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Isolation, culture and division of olive (*Olea europaea* L.) protoplasts

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ABSTRACT

Protoplasts from Olea europaea L. have been compared in terms of their yield, viability, cell division and callus differentiation. Viable protoplasts were isolated from in vitro cultured leaves and cotyledons by an overnight incubation in an enzyme solution containing 1-1.5% driselase and 0.5M sucrose. This method allowed high yield of purified protoplasts, which floated and formed a dark green band at the meniscus, after centrifugation. Purified protoplasts were diluted to $3x10^4$ protoplasts·ml^{-1} in culture medium. After cell wall regeneration, protoplasts gradually increased their volumes under appropriate conditions. The first divisions occurred during the second week in culture. Division efficiency ranged from 5.2 to 9.8% after 20 days in culture. Two weeks later visible microcolonies developed only from cotyledon protoplasts. After 6 weeks in culture, the microcalli were transferred to a solidified culture medium with 0.6% agarose, which induced active callus growth.

Abbreviations: OM=olive proliferation medium, Rugini 1984; OMg= OM for the germination of olive embryos; OMr=OM for root induction; OMp=OM for protoplasts; OMc=OM for callus; BN=Bourgin and Nitsch medium 1967; IBA=indol-3-butyric acid; NAA=naphthalene acetic acid; 2,4-D=dichlorophenoxyacetic acid.

INTRODUCTION

The absence of cell walls around protoplasts provides an opportunity to introduce foreign genes into the plants by direct transfer methods. We are currently interested in developing a genetic transformation system to introduce foreign genes into the olive protoplasts, because -up to now- in vitro infection experiments on seedling olive explants with some strains of Agrobacterium tumefaciens were unsuccessful. A. rhizogenes strain A4 produces only root induction on shoot or hypocotyl segments. Unfortunately, induced roots were always agropine negative and their genetic transformation has not been confirmed (Cañas and Benbadis, unpublished).

Different in vitro methods to obtain micropropagation in the olive tree have been recently devised (for review see Rugini 1986) and a suitable source of plant material for protoplasts isolation (leaves, cotyledons, hypocotyls,...) has been found to be the plantlets obtained from in vitro culture of naked olive embryos (Cañas et al. 1987). On the other hand, callus culture and regeneration are necessary for plant improvement via genetic engineering. Callus induction is relatively easy in the olive and is possible from any parts of the plant as described by some authors; also, some approaches to callus organogenesis and regeneration of whole plants have been done with different results on shoot and root induction (Rugini 1986; Cañas and Benbadis 1987).

Many reports exist on plant regeneration from protoplasts of herbaceous plants. However, relatively few attempts have been made on the culture of protoplasts from woody species (the fruit trees in particular) and most of these studies are preliminary. The only arborescent species, in which plant regeneration from protoplasts has been reported, are Citrus, Santalum, Broussonetia and Pirus (Vardy et al. 1982; Rao and Ozias-Akins 1985; Oka and Ohyama 1985; Ochatt and Caso 1986). There are no reports -up to now- about successful callus induction and plant regeneration from olive protoplasts.

We report here our results on isolation, culture and division of olive protoplasts. Our long-range objetive is to enhance the yield of microcalli, as a first step towards plant regeneration, in order to develop a genetic transformation system applicable to this tree (a crop species of high economic and cultural relevance in the Mediterranean countries).

MATERIALS AND METHODS

Plant materials. Leaves and cotyledons were isolated from plantlets obtained by in vitro culture of naked olive embryos. For this purpose, seed of Olea europaea L. cv Tanche (France), which developed on OMg medium in our preliminary experiments, were chosen. Mature embryos were isolated from swelled olive seeds under sterile conditions and cultured on OMg medium (Cañas et al. 1987) at 23±1°C in a growth chamber with white cool fluorescent tubes (21 Wm^{-2} of light intensity) and 16h light per day. All media used in our experiments were adjusted to pH 5.7 before autoclaving at 115°C for 20 min and thermolabile substances were added filtered. Olive plantlets as source of aseptic leaves were also obtained from in vitro rooted shoots on OMr medium with 1mg.l^{-1} of IBA or NAA (Cañas et al. 1987).

Isolation and purification of leaf and cotyledon protoplasts. One gram of aseptic leaves or cotyledons was cut into 1-2mm wide strips with a razor blade under sterile conditions and placed into 10-ml aliquots of the enzyme solution (1-1.5% driselase respectively, 3.5 mM $CaCl_2$ and 0.5M sucrose at pH 5.6) contained in 50 ml screw-capped glass bottles. The enzyme solution was sterilized by filtration using syringe and Millex TM disposable filter unit (0.22µm pore size). Small fragments were incubated overnight (14h) at 23±1°C in the darkness. Next morning, protoplasts were released by gentle swirling (30 min at 60 rpm), and the resulting suspension was then passed through a 60-70µm nylon filter to separate protoplasts from cell debris and aggregates. Filtered protoplasts floated and formed a dark green band at the meniscus after centrifugation at 80xg for 5 min. The pellet and the

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underlaying enzyme solution were removed by mean of a capillary connected to a peristaltic pump. Protoplasts were then resuspended in a washing salt solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl and 5 mM glucose) and centrifuged again for 5 min at 70xg. Finally, purified protoplasts were diluted from 10^4 to 10^5 protoplasts.ml^{-1} with OMp culture medium (BN macroelements, OM microelements and vitamins, 500 mg.l^{-1} of glutamine, 20 g.l^{-1} of sucrose, 91 g.l^{-1} of mannitol, 0.5 mg.l^{-1} of 2,4-D, 0.5 mg.l^{-1} of zeatin, 1 mg.l^{-1} of NAA and 0.03 mM of ornithine, at pH 5.7).

Protoplast culture. Diluted protoplasts were dispensed in 3-ml aliquots per 5 cm petridishes and incubated at 23±1°C, for 4 days, in the dark. Cell wall removal and regeneration were confirmed with 0.1% Calcofluor White dissolved in culture solution. After cell wall regeneration, olive cells were cultured at low light intensities (1.5 Wm^{-2}) with 16h light per day at 23±1°C. Two weeks later, the OMp medium was gradually diluted with the same medium without auxins and supplemented only with 0.2 mg.l^{-1} of zeatin.

Yield, viability and cell division measurements. Protoplasts were counted in a Nageotte chamber (0.5 mm) adapted to an inverted microscope. Viability was assessed using fluorescein diacetate (FDA) as a test of membrane integrity and non-specific internal diesterase activity (Widholm 1972). After 2-5 min in 0.01% (w/v) FDA solution, stained protoplasts were observed under blue light with a Zeiss photomicroscope equiped with epifluorescent attachments. The viability percentage was calculated as the number of protoplasts fluorescing green per total number of intact protoplasts existing at day 0 x 100. After 3 weeks in culture, the percentage of isolated protoplasts undergoing division was calculated as the number of dividing cells, divided among the number of protoplasts plated x 100.

Callus culture. After 6 weeks in liquid culture medium, the developed microcalli (1.5-2 mm in diameter) were transferred to a callus proliferation medium (OMp base without mannitol, supplemented with 0.5 mg.l^{-1} of zeatin, 2.5 mg.l^{-1} of IBA or NAA and solidified with 0.6% (w/v) Sea Plaque Agarose). Finally, proliferated calli were subcultured on the same medium supplemented only with 0.1 mg.l^{-1} of 2,4-D and 0.1 mg.l^{-1} of zeatin riboside for callus maintenance. Undifferentiated green calli were transferred to OMc medium with 1 mg.l^{-1} of zeatin or NAA in order to direct further development and induce morphogenesis. Callus culture was performed at 23±1°C and 16h light (1.5 Wm^{-2}) per day.

Cytohistological studies. The calli were fixed in AFA (absolut ethanol:formaldehyde:acetic acid, 6:3:1), washed in 60% ethanol and dehydrated in ethanol-xylene series. Small fragments were included into paraffin, sectioned on a microtome and stained with Safranin-Orange G.

RESULTS AND DISCUSSION.

Results on protoplast yield, viability, cell wall regeneration and cell division are summarized in Table 1.

Table 1. Comparison of yield, viability, cell wall regeneration and cell division of protoplasts from leaf mesophyll and green cotyledon of Olea europaea L. cv Tanche.

	Leaf mesophyll	Green cotyledon
Yield(g/fresh wt)	2.3-2.7 x 10^6	1.4-1.6 x 10^6
Viability[a] (%)	78-83	87-91
Cell wall regen.(%)	46-51	61-68
Cell division[b] (%)	5.2-6.3	8.7-9.8

([a]) After 48h in culture by FDA method.
([b]) Protoplasts density: 3x10^4 protoplasts.ml^{-1}.

Viable protoplasts from in vitro cultured leaves and cotyledons were obtained after overnight incubation with 1-1.5% driselase and 0.5 M sucrose. This combinated method allowed high yield of purified protoplasts, which floated and formed a dark green band at the meniscus, after centrifugation. Several of the common enzymatic combinations of cellulase (Cellulase R10), pectinase (Macerozyme) and hemicellulase (Rhozyme HP150) gave only poor yield and viability with different olive tissues (Cañas 1985; Rugini 1986).

Size of freshly isolated protoplasts ranged from 40 to 60µm in cotyledon protoplasts (Fig. 1A) and from 30 to 45µm in leaf protoplasts (Fig. 1B).

The cotyledon protoplasts varied considerably with respect to their number of chloroplasts, from none (white protoplasts), to very high density (green protoplasts), reflecting the variety in cell origin (Fig. 1A).

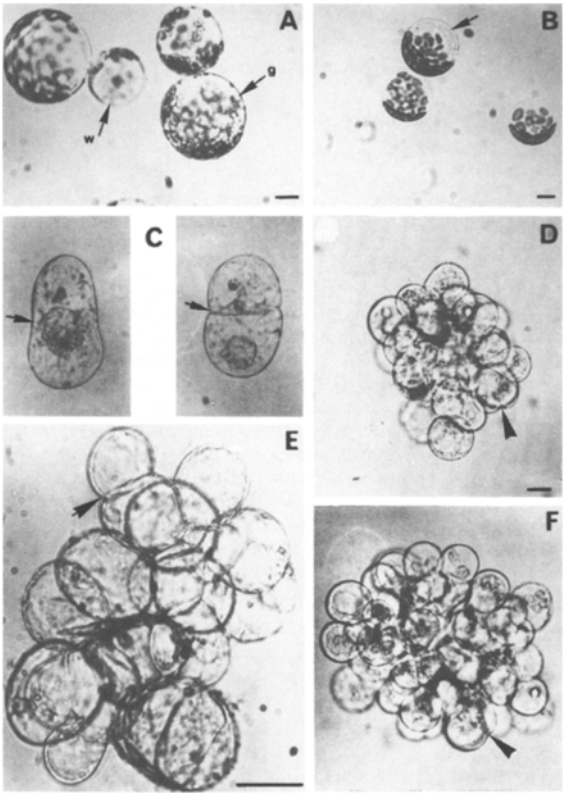

Fig.1. Culture of leaf and cotyledon-derived protoplasts from Olea europaea L. cv Tanche.
A) Freshly isolated protoplasts from cotyledon tissue, g=green protoplasts, w=white protoplasts (bar=15µm). B) Freshly isolated protoplasts from leaf tissue (bar=15µm). C) Cell wall regeneration and first division in a cotyledon protoplast. D) Cell colony from leaf protoplast after 3 weeks in culture (bar=20µm). E) Cell colony from cotyledon protoplast (bar=60µm). F) Stationary cell colony derived from leaf protoplast after 6 weeks in culture.

Protoplast viability was measured after 48h in culture medium by the FDA method (picture not shown), moreover olive protoplasts showed an endogenous fluorescence when observed under UV light (chloroplasts exhibited red chlorophyll autofluorescence and cytoplasm blue fluorescence, both in leaf and cotyledon protoplasts).

Protoplasts of various plating densities (10^4 to 10^5) were cultured in a thin-layer of OMp culture medium. Cell wall regeneration was evidenced by Calcofluor staining (picture not shown) and by the change of round protoplasts to oval or elongated cells after 4 days in culture in the darkness (Fig. 1C). Cell wall was synthesized in a large number of leaf and cotyledon protoplasts (see Table 1), but the regenerated cells failed to divide when the plating density was below 2.5x10^4 protoplasts.ml^{-1}. The results obtained from 6 experiments demonstrated that the

optimum plating density for cell division and proliferation was $3x10^4$ protoplasts.ml^{-1}.

Cell divisions were detected after 15 days in culture, both in leaf and cotyledon protoplasts, while cell clusters were obvious at approximately 4 weeks(Fig.1D and E). Microcolonies from leaf protoplasts stopped their growth in OMp diluted medium when they were formed by 40-50 cells (Fig.1F). However, cell colonies from cotyledon protoplasts developed visible microcalli (1.5-2mm in diameter) after 6 weeks in culture. It is possible that this difference between leaf and cotyledon protoplast -derived cells arises from the difference observed in viability, cell wall regeneration and cell division at the beginning of their culture. When the microcalli were transferred to OMc agarose medium, approximately the 60-70% of transferred microcalli were able to survive and two kinds of active growth calli were induced: white friable calli (12% of total formed calli) and green compact calli. Possibly friable white calli (Fig.2A) were originated from isolated white protoplasts (without chloroplasts), obtained from cotyledon tissues in the same proportion (Fig.1A). White friable callus also grew rapidly in OMc medium without phytohormones (habituated callus).

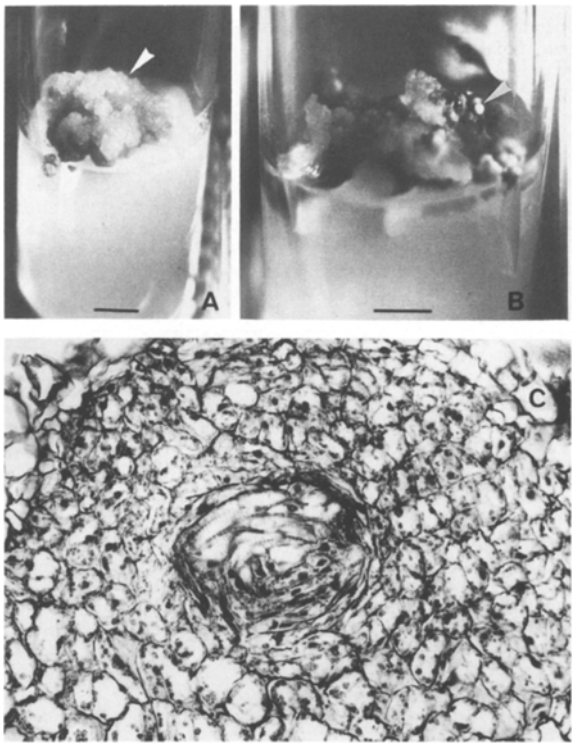

Fig.2. Active callus growth from cotyledon-derived protoplasts on OMc agarose medium. A) Friable white callus after 1 month in culture (bar=1cm). B) Compact green callus after 1 month on OMc medium with 1 $mg.l^{-1}$ of zeatin, showed white globular shapes in the surface (bar=0.5cm). C) Vascular tissue nodule with tracheids surrounded by cambium-like cells(x160).

Green calli grew more slowly than white calli on OMc medium and showed superficial proliferation and unorganized parenchima-like cells after cytohistological studies. When green calli were transferred to OMc medium with 1mg. $.l^{-1}$ of zeatin, in order to direct further development and induce morphogenesis, they developed some protuberances with white globular shapes in their surface (Fig.2B). These protuberances remind of certain embryoid or shoot primordia forms. In addition, histological studies showed periderm formation in the outer region and vascular tissue nodules with thracheids surrounded by cambium-like cells in the inner region (Fig.2C). These meristematic nodules were also detected in green calli cultured on OMc medium with 1 $mg.l^{-1}$ of NAA and they have been reported to be the origin of root primordia, in olive apical twig -derived calli (Wang et al. 1979). In our case, organogenesis from this type of nodules was not observed up to now.

Information about successful protoplast culture and division from woody crop plants (specially fruit trees) is remarkably scanty in literature. Protoplast isolation and culture, but not cell division, have been reported by some authors from different woody species, as Betula and Rhododendron (Smith and Mac Cown 1983) or Fagus sylvatica (Ahuja 1984). Calli and cell cluster formation have been reported from Pseudotsuga menziesii (Kirby 1982), Pinus pinaster and P.coulteri (David et al. 1984 and Patel et al. 1984), Prunus dulcis (Wu and Kuniyuki 1985), Alnus incana and A.glutinosa (Tremblay et al. 1985 and Huhtinen et al. 1982) and Ulmus sp.(Sticklen et al. 1985 and Dorion et al. 1986). The only arborescent species in which plant regeneration from protoplasts has been reported are Citrus, Santalum, Broussonetia and Pirus (Vardy et al. 1982; Rao and Ozias-Akins 1985; Oka and Ohyama 1985; Ochatt and Caso 1986).

We report here for the first time the successful culture and division of olive protoplasts. The increase of plating efficiency is necessary as a first step towards plant regeneration. The composition of the culture medium, the environmental conditions and the stage of the cell cycle, at the time of protoplast isolation, are also important factors that will be necessary to check in our future experiments.

Having established a standard procedure for isolation, culture and division of olive protoplasts, we are now engaged in a project to define the factors governing the development of cell colonies, and the conditions under which plant regeneration from protoplast-derived callus would be possible.

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REFERENCES

Ahuja MR (1984). Silvae Genet. 33: 37-39
Bourgin JP, Nitsch JP (1967).Ann.Physiol.Vég.9:377-382
Cañas LA, Carramolino L, Vicente M (1987). Plant Sci. 50:85-90
Cañas LA (1985). Abstracts of the First Internat.Congress on Plant Molecular Biology. Galau Ed.Athens,USA:102
David H, Jarlet E, David A (1984).Physiol.Plant.61:477-482
Dorion N, Danthu P, Bigot C (1986). Abstracts of the 4th Internat.Congress of Plant Tissue and Cell Culture. Minnesota,USA:43.
Huhtinen O, Honkanen J, Simola K (1982). Plant Sci.Lett. 28:3-9
Kirby EG (1982). Physiol. Plant. 56: 114-117
Ochatt SJ, Caso SH (1986). J.Plant Physiol.122:243-249
Oka S, Ohyama K (1985). J.Plant Physiol.119:455-460
Patel KR, Shekhawat NS, Berlyn GP, Thorpe TA (1984). Plant Cell Tissue Organ Cult. 3: 85-90
Rao P, Ozias-Akins P (1985). Protoplasma 124:80-86
Rugini E (1986). In: Bajaj Ed. Biotechnology in Agriculture and Forestry, vol.I. Springer-Verlag,Berlin pp 253-267
Smith MA, Mac Cown BN (1983). Plant Sci. Lett.28:149-156
Sticklen MB, Lineberger D, Domir SC (1985). Plant Sci. 41: 117-120
Tremblay FM, Power B, Lalonde M (1985).Plant Sci.41:211-216
Vardy A, Spiegel-Roy P, Galun E (1982). Theor.Appl.Genet. 62: 171-176
Wang KJ, Zhang PF, Ni DX, Zhu XZ, Yang WQ, Bao ZH (1979). Acta Bot. Sinica.21: 225-230
Widholm JM (1972). Stain Tech. 47: 189-194
Wu SC, Kuniyuki AH (1985). Plant Sci. 41: 55-60