



Agriculture

Protoplast isolation, culture and application to genetic improvement of woody plants

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Abstract

Traditional breeding of most woody plants is more or less confronted with certain barriers. Protoplast isolation and culture pave way for genetic improvement of woody plants. Since the first try on protoplast isolation and culture was conducted much progress has been made in woody plants, covering most of the important species including fruit trees, such as citrus, apple, pear, mango, kiwifruit, litchi, and forestry trees, such as sandalwood, poplar, eucalyptus, mulberry, conifer, etc. The present paper reviews the general protocols concerning protoplast isolation and culture, summarizes the factors affecting protoplast isolation and culture, introduces the variations, inclusive of chromosome number, morphology and resistance, occurring in protoplast-derived plants. In addition, application of protoplast to genetic improvement and basic study of woody plants are discussed herein, such as cryopreservation, somatic hybridization, genetic transformation and other basic researches.

Key words: Protoplast isolation, protoplast culture, woody plants, genetic improvement.

Introduction

Most of the woody plants are characterized with long juvenility periods, a high degree of heterozygosity, along with inadequate information concerning genetic background. conventional breeding does make great contributions to genetic improvement of the woody plants. However, certain difficulties exist in conventional breeding, such as cross or self-incompatibility, polyembryony, male and/or female sterility and differential flowering periods. So, exploration of other breeding alternatives is imperative and necessary to meet the needs of genetic improvement of woody plants. Since the first attempt on protoplast isolation was conducted in *Acer pseudoplatanus*, much progress has been made^{1,2,3,4}. Vardi et al in Israel first produced plants from cultured protoplasts of ovule-derived embryogenic callus of sweet orange⁵. Later on, great efforts were made on protoplast manipulation and plants were regenerated from many woody plants, including citrus cultivars and its relatives^{6,7,8,9,10,11,12}, apple^{13,14,15,16,17,18}, avocado¹⁹, colt cherry²⁰, grape^{21,22,23}, persimmon^{24,25}, passion fruit^{26,27}, pear^{28,29,30}, kiwifruit^{31,32,33,34,35,36,37}, papaya³⁸, sour cherry³⁹, loquat⁴⁰, litchi⁴¹, mango⁴², *Zizyphus jujube*⁴³, *Lycium barbarum*^{44,45}, *Abies alba*⁴⁶, sandalwood⁴⁷, elm⁴⁸, *Hibiscus syriacus*⁴⁹, poplar^{50,51,52,53,54,55,56}, *Rauvolfia vomitoria*⁵⁷, *Pithecellobium dulce*⁵⁸, paper mulberry⁵⁹, *Platanus orientalis*⁶⁰, conifer^{61,62,63}, white spruce⁶⁴, mulberry^{65,66}, eucalyptus⁶⁷, *Liriodendron tulipifera*⁶⁸, *Paulownia fortunei*⁶⁹, and so on (Table 1). The present paper focuses on recent progress in protoplast isolation, culture of woody plants and its application to genetic improvement and basic study of woody plants.

Protoplast Isolation

Protoplast culture and genetic manipulation depend on the production of large numbers of viable protoplasts. Rather than mechanical isolation, the more efficient enzyme digestion has become the usual method for protoplast liberation.

Source of explants and sampling: Many explants have been involved in protoplast isolation from woody plants, including leaf, shoot stem, callus, cotyledon, fruit, mesocarp, suspension cultures, needle, tetrad, Female gametophyte, and xylem^{15,17,26,41,70,71,72,73}. But young and tender leaf and callus or cell suspensions give rise to the highest yields of quality protoplasts. Leaves can be sampled from the plants grown in the greenhouse, outdoor field, growth chamber or in vitro, but in vitro leaves are mainly used since they are devoid of requiring decontamination treatment necessary and produce more protoplasts compared with field materials^{27,28,37,41,50}. The callus and suspension cultures could be induced from meristem⁴¹, zygotic or somatic embryo and nucellus^{19,38,74,75}, seed⁵¹, immature inflorescence⁷⁶, meristem¹⁷, leaf⁷⁷, root²⁰ and anther⁷⁸. Cultured callus and cell suspensions that grow rapidly are amenable to reproducible results and are always sampled in the logarithmic phase.

Enzyme solution preparation: The enzymes used in protoplast isolation of most woody plants are primarily cellulase (Onozuka R-10, namely Cellulysin, Cellulase Onozuka RS and Driselase), hemicellulase and pectinase (Pectolyase Y-23 and Macerozyme). Usually for protoplast isolation of most plants 1-2% cellulase and 0.1-1% pectolyase are used^{24,46}. When the enzyme solution is prepared, some chemicals are optionally added, such as CaCl₂·2H₂O, KH₂PO₄ or dextran sulfate potassium for maintaining stability of cell membrane and viability of protoplasts, 2-(N-morpholino) ethanesulfonic acid (MES) for pH stability and bovine serum albumin (BSA) for mitigation of organelle damage^{19,50,79}. In addition, for the maintenance of certain osmotic pressure, some regulators are added, such as glucose, fructose, sucrose, sorbitol and mannitol, singly or combined with a total concentration of 0.6-0.8 mol/l^{10,19,24,50,51}. The pH of the enzyme solution is adjusted between 5.6 and 5.8 and the enzyme solution should be filter sterilized.

Protoplast isolation: Explants are generally mixed directly with the enzyme solution in one step. A two-step incubation, in which the explant is treated with pectinase and cellulase consecutively, has also been used to isolate protoplasts from apple cotyledon⁸⁰. The ratio of the explants to enzyme solution is possibly different, depending on the physiological status, the age of the explants and the enzyme solution contents. For citrus protoplast isolation, usually 1 g callus or 0.1 g leaflets is incubated in 3 ml enzyme solution^{2,6}. The explants and the enzyme solution are put in petri dishes or in small flasks, which are generally put on a gyratory shaker at 25-28°C. Incubation is mainly carried out in dark for woody plants^{2,6,42,81} with few exceptional case²⁰. The optimum revolution speed differs among species, for instance, 25-30 rpm for citrus, 40-50 rpm for mango and 40-100 rpm for apple, pear and peach^{6,28,42,82}. The incubation time varies from several hours to one day, depending on the plant species and the explant.

Protoplast purification: After the incubation the digested mixture contains enzyme solution, protoplasts, multiple cell clusters, debris and undigested tissues. Purification is necessary so as to remove the unexpected parts. For purification, the mixture is first passed through a stainless steel or nylon sieves with appropriate mesh diameter, followed by wash with CPW salt solution². The filtered solution is then centrifuged with either of two methods, sinking or floating^{2,54,82,83}, in order to remove the enzyme solution and the washing solution and to enrich protoplast, which is then adjusted to an appropriate density with culture medium.

Protoplast viability analysis: Viability of purified protoplast is usually determined by FDA (fluorescein diacetate) staining. FDA is prepared in acetone at 2 mg/ml and stored as a stock solution at 4°C. With the illumination of UV light using a fluorescent microscope, green fluorescence can be observed in the viable cells. In addition Evans Blue was also used for the protoplast viability estimation⁵¹.

Factors affecting protoplast isolation: As has been documented in many reports, it is difficult to consistently obtain high yields of quality protoplasts. This is impacted by many factors including plant species and genotype^{84,85}, source and physiological status of explant⁸⁶, enzyme solution⁸² and pretreatment⁸⁷.

Genotype: As is well known, protoplast yields are to, a large degree, dependent on the genotype. Assani et al carried out protoplast isolation from 7 banana genotypes and only two of them gave good results⁸⁸. Cao and Fu investigated the protoplast yield of 3 apple genotypes, Royal Gala, New Johngold and Spur Fuji. Though the protoplasts' viability were the same from these 3 genotypes significant differences in the yield were detected. The highest yield was obtained in Royal Gala, followed by New Johngold and Spur Fuji⁸⁹.

Enzyme solution, components and incubation time: In general, protoplast yields increase with high enzyme concentrations and long incubation time^{82,90}. However, it has been reported that the protoplast yield was reduced when the explant is treated for such a long time that the isolated protoplasts are damaged by the solution. Therefore, when the explant is treated with enzyme, combination of long incubation time and low enzyme concentration or short incubation time and high enzyme concentration should be avoided. Addition of antioxidants to the enzyme could influence protoplast

release as well^{61,69}. For example, addition of PVP in the enzyme solution enhanced protoplast yield and viability of *Prunus davidiana* Franch and wild apricot^{91,92}.

Explant: It has been well documented that explant poses great impact on protoplast release. The physiological status, age and source of explant are important factors affecting protoplast isolation. In apple more protoplasts were released from callus that has been kept on fresh medium for shorter rather than longer periods^{78,84}. Similar results have been shown in *Pyrus persica* cell suspensions⁸², grape callus⁹³, elm⁴⁸ and persimmon callus²⁴. Moreover, different explant sources lead to disparate isolation results. It has been reported that suspension cultures provide higher yields of protoplasts than callus, cotyledon, seedling and field leaves in many kinds of woody plants^{43,48,84,85,88,94,95,96,97}. But in vitro seedling leaves are better for protoplast release than greenhouse-grown plants in both yield and viability⁸⁶. In addition protoplasts derived from leaf are considered to be more genetically uniform with satisfactory genetic integrity, as they contain little pre-existing somaclonal variation. Plasmolysis with salt or sugar solution prior to incubation and pretreatment of the explant in the condition medium was of help to protoplast isolation of wild pear, strawberry, apple and grape^{87,98,99,100}.

Protoplast Culture and Plant Regeneration

Culture methods: Several methods have been used for protoplast culture of woody plants, such as liquid thin layer culture, solid embedding culture and liquid over solid culture. Liquid thin layer culture is most commonly used for protoplast culture^{37,42,51,76}. Nearly 2 ml of the suspended protoplasts described above are put in the petri dish (6cm×6cm) to form a thin layer. This method is very simple and easy for the addition of new culture medium. However, protoplasts aggregate easily with each other⁴¹. In addition, contamination might occur due to frequent addition of fresh medium. With solid embedding culture the protoplast suspensions are mixed with 1.2% agar or low melting temperature (LMT) agarose, which is kept in 30-40°C water bath, at a certain ratio (1:2). With liquid over solid culture system solid medium is put into the petri dish, on which protoplasts suspended in liquid medium are added^{27,38}. In the culture systems the petri dish is sealed with parafilm and put in the growth chamber at 25-28°C, which is free of light in the beginning with exception in some cases. In addition feeder layer or nurse cell culture system is also employed for some woody plants^{41,76,88,101}.

Protoplast culture medium: Usually the media used for cell and tissue culture of woody plants can also be applied to protoplast culture with minor modification. The most widely used basal media for woody plants are MS or MT^{7,10,19,37,71}, B₅⁴², DCR^{102,103}, KPR⁷⁶, WPM^{51,52}, K_{8p}⁵⁶ and KM_{8p}^{17,27,38}, and other media are modified from these, such as BH₃ medium for citrus protoplast culture². Sometimes for the same species different medium had to be used during the course of protoplast culture. With *Abies alba* protoplasts KM medium is necessary for protoplast sustained division, while the development of proembryos could be fulfilled only when SH medium was used⁷⁵. In the medium, some sugars or sugar alcohols, such as sucrose, mannitol¹⁶, sorbitol^{7,92,104} and glucose^{75,97}, with the concentration of 0.4-0.6 mol/L, are added, which act as either osmotic regulators or carbon sources for the cultured protoplasts^{50,60}.

In addition, plant growth regulators (PGR), especially auxins and cytokinins, are often needed in the media^{13,52}, whereas, PGR is not needed for citrus protoplast culture with very few exceptions^{5,6,7,8}.

Aggregation of protoplast: During the course of protoplast culture, protoplast aggregation is often observed. It can influence the protoplasts' growth either positively or negatively. For instance the aggregation of litchi protoplasts accounted for the failure of sustained division⁴¹. But in citrus the aggregation always means good division activities of the protoplasts (Liu and Deng, unpublished). The underlying reason for protoplast aggregation is possibly the surface negative charge in the protoplasts¹⁰⁵. Protoplasts with weak negative charges tend to aggregate, whereas the protoplasts with strong negative charges rarely aggregate. Moreover, the aggregation can be attenuated by ABA and promoted by Ca²⁺.

Regeneration of cell wall: Protoplasts regenerate cell walls after being cultured for certain period. The phenomenon that protoplasts become elliptical always indicates the regeneration of cell walls, which can be investigated by Calcofluor White. The time for cell wall regeneration is not fixed for different plants, and ranges from several hours to several days. For example, cell wall was formed in 24 h for banana protoplasts, while cell wall regeneration was completed 12-13 days for pear protoplast^{81,106}.

Formation of cell clusters and regeneration of plantlets: Shortly after the regeneration of cell walls, protoplasts recover their first mitotic division. For some woody plants the division can be observed within 1-2 days¹⁰⁷, while others may require 4-7 days or even longer^{23,108,109}. The protoplasts with regeneration capability can keep dividing and finally develop into multi-cell clusters. At this time it is usually necessary to supply fresh culture medium to the culture system because nutrition and the osmotic pressure in the culture system cannot meet the needs of the growing cells⁶. When the cell clusters develop into callus they should be transferred to proliferation medium. The callus can regenerate into complete plantlets in two ways, organogenesis, such as apple, pear, chinara, and poplar, etc^{13,16,30,52,60}, and embryogenesis, such as *Abies alba*, *Pinus*, *Larix*, *Picea*, papaya, citrus, avocado and banana^{6,19,38,42,63,75,88}, in the differentiation medium. In some species proembryos could develop directly from embryogenic protoplasts or from 2-3 cell clusters, which subsequently developed into somatic embryos^{19,38,106}. For the development of protoplast-derived calli, different media are needed, some containing PGR^{17,42} and others don't¹¹⁰. The commonly used regulators were auxin and cytokinin^{20,28,37,51,82,88}, but ABA and GA were also used for some species^{6,38,42,64,75,83}.

Variation of the protoplast-derived plants: In theory the protoplast-derived plants should be true-to-type with the donor plants⁵¹. But variations (protoplast variation) in cytology, morphology and resistance have been observed in the regenerated plants^{23,111,112,113}. Chromosome variation is the most common variation encountered in protoplast culture. Chromosome numbers of the plants regenerated from *Actinidia deliciosa* protoplasts varied from 143 to 310, and 72.4% and 20.7% of the cells were aneuploids and hexaploids, respectively¹¹². Similarly, of the 18 plants derived from kiwifruit protoplasts, 6 were diploids, 6 were tetraploids and the rest were aneuploids with chromosomes ranging from 59 to 203. In addition, many cells with 2-7 nuclei were detected in the

interphase of mitosis²³. Aside from variation in chromosome number morphology is also a significant variation. The leaves from different plants regenerated from protoplasts of *Actinidia deliciosa* or even from the same plants were not identical. Significant differences were detected in internode length, leaf length and width and petiole length between the protoplastic plants and the control plants¹¹³. As for resistance plants resistant to *Phoma tracheiphia* were obtained when lemon protoplasts were cultured¹¹¹. Another interesting variation was change in sex of kiwifruit. 1/3 of the plants regenerated from female *Actinidia deliciosa* were male¹¹². These variations are, on one hand, not conducive to the maintenance of genetic integrity of resources. But, on the other hand, it paves way for selection of novel germplasms.

Factors affecting protoplast culture: Protoplast culture is a very complex process and is affected by many factors, including protoplast source, genotype, culture medium, culture density, osmolarity, culture methods, and regeneration procedure etc^{19,56}.

Genotype: It has been well documented that morphogenesis is dependent on genotype. Protoplasts from different genotypes or species have different response to culture. The regeneration capability of different genotypes is possibly diverse even when the same culture conditions are used. The impacts posed by genotype on sustainable division and plant regeneration have been reported in citrus and poplar. For instance, in poplar it is reported that hybrid species were more responsive to regeneration than non-hybrid species^{114,115}.

Protoplasts source: Explants used for protoplast isolation have significant effects on protoplast culture. When *Prunus davidiana* Franch. protoplasts derived from suspension cultures, embryo callus, and aseptic seedlings were cultured, the highest division frequency and plating efficiency were observed in the protoplasts from the first type, 8.1% and 2.2%, respectively, followed by the last two types, 6.2% and 1.4% for the second explant source and 2.7% and 0.4% for the last one⁹². When protoplasts derived from leaf, callus, and embryogenic suspensions of banana were cultured; only those from the latter source could divide and undergo embryogenesis⁸⁸. Chinese plum protoplasts derived from suspension cultures, callus, and axenic leaves had different plating efficiencies, those from suspension being the highest, followed by those from callus and leaves, respectively⁹⁷. Protoplasts released from apple leaves treated with ABA had higher division rates⁹⁸. It is worth mentioning that for citrus, only protoplasts from embryogenic suspensions or callus can undergo embryogenesis and further development, unless cybridization occurs^{2,12}.

Culture density: Culture density is reported to affect cell division, and subsequent formation of cell clusters and embryoids⁵¹. There is no standard and fixed density for protoplast culture of different species or different genotypes. With citrus when the density of citrus protoplast was less than 5 x 10⁴/ml, few cell clusters could be observed and no embryoids were obtained. If the density was larger than 1 x 10⁶, high frequency of cell division and cell cluster formation was observed, but no embryoids were regenerated. Only when the density was 5 x 10⁴-1 x 10⁶ could embryoid formation be observed¹¹⁵. For *Vitis vinifera* cv. Shengli protoplast culture, a high division frequency (nearly 1%), could be observed when the density was 2-5 x 10⁵/ml, whereas few protoplasts could divide if the density

was less than 2.5×10^5 /ml. On the contrary, if the culture density was too high, protoplasts grew slowly and some broke¹¹⁶. In previous reports optimum density for apple is higher than 5×10^4 since protoplasts plated at low densities are apt to brown or stop division very quickly⁸¹, 2.5×10^4 ml⁻¹ and 5×10^3 ml⁻¹ for *Ulmus pioneer* and *Populus nigra* × *P. trichocarpa* NC-5331, respectively^{48,53}.

Culture medium: Difference in growth response was also detected with different culture media. When protoplasts derived from loblolly pine (*Pinus taeda* L.) suspension cultures were cultured in four media, D₂, DCR, KM_{8p} and TE, the highest plating efficiency, along with the best somatic embryogenesis response, was produced in DCR, whereas no embryoids were observed in D₂ and TE¹⁰³. Four kinds of media, KM_{8p}, MS (1/2NO₃⁻), NT and modified MS, were employed for *Zizyphus jujube* protoplasts, and only those in the second one stopped growing after 1-2 divisions¹¹⁷. In addition, additives such as phytohormones with appropriate combinations and concentrations in the basal medium always affect protoplast division and subsequent growth and development^{31,118}.

Inorganic salts in the medium can affect protoplast development as well. It has been reported in many papers that reduction or removal of NH₄⁺ could enhance protoplast culture^{19,20,28,30,51,52,81,97,119,120}. Some investigation showed that the negative effect of NH₄⁺ was mainly related to initial division rather than sustainable division⁹⁷. In addition, organic compounds are reported of benefit to apple protoplast culture¹⁴.

Culture method: The regeneration responses are also quite different with different culture methods. Meanwhile, a method suitable for one species may not be suitable for another. Park and Son reported that a liquid culture system was superior to other culture methods for hybrid poplar protoplasts⁵⁰. In general embedding culture system is of more help to protoplast culture. When litchi protoplasts were cultured with three culture methods, shallow liquid layer, calcium-agarose and calcium-alginate, only those in the last one could undergo sustained division and subsequently developed into embryos⁴¹. Similar result was demonstrated in citrus^{115,121}. Solid embedding culture system led to higher plating efficiency and better regeneration responses for Hamlin sweet orange and tangerine protoplasts as compared to liquid medium. In addition feeder layer or nurse culture were found useful for some species. For example, three culture methods were used for banana protoplasts, liquid medium, alginate bead and feeder layer culture. Only those cultured in the latter one could undergo division¹²². Sustained mitotic division of rubber protoplasts could be realized only when a nurse culture system was used⁷⁶.

Electrical current treatment: Electrical pulse treatment could stimulate cell division and plant differentiation of cherry protoplasts derived from embryogenic suspension cultures. The stimulatory effect may result from the increase in the interior current and the growth of the cultured cells was accordingly promoted¹¹⁹.

Application to Genetic Improvement and Study of Woody Plants

As mentioned above since the first try on protoplast manipulation of woody plants was performed substantial progress has been made. More and more plant species are covered and standard and

systematic protocols have been developed for some species. These lay groundwork for further research work and play a great role in genetic improvement and will contribute a lot to basic research of woody plants. Firstly, protoplasts-to-plant regeneration can be used for cryopreservation of germplasm. Cryopreservation via protoplast takes on all of the advantages of cell cryo-preservation. For the time being, such work has been carried out in apricot⁹² and citrus¹²³. Secondly, somatic hybridization via protoplast fusion plays a significant role in circumventing reproductive barriers encountered with sexual propagation, realizing combinations of diverse traits of agronomic interest and producing novel combinations of nuclear and cytoplasmic genomes. To date successful somatic hybridization has been tried or fulfilled in citrus^{2,14,101,125}; kiwifruit¹²⁶, banana¹²⁷, apple¹²⁸, persimmon¹²⁹, wild pear and colt cherry¹³⁰, passion fruit^{131,132}, larch¹³³, Douglas fir and loblolly pine⁶², poplar¹³⁴ and rose¹³⁵. Thirdly, mutants can be selected from protoplast-derived cultures via the pathway of somaclonal variation, which has been found in citrus¹³⁶ and kiwifruit¹²². In vitro culture combined with chemical treatment or irradiation has also been used to obtain some mutants¹²⁰. Fourthly, protoplasts can be employed in production of genetic transformants, which has been successful in citrus^{137,138}, apple^{139,140}, *Picea glauca*^{141,142}, *Alnus incana*¹⁴³, eucalyptus¹⁴⁴ and *Pinus radiata*¹⁴⁵. Last but not the least, protoplasts are desirable experimental materials for basic studies on virus or viroid replication^{146,147}, respiration metabolism¹⁴⁸, fruit ripening¹⁴⁹, the relationship between expression of totipotency and the activity of cellular antioxidant machinery¹⁵⁰.

Conclusions

Plants have been regenerated from many woody plants. But protoplast isolation and culture are affected by many factors. Reproducible results can be obtained with different methods. Genetic variations exist in protoclinal plants. Meanwhile, somatic protoplast can be used for genetic improvement and basic study of woody plants.

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Table 1. List of woody plants involved in successful reneration into plants from protoplasts.

| Species | Protoplast source | References |
|--|---------------------------------|---|
| <i>Abies alba</i> | Suspension culture | Hartmann et al. 1992 |
| <i>Actinidia chinensis</i> | Petiole-derived callus | Oliveira and Pais, 1990 |
| <i>Actinidia chinensis</i> | Cotyledon-derived callus | Xiao et al. 1992 |
| <i>Actinidia deliciosa</i> | Leaf-derived callus | Tsai, 1988 |
| <i>Actinidia deliciosa</i> | Cotyledon-derived callus | Xiao et al. 1993 |
| <i>Actinidia deliciosa</i> | Cell suspension | Mii and Ohashi, 1988 |
| <i>Actinidia eriantha</i> | Leaf | Zhang et al.1995, 1998 |
| <i>Amerniaca vulgaris</i> Lan. | Suspension culture | Ma and Li, 1998c |
| <i>Atlantia bilocularis</i> (Pieree ex Guill.) | Embryogenic callus | Jumin and Nito, 1996 |
| <i>Broussonetia kazinaki</i> | Leaf | Oka and Lhyama, 1985 |
| <i>Carica papaya</i> × <i>C. cauliflora</i> | Embryoid suspension culture | Chen and Chen, 1992 |
| <i>Citropsis schweinfurthii</i> | Embryogenic callus | Jumin and Nito, 1996 |
| <i>Citrus aurantium</i> | Embryonic callus | Vardi and Spiegel-Roy, 1982 |
| <i>Citrus lemon</i> | Embryonic callus | Vardi and Spiegel-Roy, 1982 |
| <i>Citrus madurensis</i> | Cell suspension culture | Ling et al..1989 |
| <i>Citrus mitis</i> | Embryonic callus | Sim et al..1988 |
| <i>Citrus paradisi</i> | Embryonic callus | Vardi and Spiegel-Roy, 1982 |
| <i>Citrus reticulata</i> | Embryonic callus | Vardi and Spiegel-Roy, 1982 |
| <i>Citrus reticulata</i> | Embryonic callus | Vardi and Spiegel-Roy, 1982; Deng et al. 1988 |
| <i>Citrus reticulata</i> | Embryonic callus | Vardi et al. 1975 |
| <i>Citrus reticulata</i> | Embryonic callus | Vardi and Spiegel-Roy, 1982 |
| <i>Citrus sinensis</i> | Embryonic callus | Vardi and Spiegel-Roy, 1982 |
| <i>Citrus sinensis</i> | Embryogenic callus | Niedz, 1983 |
| <i>Citrus sinensis</i> | Embryonic callus | Kobayashi et al. 1983 |
| <i>Citrus sinensis</i> | Embryonic callus | Deng et al. 1988 |
| <i>Citrus sinensis</i> | Embryonic callus | Deng et al. 1990 |
| <i>Citrus sinensis</i> | Embryonic callus | Hidaka et al. 1988 |
| <i>Citrus unshium</i> | Embryogenic callus | Kunitake et al. 1991 |
| <i>Citrus yuko</i> | Embryonic callus | Hidaka et al. 1988 |
| <i>Coffea arabica</i> L | Embryogenic suspension cultures | Acuna and Pena, 1991 |
| <i>Coffea canephora</i> | Suspension cultures | Schopke et al. 1987 |
| <i>Diospyros kaki</i> | Callus | Tao et al. 1991 |
| <i>Diospyros kaki</i> | Shoot-tip derived callus | Gu and Luo, 2002 |
| <i>Eriobotrya japonica</i> Lindl. | Embryo-derived callus | Lin and Chen, 1996 |

| | | |
|---|---------------------------------|---|
| <i>Euphoria longana</i> Lam. | Embryogenic suspension culture | Lai and Chen, 1996 |
| <i>Fortunella hindsii</i> | Embryonic callus | Deng et al. 1989 |
| <i>Fragaria ananassa</i> | Leaf | Nyman et al. 1988 |
| <i>Fragaria ananassa</i> | Leaf, Petiole | Nyman et al. 1992 |
| <i>Fragaria vesca</i> | Leaf, Petiole | Nyman et al. 1993 |
| <i>Hevea brasiliensis</i> | Embryogenic callus | Sushamakumari et al. 2000 |
| <i>Hibiscus syriacus</i> | Callus | Zhao and Yao, 1993 |
| <i>Larix × euro-lepis</i> | Embryogenic, callus or cells | Klimaszewska, 1989 |
| <i>Larix decidua</i> | | Korlach et al. 1995 |
| <i>Liriodendron tulipifera</i> | Embryogenic suspension | Merkle and Sommer, 1987 |
| <i>Litchi chinensis</i> Sonn. | Embryogenic suspension | Yu et al. 2000 |
| <i>Malus × domestica</i> | Suspension cultures | Ding and Cao, 1992 |
| <i>Malus × domestica</i> | Leaf | Ding et al. 1995 |
| <i>Malus × domestica</i> | Leaf, Etiolated shoot apices | Perales and Schieder, 1993 |
| <i>Malus × domestica</i> | Meristem-derived callus | Saito and Suzuki, 1999 |
| <i>Malus pumila</i> | Leaf | Pata-Ochatt et al. 1988 |
| <i>Malus pumila</i> | Leaf | Wallin and Johansson, 1989 |
| <i>Malus pumila</i> | Leaf, shoot segment | Pata-Ochatt et al. 1993 |
| <i>Mangifera indica</i> L. | Proembryogenic mass | Ara et al. 2000 |
| <i>Microcitrus australis</i> × <i>M. australasica</i> | Embryonic callus | Vardi et al. 1986 |
| <i>Morus alba</i> L. | Leaf | Chen et al. 1995 |
| <i>Morus alba</i> L. | Leaf | Wei et al. 1992 |
| <i>Murraya paniculata</i> | | Jumin et al. 1995 |
| <i>Passiflora edulis</i> | Leaf | Manders et al. 1991 |
| <i>Passiflora edulis</i> fv. <i>flavicarpa</i> | Leaf | d'Utra Vaz et al. 1993 |
| <i>Passiflora edulis</i> var. <i>flavicarpa</i> | Deg. | Dornelas and Vieira, 1993 |
| <i>Passiflora amethystina</i> Mikan. | | Dornelas and Vieira, 1993 |
| <i>Passiflora cincinnata</i> Mast. | | Dornelas and Vieira, 1993 |
| <i>Persea Americana</i> Mill. | Embryogenic suspension culture | Witjaksono et al. 1998 |
| <i>Pinus caribaca</i> | Embryogenic suspension culture | Laine and David, 1990 |
| <i>Pithecellobium dulce</i> Benth. | Leaf | Saxena and Gill, 1987 |
| <i>Platanus orientalis</i> | Leaf | Wei et al. 1991 |
| <i>Populus alba</i> L. | Suspension cultures | Qiao et al. 1998 |
| <i>Populus alba</i> L. × <i>P. grandidentata</i> | Leaf | Russell and McCown, 1986 |
| <i>Populus deltoids</i> × <i>P. simonii</i> | Leaf | Wang et al. 1995 |
| <i>Populus nigra</i> × <i>P. maximowiczii</i> | Leaf | Park and Son, 1992 |
| <i>Populus nigra</i> × <i>P. trichocarpa</i> | Leaf | Russell and McCown, 1988 |
| <i>Populus simonii</i> | Suspension culture | Wang et al. 1995 |
| <i>Populus tomntosa</i> | Leaf | Wang et al. 1990 |
| <i>Populus tremula</i> | Leaf | Russell and McCown, 1988 |
| <i>Prunus avium</i> × <i>pseudocerasus</i> | Leaf, root cell suspension | Ochatt et al. 1987 |
| <i>Prunus cerasifera</i> | Leaf | Ochatt et al. 1992 |
| <i>Prunus cerasus</i> | Leaf, root callus | Ochatt et al. 1988, 1990 |
| <i>Prunus communis</i> L. | Leaf | Ochatt, 1988 |
| <i>Prunus davidiana</i> Franch. | Suspension cultures | Ma and Li, 1999 |
| <i>Prunus salicina</i> Lindl. | Suspension culture | Ma and Li, 1999 |
| <i>Prunus spinosa</i> | Leaf | Ochatt et al. 1992 |
| <i>Pyrus communis</i> L. | Leaf | Ochatt and Caso, 1986 |
| <i>Pyrus communis</i> L. | Leaf | Ochatt et al. 1992 |
| <i>Pyrus communis</i> L. | Embryogenic callus | Ochatt and Power, 1988 |
| <i>Rauvolfia vomitoria</i> | Leaf | Tremouillaux-Guiller and Chenieux, 1991 |
| <i>Santalum album</i> | Suspension culture | Rao and Ozias-Akins, 1985 |
| <i>Ulmus pioneer</i> | Leaf-derived callus | Sticken and McCown, 1986 |
| <i>Vitis vinifera</i> | Cell suspension | Kobalenko et al. 1990 |
| <i>Vitis vinifera</i> | Leaf-derived embryogenic callus | Zhu et al. 1997 |
| <i>Vitis vinifera</i> | Embryo and embryoid | Reustle et al. 1995 |
| <i>Vitis vinifera</i> | Filament-derived callus | Yu et al. 1999 |
| <i>Ziziphus jujube</i> | Suspension culture | He et al. 1999 |