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Isolation of mesophyll protoplasts of the genus *Coffea*.

Resumen. Se describe un método rápido para el aislamiento de protoplastos a partir de hojas de café, probándose diferentes enzimas en varias combinaciones. Es posible liberar abundante cantidad de protoplastos a partir de hojas jóvenes, provenientes de varias líneas y cruzamientos, de las especies *Coffea arabica* y *C. canephora*, mediante su incubación durante 4 horas en celulasa (3%), pectoliasa (0.5%) y manitol (0.6 molal) a pH 5.8. Los protoplastos se filtraron y lavaron varias veces en agua de mar (85%), y fue necesario resuspenderlos en percoll (70%) debido a su densidad. Los protoplastos sobreviven varias semanas, y regeneran pared celular. En uno de los medios (A 43), después de 2 semanas, se observan algunas divisiones. Se están probando varios medios de cultivo con diferentes concentraciones de hormonas.

The potential of protoplast culture and fusion in plant breeding is well known (8, 9) and this is not an exception for the genus *Coffea*. In this genus several genetic and chromosomic barriers exists between the

cultivated tetraploid species *Coffea arabica* and the diploid wild type species. Therefore it is difficult to transfer desirable genetic traits from the wild type species to the cultivated *Coffea arabica*. Somatic hybridization via protoplast fusion could be an excellent complement to conventional coffea breeding programs.

Only a few attempts have been made in coffee to isolate and culture protoplasts, and this with only little success. Söndahl *et al.* (6), reported protoplast liberation and possible callus formation of coffee protoplasts derived from callus tissue. In another short communication, the isolation of few mesophyll protoplasts after a long period (13-16 h) of enzyme treatment was reported (7). It is obvious that it could be of great advantage to have also for coffee methods which allow the isolation of mesophyll protoplasts in large numbers during a relative short time of enzyme incubation. The present technical note describes a method which leads in a short time to large numbers of mesophyll protoplasts of several coffee lines and species.

Leaf protoplasts in general have the advantage that they possess a defined chromosome number in comparison to callus or cell suspension derived protoplasts which make them more useful for somatic hybridization experiments.

Material and methods

Lines of the following species were used for the experiments: *Coffea arabica*, *Coffea canephora*, a sexual hybrid line between *Coffea arabica* and *Coffea canephora*, all obtained from Cenicafé (Colombia). These lines and species were cultivated in the greenhouse. Another sexual hybrid between *Coffea arabica* and *Coffea canephora* (Arabusta) which were obtained from the Laboratoire de Culture in Vitro, GERDAT (France) were cultivated as aseptic shoot cultures on MS agar medium (3), supplemented with 40 g/l sucrose and 1 mg/l BAP (6-benzylaminopurine). Before use, leaves from greenhouse plant material were surface sterilized by 7% sodium hypochlorite for 10 min. and subsequently washed 3 times with autoclaved tap water. For protoplast isolation leaves of different developmental stages were cut with a razor blade into small pieces (2-3 mm^2) in the presence of 0.3 M mannitol and were subsequently transferred into the various enzyme mixtures. The following enzymes dissolved in 0.6 M mannitol (ca. 730 mOsm), pH 5.8, were tested in various concentrations and combinations: Cellulase "Onozuka" R 10 and macerozyme R 10 (Kinki Yakult, Japan), cellulase 2230A (Röhm, FRG) pectolyase Y-23 (Seishin Pharmaceutical Co., Japan), driselase (Kyowa Hakko Kogy, Japan) and lysozyme (Worthington Biochem. Corp., USA).

The enzyme incubation was carried out for 4-24 h on a roller (2 rpm) at 25°C. After incubation the protoplast suspensions were sieved to remove the undigested leaf material and washed two times with seawater (ca. 730 mOsm) by centrifugation. After washing the protoplasts were resuspended in 0.6 M sucrose or 70% percoll dissolved in 0.6 M mannitol and centrifuged for 10 min. The protoplast containing supernatant was diluted with 85% seawater (1:5) and recentrifuged to remove percoll. The pellet containing the protoplasts was finally suspended in the protoplast regeneration media V 47 according to Binding (1) or A 43, according to Poirier-Hamon *et al.* (5).

Results and discussion

The enzyme combinations and concentrations tested for protoplast isolation of coffee are listed in Table 1. No protoplast release could be obtained after enzyme treatment up to 24 h of old and fully expanded leaves with all enzyme mixtures used. However, by 5 h treatment of young leaves with cellulase

Table 1. Treatment of fully expanded old and of young leaves from coffee plants with various enzyme combinations for protoplast release.

Enzyme	Old leaves	Young leaves
Driselase (2.5%) Macerozyme R 10 (2%)	–	–
Lysozyme (2%) Macerozyme R 10 (1%)	–	–
Cellulase R 10 (3%) Macerozyme R 10 (1%)	–	+
Cellulase 2230 (3%) Macerozyme R 10 (1%)	–	+
Driselase (2.5%) Pectolyase Y-23 (0.3%)	–	–
Lysozyme (2%) Pectolyase Y-23 (0.3%)	–	–
Cellulase R 10 (3%) Pectolyase Y-23 (0.5%)	–	+++
Cellulase 2230 (3%) Pectolyase Y-23 (0.5%)	–	+++

+ = few, +++ = satisfactory

R 10 or cellulase 2230 in combination with macerosyme R 10, a few protoplasts could be obtained Longer treatment, however, gave no higher yield of protoplasts. Much higher yields of protoplasts could be obtained with all lines and species tested if young leaves, less than one month old, were treated for 4 h with cellulase R 10 or cellulase 2230 in combination with pectolyase Y-23. Short time (25 min) treatments with these enzyme mixtures were not successful as it is for example the case for tobacco (4), *Datura* and *Petunia* (Schieder, unpublished)

After sieving and washing the protoplasts, the suspensions contained still broken protoplasts and undigested cells. To separate them from the protoplasts, the pellets were resuspended in 0.6 M sucrose and centrifuged. However, the protoplasts did not float in the supernatant as is observed for protoplasts of most other species (2). Much better results could be obtained if the protoplasts were suspended in 70% percoll dissolved in 0 6 M mannitol The protoplasts of coffee, though relatively small seem to be more dense than protoplasts of other species which makes it necessary to centrifuge them for floating in a solution with a higher density

The washed protoplasts suspended and cultured in the V 47 or A 43 medium survived for more than 3 weeks. They showed cell wall resynthesis and changes in their shape In the A 43 medium after 3 weeks some divisions could already be observed. Further cultivation experiments with different hormone concentrations and combinations and also with other protoplast regeneration media are under way.

Summary

A quick test for coffee mesophyll protoplasts isolation is described. By using combinations of enzymes it was possible to isolate numerous protoplasts of young leaves of hybrids of *Coffea arabica* and *C canephora* Treatments were incubated for four hours with 3% cellulase. 0.5% prectolyase and 0 6 molal manitol, at pH 5.8 Protoplasts were filtered, washed with 85% see water and resuspended in 70% percoll due to this density The protoplasts were able to survive for a few weeks and regenerated the cell wall. In media A 43 the protoplasts with some sell division was observed. New analysis are in the way trying different hormons concentration in the medio.

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