Callus formation from *Malus x domestica* cv. 'Jonathan' protoplasts

Mohamed Kouider¹, Randal Hauptmann², Jack M. Widholm², Robert M. Skirvin³, and Safi S. Korban⁴

¹ Mission Scientifique, Ministère Industrie et Recherche, 5 rue Descartes, F-75005 Paris, France
² Department of Agronomy, University of Illinois, 1102 S. Goodwin Ave., Urbana, IL 61801, USA
³ Department of Horticulture, University of Illinois, 1707 S. Orchard St., Hort. Field Lab., Urbana, IL 61801, USA

Received February 14, 1984 / Revised version received June 25, 1984 – Communicated by O. L. Gamborg

Abstract:

Protoplasts could be successfully isolated and cultured from callus and suspension cultures of *Malus x domestica* cv. 'Jonathan'. Protoplast-derived colonies were recovered when the somatic (glucose) was gradually reduced in semi-solid 8p medium or by the use of feeder plates. Formation of embryo-like structures was induced from the protoplast-derived callus on media supplemented with IAA and BA. These structures formed roots but plants failed to develop. Protoplasts could be isolated from leaves, but not from stems or petioles. The leaf protoplasts failed to divide.

List of abbreviations: BA = benzyladenine, 2,4-D = 2,4-dichlorophenoxyacetic acid, ABA = abscisic acid, IAA = indole acetic acid.

Introduction:

While there are a few reports describing apple protoplast isolation, in no case have the protoplasts divided to form colonies. Anderson et al. (1979) isolated protoplasts from 'Golden Delicious' fruit parenchyma for use in characterizing ethylene production. Yamaki (1981) used apple cotyledon protoplasts to study the role and localization of sorbitol dehydrogenase. Jazem et al. (1983) isolated protoplasts from leaves of several apple cultivars but colony formation was not observed.

Protoplast technology might be useful for the solution of practical problems involving apple cultivar improvement. The transfer of disease resistance and other traits via protoplast fusion may be of value once the technical problems of protoplast isolation and regeneration are solved. We describe a procedure for isolating protoplasts which will divide to form callus and embryo-like structures.

Materials and Methods:

Whole Plant Material: Young leaves of *Malus x domestica* cv. 'Jonathan' were harvested from orchard or greenhouse material and surface sterilized with 10 to 20% Chlorox® containing one drop of Tween® 80/100 ml. The solution was agitation for 10 to 20 minutes. Leaves were washed 3 times with sterile distilled water, 5 minutes each. Shoots were placed in water at room temperature to initiate growth and surface sterilized as described above for leaves. The shoots tips were placed on a modified MS (Murashige and Skoog, 1962) medium and were grown under ca. 3000 lux of cool white fluorescent light (ITT, F48T12/CW) with 16 hour daylight at a temperature of 20 +/- 2°C (Skirvin and Chu, 1979). The axillary shoots which developed were sub-cultured every three weeks. Leaves, petioles, and stems were harvested for protoplast isolation.

Callus and Suspension Cultures: Callus cultures were initiated from leaves on MS medium supplemented with 2,4-D (1.0 mg/l) and BA (8.0 mg/l). The cultures were grown as previously described (Skirvin and Chu, 1979) and subcultured every three weeks. The fastest growing and most friable calli which developed from leaf tissue were transferred into 50 ml liquid MS medium in 125 ml flasks, supplemented with 2,4-D (0.4 mg/l) to initiate suspension cultures. The cultures were grown at 27°C in low light and shaken at 130 rpm on a New Brunswick rotary shaker. Cell suspensions were maintained by transferring 1 ml packed cell volume into 50 ml of fresh medium weekly.

Protoplast Isolation and Culture: Protoplasts from leaf, stem or petiole tissue were isolated by incubating transverse sections (<1.0 mm) for 15 to 24 hours in an enzyme solution containing 0.5% Macerozyme R-10 and 2% Cellulase Onozuka R-10 (Kinki Yakult Mfg. Co., Ltd., Japan) in CPW solution (Frearson et al., 1973) with 9% Mannitol (w/v) pH 5.7.

Protoplasts were isolated from leaf, stem, or petiole tissue to study the role and localization of sorbitol dehydrogenase. Jazem et al. (1983) isolated protoplasts from leaves of several apple cultivars but colony formation was not observed. Protoplast technology might be useful for the solution of practical problems involving apple cultivar improvement. The transfer of disease resistance and other traits via protoplast fusion may be of value once the technical problems of protoplast isolation and regeneration are solved. We describe a procedure for isolating protoplasts which will divide to form callus and embryo-like structures.
semi-solid agar media were transferred to solid MS with sucrose (30 or 40 g/l), 2,4-D (0.4 mg/l) or to 8p medium with IAA (1.0 mg/l), BA (1.0 mg/l) with no 2,4-D.

Osmoticum and cell density studies utilized the semi-solid 8p medium only.

Differentiation Studies: Approximately 0.5 cm³ of protoplast-derived callus which had been subcultured on MS with 0.4 mg/l 2,4-D, was then subcultured to MS medium with IAA (1 mg/l) and BA (1 mg/l), denoted MSF, with and without 150 mg/l polyvinylpyrrolidone (PVP-40) two times at one week intervals. The callus was then transferred to the same medium plus 0.2 mg/l abscisic acid (ABA). In one week the callus was transferred to a medium containing 0.2 mg/l ABA, 0.5 mg/l IAA, 0.5 mg/l BA, 5% coconut milk and 20 g/l glucose and transferred weekly thereafter. Root formation was induced by subculturing on Nitsch and Nitsch (1969) medium with glucose (20 g/l), IAA (0.1 mg/l) and BA (0.08 mg/l) with no coconut milk.

Results and Discussion:

Attempts at isolating protoplasts from 'Jonathan' petiole or stem sections were unsuccessful; however, protoplasts could be obtained from in vitro cultured leaves but these failed to grow. Pretreatments such as dark incubation and incubation on culture medium (data not presented here) did not improve the yield or culturability of protoplasts from whole plant material.

In these studies, the best source of protoplast material was young and fast growing suspension cultures. Large numbers of healthy protoplasts could be isolated which, in turn, divided to form colonies.

Purified protoplasts plated in liquid medium (Figure 1) developed cell walls in 5 to 10 days, however, budding was frequent and only a few cell divisions could be observed in MS and 85 media while 8p gave better results. Division in semi-solid medium could be obtained between 2 to 3 days (Figure 2).

Sustained growth could be obtained only by transferring protoplast-derived colonies from the 8p medium to either feeder plates or onto solid MS medium (0.4 mg/l 2,4-D) (0.8% agar) + 20 g/l glucose. The feeder plate method (Fraley et al., 1983) was found to be effective in the rapid establishment of colonies from 'Jonathan' protoplasts.

The division efficiency of the protoplasts in liquid 8p medium was initially poor. To improve the efficiency, various levels of glucose were added. Freshly separated protoplasts ruptured when transferred to a medium containing less than 50 g/l glucose. Those grown in 50 to 90 g/l glucose survived and maintained their spherical shape. At 70 g/l, the protoplasts developed a cell wall in less than 7 days. Some cells elongated and a few began to divide. However, if the cultures were maintained at 70 g/l glucose, the total number of divisions was low (Figure 3).