with Sterilin tubes, when slowly pouring off supernatants after centrifugation, it is easy to keep an eye on the pellets so that they not accidently slip away, out of the tube. Important is also the behaviour of the protoplasts with the polystyrene walls of the tubes. Protoplasts are charged and upon gentle centrifugation (10 min; 640 x g) attach comparably well as a loose pellet to the walls. Centrifugation in other plastic tubes (such as centrifuge tubes made of polypropylene) might not be much a problem with the undigested spores but the slippery protoplast pellets will repel from the plastic with a great danger of loss during handling.

**Protoplasting:** After harvesting and washing spores with sterile MM buffer, an enzyme mix of cellulase “Onuzuka” R-10 and Sigma C6137 chitinase in MM buffer as osmotic stabilizer is added to digest the spore cell walls. Oidia of *C. cinerea* have bilayered hyphal cell walls and single-layered septal cell walls and are surrounded by a gelatinous mucilage ([2,37]; Fig. 1). From other cells of the fungus (38-41) and from cell wall analyses of the basidiomycete *Schizophyllum commune* (42), it is expected that the cell walls contain chitin and glucans (mainly with β-1,3-linkages but also with α-1,4- and β-1,6-linkages) and that the mucilage is also composed of glucans. “Onozuka” R-10 cellulase is an impure enzyme mixture from the ascomycete *T. viride* that next to its main cellulolytic activities confers other enzymatic activities such as of hemicellulase, protease, amylase and pectinase (Serva Electrophoresis product information sheet; 43). *T. viride* enzyme preparations have further activities such as different glucanase activities that will assist in degradation of the chitin-glucan cell walls of the

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C. cinerea spores (44, 45). Sigma C6137 chitinase contains a chitodextrinase-chitinase, a poly(1,4-β-[2-acetamido-2-deoxy-D-glucoside])-glycanohydrolase which at 37°C detaches chitobiose units from chitin and a N-acetylglucosaminidase-chitobiase which splits chitobiose into its monomers N-acetyl-D-glucosamine (Sigma product information sheet). We never experienced failure of protoplasting with newly bought batches of enzymes, but only with expired chitinase after storage for 5 years at -20°C. There is thus no urgent need for testing.

Fig. 4. Protoplasting of oidia. A. Protoplasts (with vacuoles; white arrows) and oidia (black arrows) of strain 7K17 (60% protoplasts; photographed by J.D. Granado) obtained with 2 mg/ml chitinase (encircled: a group of cells that start to aggregate with each other; size bar = 10 µm). B. Oidia suspension of strain FA2222 prior to addition of enzymes, C. and D. after 3 h incubation with 0.2 mg/ml chitinase (white arrows: cell releasing its protoplast), E. and F. respectively 6 h incubation with clumps of spheroids and protoplasts, G. after enzyme incubation and washing with MMC buffer, and H. after PEG addition (size bar for D and F = 10 µm; all others = 20 µm).

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or applying other enzymes. However, Binninger et al. (4) replaced “Onozuka” R-10 cellulase with Novozyme 234 and succeeded in C. cinerea transformation with rates of 30 clones/µg pCc1001. In spite of this, Novozyme 234 was known to be very variable from batch to batch in quality of fungal protoplasting and in toxicity and it is now not anymore on the market (46).

The standard enzyme cocktail with 40 mg/ml cellulase and 1 mg/ml chitinase works very well in protoplast formation of up to $1-5 \times 10^9$ C. cinerea spores in 2 ml enzyme mix, with protoplasting rates of 50-70% in about 4 h incubation [([10]; Fig. 4A]. However, each batch of spores is new and thus individual. Two transformation experiments performed with strain PG78 at two consecutive days gave so different results. In the first experiment after 4 h of incubation, a protoplasting rate of 75% was achieved and subsequent transformation with 1 µg pST17 mini-prep DNA resulted in total in 653 transformants. At the next day, protoplasting after 4 h was not satisfying and another 1 ml of enzyme of standard concentration was added for further 2 h of incubation when 50% of spores were protoplasted. Transformation of this second lot of protoplasts with 1 µg pST17 mini-prep DNA gave in total 382 transformants. An important question is when to best stop the digest. To decide this with confidence requires good experience of the researcher. Healthy protoplasts will look under the light microscope round and slightly more greenish than the spores, due to an altered light reflection at the protoplast membrane. First, protoplasts tend to be filled relatively equal with granular cytoplasma. Over the time, more protoplasts are generated and protoplasts might increase in size under development of large vacuoles (Fig. 4A). This stage likely reflects protoplasts where all cell wall has fully been degenerated. In S. commune, such type of naked protoplasts regenerates well (47). When C. cinerea protoplasts are harvested when this type appears, excellent transformation rates are usually observed. When further incubating with enzymes, this might not be anymore the case.

Cells in absence of enzyme are well suspended (Fig. 4B). When protoplasting spores of C. cinerea, after some time of enzyme incubation, cells still covered by cell walls start to clump together and with available protoplasts (Fig. 4A,E,F). We believe that this behaviour is induced by enzymatic alterations of the cell walls, marking partial cell wall digestion. The appearance of these clumps can also be used as a good indication that the cells are ready for transformation. In other fungi, protoplast regeneration might be accelerated when the cell wall is not completely degraded and transformation rates can be increased when using spheroplasts with cell wall remnants instead of naked protoplasts (48-50). Our more recent experiments show that chitinase concentrations might be reduced to 0.1 mg/ml for digestion of about $10^8$ cells in 2 ml (in total 0.1 to 0.16 U) with the consequence, that protoplasting slows down (Fig. 4B-F). We observed in different experiments with strain FA2222 after 3 h of incubation regularly about 5% of protoplasted spores. Many other cells are then at a stage close to release the protoplast from the cell wall. Cells often swell at one end at which the protoplast finally escapes (Fig. 4C,D). With further incubation, 30-35% protoplasting can be observed after 6-7 h. At this stage, protoplasts and partially digested spores (spheroplasts) clump in groups together (Fig. 4E,F). Nevertheless of the lower rate of protoplasting, such spheroid-/protoplast mixtures might be harvested for transformation. In 10 independent experiments with strain FA2222, 34 to 208 [in average $96 \pm 69$] trp1+ transformants per 1-2 x $10^7$ cells and 1 µg pCc1001 were so obtained.

Not unexpected, the time required for protoplasting is much influenced by the amount of chitinase added to the spores. Binninger et al. (1) digested spores in 2.5 h in 1 ml enzyme mix with 20 mg cellulase and 1 mg chitinase, stated by the authors to represent 4 U. Different batches of Sigma C6137 chitinase differ in total U/mg Dörnte and Kües
which, in our hands, ranged between 0.5-0.8 U/mg enzyme powder. The standard procedure (10,23) with in total 80 mg cellulase and 2 mg (= 1-1.6 U) chitinase takes about 3 to 4 h (occasionally up to 5 h) for adequate protoplasting. Overdigestion of the protoplasts might easier be controlled in longer incubation times. Since the times of incubation might well be used such as to prepare regeneration agar or, if required, also DNA, a longer incubation time might be of comfort for the experimenter. The steps following upon spore digestion are not as time consuming and the whole procedure can still well be performed within a working day when only 0.2 mg chitinase is applied.

Protoplasting is stopped by adding MMC buffer and subsequent centrifugation. The pellets of protoplast-spore mixtures are pearly with a slimy appearance. The supernatant is carefully poured off, thereby trying not to loosen the pellet at the bottom of the tube. If this nevertheless happens, it is advisable not to try to pipet the remaining liquid away but to add further MMC for another round of centrifugation to bring the Ca\(^{2+}\) concentration to 25 mM. The Ca\(^{2+}\) in the buffer causes the protoplasts to better stick to each other (Fig. 4G) and in consequence to better pellet. Also after the last centrifugation (after a second wash) it is better to carefully pour off the buffer with some MMC liquid remaining with the protoplasts in the tube. Trying to take off the rest with a sterile pipette tip will only cause dispersal of protoplasts when sucking the liquid and with it loss of protoplasts. The final pellet size and the left MMC volume will determine how much extra MMC might be added to the protoplasts. As a rule of thumb, about 10\(^6\)-10\(^7\) cells should be present per 50 µl protoplast suspension (1,10,23) used in the next step for transformation. Note that it is not the exact number of cells that will guarantee the success in transformation but the individual valuation of the quality of protoplasts by the researcher. An additional dilution of the suspension, below 10\(^6\) cells per 50 µl, should better be avoided. If more protoplasts are required for more transformation samples, more than one plate should be harvested. In the case that an excess of protoplast suspension exists, it is possible for later use to store the protoplasts overnight at 4°C or for a longer time at -80°C by addition of 50 µl sterile PEG/Ca\(^{2+}\) solution per 50 µl cell suspension.

Transformation: Generally, 1 µg mini-prep DNA per plasmid can directly be used in transformation. Application of larger DNA quantities (up to 50 µg plasmid DNA) does not necessarily lead to higher transformation efficiencies (1,7,15). The protoplasts are transformed by incubation with Ca\(^{2+}\) and PEG in ice at cold temperature and a following heat shock. Addition of Ca\(^{2+}\) and of PEG promotes cell agglomeration (Fig. 4G,H). Strengthened by the sudden temperature shift, Ca\(^{2+}\) and PEG are believed to take influence on the physiological properties of cell membranes in spheroplast transformation. Ca\(^{2+}\) and PEG may help to attach DNA to the cell surface and may enhance the permeability of the membrane and promote DNA internalization. However, in no fungus their exact function is so far understood (51,52).

Upon transformation and addition of 500 µl PEG/Ca\(^{2+}\) for further membrane interactions, addition of 1 ml sterile STC buffer (stabilized with 1 M sorbitol) dilutes the viscous PEG-protoplast mixture which eases the following plating on sucrose-stabilized regeneration medium. Cell wall regeneration needs time in an osmotically stabilized environment. It is therefore important that the protoplasts are brought onto plates in sufficient amount of stabilized liquid and that the liquid last a few days on the plates. Therefore at least 300 µl, better up to 400 µl of STC buffer-diluted protoplast solutions should be plated per Petri dish. Usually, one transformation sample results in four plates for regeneration. During plating, care should be taken for not diluting the sugar solution, i.e. that mixing is avoided with any condensate water that possibly accumulated on the plastic edges of the freshly poured Petri

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dishes. Plates must not be dried if such condensation is present to avoid disturbing the osmotic balance and reducing the $a_w$ value in the agar.

**Regeneration:** Regeneration of protoplasts is done by incubation at 37°C. Important here is a first phase of keeping the agar surface of the regeneration plates wet for 2 to 3 days with a liquid layer. Thereafter, the plates should be shortly dried to a level at which the liquid layer is just gone away. It is to note that too extensive drying can negatively influence the further growth of the clones. With dryer plates, few transformants tend to grow. In some of our transformation experiments of strain FA2222 and pCc1001, regeneration plates dried already in the box during the first 2 days of incubation. In two different examples, only 10, 12, 6, and 3 (in total 31) transformants, respectively 15, 8, 2, and 3 (in total 28) clones could be harvested at the days 4 to 7 of incubation. This was not much different in a parallel cotransformation with pCc1001 and pYSK7 where 26, 10, 11, and 1 (in total 48) clones grew on the plates. In another experiment with FA2222 and pCc1001 as a typical example where the liquid was kept until active drying of the plates' surfaces at day 3 of cultivation, 49, 78, 51 and 18 (in total 196) transformants were harvested at days 4 to 7 of incubation. It remains thus important to keep the $a_w$ value in the agar as high as possible. In the protocol of Granado et al. (10) an agar concentration of 1.2% is used. A reduction to 1.0% can assist to keep a high $a_w$ value and will still give the agar a required strength for plating.

Removing the liquid from the surface of regeneration plates after the initial three days of incubation reduces the risk of spreading bacterial contaminations. Even a single bacterial cell on a regeneration plate can spoil the whole transformation by rapid cell divisions and by the easy dispersal of the increasing number of bacterial cells in the liquid over the whole plate (Fig. 5A). As a further advantage, drying of the plates enhances the oxygen supply for the fungus to improve growth. Also, the contours of the small light-coloured fungal colonies become more clearly visible within the agar (Fig. 5B).

Picking emerging clones should be done as fast as possible upon appearance of transformants in order to avoid them to grow into each other. Small colonies and colony edges can best be recognized by holding the plates upright towards light (evenly distributed and not too strong light, such as natural day light from a window) whilst cutting out agar pieces with mycelium with a sharp tungsten needle. Alternatively, very small colonies might be

![Fig. 5. Plates with regeneration agar after plating of transformation mixtures of strain FA2222 with pCc1001 and pYPH3 and 4 days incubation at 37°C with A. bacterial contamination and B. young trp1+ transformants on regeneration medium made up with 1% agar.](image)
harvested with the needle while observing the colonies under a binocular with the plate illuminated from below from an indirect light source. For further cultivation and appearance of more transformants, care has to be taken that from all colonies all material is removed since any remaining hyphal debris will further grow into a new colony. All picked clones should be transferred to suitable selection medium, usually minimal medium (with specific supplements added if required). Per plate of fresh medium, about 15 transformants might be inoculated (using a grid for equal distribution) to grow them for up to 2 days at 37°C without a danger that colonies grow into each other.

Conclusions

Transformation of *C. cinerea*, first presented by Binninger et al. in 1987 (1), is an essential tool for molecular analysis of this fungus. In this paper, we discuss important aspects for success in the transformation – DNA sources and quality, fungal cultivation and age of cultures, the procedure of enzymatic protoplasting and quality criteria of protoplasts, and best handling in regeneration. Well understanding details in the protocol can help to avoid failure in handling. Transformation of *C. cinerea* needs some practice. In particular, a good sense needs to be developed for recognition of high quality protoplasts.

Acknowledgements

We thank J.D. Granado for the photo in Fig. 4A and P.J. Hoegger for plasmid pYPH3 and all present and former colleagues in the lab for sharing experiences on *C. cinerea* transformation with us.

References


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