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CIP Research Guide 1

TISSUE CULTURE: MICROPROPAGATION, CONSERVATION, AND EXPORT OF POTATO GERMPLASM

1992

Nelson Espinoza, Rolando Lizarraga, Carmen Sigüeñas, Fausto Buitron, James Bryan, John H. Dodds

INTERNATIONAL POTATO CENTER (CIP)
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TISSUE CULTURE: MICROPROPAGATION, CONSERVATION, AND EXPORT OF POTATO GERMPLASM

Tissue culture allows the rapid clonal propagation of large numbers of plantlets in a short period and the conservation of potato germplasm under controlled conditions requiring reduced space and labor. The pathogen-free status of many in vitro cultures greatly facilitates international germplasm exchange.

This document describes advantages, methodology, and materials of tissue culture techniques applied at the International Potato Center (CIP) and discusses the techniques of meristem isolation, micropropagation, long-term storage, and in vitro export of germplasm.

1 ADVANTAGES OF TISSUE CULTURE TECHNIQUES

CIP maintains a potato germplasm collection with some 5,000 clones which serve as a rich source of genetic diversity for use by breeders in potato improvement programs. The maintenance of this collection in the field is expensive, and the collection is susceptible to a wide range of risks such as disease infection or adverse weather conditions. Maintenance of the collection in vitro holds a number of advantages over growing the collection each year in the field:

- reduction of labor costs
- avoidance of field infections
- avoidance of environmental hazards, e.g. hail, frost
- easy availability of material for micropropagation
- easy availability of material for pathogen elimination
- availability of material for propagation and export all year round

The transfer of the potato germplasm collection to in vitro (tissue culture) conditions has been completed; new accessions are routinely included.

In this document we will follow the path of a potato clone through meristem culture, micropropagation, storage, and eventual export as shown in Figure 1.

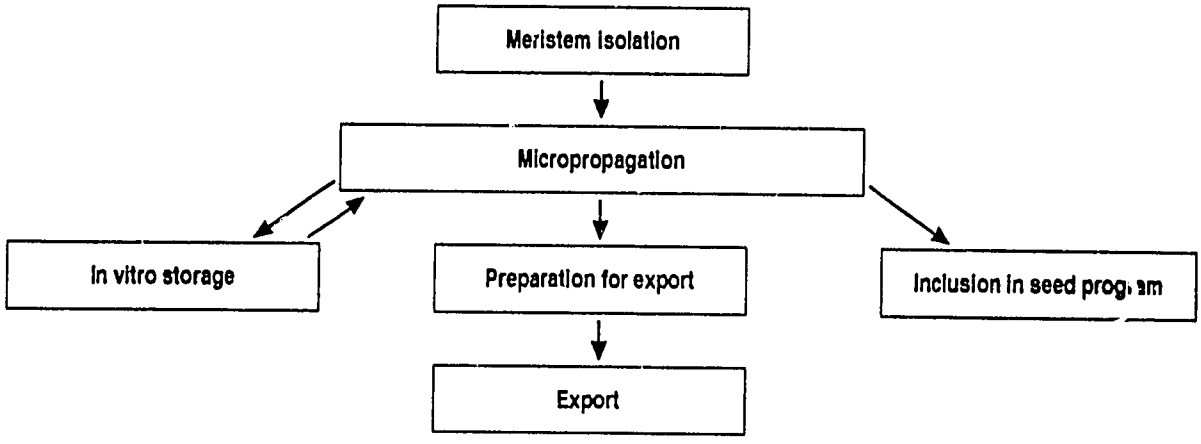


Fig. 1. Flow diagram showing the stages that plants pass through before eventual in vitro export.

2 MERISTEM ISOLATION

The active growing point of the plant shoot is the meristem. This is a small organ composed of rapidly dividing (meristematic) cells. For propagating potato shoot cultures, it is the ideal starting material as it has two favorable characteristics:

- The isolated meristem develops in culture in a genetically stable form. This is not the case, for example, in disorganized callus cultures that show major genetic irregularities.
- The isolation of meristems reduces the level of viral infection in the tissue and, under appropriate conditions, can be used for complete pathogen eradication.

The dome of a shoot apical meristem contains the truly meristematic cells and is surrounded by leaf primordia and primary leaves. Since the more differentiated vascular tissues occur away from the meristem (towards to older tissues of the stem), the vascular elements of the leaf primordia are still very incipient and have not yet made contact with the main strand of the vascular system in the stem. Therefore, virus particles, which may be present in the vascular system, can reach the meristematic region of the apex only through movement from cell to cell. This is one of the main reasons why in a virus-infected plant, virus concentration decreases acropetally toward the meristem of the apical as well as the axillary buds. This situation is, in principle, the same for many crops, the only difference being the shape of the meristem and the leaf primordia.

Whether other factors, such as the production of virus inhibitory substances by the meristematic cells or the effect of hormones in the culture medium, play a role in the elimination of virus by meristem culture has not been proven.

Isolation of the apical part, called the meristem tip, under aseptic conditions and its culture on an adequate aseptic medium leads to the development of plantlets. This development, in principle, follows a pattern similar to that in the entire plant; cells

of the meristem divide and differentiation of tissues continues. The nutrition of the excised portion of the plant is supplied by the artificial medium. This technique, called meristem culture, was first applied by Morel and Martin (1952) on dahlia, and can be used to produce pathogen-free plants.

The aseptic dissection of the meristem is a delicate process and requires many hours of practice. The sequence of dissection is shown photographically (Figure 2) and is carried out as follows: Cut stems into segments each containing one node with its axillary bud. Carefully remove the leaves. Desinfect pieces for 30 seconds in 70 % alcohol, followed by 2.5 % calcium hypochlorite for 15 minutes. Wash four times with sterile distilled water to remove excess hypochlorite.

Under a binocular dissecting microscope, cut and remove the leaflets surrounding the growing point until only the apical dome and a few leaf primordia remain. Cut off the dome and two leaf primordia and transfer to Medium A (Section 6). Transfer weekly to fresh Medium B. After 6-8 weeks, transfer the small plantlets to larger tubes for further growth. When the plants are 4 cm high, micropropagation can begin.

Sometimes it may be necessary to introduce buds or sprouts from the field--without meristem isolation--to in vitro conditions. After surface sterilization (see Section 2), explants can be inoculated onto Medium G. When plants reach 5 to 6 cm, continue micropropagation in Medium C. Preferably use an acaricide (Morestan 2/00; 20 minutes) before surface sterilization.

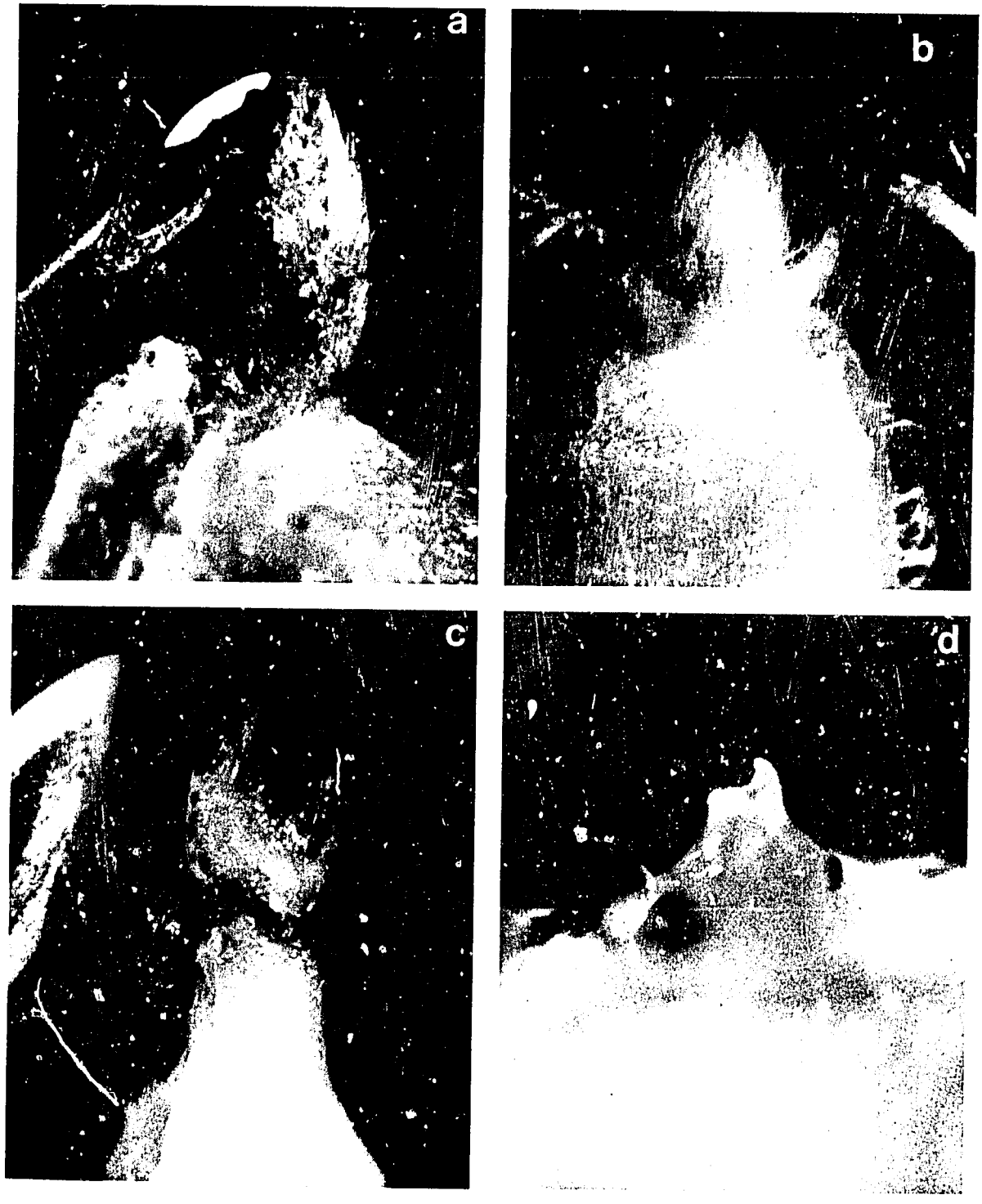


Fig. 2. Photographic sequence of meristem dissection:
a - The isolated surface-sterilized bud.
b, c - Various stages in the dissection where primary leaves are removed.
d - The fully dissected meristem with two leave primordia.

3 MICROPROPAGATION

The objective of micropropagation is to obtain large numbers of clonal plants in a short period. At CIP, micropropagation is carried out by two methods:

- semi-solid culture
- liquid culture

Semi-solid culture. Single nodes with leaves are excised from small in vitro plantlets and the large leaves are carefully removed. Each node is then inoculated onto the surface of propagation Medium C or D. The axillary bud quickly grows out (Figure 3), and in 3-4 weeks a plantlet with six or seven more nodes will be available for transfer.

Liquid culture. In vitro plantlets are cut in single nodes and the large leaves are removed. Ten single nodes are placed in 25 cm^3 of liquid Medium D (or without gelling agent) and the flask is mantained without shaking. After 3-4 weeks of rapid growth each flask contains 60 or 70 nodes (Figure 4 and 5), available either for another propagation or for transfer to soil.

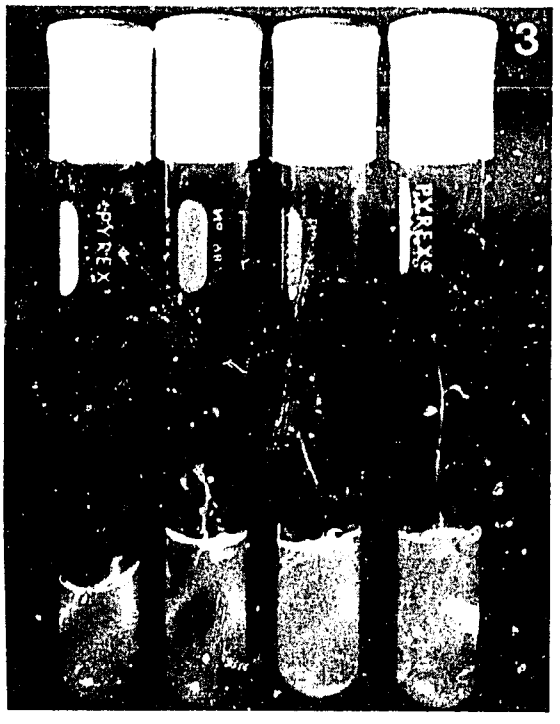
Once a suitable number has been produced, plantlets are transferred to non-sterile conditions.

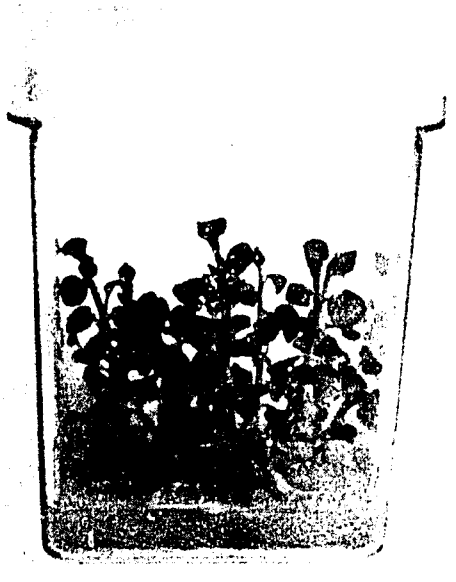
If it should be necessary to shorten time of propagation, Medium I can be used with shaking.

Environmental conditions for tissue cultures. In vitro plantlets are maintained under the following conditions:

Temperature:	18-22°C
Photoperiod:	16 hours/light
Relative humidity:	60-70%
Light intensity:	3000 Lux (45 $\mu E/m^2/seg^2$)

Transfer to non-sterile conditions. When the plantlets are 3-5 cm high and have developed a good root system they are ready for transplanting into pots or beds containing a suitable compost mixture (Figure 6). Care must be taken to avoid damage to the roots, and ensure good contact between roots and planting media. The plants must be kept in an environment with high relative humidity for the first few days.





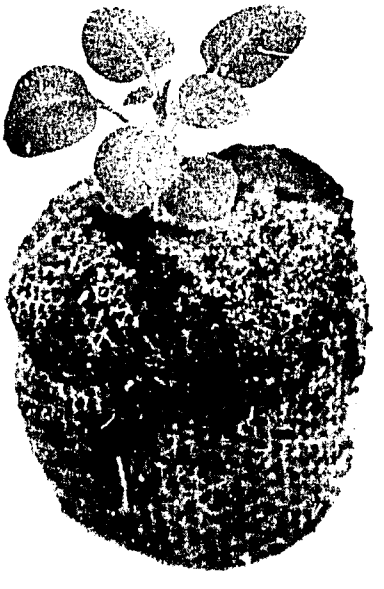


Fig. 3. Sequence of in vitro growth.
Fig. 4. Liquid culture for rapid propagation.
Fig. 5 Box containing 25 individual rooted plantlets ready for transfer from the sterile environment to compost.
Fig. 6. Plantlet a few days after transfer to compost.

4 MAINTENANCE AND LONG-TERM STORAGE

In vitro material can be kept in culture indefinitely, provided sufficient care is taken to avoid contamination and transfers to fresh medium are made at appropriate intervals. In this way a pathogen-tested stock can be kept as a reserve for future use.

For short-term maintenance, plantlets are grown on Medium C or D in tubes sealed with autoclavable plastic caps. Cotton wool plugs are easily penetrated by fungi and, although suitable for short-term storage, are not recommended for medium- or long-term storage.

The growth rate of the plantlets depends on incubation temperature, medium composition, and cultivar. It follows, therefore, that for long-term storage, modifications to the media and incubation temperature are required.

Medium E exerts an osmotic stress and can be used for storage at 25°C: the stress reduces the growth rate and produces short internodes. Many nodes are thus available when propagation of the stored material is required. Under these conditions material only needs to be transferred annually. However, when using Medium E the temperature can be lowered to 8°C, thereby significantly reducing the growth rate of the plantlets. At this low temperature transfers are only required once every 2 or 3 years.

It is possible, therefore, to maintain a germplasm collection under the storage conditions outlined above. When a request is received for a particular genotype, it can be removed from storage and micropropagated.

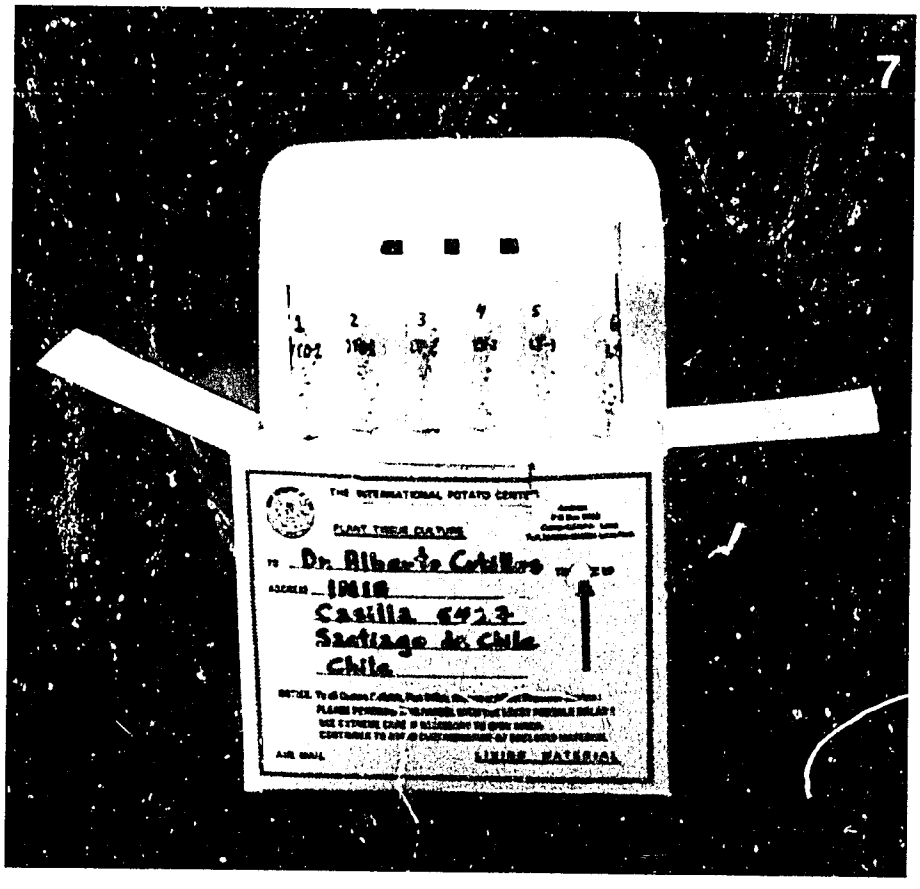
Also Medium H may be useful for long-term storage.

5 IN VITRO SHIPMENT

Material from the pathogen-tested collection can be exported to other countries as in vitro plantlets. Single node segments can be obtained from micropropagated material and transferred to tubes containing Medium F. When the in vitro plantlets are 2 cm high and have a well-developed root system, the tubes with plantlets are carefully packed (Figure 7) for air shipment. The consignee is informed of the flight and airway bill number by telex or telegram, so that the shipment can be quickly released from customs. Attached to every in vitro shipment is a booklet explaining the handling of material after receipt, a phytosanitary certificate, and a card to return to CIP giving details about the condition of the material on arrival.

The in vitro plantlets received in this way can be transferred directly to compost in plant pots or further micropropagated (Section 3).

At the present time most in vitro shipments are in the form of 2 cm high plantlets; however, exportations can be complemented with in vitro tubers (Figure 8). These are very small, aseptic, pathogen-free tubers that can be induced in culture (Tovar et al., 1985). They are easier to transport and handle than in vitro cultures.



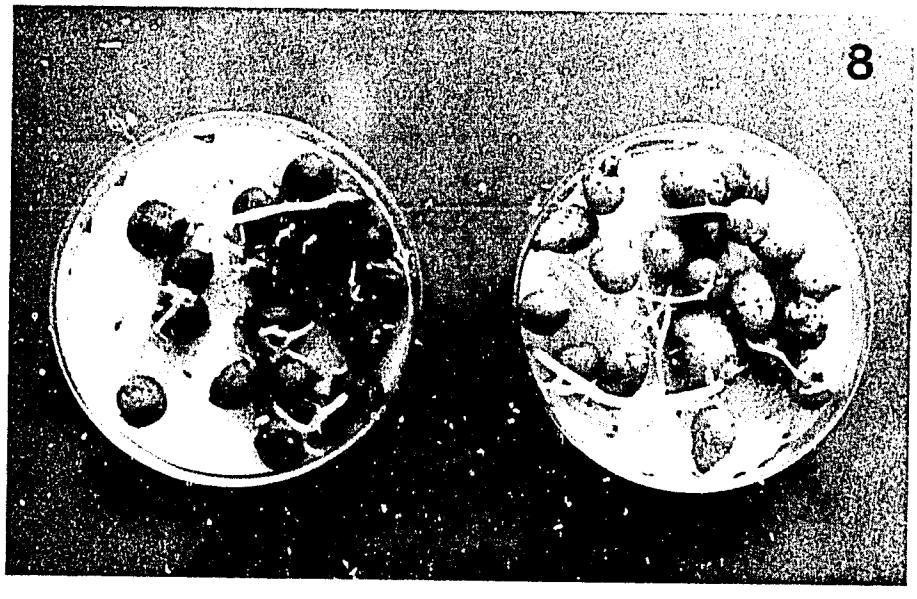


Fig. 7. In vitro plantlet export package.

Fig. 8. In vitro tubers sprouted after storage. The 9 cm petri dish gives an idea of average tuber size.

6 MEDIA

All media used for this work are based on the salts of Murashige and Skoog (1962). The salt stock solutions are normally prepared in four separate parts:

a) Salts
b) $MgSO_4$
c) Iron
d) Vitamins

a) **Salt** stock solution: dissolve each in 200 cm^3 glass distilled water

- NH_4O_3	35.0 g
- KNO_3	40.0 g
- $CaCl_2.H_2O$	9.0 g
- KH_2PO_4	3.5 g
- H_3BO_3	0.1 g
- $MnSO_4.4H_2O$	0.5 g
($MnSO_4.H_2O$	0.4 g)
- $ZnSO_4.7H_2O$	0.2 g
($ZnSO_4.H_2O$	0.1 g)
- KI	0.02 g
- $Na_2MoO_4.2H_2O$	0.005 g

Dissolve 5 mg (0.005 g) of the following salts together in 10 cm^3 of water; add 1 cm^3 of this solution to 200 cm^3 water for the stock solution

- $CuSO_4.5H_2O$ and $CoCl_2.6H_2O$

Mix the ten salt solutions together to make 2 dm^3 of the salt stock solution.

b) **$MgSO_4$** stock solution

- $MgSO_4.7H_2O$	3.7 g in 100 cm^3 distilled water

c) **Iron** stock solution

- Na_2EDTA	0.75 g
- $FeSO_4.7H_2O$	0.55 g

Dissolve $FeSO_4.7H_2O$ in 20 cm^3 distilled water; Na_2EDTA in 20 cm^3 distilled water heating it up. Mix the solutions, cool, and make up to 100 cm^3 with water.

d) **Vitamin** stock solution

- Thiamine HCl	20 mg
- Glycine	100 mg
- Nicotinic acid	25 mg
- Pyridoxine HCl	25 mg

Dissolve each vitamin and complete to 500 cm^3 with distilled water. Dispense 10 cm^3 of stock solution in small vials and keep frozen. Use one vial for 1 dm^3 of medium.

Medium preparation. Prepare 1 dm^3 of the Murashige and Skoog basic medium (MS) by mixing the stock solutions with Inositol in the following proportions:

Salts	100 cm^3
$MgSO_4$	10 cm^3
Iron	5 cm^3
Vitamin	10 cm^3
Inositol	100 mg

Add the relevant hormones and sucrose (see below). Sterilize the mediums with or without gelling agents in autoclave at 121°C during 15 minutes and at 103.4 KP of pressure (15 pounds/square inch)

Additions to the basic MS medium for preparation of specific media types:

MEDIUM	MS media	+	the following additions
A	0.1	mg/dm^3	Gibberellic acid
(meristems)	0.04	mg/dm^3	Kinetin
	2.5	%	Sucrose
	0.6	%	Agar*
B	0.1	mg/dm^3	Gibberellic acid
(meristems)	20.0	mg/dm^3	Putrescine HCl
	2.5	%	Sucrose
	0.6	%	Agar*
C**	1.0	mg/dm^3	Folic acid
(propagation)	50.0	cm^3	Coconut water
	4.0	mg/dm^3	L-Arginine HCl
	2.0	mg/dm^3	Calcium pantothenate
	10.0	mg/dm^3	Putrescine HCl
	3.0	%	Sucrose
	0.8	%	Agar* (semi-solid medium)
D**	0.1	mg/dm^3	Gibberellic acid
(propagation)	2.5	%	Sucrose
	0.8	%	Agar* (semi-solid medium)
E	4.0	%	Sorbitol
(storage)	2.0	%	Sucrose
	0.8	%	Agar*
F	0.1	mg/dm^3	Gibberellic acid
(export)	2.5	%	Sucrose
	1.0	%	Agar*

Other useful media for potato tissue culture:

Medium	MS media	+	the following additions
G	1.0	mg/dm^3	Gibberellic acid
(tuber sprouts)	100.0	mg/dm^3	Ascorbic acid
	4.0	mg/dm^3	L-Arginine HCl
	2.0	mg/dm^3	Calcium pantothenate
	3.0	%	Sucrose
	0.6	%	Agar*
H	4.0	%	Mannitol
(storage)	3.0	%	Sucrose
	0.8	%	Agar*
I	0.4	mg/dm^3	Gibberelic acid
(propagation)	0.01	mg/dm^3	Naphtalen acetic acid
	0.5	mg/dm^3	Bencylamino purine
	2.0	mg/dm^3	Calcium pantothenate
	2.0	%	Sucrose

* Other gelling agents as phytagel orgelrite (trade mark) can be used instead of agar. Add phytagel at half of agar concentration and use gelrite at a quarter of the same concentration.

** Medium C or D can be used with or without gelling agents.

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