ACIDIFICATION OF CULTURE MEDIA BY ISOLATED
PLANT PROTOPLASTS

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SUMMARY

We report the observation of a decrease in media pH caused by isolated protoplasts after
alkalinization of the culture medium. Additions of other cations or anions did not produce
a similar response. Dinitrophenol immediately terminated the response. The acidification
response was larger in suspensions that were cultured in auxins. The responses of proto-
plasts to changes in external pH may provide a means for assessing viability of nondividing
protoplasts.

Key words: plant protoplasts; H⁺ ions; viability.

INTRODUCTION

Enzymatically isolated plant protoplasts have
gained wide acceptance as a vehicle for the altera-
tion of the genetic composition of plant cells (1). Lacking a cell wall, protoplasts are amenable to
various strategies for the incorporation of foreign
DNA; and under proper culture conditions cer-
tain plants can be regenerated from a single proto-
plast. However, protoplasts from many plant
species fail to develop in culture and much effort
has been expended to alter isolation techniques and optimize culture media to improve the fre-
quency of cell division in these plants (2). The
major problem with this approach, however, is
that the relative effectiveness of a particular ex-
perimental protocol to enhance development is
scored on its ability to promote the “all or
nothing” response of cell division. Cytokinesis is a
complex, culminating event in cellular physiol-
ogy; thus, observation of division is a reasonably
clear indication that the cell is functional. How-
ever, lack of division provides few clues as to the
nature or severity of cellular malfunction that
precludes the division process.

For this reason a number of methods are used to
determine the viability of cultured cells. These
include: visual assessment of integrity, shape, and
color (3), response to osmotic changes (4), rejec-
tion of dyes (5), and enzymatic conversion of dyes
(6). Of these, some may be criticized on the basis
that they are too subjective (visualization of
integrity, shape, color) and others on the basis
that the parameter on which the evaluation is
based may be a passive characteristic of a cell. For instance, both responses to osmotic changes
and rejection of large dye molecules depend on the
permeability properties of the plasma membrane,
and conversion of a dye to a fluorescent analog de-

cends on the size and activity of an esterase pool
in the cytoplasm.

During the time in which investigators have ex-
plored the potential for using protoplasts to gain
access to the plant genome a growing body of
information has developed concerning the phy-
siologic behavior of protoplasts. Protoplasts from
a variety of plant sources have been used to ob-
serv e responses to osmotic changes (7), ion trans-
port (8), respiration (9) and molecular synthesis
(10). In this paper we report the measurement of
another physiologic parameter, the energy-
dependent efflux of hydrogen ions. Because the
technique involves recording pH changes from a
small volume of suspended mesophyll protoplasts
we feel that it may provide a rapid and direct
method for assessing both the viability of proto-
plasts in culture as well as the responsiveness of
protoplasts to physiologic stimuli.

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MATERIALS AND METHODS

Protoplast isolation. Oat protoplasts were obtained from the mesophyll of 7 to 11-d-old leaves of *Avena sativa* (var. *Windsor*). The leaves were surface sterilized, the abaxial epidermis removed with forceps, and incubation carried out in B-5 media (11) with 1% cellulysin wt/vol (Calbiochem, La Jolla, CA). The B-5 medium, also used for culture, was modified from the original formulation in that it contained a carbon source/osmoticum of 0.55 M glucose and 0.12 M sucrose. The pH of the media was adjusted to 5.5. The auxin, 2,4-dichlorophenoxyacetic acid at a concentration of 2 mg/l, was present in the media with hormones and omitted from the media without hormones. After a 2 to 4 h incubation in the enzyme solution at 25°C, protoplasts were collected by centrifugation at 500 ×g for 5 min and washed twice with the appropriate B-5 media. The final pellet was resuspended in B-5 media with or without hormones at a concentration of 10⁶ protoplasts/ml.

Tobacco protoplasts were obtained from the mesophyll of 40 to 100-d-old *Nicotiana tabacum* (var. *xanthi*) after surface sterilization, removal of the lower epidermis, and incubation for 18 to 24 h in a media of 0.7 M mannitol, 2% cellulysin, and 0.4% macerase (wt/vol) at pH 5.7. Collection of protoplasts was made by filtering through a 61 μm stainless steel filter followed by three centrifugation steps at 500 ×g for 5 min in N and T media (12), with or without 3 mg/l α-naphthaleneacetic acid and 6-benzyladenine. The final pellet was resuspended in N and T media (pH 5.7), with or without hormones, at a concentration 10⁶ protoplasts/ml.

pH Measurements. The pH was measured with an antimony-film microelectrode (Transidyne General Corp., Ann Arbor, MI) inserted into a 30 μl drop of protoplast suspension. An alkalinization step was administered by addition of 5 to 15 μl of the suspension media at pH 8.5. Additions of 1 mM DNP (2,4-dinitrophenoD, 100 mM KCl, and 100 mM NaCl also were made by inserting 5 to 15 μl amounts into the drop of protoplast suspension.

RESULTS AND DISCUSSION

Acidification response. Alkalinization of a microdrop of the suspension medium without protoplasts produced an instantaneous change in pH with no subsequent change in pH after 10 min. When a 30 μl drop of 0 to 12 h oat or 4 to 18 h tobacco protoplasts was subjected to the addition of basic media there was an immediate increase in media pH, followed by a slower decrease in pH. We have termed the slower decrease in pH, in response to the alkalinization step, an acidification and interpret it as a response of viable protoplasts to the increase in external pH. The responses of both oat and tobacco are qualitatively similar (Fig. 1). The acidification began within 30 s of the media alkalinization and proceeded quickly (0.064 pH/min in tobacco without hormones) before leveling off after about 5 to 7 min.

Although the magnitude of the responses probably was affected by proximity of protoplasts to the electrode and the density of protoplasts, under all conditions we observed larger acidification responses in oat protoplasts than in tobacco protoplasts. After the first acidification response, subsequent addition of the alkaline media produced similar but smaller responses by the protoplasts.

![Fig. 1](image-url)