Initiation, growth and cryopreservation of plant cell suspension cultures

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Methods described in this paper are confined to *in vitro* dedifferentiated plant cell suspension cultures, which are convenient for the large-scale production of fine chemicals in bioreactors and for the study of cellular and molecular processes, as they offer the advantages of a simplified model system for the study of plants when compared with plants themselves or differentiated plant tissue cultures. The commonly used methods of initiation of a callus from a plant and subsequent steps from callus to cell suspension culture are presented in the protocol. This is followed by three different techniques for subculturing (by weighing cells, pipetting and pouring cell suspension) and four methods for growth measurement (fresh- and dry-weight cells, dissimilation curve and cell volume after sedimentation). The advantages and disadvantages of the methods are discussed. Finally, we provide a two-step (controlled rate) freezing technique also known as the slow (equilibrium) freezing method for long-term storage, which has been applied successfully to a wide range of plant cell suspension cultures.

INTRODUCTION

Plant cell culture is an important tool for basic studies on plant biochemistry and molecular biology, and available methods include regeneration of differentiated cultures (the whole plant and organ cultures; shoots, roots and adventitious roots) or dedifferentiated cultures (e.g., calluses, cell suspensions and protoplasts)[1]. Differentiated cultures can be useful for studying tissue-specific biosynthetic pathways, which are not always expressed in cell suspension cultures. *In vitro* dedifferentiated plant cell suspension cultures are more convenient for the large-scale production of fine chemicals in bioreactors and for the study of cellular and molecular processes as they offer the advantage of a simplified model system for the study of plants. Cell suspension cultures contain a relatively homogeneous cell population, allowing rapid and uniform access to nutrition, precursors, growth hormones and signal compounds for the cells.

This protocol covers all steps from plant to cell suspension culture, and includes callus initiation from which cell suspension cultures can be obtained. The maintenance and methods for characterization of growth of cell cultures are described. Cell suspension cultures are maintained by subculturing using inocula prepared by weighing cells, transferring pipette-measured volumes of cell suspension or pouring cell suspension into fresh medium. Growth is measured in a time course (e.g., every 1–2 d for 2–4 weeks), which can be carried out using invasive methods such as fresh-weight (FW) or dry-weight (DW) cells per volume, or by non-invasive methods such as the determination of loss of weight (dissimilation curve) or cell volume after sedimentation (CVS). A highly successful technique for cryopreservation of plant cells is described; the two-step (equilibrium) freezing technique includes pretreatment, freezing and thawing. **Figures 1** and **2** provide schematic overviews of the steps involved.

Initiation of plant cell suspension culture

Plant cells are totipotent, which means that, in principle, every cell contains all genetic information to grow a new plant. Plant cell and tissue culture exploit this characteristic for, e.g., micropropagation of plants or to produce plant compounds by means of cell cultures. Because of the totipotency, in principle one may initiate cell cultures from any part of the plant or germinating seeds by growing an explant on a suitable medium supporting cell growth. Here we will describe the commonly used methods for initiation of cell cultures.

A plant cell suspension culture is a sterile (closed) system normally initiated by aseptically placing friable callus fragments into a suitable sterile liquid medium[2,3]. A callus is initiated from pieces of tissue cut from surface-sterilized plants. The explants are placed on different solid growth media; in successful media, callus tissue will appear on the explants in the course of 2–6 weeks. A callus is subsequently cut from the explant and further subcultured. Callus material can be inoculated in liquid medium (in flasks), and through continuous shaking cell suspension cultures are obtained. The whole process—from plant to a stable cell suspension culture—may take 6–9 months. The cells eventually become stabilized[4] in the 'new' regime, after which products accumulated in the first months after initiation may no longer be accumulated. Similarly, effects of medium changes can also be observed only after several subcultures[5] (see **Fig. 3**). Therefore, it is essential to let a cell culture first stabilize before studying the production of desired compounds.

Aggregates or clumps are commonly formed in plant cell suspension cultures,[2] as a certain degree of cell aggregation is often required for cell growth and production of metabolites[6]. This protocol also includes some techniques to provide a fine cell suspension culture, by filtering, pipetting/decantation, or by addition of a low concentration of pectinase[2]. As pectinase can disrupt the outer plant cell walls, a low concentration of pectinase will break up the aggregates by dissolving the intercellular pectins. As a model system, a fine cell suspension culture is preferable to a mixture of different sizes of cell aggregates as it is more convenient to work with (e.g., in subculturing) and a more homogeneous system provides higher reproducibility in experiments. On the other hand, aggregation may in some cases improve product formation; e.g., a positive

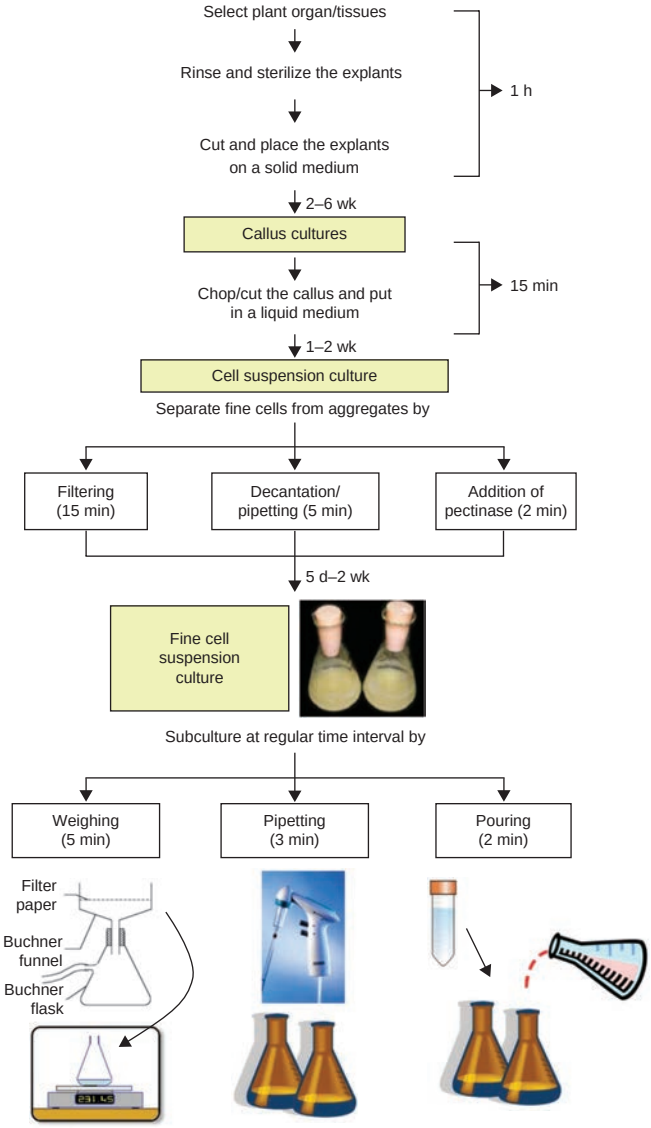

Figure 1 | Scheme of the procedure of initiation and maintenance of plant cell suspension cultures.

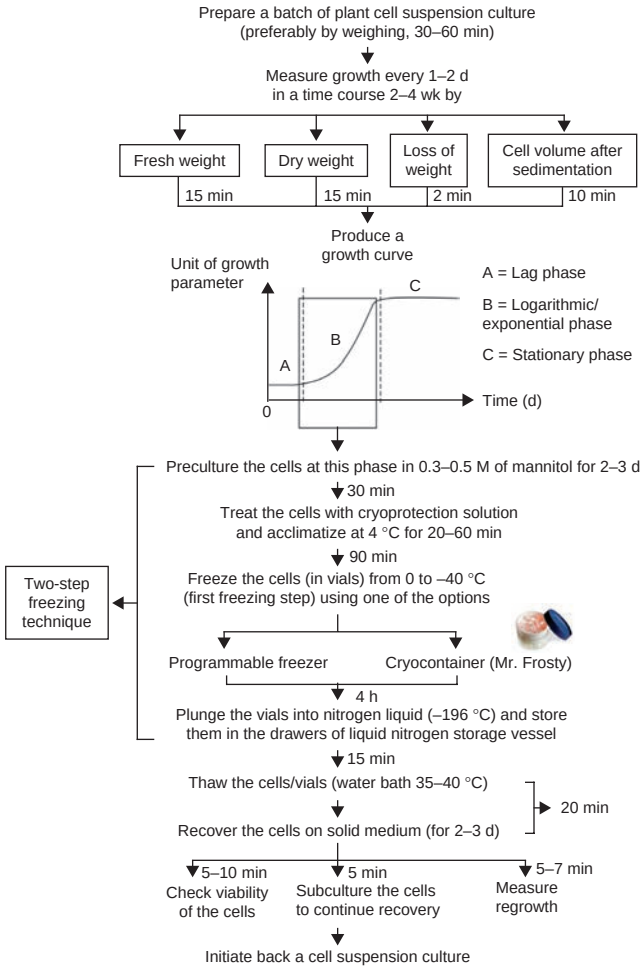

Figure 2 | Scheme of the procedure of growth measurement and cryopreservation of plant cell suspension cultures.

correlation between cell-aggregate formation and the production of anthocyanins and other phenylpropanoid metabolites was observed in a strawberry cell suspension culture[7]. Cell suspension cultures maintained in flasks (discussed in this protocol) are called batch cultures[2]. Continuous cultures of plant cells in bioreactors, in which a steady state is achieved by continuous collection of biomass and replacement with fresh medium, have been used to study effects of various limiting factors in the medium[8,9].

Maintenance of plant cell suspension culture

Maintenance of cell suspension cultures refers to the provision of fresh medium to cells at regular intervals in order to keep the cells alive and to prevent browning and loss of viability. There are three ways of subculturing, and experimental details for each are provided in the PROCEDURE. The three methods are as follows:

- *Weighing cells:* collect cells and inoculate a certain mass of these cells into a certain volume of fresh medium. This technique has the advantage that the cell density is similar for every subculture, and thus results in better reproducibility; however, the weighing step increases the chance of contamination.
- *Pipetting cells:* transfer by pipetting/pouring a determined volume (ml) of cell suspension into a certain volume of fresh medium. This approach is not as accurate as weighing cells because of the problem of nonhomogeneous cultures. It requires continuous gentle shaking of the flask with one hand while pipetting with the other in order to have a representative group of cells from the different sizes of cell aggregates present in the culture.
- *Pouring cell culture:* add a certain volume of fresh medium into the cell suspension culture and distribute this among more flasks. Compared with weighing or pipetting, the pouring technique is simpler (fewer tools used), faster and has less chance of contamination, but low reproducibility.

These three methods obviously have different levels of precision. For highest reproducibility of the inoculum density, the first one is preferred. The time interval required for subculturing depends on the growth cycle of the culture. The inoculum size has an influence on the growth curve. The smaller the inoculum size, the longer the lag phase, and below a minimum amount (which can be different for each line) the culture will not grow[2]. A larger inoculum leads to an earlier stationary phase[10] (see **Fig. 4a**). By always subculturing at the end of the logarithmic growth phase, the largest biomass production per time will be achieved. Subculturing of cells that

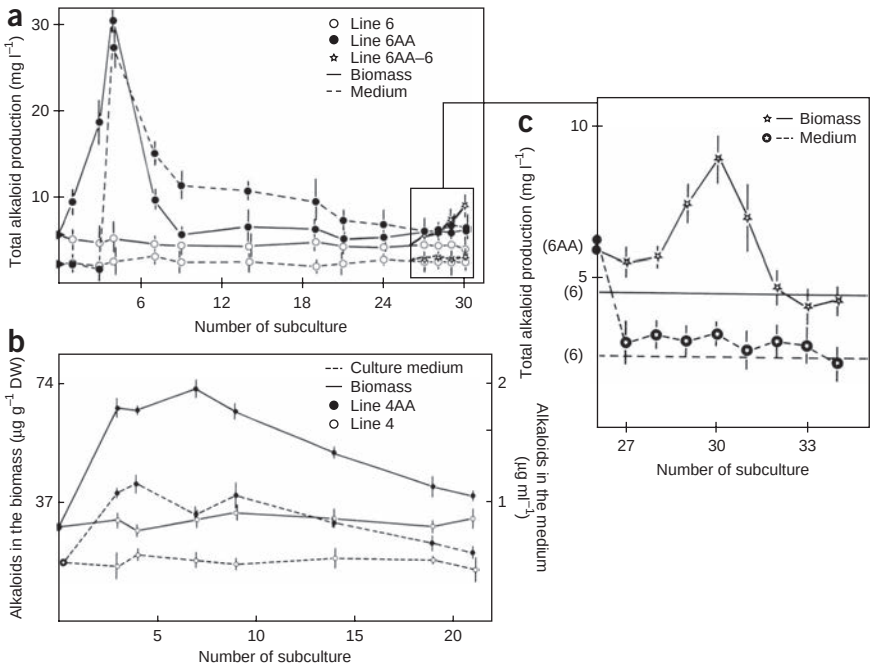

Figure 3 | Stabilization of cell suspension culture after change of 2,4-D (line 6) to NAA (line 6AA) as auxin in maintenance medium; the production of alkaloids in *Tabernaemontana divaricata* cell suspension cultures in 1 year. The alkaloid content was always determined on day 14 of the growth cycle (data are shown with the standard error for four determinations). (**a**) Alkaloid production in line 6 and line 6AA are shown. (**b**) Alkaloids in line 4 and line 4AA are shown. (**c**) Alkaloid content in line 6AA in cells (star symbol) and in the medium (circle symbol) after changing back to medium 6 during the first eight subculturing processes. In the case of line 6AA (**Fig. 3a**), alkaloid accumulation increased and reached the highest level (630% higher than in the original cell line) after subculturing twice from the initial (new) medium; subsequently it decreased and a stabilization process seemed to occur after a prolonged time of maintenance. After stabilization, the alkaloid level was still a bit higher than that found in cells grown in the original medium; thus, plant cells maintain a certain level of secondary metabolites depending on the nature and concentration of growth regulators in the medium[5]. Data reprinted with permission from reference 5.

have entered the late stationary phase will result in longer lag phases because of lower cell viability. Consequently, the subculturing regime depends on the goal[11], e.g., optimal cell viability, efficiency in maintenance, or use in further experiments to optimize biomass levels, production of proteins, RNAs or secondary metabolites[11].

Growth measurement of plant cell suspension culture

Measuring growth parameters is important if reproducible growth cycles of batches of cell suspension cultures are to be achieved, either for experiments in the laboratory or in industry to ensure the reproducibility of production. However, there is no uniform and clear definition of growth of plant cell cultures as it can be related to the number of cells, the cell mass, the volume of cells and so on[10]. Consequently, cell growth can be measured by several methods based on different parameters, e.g.:

- *The FW and DW of cells.* These methods require harvesting cells to determine the DW or FW of cells per volume of cell suspension culture; they are thus invasive methods. These are the most often used growth parameters, which can also be expressed in, e.g., biomass doubling time. For examples of growth curves based on FW, see **Figure 5a** (ref. 12) and **Figure 6a** (ref. 13) and for those based on DW, see **Figure 6b** (ref. 13) and **Figure 7** (ref. 14).
- *The number of cells.* Cell division increases the number of cells; this parameter can be used to measure the growth of cell suspension culture through a time course. Counting the number of cells per volume of suspension can be done for fine cell suspension cultures with a hemocytometer; however, cell suspension cultures containing larger cell aggregates require some treatments to break up the aggregates before counting the cells[2].
- *Packed cell volume (PCV).* An increased number of cells elevates the volume of cells occupied per ml of cell suspension culture; this is also a parameter with which to measure growth of cell suspension cultures. PCV is measured after cells are allowed to settle in a sterile conical tube. Centrifugation has been proposed to measure PCV; in this case, PCV is the volume of the pellet[2,15,16] as a function of the volume of culture normally expressed as a percentage.
- *Medium conductivity/osmolality.* Cells absorb nutrients from the medium, including the macro- and microelements that are supplemented in the medium as ions/salts. As a result, when cells grow, the concentration of ions decreases, which also decreases the medium conductivity. Thus, the conductivity of the culture medium is inversely proportional to the FW of cells[17]. The changes in medium conductivity are caused mainly by nitrate[16,17]. The methods can be made non-invasive, and in a bioreactor this can easily be measured continuously.
- *Determination of the concentrations of nutrients or metabolites*[16,18,19]. Some nutrients/metabolites in the cell suspension culture medium show correlation with growth in a single culture flask. For example, total nitrate[14] and sugar[14] levels in the medium can be used to measure growth (see **Fig. 7**)[14]. Some examples of the relationship between growth and accumulation of secondary metabolites in cells are shown in **Figure 6** and **Figure 8** (refs. 5,13).
- *Protein content.* Protein content can be measured per gram of FW cells or per PCV. This technique requires sampling/harvesting of cells and protein extraction. The total protein in the extract can be determined by different methods, e.g., by Bradford assay[20], which uses BSA as a standard[15]. In the lag phase, the protein content in cells increases slowly, subsequently increases exponentially and shows a decline when the cell biomass enters the stationary phase[15].
- *DNA content.* Nuclear DNA content correlates with ploidy; estimation of DNA content in relative units can be used for the determination of ploidy level and the detection of mixoploidy and aneuploidy[21]. During the passage through the cell cycle, the nuclear DNA content changes characteristically, allowing the determination of the growth phase of the cells[22].
- *Cell viability.* The numbers of living cells per volume of cell suspension culture can be used as a parameter to measure growth.

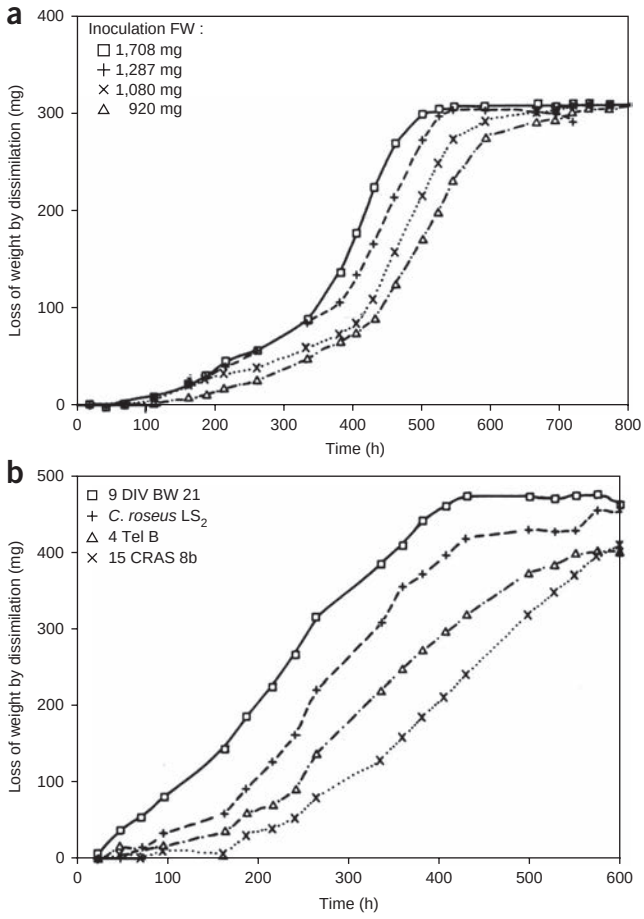

Figure 4 | Growth of plant cell suspension cultures determined by loss of weight by dissimilation. (**a**) Four dissimilation curves obtained from four flasks of *Tabernaemontana divaricata* cell suspension culture with different cell inoculum density showing that the higher the inoculum density of the cell culture, the earlier stationary phase is achieved as the carbon source is depleted earlier. In the logarithmic phase, most carbon/energy is used for cell division, at which time primary metabolism is particularly very active and delivers the most carbon dioxide (a high rate of dissimilation) as a result. In the stationary phase the energy in the cells is utilized more by other metabolic pathways, e.g., for cell enlargement/elongation and in many cases for production of secondary metabolites. (**b**) Dissimilation curves of four different cell suspension cultures are shown; *Tabernaemontana divaricata* (square), *Catharanthus roseus* (+), *Tabernaemontana elegans* (triangle), *Tabernaemontana crassa* (×). Curves show that each cell line/plant species has a different rate of growth. Data reprinted with permission from reference 10.

It can be assessed by staining the cells, e.g., with fluorescein diacetate (FDA)[23] or Evan's blue[24] followed by counting viable and dead cells under a microscope or by measuring absorbance with a spectrophotometer[24]. Another method of assessing cell viability is staining with triphenyltetrazolium chloride[2,25]; this colorless compound is converted by dehydrogenases, which are abundant in the mitochondria of living cells, to produce red formazan, which gives absorbance at 485 nm (ref. 2).

- *Loss of weight by dissimilation*[10,14]. This is a non-invasive method to characterize the growth of plant cell suspension cultures in a single flask. A dissimilation curve is produced by simply weighing the flask containing the cell suspension culture throughout a time course (see **Fig. 4** for an example). The principle is that the carbon balance in a cell suspension culture involves three pools: sugar in the medium, biomass (including stored carbohydrates), and CO_2 released and leaving the flask because of dissimilation of sugars for energy production in the cells. Control flasks containing medium only are used to correct losses due to water evaporation.
- *Cell volume after sedimentation*[12] is another way of determining growth in a single flask without killing the cells. The measurement is taken using a simple device that guarantees a fixed position of the Erlenmeyer flask and a quick reading of the height along the glass wall of the cell mass (sediment) by using a ruler fixed onto the device (see **Fig. 9**). The optimal angle for tilting the flasks is the angle that provides the largest vertical height of sediment cells, which is 60° for the commonly used 250-ml Erlenmeyer flasks. A linear correlation between CVS and FW was found for all cell lines observed[12] (see **Fig. 5c**). However, the accuracy of the CVS method appeared lower than that of FW obtained by harvesting and weighing cells. The CVS measurements are particularly less accurate at low FW[12] (measurement of volumes lower than 50 ml is less accurate than those of larger volumes) because of the shape of the Erlenmeyer flask (see **Fig. 10**). The size of cell aggregates also contributes to the accuracy of the CVS method; a relative deviation of up to 60% was observed at low FW (30 g l^{-1}) for a *Humulus lupulus* cell line with aggregates of ~5 mm and up to 25% for < 0.5-mm aggregates[12]. Despite this drawback, for a fast routine analysis without scarifying cells, the CVS method is recommended.

The methods of determining growth described in the PROCEDURE section are measurement of FW and DW, loss of weight by dissimilation and CVS.

Obviously, each of the different methods has certain disadvantages. In bioreactors, some non-invasive methods are easy to implement, whereas others require a collection of representative samples. In cell culture experiments using Erlenmeyer flasks, some of the methods require the harvesting of a whole flask to measure FW, DW, PCV, DNA and proteins. On the contrary, others require only a representative sample of the culture (viability) or medium (concentration medium components), only cell sedimentation and dissimilation curves are completely non-invasive, thus allowing the

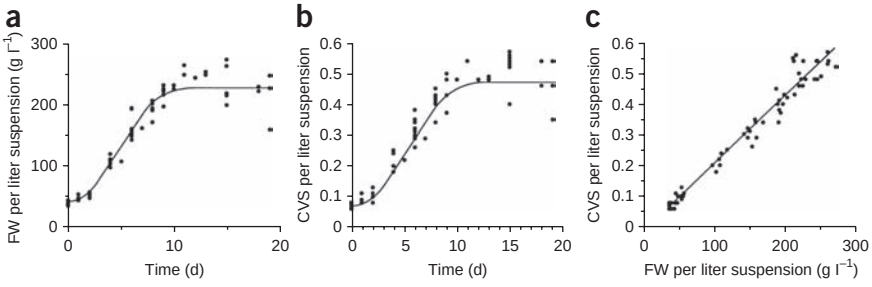

Figure 5 | Growth of a batch of *Cinchona* cell suspension cultures. (**a**–**c**) Growth determined by the fresh weight (FW) (**a**) and the cell volume after sedimentation (CVS) method (**b**). The CVS method can provide similar accuracy compared with the fresh weight (FW) method, as is confirmed by the linear relationship between CVS and FW shown in **c**. However, at small CVS (low cell suspension density) the CVS method has lower accuracy. Data reprinted with permission from reference 12.

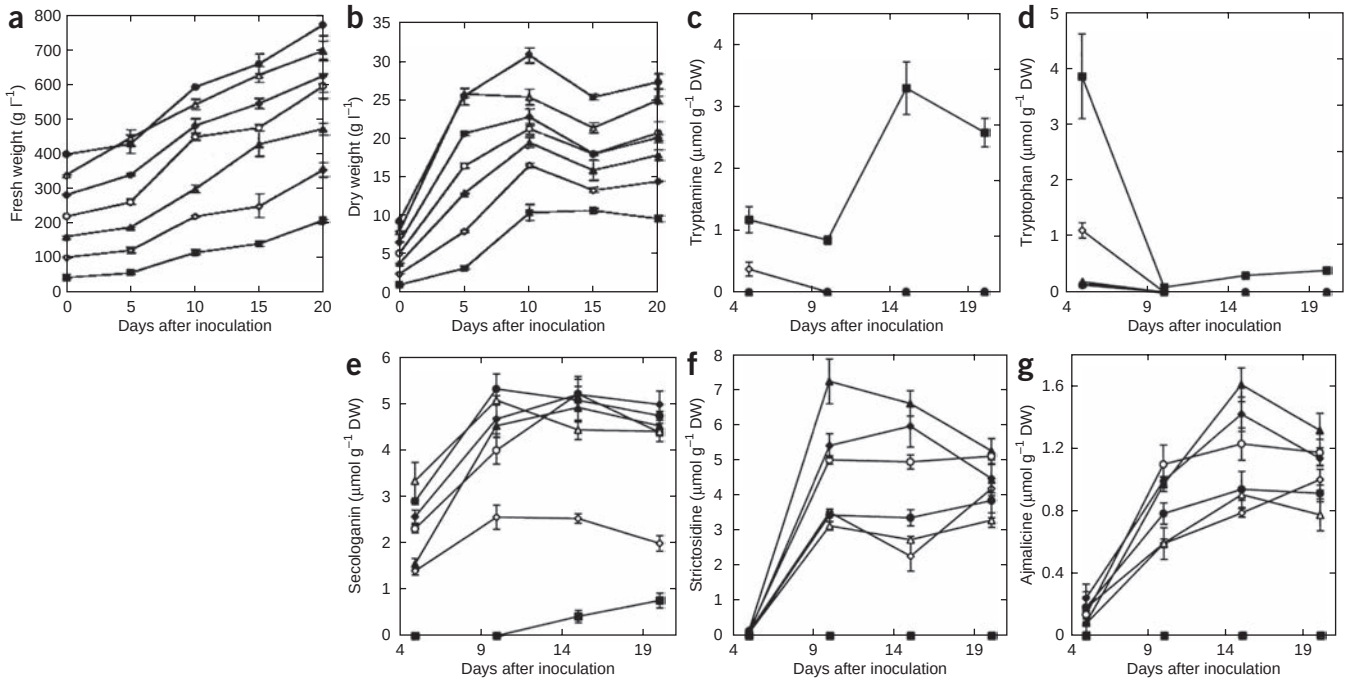

Figure 6 | The effect of inoculum density on cell metabolism (different accumulation of metabolites) of *Catharanthus roseus* cell suspension cultures. (**a**–**g**) Effect of inoculum size on cell biomass (**a**, fresh weight; **b**, dry weight) and accumulation of terpenoid indole alkaloid (TIA) precursors (**c**, tryptamine; **d**, tryptophan; **e**, secologanin) and some TIAs (**f**, strictosidine; **g**, ajmalicine) in a time course/growth cycle, detected by HPLC-PDA (data are shown with the standard error for four determinations). Inoculum size (g l^{-1}): 40 (black square), 100 (white diamond), 160 (black triangle), 220 (white circle), 280 (black diamond), 340 (white triangle), 400 (black circle). Low inoculum density cells produced relatively high amounts of TIA precursors (tryptophan and tryptamine), which were not detected in the higher inoculum density cells. Higher inoculum density cell cultures, however, produced a relatively higher level of secologanin and the alkaloid strictosidine and ajmalicine, which were (almost) not detectable in the lowest inoculum density cell cultures (40 g l^{-1}). Data reprinted with permission from reference 13.

measurement of a growth curve for each individual flask. In the other methods, for every point of a growth curve, two or more flasks need to be harvested. The medium sampling procedure itself may lead to changes in the environment of cells, as a reduction of volume of culture medium may lead to better oxygen availability or the opening of a flask may produce a different gas phase in the flask in the case of closures that limit gas exchange between flask and ambient environment, such as aluminum foil or silicone stoppers[10]. The advantages and disadvantages of each method are discussed in **Table 1**.

Cryopreservation of plant cell suspension culture

Prolonged subculturing has higher risks of contamination, is elaborate and costly. Moreover, it can cause loss of cell characteristics because of epigenetic instability[26] such as structural changes in chromosome morphology, ploidy, cell morphology and the biosynthetic capacity of a cell line. Cryopreservation (storage of biological material at ultralow temperatures below −120 °C, usually at −196 °C in liquid nitrogen) is the only method available for genetic conservation of animal or plant organ and cell cultures[27]. A recent review extensively discussed all aspects of cryopreservation of phytodiversity (plants, algae and cyanobacteria), particularly applying cryobiology theory to cryobanking practice[28].

The essence of cryopreservation is a controlled dehydration of cells to avoid formation of ice crystals in the cells during freezing and thawing, which is achieved by the addition of so-called cryoprotectants to plant cells/tissues. At present, there are three main techniques available for cryopreservation of plant cell cultures: controlled rate cooling, also called two-step/equilibrium (slow) freezing[29]; vitrification[30]; and desiccation with[31] or without[32] encapsulation in alginate beads.

- *The conventional controlled rate/two-step/equilibrium (slow) freezing technique* produces dehydration of cells by the formation of ice crystals (thus increasing the salt concentration) in the external medium during the slow cooling step (1 °C per min) to an intermediate low temperature (usually −30 to −40 °C). Subsequently, in the second step, quick cooling in liquid

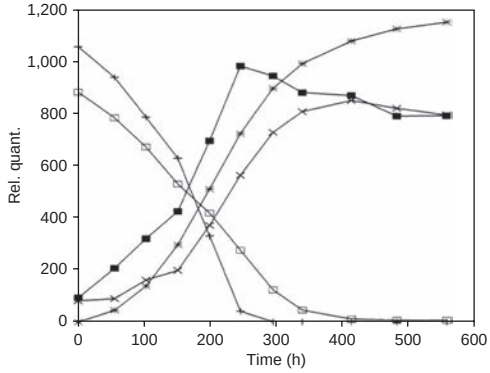

Figure 7 | Comparison of different methods of growth characterization (time-course experiment). DW (1,000 = 1 g per flask; shown by black square), carbohydrates in the medium (1,000 = 8 mmol monomer per flask; shown by '+'), nitrate in the medium (1,000 = 3 g l^{-1}; shown by white square), loss of weight by dissimilation (1,000 = 500 mg; indicated by '*'), and fresh weight (1,000 = 20 g per flask; indicated by '×'). The *Catharanthus roseus* cell suspension cultures were grown in 250-ml Erlenmeyer flasks containing 50 ml of medium. Data reprinted with permission from reference 14.

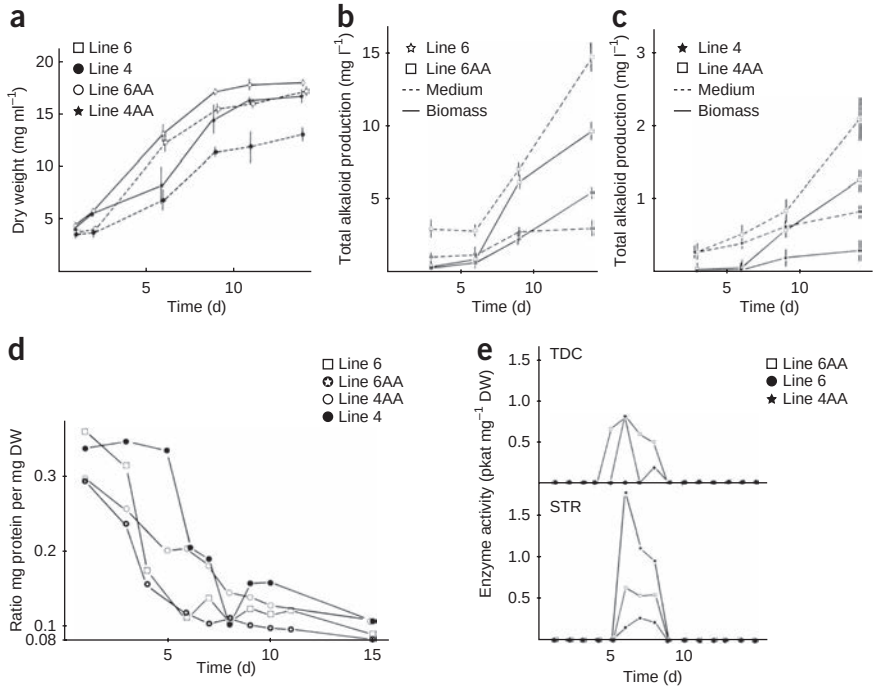

Figure 8 | Growth curves, alkaloid production and some enzyme activities of four alkaloid producing lines of *Tabernaemontana divaricata*[5] cell suspension cultures (data are shown with the standard error for four determinations). (**a**) Growth of *Tabernaemontana divaricata* cell suspension cultures determined by dry weight (white square/dashed line: cell line 6; black circle/dashed line: cell line 4; white circle: cell line 6 AA, star symbol: cell line 4 AA). (**b**) The total alkaloid production during the growth cycle of the highly producing cell lines of 6 (star symbol) and 6AA (white square), dashed line indicates in the medium, full line indicates in cells. (**c**) The total alkaloid levels during the growth cycle of the low producing cell lines of 4 (star symbol) and 4AA (white square), dashed line indicates in the medium, full line indicates in cells. (**d**) The total protein content of line 6 (white square), line 6AA (open thick-circle symbol), line 4 (black circle) and line 4AA (white circle). (**e**) Indole alkaloid pathway-related enzyme activities (tryptophan decarboxylase (TDC) and strictosidine synthase (STR)) in the cells of line 6AA (white square), line 6 (black circle) and line 4AA (star symbol) during the growth cycle. All cell lines were grown in MS medium but supplemented with different compositions of growth hormones, and were then transferred into MS medium containing NAA/BAP (1:1). Transferring the cells into the latter medium increased the alkaloid production considerably (**b** and **c**), but did not change the growth pattern (**a**). This study showed that NAA increased the production of secondary metabolites (see also **Table 3**) without losing the capacity for cell division (in combination with BAP), whereas 2,4-D was known to increase mitotic activity and commonly suppress the production of secondary metabolites[11]. In the lag phase (1–3 d), the total protein content was found to be higher in the cells grown in MS media with 2,4-D and kinetine/zeatin (lines 4 and 6) than that with NAA-BAP (lines 4AA and 6AA), but it rapidly decreased to reach a relatively low level after 5 or 7 d (**d**). TDC, which converts tryptophan into tryptamine (the precursor of terpenoid indole alkaloid/TIA from the shikimate pathway), showed activity only between 5 and 9 d (maximum level at 6 d), which was in the logarithmic phase (**e**). The same also occurred with strictosidine synthase (STR), the enzyme responsible for coupling tryptamine and secologanin into strictosidine, the first TIA in the pathway for precursor of many different TIAs. With regard to a change in the environment (media) in relation to the production of secondary metabolites, this study showed an example of the plasticity of plant cells with respect to the ability to differentiate into alkaloid accumulating cells when exposed to an appropriate stimulus. Thus, the capacity for high production of alkaloids was not lost, but can be restored by a change in growth regulators. Data reprinted with permission from reference 5.

nitrogen vitrifies the cell content (i.e., it forms a glass) without ice crystal formation. The two-step freezing technique consists of pregrowth (conditioning the culture to provide cells with a maximum of freeze tolerance by culturing cells into a so-called 'pretreatment' medium containing one or more osmotic agents for a few days), cryoprotection (application of 1 M to 2 M cryoprotectants to the cells), freezing (cooling process with an optimal cooling rate) and storage (to store the frozen culture at an adequate low temperature of at least −120 °C, or practically in liquid nitrogen)[29]. Thus, this technique requires a programmable freezer or an alternative tool to conduct a slow freezing process before storage. Cells are recovered by thawing (e.g., placing vials in a warm water bath for few minutes, e.g., 2 min at 35–40 °C) and post-thaw recovery on (preferably) semisolid medium with or without washing/removal of cryoprotectants.

- *Vitrification* is the process in which the material is exposed to a high (5–8 M) concentration of cryoprotectants at nonfreezing temperatures, resulting in dehydration of material (plasmolysis). Subsequently, an ultra-rapid cooling by direct immersion in liquid nitrogen ensures vitrification of the surrounding medium and cell content[30,33]. Plant vitrification solutions (PVS2 and PVS3)[34] have been successfully used for vitrification of (mostly) shoot tips of different plant species, but are not widely applied to plant cell suspension cultures. Cells are recovered by rapid warming in order to prevent devitrification (crystal formation) during thawing. This technique was first developed successfully for plant cell suspension culture in 1989 (refs. 35,36).
- *Encapsulation-dehydration* is another cryopreservation technique consisting of the encapsulation of plant cells in alginate beads, which are then incubated several days in a medium with increased sucrose concentration. Subsequently, the system is dried using silica gel or the airflow of a flow cabinet before transferring it to liquid nitrogen. The cells are recovered by slowly warming the beads. It was first developed for shoot tips[37], but was later applied to plant cell suspension cultures as well[31].

In the PROCEDURE, we present a two-step freezing technique for cryopreservation of plant cell suspensions (single cells and aggregates in liquid medium); it was developed based on a classical approach[38] and successfully applied in our laboratory for cell suspension cultures of several different species with a good survival rate, as determined by FDA staining and regrowth measurement[29,39]. However, the most convenient technique can be chosen by considering the available facilities, equipment, personnel, expertise and the plant/plant culture species and type[40]. It seems that a wider range of plant cell suspension cultures has been reported to undergo cryopreservation using the two-step freezing than with the other techniques[29,30,34,41]. The advantages and disadvantages of each technique are discussed in **Table 2**.

Figure 9 | Device to measure cell volume after sedimentation (CVS) as a characteristic of the growth of a cell suspension culture without sacrificing cells. The height of the cell mass along the glass wall represents the volume of cells (calibrated by measuring different known volumes of water). The measurement is taken after the cells are settled in a 250-ml Erlenmeyer flask tilted on the device with an angle of 60° for at least 5 min.

To improve survival rates, most research in cryopreservation has focused on the starting material, pretreatment/preculture, cryoprotection step and post-thaw treatment[30]. For example, the cell growth phase of the starting material is very important for the survival rate. Optimal conditions must be determined for each single cell line. Normally, exponentially growing plant cells are more tolerant to freezing than are cells in the lag or stationary phase, as these cells have the least volume, small vacuoles and contain relatively less water. For tobacco cells[42] and Bromegrass cells[43], late-exponential (early stationary) phase cells gave the best results, whereas for millet cultures[44] and *Taxus* cultures[39], late-lag phase or early exponential phase cells were better. On the other hand, the optimal growth phase of rice[45] and *Papaver*[46] is the mid-exponential phase.

Some methods can be used to determine the survival of post-cryopreserved cells, e.g., the viability test using FDA[23] or 2,3,5-triphenyltetrazolium chloride[25] staining, and regrowth measurement[39]. It is recommended that the FDA ratings be confirmed with the regrowth experiments[42], as in some studies FDA staining was not correlated with regrowth[47] (see **Fig. 11** for an example[39]). Genomic and metabolomic stability after cryopreservation can be determined by analysis of the relative nuclear DNA content using flow cytometry[39] and NMR-based metabolic profiling[48] (see **Fig. 12**). In the protocol, we describe the regrowth procedure and the cell viability test by FDA.

Experimental design and other practical considerations

Optimizing growth media. Various media may be used[1–3]. However, the best (most suitable) medium for an *in vitro* culture of, e.g., plants, plant tissues, plant cells and protoplasts can be different between species/genotype, and it also depends on the goal of the experiment. In principle, a medium for plant cell cultures contains a carbon source(s), macroelements, microelements, vitamins, growth regulator(s) and water.

- *Carbon sources.* Examples of carbon sources normally used for cell suspension cultures are sucrose and glucose. Other carbohydrates, e.g., fructose, can be used, but may result in slower growth, as commonly plant cells absorb glucose faster (after hydrolysis of sucrose) than fructose[14,49].
- *Macroelements (Mg, Ca, P, S, N and K).* These are required by plant cells in relatively large amounts (in the mmol range). Nitrogen should be added in both anionic and cationic forms, e.g., KNO_3, NH_4NO_3, $Ca(NO_3)_2$.
- *Microelements (Fe, Cu, Mn, Co, Mo, B, I, Ni, Cl and Al).* These are required in µmol amounts[1,2].
- *Vitamins (e.g., thiamine and inositol).* These are essential for many biochemical reactions, and can be added to the medium in several forms and concentrations.
- *Growth hormone(s)*[1–3,50]. These are normally required in growth or production media; however, the optimum concentrations for each plant species and even genotype have to be established experimentally. Moreover, different plant growth hormone(s) can cause different effects on the biomass and/or target metabolites in a cell suspension culture[5] (**Table 3**). The growth hormones most commonly used are auxins, which stimulate root formation, and cytokinins, which stimulate the formation of shoots. The most common auxins are indole-3-acetic acid (IAA, a natural auxin) and the synthetic auxins, 1-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D). Examples of cytokinins are kinetin, zeatin and benzylaminopurine. The range of concentrations of a growth hormone used in a culture medium is typically 10^{-7}–10^{-5} M (ref. 2). Either an individual auxin or cytokinin or a combination of both may be used. IAA and zeatin are thermolabile and cannot be autoclaved, thus requiring filtration for sterilization[2]. The pH of the medium is normally adjusted between 5.75 and 5.85 before sterilization of the medium.

The most common basal media used for plant cell cultures are Murashige and Skoog[51] (MS), Gamborg's B5-[52], Linsmaier and Skoog[53], and Schenk and Hildebrandt medium[54], which are further optimized with growth hormones for a specific plant species.

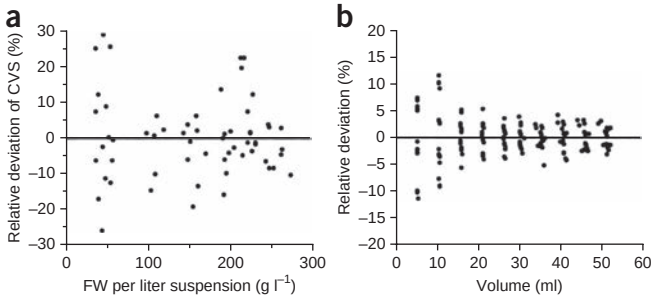

Figure 10 | Effect of biomass density and volume on the accuracy of the CVS growth measurement. (**a**) Relative deviation from the regression line of CVS at different FW for *Cinchona* cell suspension cultures. The influence of volume or cell density of a cell suspension culture on the accuracy of the CVS method is shown. Similar results were obtained for the other cell lines. The relative deviation showed no tendency to bend from the constant. The larger deviation at low values for FW indicates a lower accuracy of the method at small CVS. (**b**) Relative deviation from the regression line of volumes of water in Erlenmeyer flasks measured with the apparatus at different actually added volumes of water. Volumes were measured in the range of 5–50 ml. Data reprinted with permission from reference 12.

TABLE 1 | Methods for growth measurement of cell suspension culture in shake flasks.

Type of method	Example of method	Advantage(s)	Disadvantage(s)
All biomass harvested		**Biomass available for measuring metabolites during growth**	**Needs a large number of flasks for constructing a growth curve**
	Fresh and dry weight of the cells	Does not need aseptic conditions for sampling since all cells in the flask are harvested at once	Vacuum suction may result in irreproducible water content, thus of the fresh weight of biomass Drying in the oven and weighing the dried cells regularly until a constant weight, is time consuming In case of freeze-drying, equipment needed
Biomass sampling		**Only small sample size needed** **Experiments do not need large number of flasks** **Less variables due to flask number**	**Sampling of the same flask disturbs the gas-phase inside the flask and enhances risk for contamination** **Limited number of samples per flask**
	The number of cells[2]	Needs only very small volume of biomass as a sample, thus the same flask may be used to measure growth in a time course	Big cell aggregates need treatment to break the aggregates before counting
	Packed cell volume (PCV)[2,15,16]	Relatively fast and simple method	Centrifuge required
	Protein content[15]	Measure for metabolic activity	Protein extraction is laborious Needs further analytical method for total protein determination (e.g., Bradford method)
	DNA content[21,22]	Needs only small volume of cells Gives information about stage of development cells	Requires a flow cytometer Needs a highly pure sample of nuclei; contamination with RNA or cytosolic compounds can decrease fluorescence intensity resulting in low resolution Only a few fluorochromes are suitable for staining plant DNA Interpretation of result (relative fluorescence intensities) in terms of ploidy and genome size requires a reference standard
	Cell viability[2,23,24,25]	Gives information about stage of development of cells	Elaborate counting method requires a microscope or a spectrophotometer
	Determination of the concentrations of certain nutrients or metabolites[16,18,19]	Information about metabolome	Needs further analytical tools to measure metabolite(s)
Non destructive, non invasive		**Non-invasive method** **Can utilize a single flask to provide a growth curve** **Simple and fast methods**	**No analysis of other key features possible**
	Loss of weight by dissmilation[10]	Overall metabolic and growth measurement	Needs standardized flask closures (e.g., silicon stopper) to control for loss by evaporation Growth and metabolism can not be distinguished
	Cell volume after sedimentation (CVS)[12]	Needs only a simple home-made tool to measure CVS	Needs a similar size and shape of Erlenmeyer flasks (preferably 250 ml-flasks) At low CVS relatively big deviation from fresh/dry weight method Difficult to settle cells of fine and thick consistency of cell suspension cultures
	Medium conductivity/ osmolality	No loss of medium or cells Particularly useful in bioreactor for continuous monitoring	Requires special closures for continuous monitoring, or regular opening of flasks (see above), or otherwise (conventionally) harvesting some volume of medium[16,17]

TABLE 2 | Methods for cryopreservation of cell suspension cultures.

Method	Advantage(s)	Disadvantage(s)
(1) Two-step (equilibrium) freezing[29,39,48]	Requires a low molarity of cryoprotectant(s), which is relatively less toxic to a wide range of plant cells Can be applied to a large number of samples processed simultaneously[28,40] Computerized freezer may be applied in this technique[28] A relatively cheap cryocontainer (Mr. Frosty) can be an option to replace the controlled-freezing system[29] and is more suitable for small batches of vials[28]	May need an expensive programmable freezer/controlled-freezing system
(2) Vitrification[30,35,36,40,42]	Does not require an expensive controlled-freezing system Relatively fast More suitable (a method of choice) for plant cells which are sensitive to chilling[30,42]	Requires a relatively high molarity of cryoprotectant(s) that can be toxic for many plant cells Some steps of the procedure require a skilled and experienced technician to reduce the risk of contamination, if compared with the two-step freezing technique
(3) Encapsulation[31,32,34,37,40]	Does not require an expensive controlled-freezing system Does not employ a high concentration of cryoprotectants (less toxic to plant cells) Involves a simple thawing procedure[40] Fast recovery[40]	Needs a high concentration of sucrose that cannot be tolerated by some plant species Requires manipulation of each bead with increased sucrose concentration several times

These media, including those that have been modified/optimized for certain applications, are commercially[55] available (e.g., from Duchefa). In this protocol, we give an example of the composition of MS and Gamborg's B5 medium.

Optimizing growth conditions. The growth conditions are very important; light, temperature, shaker and gas phase are crucial parameters to be controlled. The flasks containing cell suspension cultures (e.g., 50- to 100-ml cell suspension in a 250-ml flask) can be kept in the dark, in fixed dark and light periods or in continuous light on a shaker at 90–125 r.p.m. at a certain temperature (normally at room temperature, 24–25 °C).

- *Light*: Permanent light is easiest as full darkness is difficult to achieve. Special lamps are available that emit light of a wavelength that should not affect plant cells.
- *Shaker speed*: Plant cells may be sensitive to shear, as a result of which high shaker speeds may have a negative influence on their viability. Optimal shaker speed depends on the type of shaker.
- *Gas phase*: The gas phase has several aspects; one concerns the room atmosphere (see critical factors of culture room), and the other is related to the gas phase within the flasks (see under growth measurement below).
- *Humidity*: Humidity control is important in connection with the loss of water from cultures as can be clearly appreciated when determining growth by a dissimilation curve. Cell cultures exposed to low humidity conditions lose more water and thus reach the stationary phase faster.

Below we have provided protocols for plant cell suspension cultures. Although every plant cell culture will have specific demands of the culture conditions and media, the technology of initiation and subculturing is similar for all. Our protocols refer to this part specifically. For plant tissue culture, particular conditions can be found in literature relating to the plant of interest. Several books[56,57] have been published summarizing information on specific plant tissue culture conditions.

Initiation of callus from plant. Callus can be initiated from different plant parts (called explants), e.g., buds, stems, leaves, petioles, flowers, flower stalks, roots, bulbs or germinated seeds/seedlings. Meristematic tissues (buds, young leaves or seedlings) are the most suitable explants for callus initiation. The time needed to rinse and sterilize the explant surface can be different depending on the part of the plant and the degree of contamination; normally, aerial parts of the plant (stems, leaves, flowers, buds, seeds) are less contaminated than those found below ground (roots, bulbs). For example, 5–30 min of rinsing with tap water is enough for aerial explants; however, more than 1 h or even overnight rinsing can be necessary for roots. The other steps that can vary slightly are, e.g., the duration of immersion of the explants in 70% (vol/vol) ethanol (0.5–1 min); the commercial sodium hypochlorite solution used (diluted 50–90% with water (Millipore) before use); and the duration of immersion in the hypochlorite solution (5–15 min).

Initiation of cell suspension culture from callus. Following initiation by placing callus fragments in flasks with liquid media on a shaker, suspension cultures will form over the next few days or weeks depending on the medium, growth conditions and the plant species. Addition of casein enzymatic hydrolysate to the medium can increase the rate of cell growth. The density of the culture increases with time (days), and care should be taken to filter and subculture at

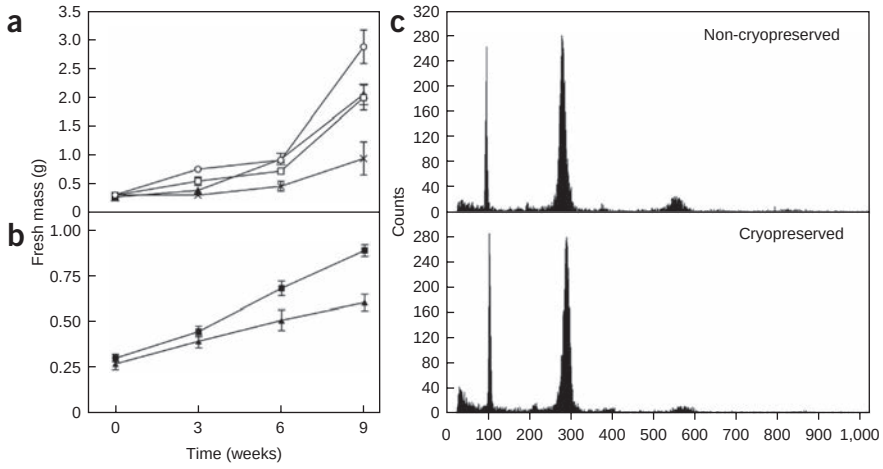

Figure 11 | Regrowth and relative nuclear DNA content of *Taxus* × *media* Rehd. cell lines after cryopreservation using the two-step freezing technique. (**a**) Line 3 (7-d-old non-cryopreserved/control cells (white circle); 7-d-old cryopreserved cells (white square), viability 80–90%; 15-d-old non-cryopreserved/control cells (black triangle), and 15-d-old cryopreserved cells ('×'), viability 40–60%). (**b**) Line 15 (7-d-old non-cryopreserved/control cells (black square) and 7-d-old cryopreserved cells (black triangle), viability 50–80%). (**c**) Histograms showing almost identical positions of relative DNA amount (measured by flow cytometry) of nuclei released from non-cryopreserved and cryopreserved cells of line 3 (left peak—internal standard *Pisum sativum*, peaks at channel 280 and 560—*Taxus* × *media* G1 and G2, respectively). The results show that the two-step freezing technique provides successful regrowth and non-altered nuclear DNA content for *Taxus* × *media* cell suspension cultures. The 7-d-old cryopreserved cells of line 3 provided both the highest viability (determined by FDA staining) and regrowth. However, a lower viability does not always reflect a lower regrowth as shown by the 15-d-old cryopreserved cells of line 3 (0.9 g FW biomass after 9 weeks of recovery, **Fig. 11a**) and the 7-d-old cryopreserved cells of line 15 (0.6 g FW, **Fig. 11b**). Regrowth shown as means of five to ten replicates ± s.e.m. Data reprinted with permission from reference 39.

the appropriate time, i.e., when the cell suspension is dense enough (comparable with a thick applesauce with no visible free medium) but not yet in the poststationary phase (often detected by a change in color (brownish) because of oxidation and/dead cells). It is also suggested that replicates be generated and that the next step be performed on each replicate at different times, thus avoiding the loss of the whole culture because of contamination.

Cell suspension cultures may be maintained in the dark, periodic dark and light intervals or in continuous light depending on the goal of the experiment. Light does affect production of compounds in the culture. Even short exposure to light may induce biosynthetic pathways; therefore, complete darkness requires special measures for reproducible results. For example, in some studies, it was observed that fluorescent light or the combined effect of light and medium composition stimulated the production of serpentine and anthocyanins in a *Catharanthus roseus* cell suspension culture[58]. For large-scale production in bioreactors, it is not possible to apply light. The characteristics and intensity of light should always be mentioned while describing experiments; moreover, it is suggested that in experiments using flasks on shakers light intensity on different areas of the shaker is measured with a digital lux meter, as the flasks on shakers will receive different amounts of light, which may also cause difference in the temperature of the flasks.

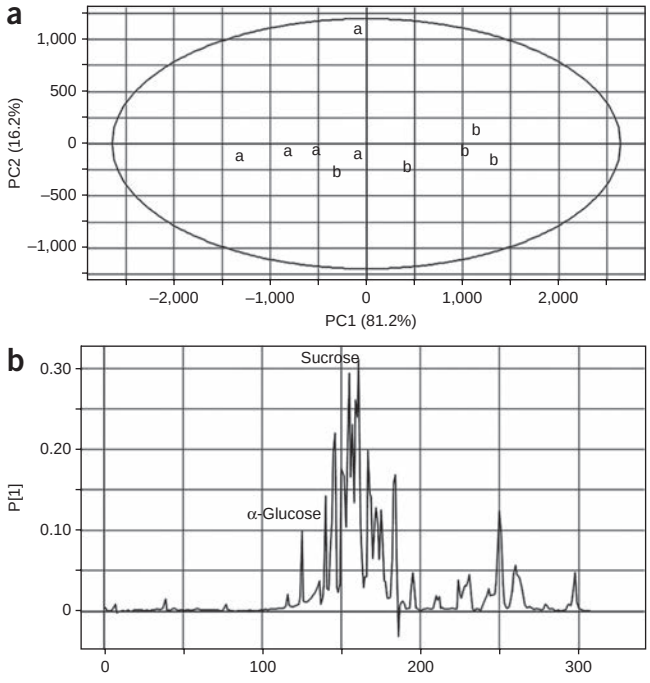

Figure 12 | Differences between control and cryopreserved cells of *Tabernaemontana divaricata* determined by NMR-based metabolomics. (**a**) Scatter plot showing the maximum separation of the NMR data for the two groups (5-d-old cells of a = control/non-cryopreserved cells and of b = cells after 3-month recovery from cryopreservation). (**b**) The corresponding loading plot, showing the responsible proton signals of the NMR data for the separation. Data reprinted with permission from reference 48.

Subculturing. The interval of time before subculturing is required will differ according to the cell line. Inoculum density also influences the subculturing interval; the higher the inoculum density, the earlier the stationary phase is achieved (see **Fig. 4a**) and the shorter the interval time can be. Subculturing is normally performed on a cell suspension culture at the end of the logarithmic phase (for fast-growing cells, the stationary phase is normally achieved 5 d after subculturing) or at the early stationary phase (can be 1–4 weeks after subculturing), but not after the late stationary phase when cell viability is low.

The number of flasks that should be subcultured depends on whether it is for maintenance or for an experiment. Two flasks are normally used for maintenance. To avoid losing cell lines because of contamination, some flasks of the batch of the old cell culture should be kept untouched, so that these can be used later in case of contamination of the new batch of subcultured cells.

The number of flasks used for an experiment should be determined in advance on the basis of the time course and the number of replicates included by the experimental design. The time course for a growth curve is normally between 2 and 4 weeks, with observations every 24 h for the first 7–10 d and every 2–7 d after 10-d subculturing. For an experiment such as

TABLE 3 | Effect of NAA on the excretion of alkaloids into the culture medium in cell line 6.

Regulators	2,4-D (1), Kn (1)	2,4-D (1) BAP (0.2)	2,4-D (2) BAP (0.2)	NAA (1) Kn (1)	NAA (2) Kn (1)	NAA (2) Kn (0.2)	NAA (2) BAP (0.2)
Total alkaloids production	8.3	14.2	13.7	15.5	16.2	18.2	24.3
Alkaloids in the medium (A)	2.9	5.4	5.0	8.2	8.2	9.7	14.7
Alkaloids in the biomass (B)	5.4	8.8	8.7	7.3	8.0	8.5	9.6
Ratio B/A	1.9***	1.6***	1.7***	0.9**	1.0***	0.9**	0.7

Kn, kinetin. Alkaloid production is expressed in mg per liter. Differences in ratio B/A were tested by comparing each of them with the one obtained in the medium containing NAA (2), BAP (0.2). ***$P \leq 0.001$, **$P \leq 0.01$. Data reprinted with permission from reference 5.

a growth, feeding or elicitation experiment, at least three flasks (triplicate) per observation should be used to be able to calculate a standard deviation value. For improved reproducibility, subculturing by weighing cells is suggested.

Cryopreservation. The cell suspension culture in a growth phase that has the highest freezing tolerance should be prepared for cryopreservation. The optimal growth phase can differ for each different line or species of cell suspension cultures; e.g., at the late

TABLE 4 | Regrowth of some cell lines that were cryopreserved with the two-step freezing technique in the laboratory at Leiden University, The Netherlands.

Species	Requirements for preculturing: Age of cells used (d) (Step 17)	Requirements for preculturing: Concentration of mannitol (M) (Step 19)	Requirements for preculturing: Preculturing/ incubation time (d) (Step 20)	Shaking at cryoprotection step (on ice, in the dark) (Step 24)	Regrowth[a] 10–14 d after plating (Box 1)
Arabidopsis thaliana (3 lines)	7	0.33	3	45 min	+ +
Catharanthus roseus wild and transgenic lines (33 lines)	3–4	0.5	2–3	20 or 45 min	+ +
Cinchona ledgeriana	3–7	0.5 or 0.33	2 or 3	20–60 min	–
Cinchona robusta	3–7	0.5 or 0.33	2 or 3	20–60 min	–
Nicotiana SR1	5	0.5	2	45 min	+
Nicotiana transgenic lines (6 lines)	4	0.33	3	45 min	+ +
Nicotiana BY2 (3 lines)	5	0.5	2	20 min	+
Oryza sativa	7	0.33	3	45 min	+ +
Petunia hybrida	4	0.5	2	20 min	+ +
Tabernaemontana divaricata	3–4	0.5 or 0.33	2 or 3	20 min, 150 r.p.m.	+ +
Taxus floridana Nutt.	7	0.5 or 0.33	4 or 7	60 min, 100 r.p.m.	+ +
Taxus × *media* (5 lines)	7	0.5	2	60 min, 100 r.p.m.	+ +/–
Vicia sativa	7	0.5	3	45 min	+ +

[a]Visually determined; minus (–) indicates browning and dead cells, plus (+) means no browning and new cells appear (+ + shows a relatively higher number of new cells than + does).

lag/early exponential, the mid-exponential or the early stationary phase. For fast-growing tobacco and *C. roseus* cell cultures, the optimal growth phase is the early exponential phase, which means that the sample should be prepared 3 or 4 d after subculturing, whereas in the case of the relatively slow growing *Arabidopsis* and *Taxus* cell lines the sample should be prepared 7 d after subculturing. See **Table 4**.

In the preculturing step, cells are treated for a few days with a so-called "pretreatment" medium containing one or more osmotic agents, which can be saccharides or saccharide derivatives (glucose, fructose, sucrose, maltose, mannitol, sorbitol, trehalose) or amino acids (proline or proline derivatives)[59]. The compounds differ in solubility, and at the same time different compounds may enter the cells in different degrees. In our protocol, we use mannitol[29]. This osmotic agent was found to work quite well for a wide range of plant cell lines in our laboratory. The optimal concentration of mannitol for different plant species should be determined experimentally in advance by testing several different concentrations in the range of 0.3–0.5 M.

Different plant cell suspension cultures may require different cooling rates. The first freezing step can be performed using a relatively expensive programmable freezer or a simple and economical Nalgene Cryo 1 °C freezing container 'Mr. Frosty'. If a programmable freezer is used for the first freezing step, the cryogenic vials are placed in a rack, precooled at 0 °C and immediately incubated for 20–30 min at 0 °C, followed by the freezing process to −35 °C at a freezing rate of 1 °C min^{-1} and subsequently maintained at −35 °C for 30 min. In the case of the cryocontainer Mr. Frosty, it should be filled with 250 ml of isopropanol (can be replaced by ethanol[29]) and placed at 4 °C overnight before use. This tool provides the average freezing rate within the vials close to 1 °C min^{-1} over the first −40 °C, which is slightly higher than with that of the programmable freezer. The final temperature within the vials during this step comes close to −80 °C. Unlike the case of a programmable freezer, no freezing plateau can be maintained. From some cell lines tested in our laboratory, no difference was observed considering the survival of the cell suspension cultures after the cryopreservation using both freezing techniques; however, it cannot be excluded that some plant cell suspension cultures are possibly more sensitive to this freezing protocol.

The management of the storage of cryopreserved cells should be organized carefully to avoid mixing samples. For example, samples should be stored separately in different containers based on the cell line and the year of initiation in such a way that the most often used cryopreserved cells are easy to access. Cryopreserved transgenic cell lines should be stored in a different container completely segregated from wild-type lines.

In the recovery step, growth can be observed 2 weeks after plating. Transferring cells from the plate culture into a liquid medium can usually be carried out 3–9 weeks after plating, after which subculturing should (normally) be carried out more frequently until about 12 weeks before subculturing for routine maintenance.

Staining with FDA does not reflect absolute viability. In untreated cultures, the percentage of cells reacting positively with FDA is usually >85%, and the preculture treatment does not appreciably lower this percentage. During cryoprotection, the staining level (the percentage of cells positive for FDA) decreases by 10–30%. Staining levels immediately after thawing are commonly 50–60%. It reduces further during the first 2–3 d after plating, and thus the most crucial factor appears to be the level on the day of transfer. A staining level of 40–70% is generally considered as being essential for a successful regrowth, although cultures may be recovered with levels of only 10% FDA positive at this stage! Because of the reasons mentioned above, the moment for determination of the percentage of viable cells after cryopreservation can be fixed at 2 d after plating[29].

MATERIALS

REAGENTS

- Aerial parts of plant such as leaf or flower buds, stems, leaves (preferably young leaves), flower petals and/or stalks, seedlings (hypocotyl and/or cotyledon parts) or roots.

Reagents for medium preparation (MS and Gamborg's B5 medium) and callus initiation

- Sucrose (>99.7%; Duchefa Biochemie, cat. no. S 0809) ▲ CRITICAL Although the sugar available in the supermarket can be used as a carbon source, it may give different results.
- D(+)-glucose monohydrate (≥99.0%; Fluka Chemie, cat. no. 49159)
- $CaCl_2.2H_2O$ (min. 99%; Riedel-de Haën, cat. no. 31307)
- KNO_3 (≥99%; Merck, cat. no. 1.05063.0500)
- $MgSO_4$ exsiccatus BP (OPG Farma, cat. no. 800598)
- KH_2PO_4 (min. 99.5%; Merck, cat. no. 1.04873.1000)
- $NaH_2PO_4{\cdot}H_2O$ (99.0–102.0%; Merck, cat. no. 1.06346)
- NH_4NO_3 (≥99%; Fluka, Sigma-Aldrich, cat. no. 09890)
- $(NH_4)_2SO_4$ (min. 99.5%; Merck, cat. no. 1.01217.1000)
- H_3BO_3 (min. 99.8%; Merck, cat. no. 1.00165.0500)
- $MnSO_4{\cdot}H_2O$ (min. 99%; Merck, cat. no. 5963)
- $ZnSO_4{\cdot}7H_2O$ (min. 99.5%; Merck, cat. no. 8883)
- Na_2H_2EDTA (>99%; Duchefa Biochemie, cat. no. E 0511.1000)
- $FeSO_4{\cdot}7H_2O$ (min. 99.5%; Acros, cat. no. 20139.2500)
- KI (>99%; Duchefa Biochemie, cat. no. P 0518.0100)
- $Na_2MoO_4{\cdot}2H_2O$ (min. 99.5%; Merck, cat. no. 1.06521.0100)
- $CuSO_4{\cdot}5H_2O$ (99.0–100.5%; Merck, cat. no. 2787.1000)
- $CoCl_2{\cdot}6H_2O$ (min. 99%; Acros, cat. no. 423571000)
- Myo-inositol (>99.0%; Duchefa Biochemie, cat. no. I 0609)
- Pyridoxine hydrochloride (>99.5%; Duchefa Biochemie, cat. no. P 8666)
- Thiamine hydrochloride (>99%; Janssen Chimica, cat. no. 14.899.58)
- Nicotinic acid (98%; Sigma-Aldrich, cat. no. N785-0)
- Glycine (min. 99.7%; Merck, cat. no. 1.04201.0100)
- 1-Naphthalene acetic acid (NAA, >98%; Duchefa Biochemie, cat. no. N 0903.0025)
- Indole-3-acetic acid (IAA, >98%; Duchefa Biochemie, cat. no. I 0901)
- 2,4-Dichlorophenoxyacetic acid (2,4-D, >96%; Duchefa Biochemie, cat. no. D 0911.0100)
- Kinetin (>98%; Duchefa Biochemie, cat. no. K 0905)
- 6-Benzylaminopurine (>99.2%; Duchefa Biochemie, cat. no. B 0904)
- Zeatin (>99%; Duchefa Biochemie, cat. no. Z 0917)
- HCl p.a. (min. 37%; Sigma-Aldrich, cat. no. 30721)
- NaOH (Boom, cat. no. 21292)
- Ethanol absolute (Biosolve, cat. no. 05250501) to dissolve plant hormones
- DMSO (min. 99.7%; Biosolve, cat. no. 04470501) to dissolve IAA ▲ CRITICAL Store DMSO in the dark. Purity should be checked at 275 nm, absorbance should be below 0.03.

- Casein enzymatic hydrolysate from bovine milk, EZMix N-Z-Amine A (Sigma-Aldrich, cat. no. C 4464-250G)
- Pectinase (Polygalacturonase) from *Rhizopus sp.* (Sigma-Aldrich Chemie, cat. no. 9032-75-1)
- Agar or, preferably, Gelrite (Duchefa, cat. nos. P 1001 (Plant agar), D 1004 (Daishin agar), M 1002 (Micro agar), P 1003 (Phyto agar), or G1101 (Gelrite)) ▲ **CRITICAL** Gelrite (produced by microbial fermentation) is better than normal agar as it is a highly purified natural anionic polysaccharide without the variations commonly associated with agar obtained from natural sources. Phenolic compounds found in agar, which can be toxic to certain sensitive organisms/plant cells, is absent in Gelrite. Gelrite forms rigid, brittle agar-like gels at approximately half the concentration of agar in the presence of soluble salts such as Mg^{2+} and Ca^{2+}
- Liquid soap/detergent for cleaning the surface of explants
- Ethanol 96% L.U. (Boom BV., cat. no. 45206) for aseptic work and sterilization of the surface of explants
- Sodium hypochlorite solution for sterilization of the surface of explants (commercially available in the supermarket as household bleaching/cleaning liquids) ! **CAUTION** Hypochlorite solutions are unstable and will lose activity. Toxic chlorine gas is formed during sterilization, and thus good ventilation is required.
- Tween 20

Reagents for cryopreservation

- Liquid nitrogen ! **CAUTION** Liquid nitrogen can cause frostbite (injury due to extreme cold); avoid direct contact with liquid nitrogen. Eye protection is also required. Always work with liquid nitrogen in open containers.
- Ultrapure water (high-purity water)
- Sucrose (>99.7%; Duchefa Biochemie, cat. no. S 0809)
- D(−)-Mannitol (min. 98.5%; BDH, VWR International, cat. no. BDH8009) ▲ **CRITICAL** On the basis of experience in our laboratory, some sources of mannitol may not be suitable for cryopreservation, as they result in poor cell survival.
- Glycerol (>99%; Sigma-Aldrich Chemie, cat. no. 9032-75-1)
- Fluorescein diacetate (FDA; 98%; Sigma-Aldrich, cat. no. 201642)
- Acetone p.a. to dissolve FDA (min. 99.8%; Biosolve BV., cat. no. 01030501)
- Agarose (SFR, molecular biology reagent grade, BDH, VWR International, cat. no. BDH4104) ▲ **CRITICAL** Agarose gives better results than agar as a solidifier of the first recovery medium. Pronarose/agarose D-1, low electroendosmosis (Hispanagar, CAS 9012-36-6) may be successfully used as a cheaper alternative.
- Activated carbon (charcoal, USP, FCC grade, 90.0–100.0%, JT Baker, cat. no. JT 1560-1)

EQUIPMENT

Equipment for medium preparation

- Erlenmeyer flasks (1 liter or 2 liters)
- Wide-mouth 100-ml Erlenmeyer flasks
- Erlenmeyer flasks (250 ml)
- Silicone stoppers T-32 (Karlan, cat. no. T-32)
- Sterile (polystyrene) cell culture dishes 60 mm × 15 mm (Corning, cat. no. 430166)
- Sartorius L420P + balance (range: 420 g; accuracy 1 mg)
- Sartorius balance (range: 200 g; accuracy 0.1 mg)
- Sterile syringes BD Discardit II (10 ml; Becton Dickinson, cat. no. 309110)
- Disposable syringe filter unit MillexGP 0.2 µm diameter 33 mm (Millipore, cat. no. SLGP033RS)
- Magnetic stirrer (with hot plate)
- pH meter
- Water purification (distillation and deionization) unit
- Graded glass cylinder or media dispenser to distribute media into Erlenmeyer flasks
- Autoclave
- Refrigerator
- Freezer

Equipment for initiation of callus and cell suspension, maintenance and growth measurement

- Laminar airflow cabinet equipped with vacuum and gas
- Gas-flame burner
- Glass bead sterilizer as an alternative to gas-flame burner for sterilizing metal instruments (tweezers, scalpels and so on) Model 'Lab Associates', operating temperature of 275 °C (Duchefa, cat. no. G 3301)
- Tweezers/forceps
- Knives (Scalpels) and sterile surgical blades no. 21 (Swann-Morton, cat. no. 0207)
- Rest: stainless steel rest for holding sterile tweezers/forceps and scalpel handle in convenient position (20-cm length, 3-cm height; Duchefa, cat. no. R3002)
- Sterile (γ-radiated) paper cutting pad (Duchefa, cat. no. P3202)
- A metal sieve or a Buchner filter (funnel) with a pore diameter of 1.5–2.0 mm (for filtration of cells without a vacuum pump). Alternatively, a Collector tissue sieve (VWR, cat. no. 62399-918) with Fine Mesh Kit (VWR, cat. no. 62399-951)
- Vacuum Erlenmeyer flask (aspirator) and Buchner filter with 75-mm ID for vacuum filtration
- Vacuum pump
- Whatman filter paper circles of 70-mm diameter (Schleicher & Schuell, cat. no. 10311808)
- Glass Petri dishes (for sterilization of paper circles)
- Spatula/chemical spoon (for scraping cells)
- Sartorius balance (range: 200 g; accuracy 0.1 mg)
- Pipette filler (Pipetus-akku)
- Wide-mouth non-pyrogenic disposable pipettes (10 ml; Costar 10-ml Stripette, serological pipettes, Corning, cat. no. 4101)
- Growth chamber/culture room
- Gyratory shaker G-10-21 (New Brunswick)
- White fluorescence lamps attached on/above the gyratory shaker (e.g., Phillips-TL33, Phillips TLF 65W33) to provide the light intensity of 500–2,500 lux.
- Digital lux meter RO 1332 (Roth, cat. no. E 513.1)
- Cell packed volume sedimentation (CVS) device. This is a homemade device, not commercially available, but every laboratory can make it quite easily by referring to **Figures 9** and **13**. The materials used are stainless steel and plastic/polymers; see the detailed dimensions in the pictures
- DISCURV, program for determination of dissimilation curves. For information, contact J. Schripsema (jan@uenf.br)
- Oven (set at 60 °C) or a vacuum freeze-dryer

Equipment for cryopreservation

- Low-speed benchtop centrifuge
- Orbital shaker
- Sterile 2-ml cryogenic vials (Nalgene, Sigma-Aldrich, cat. no. V5007)
- Programmable freezer able to cool at a rate of 1 °C min^{-1} to −40 °C, or Cryocontainer, 1 °C Mr. Frosty freezing container (Nalgene, Nalge Nunc International, VWR, cat. no. 55710-200)
- Liquid nitrogen storage vessel
- Water bath 35–40 °C
- Sterile (polystyrene) cell culture dishes 60 mm × 15 mm (Corning, cat. no. 430166)
- Whatman filter paper circles, diameter of 4.25 cm (Whatman International, cat. no. 1004042)
- Parafilm 'M' (Pechiney plastic packaging, cat. no. PM-996)
- Glass Petri dishes (for sterilization of paper circles)
- Oven (set up at 35–40 °C) for drying sterilized filter paper and pipette tips
- Sterile 3.5-ml transfer pipette (Sarstedt, cat. no. 861171001)
- Fluorescence microscope with blue-UV excitation and a barrier filter >510 nm
- Microscope slides (UV quality)

REAGENT SETUP

Macro salts stock solution (10×) for MS medium To prepare 1 liter of this solution, weigh 19 g of KNO_3, 3.7 g of $MgSO_4 \cdot 7H_2O$ (or 1.82 g of water-free $MgSO_4$), 4.4 g of $CaCl_2 \cdot 2H_2O$, 16.5 g of $(NH_4)NO_3$ and 1.7 g of KH_2PO_4, and dissolve the chemicals in ~400 ml of deionized water in a 1-liter Erlenmeyer flask. Place the flask on a magnetic stirrer plate to dissolve the salts and add deionized water to a volume of 1 liter. Transfer the solution into a 1-liter glass bottle, close the bottle and sterilize by autoclaving. The sterile stock solution (in the closed container) can be stored at room temperature (20–25 °C) for a maximum of 1 year. After opening, it should be stored at 4 °C for a maximum of 6 months for repeated use.

Macro salts stock solution (10×) for Gamborg's B5 medium To prepare 1 liter of this solution, weigh 25 g of KNO_3, 2.5 g of $MgSO_4 \cdot 7H_2O$ (or 1.23 g of $MgSO_4$ water free), 1.5 g of $CaCl_2 \cdot 2H_2O$, 1.34 g of $(NH_4)_2SO_4$ and 1.5 g of $NaH_2PO_4 \cdot H_2O$, and dissolve the chemicals in ~400 ml of deionized water in a 1-liter Erlenmeyer flask. Place the flask on a magnetic stirrer plate to

dissolve the salts and add deionized water to a volume of 1 liter. Transfer the solution into a 1-liter glass bottle, close the bottle and sterilize by autoclaving. The sterile stock solution (in the closed container) can be stored at room temperature (20–25 °C) for a maximum of 1 year. After opening, it should be stored at 4 °C for a maximum of 6 months for repeated use.

Micro salts stock solution (100×) for MS medium To prepare 1 liter of this solution, weigh 1.69 g of $MnSO_4 \cdot H_2O$, 0.62 g of H_3BO_3, 0.86 g of $ZnSO_4 \cdot 7H_2O$, 3.73 g of Na_2H_2EDTA and 2.78 g of $FeSO_4 \cdot 7H_2O$, and dissolve the chemicals in ~400 ml of deionized water in a 1-liter Erlenmeyer flask. Place the flask on a magnetic stirrer plate to dissolve the salts and add deionized water to a volume of 1 liter. Distribute the solution into, e.g., four 250-ml glass bottles, close the bottles and sterilize by autoclaving. The sterile stock solutions (in the closed containers) can be stored at room temperature (20–25 °C) for a maximum of 1 year. After opening, the stock solution should be stored at 4 °C for a maximum of 6 months for repeated use.

Micro salts stock solution (100×) for Gamborg's B5 medium To prepare 1 liter of this solution, weigh 1 g of $MnSO_4 \cdot H_2O$, 0.3 g of H_3BO_3, 0.2 g of $ZnSO_4 \cdot 7H_2O$, 3.73 g of Na_2H_2EDTA and 2.78 g of $FeSO_4 \cdot 7H_2O$, and dissolve the chemicals in about 400 ml of deionized water in a 1-liter Erlenmeyer flask. Place the flask on a magnetic stirrer plate to dissolve the salts and add deionized water to a volume of 1 liter. Distribute the solution into, e.g., four 250-ml glass bottles, close the bottles and sterilize by autoclaving. The sterile stock solutions (in the closed containers) can be stored at room temperature (20–25 °C) for a maximum of 1 year. After opening, the stock solution should be stored at 4 °C for a maximum of 6 months for repeated use. ▲ **CRITICAL** In some protocols, it is suggested that stock solutions of Na_2EDTA and $FeSO_4 \cdot H_2O$ be prepared individually as they may precipitate during storage at 4 °C; however, we have never encountered this problem. Autoclaving brings the chelating reaction to completion reflected by the deep golden yellow color of the solution. FeNaEDTA can also be used as an alternative.

Micro salts stock solution (1,000×) for MS medium To prepare 500 ml of this stock solution, weigh 0.415 g of KI, 0.125 g of $Na_2MoO_4 \cdot 2H_2O$, 0.0125 g of $CuSO_4 \cdot 5H_2O$ and 0.0125 g of $CoCl_2 \cdot 6H_2O$, and dissolve the chemicals in ~200 ml of deionized water in a 500-ml Erlenmeyer flask. Place the flask on a magnetic stirrer plate to dissolve the salts and add deionized water to a volume of 500 ml. Distribute the solution into, e.g., five 100-ml glass bottles, close the bottles and sterilize by autoclaving. The sterile stock solutions (in the closed containers) can be stored at room temperature (20–25 °C) for a maximum of 1 year. After opening, the stock solution should be stored at 4 °C for a maximum of 6 months for repeated use.

Micro salts stock solution (1,000×) for Gamborg's B5 medium To prepare 500 ml of this stock solution, weigh 0.375 g of KI, 0.125 g of $Na_2MoO_4 \cdot 2H_2O$, 0.0125 g of $CuSO_4 \cdot 5H_2O$ and 0.0125 g of $CoCl_2 \cdot 6H_2O$, and dissolve the chemicals in ~200 ml of deionized water in a 500-ml Erlenmeyer flask. Place the flask on a magnetic stirrer plate to dissolve the salts and add deionized water to a volume of 500 ml. Distribute the solution into, e.g., five 100-ml glass bottles, close the bottles and sterilize by autoclaving. The sterile stock solutions (in the closed containers) can be stored at room temperature (20–25 °C) for a maximum of 1 year. After opening, the stock solution should be stored at 4 °C for a maximum of 6 months for repeated use.

Myo-inositol (50 mg ml^{-1}) To prepare 100 ml of this solution, weigh 5 g of myo-inositol and dissolve in exactly 100 ml of deionized water in a 250-ml Erlenmeyer or a glass bottle. Distribute the solution into smaller-volume brown glass bottles (e.g., 20 ml each), close the bottles and sterilize by autoclaving. Store the sterile solutions in the dark at −20 °C for long storage[2,3] or at 4 °C for a maximum of 3 months for repeated use.

Pyridoxine-HCl (1 mg ml^{-1}) To prepare 100 ml of this solution, weigh 100 mg of pyridoxine-HCl and dissolve in exactly 100 ml of deionized water in a 250-ml Erlenmeyer or a glass bottle. Distribute the solution into smaller-volume brown glass bottles (e.g., 10–20 ml each), close the bottles and sterilize by autoclaving. Store the sterile solutions in the dark at −20 °C for long storage[2,3] or at 4 °C for a maximum of 3 months for repeated use.

Thiamine-*di*-HCl (1 mg ml^{-1}) To prepare 100 ml of this solution, weigh 100 mg of thiamine-*di*-HCl and dissolve in exactly 100 ml of deionized water in a 250-ml Erlenmeyer or a glass bottle. Distribute the solution into small-volume brown glass bottles (e.g., 10–20 ml each), close the bottles and sterilize by autoclaving. Store the sterile solutions in the dark at −20 °C for long storage[2,3] or at 4 °C for a maximum of 3 months for repeated use.

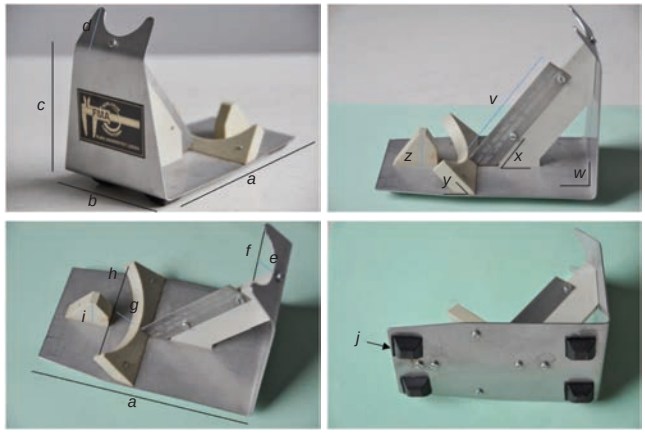

Figure 13 | The dimensions of the CVS device. The length (in cm) of *a*: 14.5, *b*: 8.7, *c*: 8.7, *d*: 2.8, *e*: 1.6, *f*: 4.4, *g*: 2.0, *h*: 7.5 and *i*: 2.4. The material used for *v* and *w* is stainless steel (*v*: a ruler of 9 cm; the angle of w is 90°). The *x*, *y* and *z* parts are made from polymer/plastic with thicknesses of 0.6, 1.0 and 1.0 cm, respectively. The angle of *x* is similar to that of *z* (50° or 60°), whereas the *y* angle is 45°. *j* is made from 2 cm × 2 cm × 0.8 cm of (black) rubber.

Thiamine-*di*-HCl (10 mg ml^{-1}) This stock solution is specifically used for Gamborg's-B5 medium, which contains ten times higher concentration of thiamine-*di*-HCl than does MS medium. To prepare 100 ml of this solution, weigh 1 g of thiamine-*di*-HCl and dissolve in exactly 100 ml of deionized water in a 250-ml Erlenmeyer or a glass bottle. Distribute the solution into small-volume brown glass bottles (e.g., 10–20 ml each), close the bottles and sterilize by autoclaving. Store the sterile solutions in the dark at −20 °C for long storage[2,3] or at 4 °C for a maximum of 3 months for repeated use.

Nicotinic acid (0.5 mg ml^{-1}) To prepare 100 ml of this solution, weigh 50 mg of nicotinic acid and dissolve in exactly 100 ml of deionized water in a 250-ml Erlenmeyer flask or a glass bottle. Close the bottles and sterilize by autoclaving. Store the sterile solutions in the dark at −20 °C for long-term storage[2,3] or at 4 °C for a maximum of 3 months for repeated use.

Glycine (2 mg ml^{-1}) To prepare 100 ml of this solution, weigh 200 mg of glycine and dissolve in exactly 100 ml of deionized water in a 250-ml Erlenmeyer flask or a glass bottle. Distribute the solution into small-volume brown glass bottles (e.g., 20 ml each), close the bottles and sterilize by autoclaving. Store the sterile solutions in the dark at −20 °C for long storage[2,3] or at 4 °C for a maximum of 3 months for repeated use. ▲ **CRITICAL** Vitamins can also be prepared together in one stock solution except myo-inositol, as this is used at a higher concentration. Fungi are often found growing in stock solutions of vitamins containing myo-inositol after several months of storage at 4 °C. In addition, it is better to prepare separate solutions of vitamins, as one can easily modify the composition or concentration of (individual) vitamins in the medium for medium optimization.

NAA (1 mg ml^{-1}) Weigh 100 mg of NAA and dissolve in 5 ml of 1 M NaOH. Adjust to 100 ml with deionized water (use a magnetic stirrer for homogenization, if necessary). Autoclave and store the sterile stock solution at room temperature (20–25 °C) in the dark for a maximum of 1 year, or at 4 °C for a maximum of 6 months for repeated use. ▲ **CRITICAL** Plant growth hormones act at low concentrations; ensure that they are dissolved completely and no microprecipitates are present in the solution, especially if they are dissolved in water. ▲ **CRITICAL** If NAA, or other growth hormones, are to be added into a sterile medium (50–100 ml of medium in an Erlenmeyer flask) that does not contain a growth regulator, one should consider possible pH changes of the medium by the addition of the stock solution. Preferably, a lower concentration of NAA stock solution should be used, e.g., 0.2 mg NAA ml^{-1}; weigh 100 mg of NAA in a 1-liter flask/glass bottle, add 20 drops (more or less 1 ml) of 1 M NaOH and 100 ml water, and then use heating and magnetic stirring to homogenize the mixture. If it does not dissolve well, heat the mixture carefully until it starts to boil and then adjust to 500 ml with water. After cooling the solution, adjust the pH to 5.80.

Sterilize the solution by autoclaving and aseptically pipette the necessary volume of the NAA stock solution into the medium to obtain the final concentration required. When using the stock solution, filter-sterilize it before adding it into the sterile medium.

IAA (0.2 mg ml^{-1}) Weigh 20 mg of IAA and dissolve in 5 ml of 1 M NaOH. Add deionized water to 100 ml (use a magnetic stirrer without heating for homogenization if necessary). ▲ **CRITICAL** IAA is unstable; the solution should be filter-sterilized (not autoclaved) before addition to the autoclaved liquid medium. This step should be performed in a laminar airflow hood. It is better to use a freshly prepared aqueous IAA stock solution (on the day of the experiment), as IAA is sensitive to light and temperature. Alternatively, it can be stored in the dark at 4 °C for a maximum of 2 weeks for repeated use. For longer storage, aseptically prepare a 1 mg ml^{-1} stock solution of IAA dissolved in DMSO and store it in the dark at −20 °C for a maximum of 6 months.

2,4-D (1 mg ml^{-1}) Weigh 100 mg of 2,4-D and dissolve in 5 ml of 1 M NaOH (or alternatively, in 12 ml of ethanol p.a.). Once dissolved, add deionized water to a final volume of 100 ml. Autoclave and store the sterile stock solution at room temperature (20–25 °C) for a maximum of 1 year, or at 4 °C for a maximum of 6 months for repeated use.

Stock solutions of kinetin, BAP or zeatin (0.5 mg ml^{-1}) Weigh 50 mg of kinetin or benzylaminopurine (BAP) or zeatin, and then dissolve in five to ten drops of 1 M HCl. Add 50 ml water, stir and heat at 60 °C (except for zeatin) to dissolve. Once dissolved, add deionized water to adjust to a final volume of 100 ml. ▲ **CRITICAL** Zeatin is unstable when heated; the solution should be filter-sterilized and not autoclaved. Store zeatin stock solution in the dark at 4 °C for a maximum of 2 months. Kinetin and BAP solutions can be autoclaved. The sterile stock solutions can be stored at room temperature (20–25 °C) in the dark for a maximum of 1 year, or at 4 °C for a maximum of 6 months for repeated use.

MS liquid medium To prepare 1 liter of MS liquid medium, add the appropriate amount of sugar (commonly 30 g of sucrose or 20 g of glucose) to 400 ml of deionized water in a 1-liter Erlenmeyer flask and stir on a magnetic stirrer. Add 100 ml of (10×) MS-Macro salts stock solution, 10 ml of (100×) MS-Micro salts stock solution and 1 ml of (1,000×) MS-Micro salts stock solution. Also add, if required, some vitamins or other additives to the mixture. Commonly, the following compounds are added: 2 ml of myo-inositol solution (50 mg ml^{-1}), 1 ml of pyridoxine-HCl solution (1 mg ml^{-1}), 1 ml of thiamine-*di*-HCl solution (1 mg ml^{-1}), 1 ml of nicotinic acid solution (0.5 mg ml^{-1}) and 1 ml of glycine solution (2 mg ml^{-1}). Growth hormone(s) may also be added to the mixture; e.g., 1–2 ml of 2,4-D solution (1 mg ml^{-1}), 1–2 ml of NAA solution (1 mg ml^{-1}) or a combination of 2,4-D/kinetin or NAA/kinetin (either 1 mg/1 mg or 2 mg/0.2 mg per liter medium). Any concentration of growth hormone(s) in the range of 10^{-7}–10^{-5} M in the medium can be used for medium optimization. In case of a poorly growing cell culture, one may add 2 g of casein enzymatic hydrolysate. Add water to just under the final volume of 1 liter, and while stirring adjust the pH by adding 0.1–1.0 M NaOH or 0.1–1.0 M HCl solution dropwise to reach a pH of 5.75–5.80. Take the mixture to the final volume and fractionate (e.g., 50 or 100 ml each) into 250 ml Erlenmeyer flasks. Label the flasks with the date and the name of medium, close with silicone stoppers and autoclave. Store the sterile media at room temperature (20–25 °C) in the dark for a maximum of 6 months; however, it can only be stored for 1 week if any unstable (filter-sterilized) hormone or compound solution is added into the sterile medium.

MS solid medium Follow the procedure indicated for the preparation of 1 liter of MS liquid medium, but after the adjustment of pH, transfer the mixture into a 1 liter glass bottle, gently pour 2.5 g of Gelrite (or 5.5–8.0 g agar), do not shake, close the bottle loosely and autoclave. After this, close the bottle tightly, shake it to homogenize the mixture while warm, and, inside the laminar airflow hood, distribute the mixture by pouring or pipetting 6.5 ml of medium into sterile 60 mm × 15 mm cell culture dishes (1 liter medium provides ~150 dishes). Leave the dishes to cool and solidify for 30 min. Close and seal the dishes with Parafilm and store them at room temperature (20–25 °C) in the dark for a maximum of 3 months if the medium contains a stable growth hormone (e.g., NAA, 2,4-D and kinetin), or for 1 week if the medium contains an unstable growth hormone (added into the medium by filter-sterilization) such as IAA.

Gamborg's B5 liquid medium To prepare 1 liter of Gamborg's B5 liquid medium, add 30 g of sucrose or 20 g of glucose to 400 ml of deionized water in a 1-liter Erlenmeyer flask and stir on a magnetic stirrer. Add 100 ml of (10×) B5-Macro salts stock solution, 10 ml of (100×) B5-Micro salts stock solution, 1 ml of (1,000×) B5-Micro salts stock solution. Also add some vitamins to the mixture as follows: 2 ml of myo-inositol solution (50 mg ml^{-1}), 0.5 ml of pyridoxine-HCl solution (1 mg ml^{-1}), 1 ml of thiamine-*di*-HCl solution (10 mg ml^{-1}) and 2 ml of nicotinic acid solution (0.5 mg ml^{-1}). Glycine is not supplemented in Gamborg's B5 medium. Growth hormone(s) may also be added to the mixture; e.g., 1–2 ml of 2,4-D solution (1 mg ml^{-1}), 1–2 ml of NAA solution (1 mg ml^{-1}) or a combination of 2,4-D/kinetin or NAA/kinetin (either 1 mg/1 mg or 2 mg/0.2 mg per liter medium). Any concentration of growth hormone in the range of 10^{-7}–10^{-5} M in the medium can be used for medium optimization. If necessary, add 2 g of casein enzymatic hydrolysate. Add water to just under the final volume of 1 liter, and while stirring adjust the pH by adding 0.1–1.0 M NaOH or 0.1–1.0 M HCl solution dropwise to reach a pH of 5.75–5.80. Take the mixture to the final volume and fractionate (e.g., 50 or 100 ml each) into 250-ml Erlenmeyer flasks. Label the flasks with the date and the name of medium, close with silicone stoppers and autoclave. Store the sterile medium at room temperature (20–25 °C) in the dark for a maximum of 6 months, or for a maximum of 1 week if any unstable (filter-sterilized) hormone or compound solution is added to the sterile medium.

Gamborg's B5 solid medium Follow the procedure indicated for the preparation of 1 liter of Gamborg's B5 liquid medium; however, after the adjustment of pH, transfer the mixture into a 1-liter glass bottle, gently pour 2.5 g of Gelrite (or 5.5–8.0 g agar), do not shake, close the bottle loosely and autoclave. After this, close the bottle tightly, shake it to homogenize the mixture while warm, and under the laminar airflow distribute the mixture by pouring or pipetting 6.5 ml of media into sterile 60 mm × 15 mm cell culture dishes (1 liter medium provides ~150 dishes). Leave the dishes to cool and solidify for 30 min. Close and seal the dishes with Parafilm and store at room temperature (20–25 °C) in the dark for a maximum of 3 months if the medium contains a stable growth hormone (e.g., NAA, 2,4-D and kinetin), or for a maximum of 1 week if the medium contains an unstable growth hormone such as IAA. ▲ **CRITICAL** Perform each step of the medium preparation procedure in the same way to ensure reproducibility of medium composition. To avoid making mistakes, add each component/ingredient according to the list, and always check it off on the list after addition. ▲ **CRITICAL** A wide-mouth 100-ml Erlenmeyer flask can be used for up to a 50-ml volume of cell suspension culture. A higher volume of cell suspension in the flask has a negative influence on homogenization and aeration, and consequently on the cell growth and viability or the formation of larger cell aggregates. ▲ **CRITICAL** Do not autoclave for longer than 20 min as sugars are affected by autoclaving; glucose will show browning, and sucrose may be partly converted into glucose and fructose.

Detergent solution Dilute 3 ml of liquid soap/detergent with 300 ml of demineralized water in an 800-ml glass beaker.

Diluted hypochlorite solution Pour 10 ml of commercial household sodium hypochlorite solution (4–6%, wt/vol) into a clean glass bottle or glass beaker, add two drops of Tween 20, dilute with 90 ml of Millipore filtered and deionized water to obtain the total volume of 100 ml; shake gently to homogenize. ▲ **CRITICAL** Hypochlorite solutions are not stable and lose activity. They produce toxic chlorine gas during sterilization. Good ventilation is thus required.

Sterile water Prepare 1 liter of deionized water in a 1-liter glass bottle, screw the bottle cap loosely and sterilize by autoclaving (screw the cap tightly after autoclaving).

Pretreatment medium (1 M mannitol in culture medium) To prepare 100 ml of this solution, warm 50 ml of 2× culture medium and gradually add 18 g of mannitol. Heat and stir gently to dissolve. Initially, the mixture is white and becomes colorless once the mannitol is well dissolved. Add water to adjust to a final volume of 100 ml. After cooling, adjust the pH by the dropwise addition of (0.1–1.0 M) NaOH or (0.1–1.0 M) HCl solution to pH 5.75–5.80 and autoclave. Store the sterile solution at room temperature (20–25 °C) in the dark for a maximum of 2 months.

Cryoprotectant (SGD) solution (2 M Sucrose, 1-M Glycerol, 1-M DMSO and 1% (wt/vol) L-proline) To prepare 100 ml of this solution, dissolve (by heating and magnetic stirring) 68.5 g of sucrose in 40 ml of water[38]. By using a

prewarmed wide-mouth glass pipette, add 7.23 ml of hot glycerol (preheated to 60 °C), then add water to a final volume of 93 ml. Autoclave and once cool, aseptically (under laminar flow) add 7.1 ml of DMSO and 2.5 ml of filter-sterilized L-proline (40%). Store the sterile solution at room temperature (20–25 °C) in the dark for a maximum of 2 months. ▲ CRITICAL The fresh solution is viscous. As it is hygroscopic, storage of longer than 2 months will result in lower density and poorer quality of the solution.

FDA Dilute in acetone to a final concentration of 2 mg ml^{-1} per aliquot; store at −20 °C for a maximum of 1 month.

Solid recovery medium (1 liter) Follow the procedure indicated for the preparation of 1 liter of MS or Gamborg's B5 liquid medium. After adjusting the pH with a pH meter, instead of Gelrite or agar add agarose (0.75%, wt/vol) and if necessary activated charcoal (0.5%, wt/vol) and autoclave for 20 min. After this, shake the flask to homogenize the mixture while warm, and under the laminar airflow distribute the mixture by pouring or pipetting 6.5 ml of medium into sterile 60 mm × 15 mm cell culture dishes (1 liter medium provides around 150 dishes). Leave the dishes to cool and solidify for 30 min. Close and seal the dishes with Parafilm and store at room temperature (20–25 °C) in the dark for a maximum of 3 months if the medium contains a stable growth hormone (e.g., NAA, 2,4-D and kinetin), or for 1 week if the medium contains an unstable growth hormone (added into the sterile medium by filter-sterilization) such as IAA. ▲ CRITICAL Activated charcoal is used in the recovery agar medium if the medium of a cell suspension culture shows a thick viscous consistency (gel-like medium), e.g., of *Taxus* cell suspension culture; however, in most cases, it is not necessary.

EQUIPMENT SETUP

Laminar airflow cabinet Working with plant cell cultures requires a much higher level of sterility compared with microbial work as almost any microbe will rapidly overgrow the plant cell culture. Never use the same room and particularly the same laminar airflow cabinet for plant cultures and microorganism cultures. When not in use, leave the flow of the laminar cabinet switched on at its minimum setting to keep the filter and the inside of the cabinet clean. Regular annual maintenance is important to detect leaks, uniformity of airflow pressure and/or to replace the filter. Before using the laminar airflow cabinet, spray its inside surface with 70% (vol/vol) ethanol, clean the bench surface with a tissue dampened in 70% (vol/vol) ethanol and switch on the flow to the maximum setting. Precondition the flow for 15 min before use. ▲ CRITICAL For thawing, clean the water bath with soap and/or a disinfectant and fill it with sterile water before using it in the laminar airflow cabinet.

Flaming and aseptic technique If flaming is done, the temperature for the instruments should be as high as possible. For this, the gas burner should emit a blue flame with a sufficiently high temperature to allow the sterilization of instruments within seconds. 'Aseptically' opening the flask means that the flask should be opened close to the flame, and the neck should be exposed to the flame for about 5–10 s while rotating the flask. Avoid leaving drops of medium in the neck of the flask, as overheating can cause charring of this remnant medium and it can even cause the glass neck to break.

Sterilizing metal instruments Instruments such as spoons/spatulas, tweezers/forceps and knives can be sterilized by immersing them in 70% (vol/vol) ethanol and heating their tips (1–3 cm from the tip) over a flame until they are red hot. The instruments should then be placed close to the flame above the surface of the cabinet bench in such a way that the burnt parts are not touched and kept sterile (e.g., use a stainless steel rest holder). Allow them to cool at room temperature in the laminar cabinet before use. Alternatively, sterilize the instruments by heating on an electric Bunsen burner[3] at 250–400 °C for 15 s or on a glass bead sterilizer with operating temperature of 275 °C (ref. 55).

Sterilizing the vacuum flask and Buchner filter Cover the mouth of the vacuum flask (including the connection to the vacuum source) and the Buchner filter using aluminum foil sealed with sterilization tape for their sterilization in the autoclave (at 121 °C for 20 min). The stripes on the sterilization tape change from white to black after heating/autoclaving. ▲ CRITICAL If vacuum filtration is used, a filter for the sterilization of air between the Erlenmeyer flask and the vacuum source can be useful to keep the contents of the Erlenmeyer flask sterile.

Sterilizing Whatman filter paper circles Place the filter paper circles in glass Petri dishes, cover the dishes with aluminum foil or seal with sterilization tape, and then sterilize them in the autoclave (121 °C for 20 min). Never use a nonautoclavable plastic container or bag for sterilization in the autoclave. Leave the sterilized filter paper circles (in the closed and sealed Petri dishes) to dry in the oven at 35–40 °C overnight before use.

Culture room The temperature of the culture room should be automatically maintained (normally it is set at 24–25 °C). Any volatiles present in the air, coming from, e.g., perfumes (most contain methyl jasmonate, a plant stress signal compound), aftershaves, kits, paints, chemicals and plants, may affect cell cultures. ▲ CRITICAL Transgenic cell cultures should be handled according to the national and international regulations applicable. This means that transgenic cell lines should be cultivated in a different certified culture room separated from other wild-type cell lines. Old or contaminated transgenic cells should be autoclaved before discarding.

Shaker The shaker speed affects aeration and cell viability. The optimal speed of a shaker depends on the type of shaker. For cell suspension culture, the speed of shaking is normally between 90 and 125 r.p.m. on a G-10 New Brunswick gyratory shaker. Check the intensity of light at different areas on the shaker using a digital lux meter. Flasks on shakers will receive varying amounts of light, which may also lead to differences in temperature of the flasks.

CVS device The 250-ml Erlenmeyer flask should be placed on the CVS device with the (optimal) angle of 60°. The materials used to make the device are stainless steel and polymers (plastic); the detailed dimensions of the CVS device can be seen in **Figure 13**.

PROCEDURE

▲ CRITICAL Label all reagents properly (i.e., register the name of the reagent, the concentration, the date and the name of the person who made them). The cell culture flasks after initiation and subculturing should be labeled with the name of the cell line, the media, the date of initiation/subculturing, the cell density and the name of the person responsible for it. Additional information can be written in a log book.

▲ CRITICAL Some of the following steps should be performed in a laminar airflow bench (indicated by *). Before starting to work, wash hands well with soap and spray them with 70% (vol/vol) ethanol. All of the implements used should be autoclaved or cleaned with 70% (vol/vol) ethanol. For information on the sterilization of knives (scalpels), forceps/tweezers and spatulas, see EQUIPMENT SETUP.

Initiation of callus culture from plants (buds, stems, leaves, flowers, seedlings or roots)

1| Select plant parts that will be used for callus initiation, place them on a sieve and rinse thoroughly under running tap water for 5 min. Longer washing may be required if you are using roots from soil-growing plants.

▲ CRITICAL STEP Steps 1–5 are not necessary when sterile *in vitro* plant cultures are used for explants.

2| Place the explants in a glass beaker containing a diluted soap/detergent solution, shake or wipe them gently for 2–3 min and rinse again with water to remove the soap.

3| *Sterilize the surface of the explants by immersion in 70% (vol/vol) ethanol for 30 s.

4| *Transfer the explants to a sterile wide-mouth flask containing a diluted sodium hypochlorite solution in water (1:9, vol/vol) + Tween 20 and immerse for 5–10 min.

5| *Transfer the explants to a sterile wide-mouth flask and rinse them five times with sterile water.

6| *Place the sterilized explants on a sterile paper cutting pad and cut the leaf or flower explant into several small squares of 0.3–0.5 cm, or lengths of 0.5–0.8 cm for stems, leaves and flower stalks or roots.

7| *Place three to four pieces of the cut explants on solid MS or Gamborg's-B5 medium containing growth hormones for callus initiation prepared in a Petri dish (60 mm × 15 mm, use separate dish for different explants) and seal the plates with Parafilm.

8| Place the dishes in the culture room at 24–25 °C in the dark, photoperiodic or 24-h-light regime depending on the goal of the experiment (e.g., light can increase the production of certain secondary metabolites).
? TROUBLESHOOTING

9| Monitor the cultures regularly for callus formation and possible microbial infections (e.g., every 2 d). Normally, callus can be separated from the explants 3 weeks after initiation and transferred to a fresh solid medium. Subculturing for maintenance of callus can be performed every 4–6 weeks.
? TROUBLESHOOTING

Initiation of plant cell suspension culture from callus culture

10| *Remove the callus grown on solid medium with sterilized tweezers, and place it on an autoclaved Whatman filter paper disc in a sterile Petri dish or on a sterile paper cutting pad.

11| *Using a sterile knife/scalpel, cut 1–2 g of FW of the callus into smaller (2–3 mm length) fragments and transfer into a 100-ml Erlenmeyer flask containing 15 or 25 ml of sterile liquid medium.

12| Place the culture under appropriate light conditions at 25 °C (climatized growth cabinet or chamber) on a shaker (90–125 r.p.m.) until a suspension culture has formed (fine cells are released into the medium causing turbidity).
? TROUBLESHOOTING

13| Once the cell suspension culture becomes dense (applesauce-like consistency with different sizes of cell aggregates), prepare a fine cell suspension from the cell culture using one of three options: filtration (A), decanting or pipetting (B) or addition of pectinase (C).
? TROUBLESHOOTING

(A) Preparing a fine cell suspension by filtration

(i) *Filter the culture in order to separate fine cells from larger cell aggregates using either a Cellector Tissue Sieve, a sterile metal sieve or a Buchner filter with a pore diameter of 1.5–2.0 mm (without vacuum); place the fine cell suspension into a sterile Erlenmeyer flask.

(ii) *Dilute it with the same volume or a half-volume (in case of low cell density) of fresh medium, distribute into two flasks and return the cell suspension cultures to the culture room in regular conditions (e.g., see footnote of **Table 5**).

(B) Preparing a fine cell suspension by decanting or pipetting

(i) *Tilt the flask for a few seconds to allow the bigger cell aggregates to settle.

(ii) *Decant or pipette carefully only the medium containing fine cells into a flask containing sufficient fresh medium for a dilution of 1:1 vol/vol.

(iii) Return the cell suspension cultures to the culture room in regular conditions.

(C) Preparing a fine cell suspension by addition of pectinase[2,60]

(i) *Add sufficient filter-sterilized pectinase solution to reach a concentration of 0.005% into the culture medium to disrupt the cell aggregates[60].

(ii) Return the suspension culture to the culture room in regular conditions until sufficient fine cells are obtained for subculturing.

Maintenance/subculturing of plant cell suspension culture

14| This step can be performed using any of the described options: weighing (A), pipetting (B) or pouring (C). The methods differ in precision and thus reproducibility; the most elaborate (A) has the highest reproducibility.
? TROUBLESHOOTING

(A) Subculturing by weighing

(i) *Pour 50 or 100 ml of the cell suspension culture from the Erlenmeyer flask onto a sterilized Whatman filter paper in a sterilized Buchner vacuum filter.

(ii) *Gently, by vacuum suction, separate the cells from the medium, leaving the cells on the filter paper.
▲ CRITICAL STEP Cell FW is influenced by the (external) water content of the wet cells; thus the filtering step is necessary, but overdoing the suction may cause cell stress and low cell viability.

(iii) *Place a 250-ml Erlenmeyer flask containing 50 or 100 ml of fresh medium on the Sartorius balance, and zero it.

(iv) *Using a chemical spoon, scrape sufficient cells from the filter to reach the weight required for the chosen inoculum density. Transfer the cells from the spoon into the flask, close the flask and swirl gently (low inoculum density: 40–60 g FW per liter; medium inoculum density: 100–160 g FW per liter; and high inoculum density: >200 g FW per liter).

(v) Place the flask(s) in the culture room in regular conditions.

(B) Subculturing by pipetting (volume measuring)

(i) *Gently shake the flask and reflux the solutions with a pipette (using a sterile wide-mouth pipette connected to an electrical vacuum pipette filler) three times in order to achieve a more homogenous distribution of cells in the pipette (for inoculum) and to break up large cell aggregates.

(ii) *Pipette and transfer 10, 20 or 30 ml of the cell suspension culture into a 250-ml Erlenmeyer flask containing a determined volume of fresh medium. The volume transferred depends on the density of culture required to provide a final volume of 50–100 ml.

(iii) Place the flask(s) in the culture room in regular conditions.
? TROUBLESHOOTING

(C) Subculturing by pouring (volume measuring)

(i) *Shake the flask to homogenize the suspension culture. Immediately pour an exact volume of the culture (10–50 ml) into a sterile disposable 50-ml tube and transfer to a flask containing a determined volume of fresh medium so as to prepare a suspension culture with the required density. Alternatively, a simpler, though less accurate, method is to pour 100 or 50 ml of fresh medium into a 250-ml Erlenmeyer flask containing, respectively, 100 or 50 ml of cell suspension culture, and swirl gently.

(ii) *Shake the flask and pour the refreshed cell cultures (100 or 50 ml each) into two empty sterilized 250-ml Erlenmeyer flasks, thus obtaining two flasks of (respectively) 100 or 50 ml of cell suspension culture by estimation. Close the flasks and swirl them gently.

(iii) Place the flask(s) in the culture room in regular conditions.
? TROUBLESHOOTING

Growth measurement

15| Prepare a batch of cell suspension cultures with a determined inoculum density in 250-ml Erlenmeyer flasks containing the same volume of fresh medium (50 or 100 ml) as described in Step 14A.
▲ CRITICAL STEP Different inoculum densities may give different growth curves.

16| Measure the growth of the cell suspension cultures, according to one of the options below (A–D). These differ in being invasive or non-invasive, and thus either allow you to measure a growth curve only by harvesting a large number of flasks (A and B) or to measure growth in a single flask (C and D).

(A) Dry weight measurements on filter paper—oven heating

(i) Using a Buchner vacuum filter, collect the cells from a flask every 24 h after inoculation (use three flasks for each observation time) on a preweighed Whatman filter paper disc.

(ii) Determine the total weight of the filter paper and the cells, and then subtract the weight of the filter paper to obtain the FW of the cells.

(iii) Place the filter paper containing the cells prepared on a glass dish and place in a 60 °C oven for, e.g., 48 h.

(iv) Weigh the filter paper and cells at intervals during the drying procedure until the weight remains constant.

(v) Subtract the weight of the filter paper to obtain the DW of the cells, or alternatively transfer and weigh the dried cells in a clean empty tube.

(vi) Plot a growth curve with time on the *x* axis and the FW and DW of cells on the *y* axis.

(B) Dry weight measurements in tubes—freeze-drying

(i) Filter and weigh cells as described in Steps 16A(i) and (ii). Transfer all freshly weighed cells from the filter paper into a preweighed 15-ml plastic tube.

(ii) Perforate the tube cap with a needle, place the tube in a rack, freeze in liquid nitrogen and rapidly place the frozen tube in a freeze-dryer for 48 h. If it is not directly freeze-dried, keep the frozen cells/tube at −80 °C and, preferably, simultaneously freeze-dry all samples (tubes) from the experiment in the same equipment.

(iii) Determine the DW of cells by subtracting the weight of the tube(s).

(iv) Plot a growth curve of the FW and DW of the cells.

(C) Growth measurement by determination of loss of weight (dissimilation curve)

(i) Immediately after inoculation (Step 15), record the weight of three flasks of the cell suspension cultures and three flasks containing the same volume of medium alone (to be used for control of water evaporation) and then at periodic time intervals (e.g., every 24 h) throughout an established time course.

TABLE 5 | Conditions for some of the plant cell suspension cultures that have been maintained in the laboratory at Leiden University, The Netherlands.

Species	Name of line/ subculturing time/method	Medium	Growth regulator (mg l^{-1})	Volume of cell suspension/volume of flask	Aspect of cell suspension (color and consistency)
Achillea millefolium L. ssp. *Millefolium*[61]	1 week/by weighing (3.5 g cells/50 ml medium)	Gamborg's B5, 2% sucrose, 30 mM NO_3^-, 1 mM NH_4^+	2,4-D/kinetin (1.5:0.1)	50 ml/250 ml flask	White/creamy cells + small aggregates
Cannabis sativa[62]	2 week/by pouring (3-fold dilution)	MS, 3% sucrose, + B5 vitamins	2,4-D/kinetin (1:1)	75 ml/250 ml flask	Green yellowish fine cells
Catharanthus roseus[63,64]	A12A2/1 week/by pouring (dilution 1:1) or pipetting (20 ml culture into 50 ml medium)	MS, 2% glucose or 3% sucrose	None	100 ml/250 ml flask or 70 ml/250 ml flask	Light green/green yellowish fine cells
Catharanthus roseus[65]	CRPM/1 week/by pouring (dilution 1:1) or pipetting (20 ml culture into 50 ml medium)	MS, 2% glucose or 3% sucrose	NAA/kinetin (2.0:0.2)	100 ml/250 ml flask or 70 ml/250 ml flask	Creamy fine cells
Catharanthus roseus[13]	A11/2 weeks/by pouring (dilution 1:1)	Gamborg's B5, 2% glucose	NAA (1.86)	100 ml/250 ml flask	Dark green fine cells
Catharanthus roseus	A11/2 weeks/by pipetting (20 ml culture into 50 ml medium)	Gamborg's B5, 3% sucrose	NAA (1.86)	70 ml/250 ml flask	Yellowish fine cells + small aggregates
Catharanthus roseus Pacifica Punch	CRPP93/3–4 weeks/ by pouring (dilution 1:1) or pipetting (20 ml culture into 50 ml medium)	Gamborg's B5, 2% glucose or 3% sucrose	NAA (1.86)	100 ml/250 ml flask or 70 ml/250 ml flask	Dark green fine cells + small aggregates
Catharanthus roseus	CAT-L/1 week/by pipetting	LS, 3% sucrose	NAA/kinetin (2.0:0.2)	70 ml or 50 ml/250 ml flask	Yellowish or green yellowish fine cells
Catharanthus roseus (white flower)	CRWF/1 week/by pouring	MS, 3% sucrose	IBA (3.0)	100 ml/250 ml flask	Creamy fine cells
Cinchona ledgeriana	25 CiLed/1 week/by pouring	Gamborg's B5, 2% sucrose	2,4-D/kinetin (2.0:0.2)	100 ml/250 ml flask	Yellowish aggregates

(continued)

TABLE 5 | (continued).

Species	Name of line/ subculturing time/method	Medium	Growth regulator (mg l^{-1})	Volume of cell suspension/volume of flask	Aspect of cell suspension (color and consistency)
Cinchona robusta (2 lines: AQ+ and AQ−)[66]	Line AQ+: 1 week/ by weighing 5 g cells/50 ml medium Line AQ−: 1 week/by weighing or pouring (dilution 1:1)	Line AQ+: Gamborg's B5 4% sucrose + caseinhydrolysate 500 mg l^{-1} Line AQ−: Gamborg's B5 2% sucrose + cysteine 50 mg l^{-1}	Line AQ+: NAA: BAP (0.1:10) Line AQ−: 2,4-D/kinetin (2.0:0.2)	Line AQ+: 50 ml/250 ml flask Line AQ−: 50–100 ml/250 ml flask	Yellowish large aggregates
Ginkgo biloba	58 *Ginkgo*/2 weeks/ by pouring	MS, 3% sucrose	NAA (3.5)	100 ml/250 ml flask	Greenish (low density) fine cells
Humulus lupulus[67] (8 lines)	2 weeks/by weighing (3 g cells/50 ml medium) or pouring (dilution 1:1)	MS, 3% sucrose or Gamborg's B5, 2% sucrose	2,4-D/kinetin (1:1) in MS, or (1.0:0.2) in Gamborg's B5 medium	50 ml/250 ml flask	White/creamy cells
Ilex dumosa[68]	1 week/pouring (dilution 1:1)	Gamborg's B5, 4% sucrose	2,4-D/kinetin (1:1)	50 ml/ 250 ml flask	Creamy fine cells
Ilex paraguariensis[72]	3 weeks for 4–5 g of inoculum or 2 weeks for 10 g of inoculum/ by weighing	MS, 3% sucrose + 1 g l^{-1} of casein hydrolysate	NAA (5.0)	50 ml/250 ml flask	Creamy fine cells
Morinda citrifolia (2 lines: AQ+ and AQ−)[69]	1 week/by pouring (fourfold dilution)	Line AQ+: Gamborg's B5, 2% sucrose Line AQ−: Gamborg's B5, 4% sucrose	Line AQ+: NAA (1.86) Line AQ−/2,4-D/kinetin (1.0:0.2)	200 ml/500 ml flask	Yellowish fine cells
Nicotiana tabaccum (2 lines; SR1 and BY2)	10 d/by pipetting	LS, 3% sucrose	2,4-D (2.0)	40 ml/100 ml flask	Yellowish fine cells
Oryza sativa	1 week/by pipetting (10 ml culture into 50 ml medium)	AA (amino acid) medium, 2% sucrose	2,4-D/kinetin: gibberilic acid (2:0.2:0.1)	60 ml/250 ml flask	White fine cells
Rubia tinctorum[69]	1 week/by pouring (fourfold dilution)	Gamborg's B5, 2% sucrose	2,4-D/NAA/ IAA/kinetin (2:0.5:0.5:0.2)	200 ml/500 ml flask	Orange fine cells
Senecio jacobaeae	1–2 weeks/by pouring	Gamborg's B5, 2% glucose	NAA (1.86)	75–100 ml/250 ml flask	A mixture of dark green fine cells and small aggregates
Tabernaemontana divaricata[5,10,48,70,71]	6DivBW101/1 or 2 weeks/by pouring	MS, 3% sucrose	2,4-D/kinetin (1:1)	100 ml/250 ml flask	White fine cells
Tabernaemontana elegans	6TelS4/1 week/by pouring	MS, 3% sucrose	2,4-D/kinetin (1:1)	100 ml/250 ml flask	Creamy fine cells
Taxus floridana[39]	11 Flori/3 weeks/by pouring (dilution 1:1)	Gamborg's B5, 2% sucrose	NAA (1.86)	200 ml/500 ml flask	Green brownish small aggregates
Vanilla planifolia[68]	1 week/by pouring (dilution 1:1)	MS, 3% sucrose	2,4-D (1.0)	100 ml/ 250 ml flask	Creamy fine cells

The cell suspension cultures were initiated from calluses (which were initiated mostly from young leaf or leaf-stem explants, except for *Catharanthus roseus* CRWF which was from flower explants) maintained at 25 ± 0.5 °C, on a gyratory shaker of 100–125 r.p.m., under continuous light of 500–2,500 lux, except for *Taxus* cultures (in 16-h photoperiod) and *Ginkgo*-, *Ilex*- and *Oryza sativa* cultures (in the dark).

(ii) Calculate the loss of weight after correction due to water evaporation with reported software[10].
(iii) Plot a growth curve with the time course (day) as the axis (*x*), and loss of weight (in g or mg) as the ordinate (*y*).

(D) Growth measurement by determination of CVS

(i) After inoculation (Step 15), take three flasks from the batch of cell suspension cultures to be used for CVS measurement.
(ii) Place the flask on the CVS device (see **Fig. 9**) designed to tilt an Erlenmeyer flask at an angle of 60°.
(iii) Maintain the flask in position for 5 min to allow sedimentation of the cells.
(iv) Using a ruler fixed on the CVS device, measure the height of the cell mass along the glass wall.
(v) Transfer a volume of water into an empty 250-ml Erlenmeyer flask placed on the CVS device in amounts such as to provide a height along the glass wall similar to that of the cells. Register the height with the corresponding volume of water; the volume of water thus determined represents the CVS.
(vi) Repeat Steps 16D(ii–iv) on the same flasks every 24 h for the first 7 d and then every 48 h for 2–3 weeks to construct a CVS curve.
(vii) Compare the growth curve provided by FW cells and CVS measurement.

Cryopreservation: preculturing

17| Measure the CVS to determine the growth phase of the cell suspension culture based on the data provided in Step 16D. If the other options have been used for measuring growth, ensure that the growth state/phase of the cells is known.
? TROUBLESHOOTING

18| *Allow the cells to settle; with a pipette, transfer all the supernatant medium above the cells into a graded tube (usually about one-third or half of the total volume of the suspension culture in the flask).

19| *Add to these cells an equal volume of pretreatment medium containing an amount of mannitol that will result in a final concentration of 0.33 or 0.5 M mannitol in the cell culture.

20| Incubate the cell suspension culture for an additional 2–3 d under its standard culture regime at 100–125 r.p.m., 24–25 °C, in the dark.
? TROUBLESHOOTING

Cryopreservation: cryoprotection

21| *Place the cryoprotectant solution and the cell suspension culture flask (Step 20) on ice. Allow the cells to settle.
▲ **CRITICAL STEP** In the case of a cell suspension culture that sediments slowly, transfer the cell culture into a sterile 50-ml conical tube and centrifuge at a low speed (i.e., 107*g* for 2 min) to sediment the cells without affecting cell survival.

22| *Pipette or decant the entire medium above the cells, transfer it into a sterile disposable graded tube (e.g., 50-ml tube) and measure the volume in order to determine the volume of cryoprotectant solution that should be added at Step 23.

23| *Substitute the discarded medium with the same volume of cold cryoprotectant solution by using a sterile disposable graduated tube and pouring it gently into the remaining cell suspension.
▲ **CRITICAL STEP** Do not mix (care should be taken to make sure the cold cryoprotectant solution rests gently in a layer on top of the suspension).

24| *Place the cryoprotected cell culture on ice and shake it slowly on an orbital shaker at 80–100 r.p.m. for 20–60 min (see **Table 4** for the requirements of some cell lines).

25| *Fill the cold 2-ml cryogenic vials (previously stored at −20 °C for 2 h) each with 1.8 ml of the cryoprotected cell suspension and close the vials.
▲ **CRITICAL STEP** After the freezing procedure, two vials of the frozen cell line are thawed and tested for viability.

Cryopreservation: freezing and storage

26| Rapidly place the cryogenic vials into a cryocontainer (e.g., Nalgene's Mr. Frosty, filled with 250 ml of isopropanol and placed at 4 °C overnight) and freeze for 2–4 h at −80 °C. Alternatively, place the vials in a programmable freezer.

27| Plunge the frozen vials rapidly into a liquid nitrogen container for 5 min; subsequently, transfer the vials to the drawers or holders of the liquid nitrogen storage vessel.
! CAUTION The extremely low temperature of liquid nitrogen is dangerous; use proper gloves, laboratory coat and protective glasses when handling liquid nitrogen.

Thawing and plating

28| Transfer the frozen vials containing cryopreserved cells from the liquid nitrogen storage vessel into a Dewar flask containing liquid nitrogen.

29| *Remove the cryovials and carefully but quickly unscrew the caps of the vials to remove any remnant liquid nitrogen inside them.

! CAUTION Care should be taken, as liquid nitrogen evaporates rapidly and there is a potential risk of vial rupture; full safety precautions should be observed.

? TROUBLESHOOTING

30| *Close the vials again, and after wiping their outer walls with 70% (vol/vol) ethanol place the vials on ice.

31| *Transfer each vial (one by one) to a clean 35–40 °C water bath and gently flip several times until the content is thawed (the last piece of ice disappears). Immediately place each vial on ice again.

▲ CRITICAL STEP Avoid contact between the lid of the vial and the water during immersion in the water bath, as microorganisms may enter the rim of the screw cap of the vial. Decontamination and filling the water bath with sterile water are recommended.

32| Centrifuge the vials at 100*g*, at 4 °C for 1–2 min.

33| *Wipe the outside of the vials with 70% (vol/vol) ethanol and remove the supernatant from each vial using a sterile Pasteur pipette.

▲ CRITICAL STEP Resuspending/washing of cells with fresh preculture medium may improve the survival rate. In this case, repeat Steps 32–33 once more (as, e.g., reported for *Taxus* × *media* cells[39]).

34| *Place two layers of sterilized Whatman filter paper discs onto a Petri dish containing solid recovery medium.

▲ CRITICAL STEP Weigh the upper filter paper disc before transferring it onto the plate medium; this information will be used for regrowth measurement (see **Box 1**).

35| *Use a sterile 3.5-ml transfer pipette, and without touching the rim of the vial transfer two-thirds' volume of the cells by spreading or placing them as a few clusters onto the filter paper. Close and seal the dish with Parafilm. Use the remaining cells in the vial for viability tests (see **Box 2**).

▲ CRITICAL STEP In most cases, place the cells as close together as possible (e.g., 5–10 clusters). Normally, one vial of cells per dish is used; however, one vial can also be used for more dishes depending on the required cell density for regrowth.

36| Cover the dish(es) with one or two sheets of filter paper to reduce the light intensity (to ~8 µmol m^{-2} s^{-1})[25], and place them in the culture room in regular conditions (24–25 °C).

Post-thaw recovery

37| *After 2 d of recovery, use a spatula (width of 4 mm) to collect some cell mass (about 100–200 mg FW) from the plate and place into a microtube for viability testing (see **Box 2**). Transfer the remaining cells with the upper filter paper to a fresh recovery dish containing recovery medium. Close and seal the dishes, cover them with filter paper and then return them to the culture room.

▲ CRITICAL STEP Viability test is fixed at day 2 of recovery, but transferring cells to a fresh dish in this step can be performed 2–4 d after recovery, depending on the sensitivity of the cell line; however, it must be done no longer than 4 d after recovery or the cells will die.

BOX 1 | TESTING REGROWTH BY FW CELL • TIMING 5–7 MIN

▲ CRITICAL All steps in this Box should be performed in a laminar airflow bench.

1. After 3 d of recovery time (Step 37), aseptically weigh the cells together with the filter paper while transferring onto fresh recovery plate medium. Seal the plate, cover the dish with a filter paper to reduce the light intensity and store in the same culture room for a further 7–14 d. Use at least three plates (triplicate) to be able to determine standard deviation.
2. Scrape and weigh all the cells/callus while transferring/subculturing directly (without a filter paper circle) onto fresh recovery plate medium, close and seal the dish, store in the same culture room in the regular/original conditions (periodic or continuous light or in the dark, at 24–25 °C).
3. Regularly weigh the callus in a time course (e.g., every 1–2 weeks for 2 months) while subculturing onto fresh solid medium. As the weight of the filter paper (Step 34) is known, the increased cell mass in a time course can be determined. Plot a growth curve of the time course (*x* axis) and the FW cells (*y* ordinate).

BOX 2 | VIABILITY TEST USING FDA • TIMING 5–10 MIN

This method is based on that described in reference 23.

1. Mix 10 µl of FDA solution with 0.5 ml of liquid medium and add this to the cells collected from Step 35 and/or Step 37.
2. With a micropipette, homogenize (by gently refluxing several times) the FDA-cell mixture and apply a drop (10 µl) of the mixture onto a microscope slide.
3. Place the slide under a fluorescence microscope, using blue-UV light excitation and a barrier filter with cutoff >510 nm.
4. Observe the slide after 5 min and count the number of fluorescent cells and the total number of cells.

▲ **CRITICAL STEP** The total number of cells[42] analyzed on the slide are between 300 and 500. The cytoplasm of viable cells shows a bright green fluorescence whereas dead cells do not fluoresce at all. Weakly fluorescent cells are not considered to be viable. Cell counting in aggregates may be difficult.

5. Calculate the percentage of cell viability as the number of fluorescing cells divided by the total number of cells × 100%.

38| Depending on their growth rates, allow cells to grow for an additional number of days (e.g., for *Taxus* × *media*[39], 7 d in the dark) in the same culture room, in regular conditions (24–25 °C). If assessing regrowth by FW, proceed to **Box 1**. Otherwise, proceed with Step 39 to continue cell recovery.

? TROUBLESHOOTING

39| *When most of the filter paper is covered with a thick layer of cells, transfer the cell mass to a fresh dish containing recovery medium without filter paper for a further 1–2 weeks under standard conditions (at this recovery stage, agarose may be replaced by agar). Normally, 3–9 weeks after recovery, cells can be transferred to a liquid medium to initiate suspension culture as described previously in Steps 10–13.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 6**.

TABLE 6 | Troubleshooting table.

Step	Problem	Possible reason	Solution
9	Contamination in callus cultures	Inappropriate cleaning, rinsing and sterilization of the surface of explants	Wash the explants properly under tap water, shake gently during immersion in ethanol 70% (vol/vol) and in the hypochlorite solution, increase the time of immersion in the range of seconds to a few minutes, but take care not to cause tissue death. Check activity of hypochlorite solution
		Inappropriate sterile/aseptic conditions, particularly during callus initiation	Wash hands properly and spray them with ethanol 70% (vol/vol) before work Properly follow the sterilization procedures of the tools and instruments; once they are sterile, place them untouched on the bench surface and close to the flame
12, 13	Contamination in suspension culture indicated by (1) dark/brownish color because of oxidative burst and cell death, (2) the presence of large white clumps of fungal colonies, (3) changing color, e.g., from cream-colored to reddish	Inappropriate sterile/aseptic conditions, particularly during initiation of cell suspension culture	Control the distance between operator and materials; do not speak or breathe close to the objects that need to be kept sterile

(continued)

TABLE 6 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
14	Contamination in suspension culture	Inappropriate sterile/aseptic conditions during subculturing	Strictly follow the procedure for aseptically opening and closing the Erlenmeyer flasks (they require longer exposure to the blue flame, especially the neck as compared with that for subculturing of microorganism cultures) When subculturing the cells by weighing, spray tissue papers and all tools including the balance with 70% (vol/vol) ethanol and wipe them with the dampened tissues Open the cover (aluminum foil) of sterilized sieve, Buchner filter, pipette or sterile microfilter only directly before use. *Note*: a low percentage of the commercially available sterile pipettes may accidentally not be sterile Work as efficiently as possible to reduce time of exposure of the sterile objects to the environment
20, 36–39	Contamination of cells after cryopreservation	Inappropriate sterile/aseptic conditions, particularly during cryopreservation and post-thaw recovery	Wash hands properly and spray them with ethanol 70% (vol/vol) before work Properly follow the sterilization procedures of the tools and instruments Be sure that the sterile pipette and/or –filter paper used is always kept sterile by not touching non-sterile surfaces Control the distance between operator and materials
8, 9, 12	No growth of cell suspension or callus culture after initiation, or low growth rate	Unsuitable medium for the plant cells/species	Initiate cell suspension cultures using different liquid media at the same time to avoid loss of time while waiting for the cell suspension culture to be formed
		Lack of growth hormone or inadequate applied growth hormone	Test single different auxins (e.g., NAA, IAA or 2,4-D) or cytokinins (e.g., BAP, zeatin, kinetin) in different concentrations. Test different combinations of auxin and cytokinin at different concentrations and ratios
		Unsuitable cultivation conditions	Change the conditions; light intensity and period are important factors, some cell suspension cultures of plant species even require absence of light to grow The shaking speed and time also influence cell growth, some cell lines can be more sensitive to shaking speed of 125 r.p.m.
		Inoculum density too low	Increase the inoculum density, use more callus (e.g., 2–3 g FW/15–25 ml medium)
		Poor medium, need a certain precursor for growth	Add casein hydrolysate 0.5–2.0 g l^{-1} of medium
		Too tight flask/plate closure, aluminum foil, parafilm or plastic are completely airtight, thus causing oxygen limitation	Use either cotton plugs, allowing equilibrium with ambient atmosphere, or silicon stoppers that have reproducible gas exchange rate
9, 12	Browning	Phenolics	Add PVPP or carbon active to absorb the phenolics
		Inappropriate subculturing time (later than required) resulting in the death of many cells	Let the still-viable cells recover by subculturing in liquid medium more frequently than usual or spreading the cells on a solid medium
		Unsuitable medium/growth hormone	Change medium with different hormone
12–14	Big aggregates/clumps/calli-like	Inadequate subculturing time (later than required)	Separate the fine cells from aggregates by pipetting, decantation or filtering using a sieve when subculturing Add 0.005% pectinase to the cell cultures[60]

(continued)

TABLE 6 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
		Inappropriate aeration/too much volume of cell suspension in the flask	Reduce the volume from 100 ml to 50–70 ml for a 250-ml flask
14B, 14C	Dissimilar amounts/ volume/density of cells in the flasks of one batch during maintenance	Different cell density while pipetting	Use pipetting only for inoculation of homogenous fine cell suspension culture. Continuously shake both the flask and the pipette while pipetting
		Different amounts of cells inoculated by pouring	Shake and immediately pour into a graduated tube/flask. Preferably apply this technique only to homogenous fine cell suspension culture; it may provide better result than pipetting
16	Nonreproducible growth curve	Different water content due to suction filtering	Optimize reproducibility: the same vacuum strength (suction power), the same time (min), the same operator
		Different water content of dry weight cells	Dry the cells from the same batch for a growth curve simultaneously in the same freeze-dryer and for the same time
		Different weight/shape of the flasks used	Select flasks with the same volume and weight for dissimilation curve Select flasks with the same volume and shape for CVS growth curve, use the same CVS device Increase settling time of CVS method in case of very fine suspensions
16	Inconsistency/high variation of analytical results between the flasks of the same batch/treatment	Different flasks of original cells used for inoculation	Mix/homogenize the cell suspensions from several different flasks into one, bigger Erlenmeyer flask; use them for inoculation of one batch suspension cultures for an experiment
17, 20, 36–39	Low viability/no regrowth after cryopreservation	Problems in the preculturing step	Try using as starting material cells growing at a different day of the growth phase (exponential to early stationary phase); try different concentrations of mannitol/other osmotic agent(s), or different incubation time
		Problems in the cryoprotection step	Change or optimize the composition and the concentration of the "SGD" cryoprotectant solution; e.g., instead of DMSO use propane-1,2-diol[29] or other cryoprotectant agents[59] Optimize the shaking speed and time
		Problems at the freezing step	Have a backup of a cryopreserved cell line in some cryovials Use different freezing equipment, e.g., 'Mr. Frosty' or a (expensive) programmable freezer; some strains could be more sensitive to one of the two cooling processes
		Problems at the thawing/plating steps	Rinse the cells with preculture medium (Step 38), particularly for a cell suspension culture showing turbidity (e.g., of *Taxus*[39]). However, rinsing the cells can also be deleterious[29] Change the way of plating; clustering the cells improves the survival (e.g., for *Catharanthus roseus* cell lines); for rice cells either spreading or clustering technique provides good survival Use larger amounts of cells for plating; e.g., one vial of cells for one medium plate

(continued)

TABLE 6 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
		Problems at the recovery step	Monitor the dishes every 2–3 d after Step 38, if the filter paper is wet, transfer it again to a fresh plate of medium
		Unsuitable cryopreservation technique for a particular plant cell species/cell line	Try another technique; e.g., vitrification[30] or encapsulation-dehydration[31,34]
29	Cryovials rupturing	Liquid nitrogen was trapped in the vials, evaporated rapidly and increased pressure in the vials	Use eye protection during this step for safety. Use cryovial with the best closure; different cryovials have different closure systems, which allow the release of evaporating nitrogen to different extents

● TIMING

The timing of the different technical operations as described in this protocol is shown below. However, the total time schedule from plant to cryopreserved cell line will vary, dependent not only on cellular growth but also on the stabilization of a cell line. To achieve this, 6–9 months are usually needed.
Steps 1–9, Initiation of callus culture from plants: 1 h
Steps 10–12, Initiation of plant cell suspension culture from callus culture: 15 min
Step 13A, Preparing a fine cell suspension by filtration: 10–15 min
Step 13B, Preparing a fine cell suspension by decanting or pipetting: 5 min
Step 13C, Preparing a fine cell suspension by addition of pectinase: 2 min
Step 14A, Subculturing by weighing: 5 min
Step 14B, Subculturing by pipetting (volume measuring): 3 min
Step 14C, Subculturing by pouring (volume measuring): 2 min.
Step 15, Preparing a batch of cell suspension cultures for growth measurement: 30–60 min
Step 16A, Dry weight measurements on filter paper—oven heating: 15 min
Step 16B, Dry weight measurements using tubes—freeze-drying: 15 min
Step 16C, Determination of loss of weight (dissimilation curve): 2 min
Step 16D, Determination of CVS: 5–10 min
Steps 17–20, Preculturing: 30 min
Steps 21–25, Cryoprotection: 90 min
Steps 26–27, Freezing and storage: 4 h and 15 min
Steps 28–36, Thawing and plating: 20 min
Step 37, Post-thaw recovery (subculturing cells): 5 min
Step 38, Post-thaw recovery (additional days for recovery/monitoring of cells): 7–14 d
Step 39, Post-thaw recovery (subculturing cells): 5 min

ANTICIPATED RESULTS

The protocol described here should allow the initiation, maintenance and long-term storage of a wide range of wild-type and transgenic plant cell suspension cultures. Labs at Leiden University have used this protocol to establish a large collection of cultures, some[61–71] of which are listed in **Table 5**, along with media requirements and culture characteristics. Plant cell suspension cultures provide researchers with the means to study the following: optimal conditions for industrial scale-up; plant metabolomics; biosynthesis of secondary metabolites; metabolic transport/compartmentation; biotransformation; plant proteomics; metabolic engineering to improve the production of valuable compounds; and signal transduction cascades in plant cell physiology.

It is recommended to keep a relatively stable stock of cell lines for various experiments and a back up of the cell lines in cryopreserved stock. These cell lines can be used over a number of years. It is important to bear in mind, though, that genetic instability[26] of cell lines may occur and environmental changes may cause changes. For example, moving to new facilities in a different floor affected all suspension cultures (about 90 different cell lines), resulting in the death of ~60% of the cell lines, whereas the surviving strains completely changed their character in terms of growth and secondary metabolite production. Cryopreservation is now performed on each new wild-type and transgenic cell line, and once a year on each cell

line maintained in our laboratory. Until 1995, 75% of the ~60 cell lines cryopreserved using the two-step freezing protocol successfully survived with growth rates varying between a tenfold increase in density in 1 week to a threefold increase in 2 weeks after thawing. The cryopreserved cells could still be recovered after 6 years of storage[29]. Analysis of cell lines after recovery from cryopreservation by regrowth and relative nuclear DNA content (see **Fig. 11**) and by NMR-based metabolomics in combination with a multivariate data analysis (see **Fig. 12**) showed that the two-step freezing cryopreservation technique provides an alternative to avoiding genetic instability or loss of productivity because of cell selection,[4] which can occur during a prolonged (years) subculture period. No differences were detected in the cell growth (data not shown), but some changes occurred in the primary metabolism in the cells (**Fig. 12**), which could be due to several factors. The cryopreservation may change the cell physiology as the cells experienced, e.g., chemical and osmotic stress, cold shock, ice crystal formation, membrane overstretch or rupture during thawing; however, it could also be that the cells were not fully recovered within 3 months after cryopreservation. However, the change was found only in sugars and alanine; the levels of tryptophan and tryptamine were similar when compared with control/non-cryopreserved cells. Additional data obtained by gas chromatography-flame ionization detection (GC-FID) analysis (data not shown) also revealed no differences in the level of some sterols between control and cryopreserved cells.

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AUTHOR CONTRIBUTIONS N.R.M., on the basis of her experience as the person responsible for the plant cell cultures in the Department of Pharmacognosy, made the draft proposals for the plant cell culture protocols and combined them for the manuscript. W.d.W. and F.v.I., who developed the methods for cryopreservation and have been applying them for the past 20 years, wrote the protocols for this method. R.V. coordinated and supervised the process of writing, and on the basis of his many years of experience in plant cell biotechnology was particularly involved in the process of identifying and describing critical steps in the protocols.

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1. Endreß, R. *Plant Cell Biotechnology* (Springer-Verlag, 1994).
2. Dixon, R.A. Isolation and maintenance of callus and cell suspension cultures. In *Plant Cell Culture; a Practical Approach* (ed. Dixon, R.A.) 1–20 (IRL Press, 1985).
3. Gamborg, O.L. & Phillips, G.C. Media preparation and handling. In *Plant Cell, Tissue and Organ Culture; Fundamental Methods* (eds. Gamborg, O.L. & Phillips, G.C.) 1–90 (Springer Lab Manual, Springer-Verlag, 1995).
4. Morris, P. Long term stability of alkaloid productivity in cell suspension cultures of *Catharanthus roseus*. In *Secondary Metabolism in Plant Cell Cultures* (eds. Morris, P., Scragg, A.H., Stafford, A. & Fowler, M.W.) 257–262 (Cambridge University Press, 1986).
5. Sierra, M.I., van der Heijden, R., van der Leer, T. & Verpoorte, R. Stability of alkaloid production in cell suspension cultures of *Tabernaemontana divaricata* during long-term subculture. *Plant Cell Tiss. Org. Cult.* **28**, 59–68 (1992).
6. Hulst, A.C., Meyer, M.M.T., Breteler, H. & Tramper, J. Effect of aggregate size in cell-cultures of *Tagetes-patula* on thiophene production and cell growth. *Appl. Microbiol. Biotechnol.* **30**, 18–25 (1989).
7. Edahiro, J.-I. & Seki, M. Phenylpropanoid metabolite supports cell aggregate formation in strawberry cell suspension culture. *J. Biosci. Bioeng.* **102**, 8–13 (2006).
8. Su, W.W. & Arias, R. Continuous plant cell perfusion culture: bioreactor characterization and secreted enzyme production. *J. Biosci. Bioeng.* **95**, 13–20 (2003).
9. Ten Hoopen, H.J.G., van Gulik, W.M. & Heijnen, J.J. Continuous culture of suspended plant cells. *In Vitro Cell Dev. Biol.* **28**, 115–120 (1992).
10. Schripsema, J., Meijer, A.H., van Iren, F., ten Hoopen, H.J.G. & Verpoorte, R. Dissimilation curves as a simple method for the characterization of growth of plant cell suspension cultures. *Plant Cell Tiss. Org. Cult.* **22**, 55–64 (1990).
11. Stafford, A., Smith, L. & Fowler, M.W. Regulation of product synthesis in cell cultures of *Catharanthus roseus* (L)G. Don. *Plant Cell Tiss. Org. Cult.* **4**, 83–94 (1985).
12. Blom, T.J.M., Kreis, W., van Iren, F. & Libbenga, K.R. A non-invasive method for the routine-estimation of fresh weight of cells grown in batch suspension cultures. *Plant Cell Rep.* **11**, 146–149 (1992).
13. Contin, A., van der Heijden, R., ten Hoopen, H.J.G. & Verpoorte, R. The inoculum size triggers tryptamine or secologanin biosynthesis in a *Catharanthus roseus* cell culture. *Plant Sci.* **139**, 205–211 (1998).
14. Schripsema, J. & Verpoorte, R. Novel techniques for growth characterization and phytochemical analysis of plant cell suspension cultures. *J. Nat. Prod.* **58**, 1305–1314 (1995).
15. David, H., Laigneau, C. & David, A. Growth and soluble proteins of cell cultures derived from explants and protoplasts of *Pinus pinaster* cotyledons. *Tree Physiol.* **5**, 497–506 (1989).
16. Hahlbrock, K. Further studies on the relationship between the rates of nitrate uptake, growth and conductivity changes in the medium of plant cell suspension cultures. *Planta (Berl.)* **124**, 311–318 (1975).
17. Hahlbrock, K., Ebel, J. & Oaks, A. Determination of specific growth stages of plant cell suspension cultures by monitoring conductivity changes in the medium. *Planta (Berl.)* **118**, 75–84 (1974).
18. Taya, M., Hegglin, M., Prenosil, J.E. & Bourne, J.R. On-line monitoring of cell growth in plant tissue cultures by conductometry. *Enzyme Microb. Technol.* **11**, 170–176 (1989).
19. Thom, M., Maretzki, A., Komor, E. & Sakai, W.S. Nutrient uptake and accumulation by sugarcane cell cultures in relation to the growth cycle. *Plant Cell Tiss. Org. Cult.* **1**, 3–14 (1981).
20. Bradford, M.M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254 (1976).
21. Doležel, J., Greilhuber, J. & Suda, J. Estimation of nuclear DNA content in plants using flow cytometry. *Nat. Protoc.* **2**, 2233–2244 (2007).
22. Pfosser, M., Magyar, Z. & Bögre, L. Cell cycle analysis in plants. In *Flow Cytometry with Plant Cells* (eds. Doležel, J., Greilhuber, J. & Suda, J.) 323–347 (Wiley-VCH, 2007).
23. Widholm, J.M. The use of fluorescein diacetate and phenosafranin for determining viability of cultured plant cells. *Stain Technol.* **47**, 189–194 (1972).
24. Baker, J.C. & Mock, N.M. An improved method for monitoring cell death in cell suspension and leaf disc assays using evans blue. *Plant Cell Tiss. Org. Cult.* **39**, 7–12 (1994).
25. Verleysen, H., Samyn, G., Van Bockstaele, E. & Debergh, P. Evaluation of analytical techniques to predict viability after cryopreservation. *Plant Cell Tiss. Org. Cult.* **77**, 11–21 (2004).
26. Deus-Neumann, B. & Zenk, M.H. Instability of indole alkaloid production in *Catharanthus roseus* cell suspension cultures. *Planta Med.* **50**, 427–431 (1984).
27. Kartha, K.K. Meristem culture and germplasm preservation. In *Cryopreservation of Plant Cells and Organs* (ed. Kartha, K.K.) 115–134 (CRC Press, 1985).

28. Benson, E.E. Cryopreservation of phytodiversity: a critical appraisal of theory & practice. *Crit. Rev. Plant Sci.* **27**, 141–219 (2008).
29. Schrijnemakers, E.W.M. & Van Iren, F. A two-step or equilibrium freezing procedure for the cryopreservation of plant cell suspensions. In *Methods in Molecular Biology Vol. 38 Cryopreservation and Freeze-drying Protocols* (eds. Day, J.G. & McLellan, M.R.) 103–111 (Humana Press, 1995).
30. Reinhoud, P.J., Van Iren, F. & Kijne, J.W. Cryopreservation of undifferentiated plant cells. In *Cryopreservation of Tropical Plant Germplasm: Current Research Progress and Application* (eds. Engelmann, F. & Takagi, H.) 91–102 (International Plant Genetic Resources Institute, 2000).
31. Bachiri, Y.C., Gazeau, J., Hansz, J., Morisset, C. & Dereuddre, J. Successful cryopreservation of suspension cells by encapsulation-dehydration. *Plant Cell Tiss. Org. Cult.* **43**, 241–248 (1995).
32. Yamazaki, H., Ayabe, K., Ishii, R. & Kuriyama, A. Desiccation and cryopreservation of actively-growing cultured plant cells and protoplasts. *Plant Cell Tiss. Org. Cult.* **97**, 151–158 (2009).
33. Steponkus, P.L., Langis, R. & Fujikawa, S. Cryopreservation of plant tissues by vitrification. In *Advances in Low Temperature Biology* Vol. 2 (ed. Steponkus, P.L.) 1–62 (JAI Press, 1992).
34. Sakai, A., Hirai, D. & Niino, T. Development of PVS-based vitrification and encapsulation-vitrification protocols. In *Plant Cryopreservation: A Practical Guide* Chapter 3 (ed. Reed, B.M.) 33–57 (Springer, 2008).
35. Langis, R., Schnabel, B., Earle, E.D. & Steponkus, P.L. Cryopreservation of *Brassica campestris* L. cell suspensions by vitrification. *Cryo-Lett.* **10**, 421–428 (1989).
36. Uragami, A., Sakai, A., Nagai, M. & Takahashi, T. Survival of cultured cells and somatic embryos of *Asparagus officinalis* cryopreserved by vitrification. *Plant Cell Rep.* **8**, 418–421 (1989).
37. Dereuddre, J., Scottez, C., Arnaud, Y. & Duron, M. Resistance of alginate-coated axillary shoot tips of pear tree (*Pyrus communis* L. cv. Beurré Hardy) *in vitro* plantlets to dehydratation and subsequent freezing in liquid nitrogen: effects of previous cold hardening. *Comptes Rendus de l'Académie des Sciences* **310** Série III, 317–323 (1990).
38. Withers, L.A. & King, P. A simple freezing unit and routine cryopreservation method for plant cell cultures. *Cryo-Lett.* **1**, 213–220 (1980).
39. Škrlep, K. *et al.* Cryopreservation of cell suspension cultures of *Taxus x media* and *Taxus floridana*. *Biol. Plant.* **52**, 329–333 (2008).
40. Reed, B.M. Cryopreservation; practical consideration. In *Plant Cryopreservation: A Practical Guide* Chapter 1 (ed. Reed, B.M.) 3–13 (Springer, 2008).
41. Heine-Dobbernack, E., Kiesecker, H. & Schumacher, H.M. Cryopreservation of dedifferentiated cell cultures. In *Plant Cryopreservation: A Practical Guide* Chapter 8 (ed. Reed, B.M.) 141–176 (Springer, 2008).
42. Reinhoud, P.J., Schrijnemakers, E.W.M., van Iren, F. & Kijne, J.W. Vitrification and a heat-shock treatment improve cryopreservation of tobacco cell suspensions compared to two-step freezing. *Plant Cell Tiss. Org. Cult.* **42**, 261–267 (1995).
43. Ishikawa, M. *et al.* Effect of growth phase on survival of Bromegrass suspension cells following cryopreservation and abiotic stresses. *Ann. Bot.* **97**, 453–459 (2006).
44. Lu, T.G. & Sun, C.S. Cryopreservation of millet (*Setaria italica* L.). *J. Plant Physiol.* **139**, 295–298 (1992).
45. Sala, F., Cella, R. & Rollo, F. Freeze-preservation of rice cells grown in suspension culture. *Physiol. Plant* **45**, 170–176 (1979).
46. Friesen, L.J. *et al.* Cryopreservation of *Papaver somniferum* cell suspension cultures. *Planta Med.* **57**, 53–55 (1991).
47. Kim, S.I. *et al.* Cryopreservation of *Taxus chinensis* suspension cell cultures. *CryoLetters* **22**, 43–50 (2001).
48. Suhartono, L. *et al.* Metabolic comparison of cryopreserved and normal cells from *Tabernaemontana divaricata* suspension cultures. *Plant Cell Tiss. Org. Cult.* **83**, 59–66 (2005).
49. Schripsema, J., Erkelens, C. & Verpoorte, R. Intra- and extracellular carbohydrates in plant cell cultures investigated by ^{1}H-NMR. *Plant Cell Rep.* **9**, 527–530 (1991).
50. George, E.F., Puttock, D.J.M. & George, H.J. *Plant Culture Media Vol. 1; Formulations and Uses* (Exegetics Limited, 1987).
51. Murashige, T. & Skoog, F. A revised medium for rapid growth and bioassays with tobacco cultures. *Physiol. Plant* **15**, 473–497 (1962).
52. Gamborg, O.L., Miller, R.A. & Ojima, K. Nutrients requirements of suspension cultures of soybean root cells. *Exp. Cell Res.* **50**, 151–158 (1968).
53. Linsmaier, E.M. & Skoog, F. Organic growth requirements of tobacco tissue cultures. *Physiol. Plant* **18**, 100–127 (1965).
54. Schenk, R.U. & Hildebrandt, A.C. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. *Can. J. Bot.* **50**, 1999–2004 (1972).
55. Kors, F.T.M. *Biochemicals Plant Cell and Tissue Culture: Catalogue 2003–2005* (Duchefa Biochemie, 2003).
56. Bhojwani, S.S., Dhawan, V. & Cocking, E.C. *Plant Tissue Culture: A Classified Bibliography* 1–65 (Elsevier Science, 1986).
57. Bhojwani, S.S., Dhawan, V. & Arora, R. *Plant Tissue Culture: A Classified Bibliography 1985–1989* 1–13 (Elsevier Science, 1990).
58. Knobloch, K.H., Bast, G. & Berlin, J. Medium- and light-induced formation of serpentine and anthocyanins in cell suspension cultures of *Catharanthus roseus*. *Phytochemistry* **21**, 591–594 (1982).
59. Kadkade, P.G., Bare, C.B., Schnabel-Preiktas, B. & Yu, B. *Cryopreservation of Plant Cells*. US Patent no. 5,965,438 (1999).
60. Lee, T.-J., Shultz, R.W., Hanley-Bowdoin, L. & Thompson, W.F. Establishment of rapidly proliferating rice cell suspension culture and its characterization by fluorescence-activated cell sorting analysis. *Plant Mol. Biol. Rep.* **22**, 259–267 (2004).
61. Figueiredo, A.C.S. & Pais, M.S.S. *Achillea millefolium* (Yarrow) cell suspension cultures: establishment and growth conditions. *Biotechnol. Lett.* **13**, 63–68 (1991).
62. Flores-Sanchez, I.J. *et al.* Elicitation studies in cell suspension cultures of *Cannabis sativa* L. *J. Biotechnol.* **143**, 157–168 (2009).
63. El-Sayed, M. & Verpoorte, R. Effect of phytohormones on growth and alkaloid accumulation by a *Catharanthus roseus* cell suspension cultures fed with alkaloid precursors tryptamine and loganin. *Plant Cell Tiss. Org. Cult.* **68**, 265–270 (2002).
64. Mustafa, N.R., Kim, H.K., Choi, Y.H. & Verpoorte, R. Metabolic changes of salicylic acid-elicited *Catharanthus roseus* cell suspension cultures monitored by NMR-based metabolomics. *Biotechnol. Lett.* **31**, 1967–1974 (2009).
65. Moreno, P.R.H. *et al.* Induction of ajmalicine formation and related enzyme activities in *Catharanthus roseus* cells: effect of inoculum density. *Appl. Microbiol. Biotechnol.* **39**, 42–47 (1993).
66. Ramos-Valdivia, A.C., van der Heijden, R. & Verpoorte, R. Elicitor-mediated induction of anthraquinone biosynthesis and regulation of isopentenyl diphosphate isomerase and farnesyl diphosphate synthase activities in cell suspension cultures of *Cinchona robusta*. *Planta* **203**, 155–161 (1997).
67. Langezaal, C.R. & Scheffer, J.J.C. Initiation and growth characterization of some hop cell suspension cultures. *Plant Cell Tiss. Org. Cult.* **30**, 159–164 (1992).
68. Yuana Dignum, M.J.W. & Verpoorte, R. Glucosylation of exogenous vanillin by plant cell cultures. *Plant Cell Tiss. Org. Cult.* **69**, 177–182 (2002).
69. Han, Y., van der Heijden, R. & Verpoorte, R. Biosynthesis of anthraquinones in cell cultures of the Rubiaceae. *Plant Cell Tiss. Org. Cult.* **67**, 201–220 (2001).
70. Schripsema, J., Dagnino, D., Dos Santos, R.I. & Verpoorte, R. Breakdown of indole alkaloids in suspension cultures of *Tabernaemontana divaricata* and *Catharanthus roseus*. *Plant Cell Tiss. Org. Cult.* **38**, 299–305 (1994).
71. Dagnino, D., Schripsema, J. & Verpoorte, R. Terpenoid indole alkaloid biosynthesis and enzyme activities in two cell lines of *Tabernaemontana divaricata*. *Phytochemistry* **39**, 341–349 (1995).
72. Kraemer, K.H., Schenkel, E.P. & Verpoorte, R. *Ilex paraguariensis* cell suspension culture characterization and response against ethanol. *Plant Cell Tiss. Org. Cult.* **68**, 257–263 (2002).