# Somatic embryogenesis and organogenesis in citrus for sanitatio and in vitro conservation

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# Somatic embryogenesis and organogenesis in Citrus for sanitation and *in vitro* conservation

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## 1. Procedures for *in vitro* sanitation of citrus germplasm

The main factors influencing somatic embryogenesis are the genotype, the composition of the culture medium, the type and developmental stage of the explant.

Generally, explants from tissues in juvenile phase of development are more inclined towards embryogenesis than those from mature tissues. Many citrus genotypes have been regenerated by somatic embryogenesis from explants derived from different plant tissues; nevertheless, there are considerable differences in the somatic embryogenic potential of them. Since many c*itrus* are polyembryonic, and adventive embryos are generated *in vivo* from the nucellar tissue, several studies on somatic embryogenesis have involved this tissue. Mitra and Chaturvedi (1972) have reported a direct correlation between the degree of polyembryony *in vivo* and the attitude to regenerate somatic embryos *in vitro*.

Somatic embryogenesis, from ovular tissues or embryos developed from ovules, has been extensively investigated in *Citrus*. Maheshwari and Rangaswamy (1958) first reported the regeneration *in vitro* of embryos from the culture of the nucellus; many subsequent improvements in the development of efficient protocols for the regeneration of somatic embryos have been reported after this observation. The regeneration of somatic embryos has been obtained from excised nucelli (Rangan *et al*., 1968), abortive ovules (Bitters *et al*., 1970), unfertilized ovules (Button and Bornman, 1971), undeveloped ovules (Starrantino and Russo, 1980; Moore, 1985), and isolated nucellar embryos (Litz *et al*., 1985). Only few reports concern somatic embryogenesis from somatic tissue neither nucellar nor ovular in origin.

Hidaka *et al*. (1981) regenerated diploid plants from anthers of sour orange. Nito and Iwamasa (1990) obtained somatic embryos from cultures derived from satsuma juice vesicles, and Carimi *et al*. (1994, 1995) induced the formation of embryogenic cultures from stigmas and styles of lemon, grapefruit, mandarin, sour orange and sweet orange. Gill *et al*. (1995) obtained somatic embryos from epicotyl, cotyledon, leaf and root segments of *in vitro* grown nucellar seedlings of mandarin. Recent studies have indicated the embryogenic potential in six different citrus species from cultures of pistil transverse thin cell layer explants (Carimi *et al*., 1999). Unfortunately the results in terms of somatic embryo induction from nucellar tissues are very low in monoembryonic citrus genotypes. Rangan *et al*. (1968) first reported the direct induction of somatic embryos from nucellus of fertilized ovules of monoembryonic *Citrus*. Button and Kochba (1977), Kobayashi *et al*. (1981) and Kobayashi *et al*. (1982) reported that somatic embryogenesis from unfertilized ovules of monoembryonic genotypes were unsuccessful. Carimi *et al*. (1999) observed that stigma and style transverse thin cell layer explants could optimize the regeneration of somatic embryos in a monoembryonic species as citron.

High concentrations (50 g/L) of sucrose are usually used to induce the formation of embryogenic callus. In some cases it was observed that the omission of sucrose from the culture medium for a single culture passage greatly stimulated embryogenesis when the following subculture was performed in presence of sucrose (Kochba and Button, 1974). An important plant growth regulator for the regeneration of citrus somatic embryos is the cytokinin BA. The use of 13.3 µM BA stimulated the embryogenic response of stigmas and styles (De Pasquale *et al*., 1994), while Moore (1985) found that levels of BA higher than 0.44 µM repressed embryogenesis in ovules. In some cases the presence of BA in the culture medium is not necessary to induce the formation of somatic embryos; Starrantino and Russo (1980) obtained somatic embryogenesis from undeveloped ovule of *Citrus* cultured on Murashige and Skoog medium (MS) (1962) supplemented with malt extract (0.5 g/L). Usually, the embryogenic calluses of *Citrus* can maintain their embryogenic competence for long periods (5-10 years).

Since somatic embryogenesis can be used to eliminate many virus diseases (Bitters *et al*., 1970; D'Onghia *et al*., 1997; D'Onghia *et al*., 2001), the plant material obtained by somatic embryos regenerated *in vitro* can be used to establish healthy citrus stocks. Somatic embryogenesis from stigma and style culture represents a very recent technique for *Citrus* sanitation; this technique proved to be highly effective in the complete elimination of psorosis, tristeza, infectious variegation, concave gum, impietratura, cristacortis, exocortis and

cachexia (D'Onghia *et al*., 2000). Moreover, as the international demand for *Citrus* is high, the *in vitro* conservation of plant material regenerated by somatic embryogenesis is very promising for the establishment of the safe international exchange of citrus germplasm (D'Onghia *et al*., 2000).

### *Protocol for regeneration of somatic embryos from citrus stigma and style culture*

Flowers are collected before opening and surface-sterilized by immersion for 5 min in ethanol (70% v/v in water), 15 min in 2% (w/v in water) sodium hypochlorite, followed by one 5-min rinse in sterile distilled. Stigma/style explants are excised with a scalpel, and placed vertically onto the media with the cut surface in contact with the medium. Explants were maintained on a Murashige and Skoog (1962) semisolid medium (7 g $l^{-1}$ agar, type M Sigma A-4800) with sucrose (146 mM) as the carbon source. Three different media are used based on the nutrients and vitamins of MS medium supplemented with 500 mg $l^{-1}$ Malt Extract (ME) (Sigma M-0383) and 13.3 μM BAP (Sigma B-3274). Growth regulators are added to the medium and the pH is adjusted to 5.7±0.1 with 0.5 M KOH before autoclaving at 121 °C and 1.1 Kg $cm^{-2}$ for 15 min. Plastic Petri dishes (100 x 15 mm) are filled with 25 ml of medium and sealed with Parafilm M. Explants and calluses are subcultured at 30-day intervals and maintained in a growth chamber at 25±1 °C with a 16 h photoperiod, and a photosynthetic photon flux of 100 μmol $m^{-2}$ $s^{-1}$ provided by Osram cool-white 18 W fluorescent lamps. Individual somatic embryos (about 2 mm in diameter) are isolated and germination is attempted in test tubes (1 embryo per 155 x 23 mm glass tube sealed with Parafilm M) containing 20 ml of MS basal solid (7 g $l^{-1}$ agar) medium supplemented with 146 mM sucrose and 500 mg $l^{-1}$ ME. When plantlets are 5-7 cm length, they are washed in distilled sterile water and planted into autoclaved plastic pots containing a 1:1 (v/v) mixture of peat-moss:sand. Plantlets are maintained for 1-2 months under 95±5% relative humidity in a climate chamber at the same temperature and light conditions as above described. Later, plantlets are transferred to the greenhouse.

## 2. Procedures for *in vitro* conservation of healthy germplasm

There have been many reports on organogenesis from different type of explants of *Citrus* and citrus rootstocks. The morphogenic responses of *Citrus* cultured *in vitro* are influenced by genotype, explant type and culture medium. Explants have included shoot tip (Barlass and Skene, 1986), stem sections (Grinblat, 1972; Chaturvedi and Mitra, 1974; Raj Bhansali and Arya,

1979; Barlass and Skene, 1982), root sections (Sauton *et al*., 1982; Burger and Hackett, 1986; Sim *et al*., 1989; Bhat *et al*., 1992), leaf sections (Chaturvedi and Mitra, 1974, Hu and Kong, 1987), stem internode (Duran-Vila and Navarro, 1989), epicotyl segments (Edriss and Burger, 1984) and transverse thin cell layer (tTCL) explants excised from stem internodes (Van Le *et al*., 1999). The regeneration of adventitious shoots has been obtained either directly from the explant or from an intermediate callus phase. The regeneration of *Citrus* and related species by organogenesis has been described in many reports (Table 1). Only the references of the last twenty years will be given here; the reader may refer to the work of Skirvin (1981) and Barlass and Skene (1986) for previous references.

Marin and Duran-Vila (1991) proposed a micropropagation protocol for the conservation of citrus germplasm *in vitro*. This method was based on establishment of primary cultures to recover *in vitro* plants from nodal stem segments (dissected from 1-2 years old plants) and the maintenance of successive cycles of secondary cultures with the recovery of rooted plantlets in each subculture cycle. The subculture cycle would last 8-12 months and would include several operations (culture of nodal segments, rooting of shoots and elongation of rooted shoots). This protocol allowed the long-term maintenance of lemon, Mexican lime, sweet orange and trifoliate orange; the plants regenerated *in vitro* can be maintained for up to 12 months on the same medium before subculture to fresh medium (Marin and Duran-Vila, 1991).

Recently, Van Le *et al*. (1999) have reported a new efficient regeneration method leading to a high frequency of plant regeneration by using transverse thin cell layer (tTCL) explants excised from the stem internodes of 1-year-old trifoliate orange (*P. trifoliata*). The Authors found that the optimal combination of growth regulators for shoot regeneration was obtained with MS medium supplemented with 10 µM BA and 1 µM N-phenyl-N'-1,2,3-thidiazol-5ylurea (thidiazuron, TDZ), leading to 90% of responsive tTCL forming on average 37 buds per tTCL. The highest percentage (100%) of shoot elongation was obtained onto MS medium supplemented with 1 µM gibberellic acid ($GA_3$), 60% of shoots formed roots on MS medium containing 5 µM α-naphthaleneacetic acid (NAA) and 100% of these plants survived. The plants regenerated from tTCL had no morphological alterations; this could be due to the direct regeneration of plants from the explant without an intermediate callus phase and therefore the reduction of somaclonal variation (Van Le *et al*., 1999). The Author's opinion related to the high efficiency of bud formation is that in tTCL the transport of nutrients and growth regulators from the medium to the cells of the explant is faster. Moreover, a higher quota of responsive cells can be directly exposed to the medium (Van Le *et al*., 1999).

**Table 1.** Regeneration of Citrus and related species by organogenesis. Hormones concentrations are expressed in µM

| Species | Common name | Explant | Medium & PGR for callus formation | Medium & PGR for Shoot formation | Medium & PGR for Root formation | Reference |
|---|---|---|---|---|---|---|
| *C. acida* | Wild orange | Epicotyl | MS + 4.5 µM BA | MS + 4.5 µM BA + 5.8 µM $GA_3$ | MS + 5.4 µM NAA | Chakravarty & Goswami, 1999 |
| *C. assamensis* | | Shoot tips | | MS + 2.2 µM BA | Soilrite | Baruah *et al.*, 1996a-b |
| *C. aurantifolia* | Mexican lime | Nodal stem segments | | MS + 0.4 µM BA | MS + 16.1 µM NAA | Duran-Vila *et al.*, 1989 |
| *C. aurantifolia* | Mexican lime | Internodal stem segments | | MS + 33.3 µM BA + 5.4 µM NAA | ½ MS + 2.7 µM NAA | Pérez-Molphe-Balch & Ochoa-Alejo (1997) |
| *C. aurantium* | Sour orange | Internodal seedlings stem sections | | MT + 22 µM BA + 5.4 µM NAA | ½ MT + 5.4 µM NAA | Moore, 1986 |
| *C. aurantium* | Sour orange | Mature embryos | MT + 9 µM 2,4-D + 22.2 µM BA | MT + 44.4 µM BA + 5.4 µM NAA | MT + 5.4 µM NAA | Beloualy, 1991 |
| C. grandis | Pummelo | Epicotyl segments | | MS + 2.2 µM BA | MS + 2.5 µM IBA | Goh *et al.*, 1995 |
| C. grandis | Pummelo | Root segments | | MS + 0.089 µM BA | MS + 2.5 µM IBA | Goh *et al.*, 1995 |
| *C. grandis* | Pommelo | Shoot-tip | | MS + 1.8 µM BA | ½ MS + 5.4 µM NAA | Paudyal & Haq, 2000 |
| *C. halimii* | | Hypocotyl segments | | MS + 4.4 µM BA | MS + 2.7 µM NAA | Normah *et al.*, 1997 |
| *C. indica* | | | | MS + 2.2 µM BA | Soilrite | Baruah *et al.*, 1996a-b |
| *C. jambhiri* | | Stem and root segments | MS + 0.9 µM KIN + 53.7 µM NAA | ½ MS + 22.2 µM BA | ½ MS + 5.4 µM NAA | Raman *et al.*, 1992 |
| *C. latipes* | | | | MS + 2.2 µM BA | Soilrite | Baruah *et al.*, 1996a-b |
| *C. limon* | Lemon | Stem and root segments | MS + 0.9 µM KIN + 53.7 µM NAA | ½ MS + 22.2 µM BA | ½ MS + 5.4 µM NAA | Raman *et al.*, 1992 |
| *C. limon* | Lemon | Shoot tips | | MS + 4.4 µM BA + 4.6 µM KIN + 2.7 µM NAA | MS + 1.1 µM BA + 2.7 µM NAA + 2.5 µM IBA | Sing *et al.*, 1994 |
| *C. limon* | Lemon | Shoot-tip and node | | DKW + 0.76 µM ABA + 8.87 µM BA | DKW + 19.6 µM IBA | Kotsias & Roussos, 2001 |
| *C. limon* x *P. trifoliata* | Citremon | Stem segments | | MS + 2.2 µM BA + 2.7 µM NAA | MS + 0.8 µM NAA | Mas *et al.*, 1992 |
| *C. limonia* | Rangpur lime | Nodal and internodal stem segments | | MS + 10 µM BA | W + 10 µM NAA | Barlass and Skene, 1982 |
| *C. medica* | Citron | Nodal stem segments | | MS + 4.4 µM BA | MS + 16.1 µM NAA | Duran-Vila *et al.*, 1989 |
| *C. mitis* | Calamondin | Epicotyl, shoot tip and nodal stem segments | | MS + 2.2-4.4 µM BA | ½ MS + 4.9 µM IBA | Sim *et al.*, 1989 |
| *C. mitis* | Calamondin | From root of whole seedlings | | MS + 2.2 µM BA | ½ MS + 4.9 µM IBA | Sim *et al.*, 1989 |
| *C. mitis* | Calamondin | Leaf from seedlings (whole or segments) | | MS + 8.9 µM BA | ½ MS + 4.9 µM IBA | Sim *et al.*, 1989 |
| *C. paradisi* x *P. trifoliata* | Swingle citrumelo | Epicotyl stem segments | | MT + 150 µM Cumarin | MT + 150 µM Cumarin | Grosser and Chandler, 1986 |
| *C. reshni* | Cleopatra mandarin | Internodal seedlings stem sections | | MT + 22 µM BA + 5.4 µM NAA | ½ MT + 5.4 µM NAA | Moore, 1986 |
| *C. reticulata* | Mandarin | Shoot tips | | MS + 4.4 µM BA + 4.6 µM KIN + 2.7 µM NAA | MS + 1.1 µM BA + 2.7 µM NAA + 2.5 µM IBA | Sing *et al.*, 1994 |
| *C. reticulata* | Mandarin | Internodal stem segments | | MS + 33.3 µM BA + 5.4 µM NAA | ½ MS + 2.7 µM NAA | Pérez-Molphe-Balch & Ochoa-Alejo (1997) |
| *C. sinensis* | Sweet orange | Nodal and internodal stem segments | | MS + 10 µM BA | W + 10 µM NAA | Barlass and Skene, 1982 |
| *C. sinensis* | Sweet orange | Nodal stem segments | | MS + 4.4 µM BA | MS + 16.1 µM NAA | Duran-Vila *et al.*, 1989 |

*continued*

| Species | Common name | Explant | Medium & PGR for callus formation | Medium & PGR for Shoot formation | Medium & PGR for Root formation | Reference |
|---|---|---|---|---|---|---|
| *C. sinensis* | Sweet orange | Nodal and internodal stem segments | | MS + 4.4 µM BA or 13.3 µM BA | MS + 54 µM NAA | Duran-Vila *et al.*, 1992 |
| C. sinensis | Sweet orange | Epicotyl and hypocotyl | | MS + 8.89 µM BA + 0.76 µM ABA | Not reported | Maggon and Singh, 1995 |
| C. sinensis | Sweet orange | Nodal stem segments | | MS + 2.2 µM BA + 0.5 µM NAA | ½ MS + 2.7 µM NAA + 2.5 µM IBA | Tapati *et al.*, 1995 |
| *C. sinensis* x *P. trifoliata* | Carrizo citrange | Shoot tips and nodal sections | | MS-KNOP + 22.2 µM BA | MT + 5.4 µM NAA | Kitto and Young, 1981 |
| *C. sinensis* x *P. trifoliata* | Carrizo citrange | Nodal and internodal stem segments | | MS + 10 µM BA | W + 10 µM NAA | Barlass and Skene, 1982 |
| *C. sinensis* x *P. trifoliata* | Troyer citrange | Epicotyl stem segments | | MS + 2.2 µM BA + 0.5 µM NAA | MS + 10.7 µM NAA | Edriss and Burger, 1984 |
| *C. sinensis* x *P. trifoliata* | Carrizo citrange | Internodal seedlings stem sections | | MT + 22 µM BA + 5.4 µM NAA | ½ MT + 5.4 µM NAA | Moore, 1986 |
| *C. sinensis* x *P. trifoliata* | Carrizo citrange | Shoot-tip | | MS + 4.4 µM BA + 2.5 µM IBA + 296 µM AD | MT + 5.4 µM NAA | Starrantino and Caruso, 1987 |
| *C. sinensis* x *P. trifoliata* | Troyer citrange | Shoot-tip | | MS + 4.4 µM BA + 2.5 µM IBA + 296 µM AD | MT + 5.4 µM NAA | Starrantino and Caruso, 1987 |
| *C. sinensis* x *P. trifoliata* | Carrizo citrange | Nodal stem segments | | MS + 4.4 µM BA + 2.5 µM IBA + 296 µM AD | MS + 5.4 µM NAA | Starrantino and Caruso, 1988 |
| *C. sinensis* x *P. trifoliata* | Troyer citrange | Nodal stem segments | | MS + 4.4 µM BA + 2.5 µM IBA + 296 µM AD | MS + 5.4 µM NAA | Starrantino and Caruso, 1988 |
| *C. sinensis* x *P. trifoliata* | Carrizo citrange | Mature embryos | MT + 9 µM 2,4-D + 22.2 µM BA | MT + 22.2 µM BA + 5.4 µM NAA | MT + 5.4 µM NAA | Beloualy, 1991 |
| *P. trifoliata* | Trifoliate orange | Nodal and internodal stem segments | | MS + 10 µM BA | W + 10 µM NAA | Barlass and Skene, 1982 |
| *P. trifoliata* | Flying Dragon | Shoot-tip | | MS + 2.2 µM BA + 1.2 µM IBA + 296 µM AD | MS + 5.4 µM NAA | Starrantino and Caruso, 1987 |
| *P. trifoliata* | Flying Dragon | Nodal stem segments | | MS + 2.2 µM BA + 1.2 µM IBA + 296 µM AD | MS + 5.4 µM NAA | Starrantino and Caruso, 1988 |
| *P. trifoliata* | Trifoliate orange | Mature embryos | MT + 9 µM 2,4-D + 22.2 µM BA | MT + 22.2 µM BA + 5.4 µM NAA | MT + 5.4 µM NAA | Beloualy, 1991 |
| *P. trifoliata* | Trifoliate orange | Hypocotyl | | MS + 44.4 µM BA | ½ MS + 0.5-5.0 µM IBA | Harada and Murai, 1996 |
| *P. trifoliata* | Trifoliate orange | tTCL from stem internodes | | MS + 10 µM BA + 1 µM TDZ | MS + 5 µM NAA | Van Le *et al.*, 1999 |

**Abbreviation used in the table**

AD = Adeinine; BA = 6-benzylaminopurine; DKW = Driver-Kuniyuki medium; IBA = indole-3-butyric acid; KIN = kinetin; MS = Murashige and Skoog medium; MS-KNOP = medium based on MS microelements and Knop's macroelements and vitamins; NAA = α-naphthaleneacetic acid; PGR = Plant growth regulators (concentrations in µM); TDZ = thidiazuron; tTCL = transverse thin cell layer; W = White medium

## Technical protocols

### Surface-sterilisation of citrus flowers

The conditions of the stock field-growing plants are very important for the establishment of explant *in vitro*. On the surface of the plant material growing in the field many microorganisms can be found (in some cases endogenous contamination are also present); they can be difficult to eliminate and can limit the initiation of axenic cultures. If the stock material is collected during the warm humid season (a period that corresponds with a vigorous growth) the bacterial and fungal contamination problems can be easily avoided in *Citrus* with a standard procedure for surface-disinfection.

Before the introduction *in vitro*, flowers collected from plant cultivated in the field or in the greenhouse are surface-disinfected. A typical protocol for citrus flower surface-disinfection is described below.

1) Collect the citrus flowers before opening and store them at 4 °C. The flowers can be maintained at 4 °C for 5-6 days.
2) For the surface sterilisation to work in a sterile environment (laminar cabinet), and manipulate the explants with sterile forceps and scalpels.
3) Surface-sterilise the entire flowers by immersion for 5 min in ethanol (70% v/v in water).
4) Sterilise by immersion for 15 min in 2% sodium hypochlorite (2% w/v in water). It is possible to use a solution 20% commercial bleach (20% v/v in water).
5) After being sterilised the flowers are open under sterile conditions.
6) Those flowers in which the sterilisation solution does not penetrate through the petals are used. For culture initiation only non-brownish pistils are use. With the scalpel, stigmas, and styles are dissected from ovaries (with a cut perpendicularly to the longitudinal axis).
7) Styles and stigmas are placed vertically on the culture medium with the cut surface of the style in contact with MS-solidified medium (containing 3 mg/L BAP).

### Murashige and Tucker medium preparation

The medium composition for culturing *Citrus in vitro* is usually based on the nutrients and vitamins of the MS medium, although in some cases better results are obtained with Murashige and Tucker medium (MT) (1969). The

MT medium contains the inorganic salts of MS medium plus 26.6 µM glycine, 554.9 µM myo-Inositol, 40.6 µM nicotinamide, 48.6 µM pyridoxine-HCl and 29.7 µM thiamine-HCl.

*Literature*

Murashige T and Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant, 15:473-497

Murashige T and Tucker DPH (1969) Growth factors requirements of citrus tissue culture. 1st Int Citrus Symp., 3:1155-1161

The Mt medium is a MS medium modified as follows:

| *Vitamin* | **MS medium mg/l** | **MT medium mg/l** | **Molecular weight** |
|---|---|---|---|
| Glycine | 2.0 | 2.0 | 75.05 |
| Myo-Inositol | 100 | 100 | 180.2 |
| Nicotinamide | 0.5 | 5.0 | 123.1 |
| Pyridoxine HCL | 0.5 | 10.0 | 205.6 |
| Thiamine HCL | 0.1 | 10.0 | 337.3 |

To prepare 200 ml of a Stock MT vitamin solution 1000x use the following quantities of vitamins:

| *Vitamin* | **Stock 1000X (g/200 ml)** |
|---|---|
| Glycine | 0.4 |
| Myo-Inositol | 20.0 |
| Nicotinamide | 1.0 |
| Pyridoxine HCL | 2.0 |
| Thiamine HCL | 2.0 |

Dissolve the vitamins in 200 ml of distilled water, (heat at 60°C). Store at -20°C.

For the preparation of 1 L MT medium add 1 ml of MT vitamin stock solution 1000x to 1 liter containing the inorganic salts of MS medium.

## Murashige & Skoog (MS) culture medium preparation

The medium was described by Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

*Macro and microelements*

To prepare 500 ml of Stock solution 100X use the following quantities of salts:

| Compound | Quantity g salt / 500 ml solution | Nome solution | Store at |
|---|---|---|---|
| $NH_4NO_3$ | 82.5 | Solution A | Room Temperature |
| $KNO_3$ | 95.0 | | |
| $MgSO_4$ $7H_2O$ | 18.5 | Solution B | 4 °C |
| $MnSO_4$ $4H_2O$ | 1.12 | | |
| $ZnSO_4$ $7H_2O$ | 0.43 | | |
| $CuSO_4$ $5H_2O$ | 0.00125 | | |
| $CaCl_2$ $2H_2O$ | 22.0 | Solution C | 4 °C |
| KI | 0.0415 | | |
| $CoCl_2$ $6H_2O$ | 0.00125 | | |
| $KH_2PO_4$ | 8.5 | Solution D | 4 °C |
| $H_3BO_3$ | 0.31 | | |
| $Na_2MoO_4$ $2H_2O$ | 0.0125 | | |
| $FeSO_4$ $7H_2O$ | 1.392 | Solution E | 4 °C |
| EDTA | 1.865 | Solution F | 4 °C |

The solutions are sterilised by autoclaving at 121 °C for 20 min or filter sterilise.

*Vitamins*

To prepare 100 ml of a Stock vitamin solution 1000x use the following quantities of vitamins:

| Vitamin | Quantity (g) |
|---|---|
| Tiamine ($B_1$) | 0.010 |
| Nicotinic Acid ($B_3$) | 0.050 |
| Myo-Inositol | 10.000 |
| Glicine | 0.200 |

Dissolve the vitamins in 50 ml of distilled water, (heat at 60 °C). Add distilled water to 100 ml of final volume and filter sterilize. Store at -20 °C.

Protocol for the preparation of 1 L MS medium:

1. Add 500 ml of distilled water in a 1 L beaker.
2. Add 10 ml of each of the 6 stock solutions (A-F).
3. Add 1 ml of vitamin 1000x.
4. Add 30-50 g of sucrose.
5. Add (if necessary) growth regulators.
6. Add distilled water to 1 Litre of final volume.
7. Adjust pH to 5.7 with 0.5 M HCl or KOH.
8. Transfer the medium in 1 Litre Erlenmeyer flasks.
9. Add 8-10 g of agar.
10. Sterilise by autoclaving for 15-20 min at 121 °C.
11. Transfer the medium in the Petri dishes (8 ml / 60 mm Ø Petri dish, or 25 ml / 100 mm Ø Petri dish).

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