SIMPLE STORAGE OF PLANT CELL CULTURES IN LIQUID MEDIA

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Introduction

Plant cell suspension cultures are mostly used for the biochemical investigation of cell physiology, growth, metabolism and for large or medium scale production of secondary metabolites. For such purposes normally suspension cultures are used which are propagated in Erlenmeyer flasks on a gyratory shaker and are maintained by regular subculturing after short intervals (usually 1 or 2 weeks). For such use callus cultures are also maintained under a mineral oil layer which reduces the subculture frequency. However, from the callus phase it takes additional time to establish a new suspension culture again and this new suspension may exhibit changed biochemical traits.

For storage purposes, several strategies have been developed, all aiming at reduction of labour and costs of maintenance, while preserving all properties of the cells. Long-term conservation of suspension cultures is usually successful by cryopreservation [1,2]. However, it requires special and expensive equippment and is not suitable for routine work as reactivation of cells requires prolonged incubation and has to pass a callus phase. Therefore, for practical work there is a need for an easy and a time-saving method that could allow the storage of actually investigated suspension cultures for relatively longer terms.

A simple procedure is described here to maintain suspended plant cell cultures for medium-terms. With this method suspension cultures of *Agrostis tenuis*, *Nicotiana tabacum*, *Nicotiana chinensis*, *Oryza sativa* and *Solanum marginatum*, could be maintained viable under reduced temperatures for more than 4 months without transfer to fresh medium [3,4]. The suspension cultures were kept without shaking at 10C (in dark or in dim light at about 50 lux) in screw-cap plastic bottles (tissue culture flasks with membranes, Fig. 1) that permitted sterile air to pass through easily. Some effective adsorption materials or stabilizers such as activated charcoal, gelatine, glutamic acid, starch, sugar etc. were added to the cell suspensions. In the case of sensitive microorganisms and unicellular green algae such substances have already shown protection during maintenance and against known freezing and drying injuries [5,6,7]. In the case of plant cell suspensions only with *Nicotiana tabacum* the addition of 0.01% charcoal + 1.0% gelatine resulted in slightly improved cell survival as compared to the cells grown without additives. However, the simple maintenance conditions used proved effective to prolong the storage life of suspension cultures as compared to control samples grown under normal conditions. The stability of the stored cells usually appeared unchanged, as evident from HPLC-fingerprints) and from growth characteristics after re-establishment of the cultures.

The method is convenient and the equipment described here is easily available. It is useful for routine work as during such long-term maintenance a ready inoculum can continuously be obtained (from the same batch of cell suspension) for immediate use.

Material and Methods

Preparation of cultures

Plant cell cultures are grown under normal conditions using routine methods. The suspension cultures should be propagated in Erlenmeyer flasks on a gyratory shaker (100rpm) at 24C under continuous light at 600 lux. These should be transferred to fresh medium after every 7-10 days.

Storage of cell suspensions

For storage 100 ml of the cell suspension from the early exponential growth phase is transferred to a 650 ml screw caps plastic bottle with membranes (Art. no, 6601755, Greiner GmbH, D-72636 Frickenhausen, Germany) which permits sterile air to pass through (see Fig. 1). Depending on the volume of cell suspension to be maintained, smaller 250 ml bottles (Art. no, 658175) with 43 ml cell suspension can also be used. However, the surface to volume ratio should remain the same as with big bottles. These bottles are supplied as tissue culture flasks with closure secure against contamination. Alternatively wide mouth Erlenmeyer flasks with new cotton plugs can also be used. The volume of the added cell suspension in the vessels should lead to a high surface to volume ratio in order to assure sufficient oxygen supply for the cells. The suspension cultures are stored without shaking in the dark and at a reduced temperature of 10C. To prolong cell survival it is recommended to add some stabilizers such as 0.01% activated charcoal + 1.0% gelatine, or doubled to tripled concentration of sugar.

Recultivation and viability assay

For recultivation an aliquot of 5ml of the stored cell suspension is transferred with a sterile pipette from the plastic bottles to 100 ml Erlenmeyer-flasks containing 25 ml of medium. The cells are cultured in these flasks for 1-2 weeks at 24C on a gyratory shaker (100rpm) under continuous light (600 lux). To determine regrowth on solid media 1ml of cell suspension is placed on a Petridish (60mm dia.).

For a perodic viability assay of the preserved cultures, the ability of regrowth is determined. After appropriate intervals of storage (every 2-3 weeks or longer depending on the sensitivity of the cell suspension) aliquots of the stored cells and the control samples (grown under normal conditions) are recultivated in equal amount of liquid media. After a definite period of growth (about 1 week when the cells are in exponential growth phase), the cells are harvested by suction filtration. The dry weight of the re-grown cells is determined which is taken as a measure of the inoculum quantity (the cell density developed from the surviving cells during storage). The resulted cell mass from the stored cells is compared after different storage intervals and with the control samples.

Staining of a very small aliquot with fluorescein diacetate (FDA) on a microscope slide and counting of a fraction of positive cells under the fluorescence microscope is also an easy tool to obtain a quick impression of the viability of the cells [8].

Stability checking

For stability checking by HPLC, 2g (fresh weight) of the harvested cells are extracted in boiling methanol for one hour. 20µl of the resulting extracts are analyzed by reverse phase high pressure liquid chromatography (HPLC). The result of the chromatographic procedure is a characteristic "fingerprint" of cell metabolites. Methanol is used as an organic solvent. Substances are eluted by a standard gradient system which runs from 0% methanol to 100% methanol within 30 minutes. The majority of cell cultures show most characteristic "fingerprints" when a slightly acidic solvent system is used. Comparability of such "fingerprints" has to be assured by extracting cells from the same growth stage of the cell culture.

High pressure liquid chromatography (HPLC) protocol

Column :	Nuleosil 100-7 C_{18}, 7µm, 4 x 100mm
Flow rate :	1ml/min
Solvent A :	Water + 0.1ml H_3PO_4
Solvent B :	Methanol + 0.1ml H_3PO_4
Gradient :	0min : 100% Sol. A / 0% Sol B
	5min : 100% Sol. A / 0% Sol B
	35min : 0% Sol. A / 100% Sol B
	40min : 0% Sol. A / 100% Sol B
	45min : 100% Sol. A / 0% Sol B
Detector wavelength :	280nm

Notes:

Plant cell suspension cultures are normally cultivated in Erlenmeyer-flasks on gyratory shakers. At normal growth temperature when gyration of these flasks is not maintained, plant cells normally settle down very fast. Under such conditions a lack of oxygen supply leads to cell death or at least a cell damage occurs within few hours for most species. However, during a systematic study we observed that such plant suspension cultures are able to survive at reduced temperatures and without gyration much longer (more than 16 weeks). The influence of doubling or tripling of the sugar content of the media (up to 6% sucrose or glucose) tested showed that one of the *Nicotiana tabacum* lines resulted in a re-established culture even after 26 maintenance free weeks at 10C (with 6% sucrose instead of normal 3% concentration), For *Cinchona robusta* cell suspensions (recalcitrant to any tested cryopreservation protocol) after 8 weeks of standing culture at 10C survival was still high, but no regrowth was obtained after 16 weeks. Nevertheless after 16 weeks with FDA staining 60% "viability" was detected. This indicates that optimized conditions may improve the regrowth.

Fig.1 Tissue culture flasks with membranes for contamination free aeration

The onset of regrowth normally occured within a few days and after one week normal growth parameters were restored. Nevertheless with *Oryza sativa* it took about 2 weeks before rapid cell growth resumed and the re-establishment of the normal growth pattern took one or two subculture stages.

One should be prepared that during maintenance of the standing culture much of the medium water may evaporate. Depending on the dryness of the air in the incubator or room climate, up to 1/3 of the volume may disappear within 16 weeks. The bottles or flasks should however, not be sealed or closed air tight to minimize evaporation because of disturbances in the aeration. To avoid this one may start with more volume if frequent samples of inoculum are required.

To optimize the maintenance conditions in one's own laboratory and to establish a maximum storage period for the cell suspension under study it is recommended to conduct perodic viability assay and the stability check of the chemical traits of the preserved cultures. To check whether cell metabolism is affected by these storage conditions or the added substances, methanolic extracts should be analyzed by reversed phase HPLC. The analysis will show characteristic peak patterns (which need not be identified chemically). The storage conditions and reduced incubation temperature should not cause any changes in the characteristic of such peak patterns.

For rice cultures regeneration experiments were carried out. Dependent on the amount of extra sugar during standing culture, regeneration was higher or lower than in the controls, but high regeneration capacity appeared to be correlated with a high incidence of aberrant plants : 20% was albino. Therefore it is recommended as with other preservation methods to check for the occurrence of somaclonal variations.

Although the stabilizers showed no remarkable increase in survival of few of the tested cultures, they might cause some kind of protection in other cultures and result in better stability and viability of such cell suspensions at least over few passages.

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