

# 1. Tissue Culture Laboratory

## 1.1 MICROPROPAGATION UNIT

A micropropagation unit includes a tissue culture laboratory and a propagation greenhouse. When planning a micropropagation unit we have to consider the following factors: available space, environment, financing, type of work to be developed, and required production capacity. According to the production capacity and the available space, we may consider three types of micropropagation units:

a) Small scale. The facilities for in vitro work can be adapted for a house setting, using the available equipment and materials to carry out the basic micropropagation activities. This method could be used to micropropagate plants for interested people, or mother plants for greenhouses but with great care to avoid contamination problems.

b) Medium scale. It is necessary to design, implement and/or prepare specific working areas, and to acquire equipment and materials to increase the efficiency and uniformity of the results.

c) Large scale. The facilities and the equipment must be designed to actually perform the work and to maintain an optimum production flow.

### Basic Processes

The basic processes normally carried out in a tissue culture laboratory are:

- a) Glassware washing
- b) Culture media preparation

- c) Media and equipment sterilization
- d) Ex-plants preparation and aseptic transference of cultivated materials
- e) Incubation and growth of cultivated materials up to maturity
- f) The rooted-plantlets transplantation, is accomplished, in part, with the help of laboratory personnel.

### **Basic Organization**

The laboratory for plant tissue culture requires a basic organization that comprises three areas:

- a) General laboratory (or media preparation area) provided with spaces for common or independent work. Some equipment and materials can be used by several workers at the same time.
- b) Area for the aseptic manipulation of plant material (or transference area).
- c) Culture maintenance area (or culture rooms) with controlled conditions for light, temperature and humidity.

There should be two separate rooms at least. One for washing, sterilization, storage and culture media preparation; and another one for culture maintenance (culture room).

The transference chamber can be located in the general laboratory or in an area specifically designed as a transference room, according to available conditions.

### **Washing and Media Preparation Area**

The area for washing should have a big washbasin (of stainless steel, and be acid and alkali resistant), tap water, tables that allow stand-up work and shelves to dry and keep the washed materials. The media preparation area must be equipped with a refrigerator to keep the chemicals and solutions used in the media culture, scales, a potentiometer, a kitchen, a media mixer, a water distiller, and an autoclave or pressure pot. The last two must be located as close as possible to the washbasin. The stove may be used to dry the materials.

### **Culture Area**

This is the culture incubation area, where optimum media conditions change according to the species in culture. So, temperature variations, light intensity and quality, relative humidity and photoperiod should be taken into consideration. Temperature is controlled with air-conditioning equipment or heaters. According to the cultivar, the average temperature of an incubation room should be  $25^{\circ}\text{C} \pm 1-200$ . For higher or lower temperatures, air-conditioning equipment should be

used to reach the appropriate temperature. It is recommended to use thermostats which prevent temperature variations in the room from exceeding the culture requirements.

The airflow must be uniform within the culture room to maintain the same temperature in the whole environment.

The air-conditioning equipment indirectly controls the relative humidity. If the relative humidity drops below 50%, there will be a water loss in the culture media, and an increase in the mineral salts concentration, which can damage the cultures. With a high relative humidity (80-100%) contaminants could enter the culture containers. The optimum average is between 50 and 70%. The light source is provided by fluorescent lamps and the photoperiod is controlled by an hourly timer. The fluorescent lamps have an advantage over the incandescent lamps because they have better light quality, distribute the light uniformly and produce less heat. However, some cultivars grow better with a combination of both types of light.

Most of the cultivars require an illumination that varies between 500 and 3,000 lux. Some of them need more than 5,000 lux, and others just need darkness as in the case of in vitro tube induction. When using fluorescent lamps we should consider that the ballast generates heat which affects the culture room temperature. That is why they must be installed outside the room.

The arrangement and number of the shelves, where magenta vessels and tubes with the cultures are placed, will vary according to the room's dimensions. Shelves can be metallic or wooden, and should be painted white.

The shelves' dimensions may vary. However, it is recommended to have an incubation platform of 0.45 x 0.90 m, with a height of 0.30 m among the shelves because it allows good illumination, access to, and control of the incubated materials. The space between the soil and the first platform must be 0.15 m to facilitate soil cleaning. A distance of 0.05 to 0.10 m must be kept between the wall and the shelves to allow the free circulation of air.

For the laboratory walls antifungal-epoxy-paint (used in cool temperature chambers) is recommended as a preventive measure.

It is recommended to put a tray on the floor, with a rug containing an acaricide and a fungicide to impregnate the shoes of those who enter the culture room.

The culture room must be isolated from the external environment to maintain the appropriate temperature and the relative humidity and to avoid the entrance of contaminants. Just in case, the windows should also be sealed. Access to the culture room will be allowed only to the people who work there.

A tissue culture laboratory can be located in any geographical area. The internal controlled environmental conditions allow isolation with a minimum of external influence.

To decide where to build the tissue culture laboratory, the following factors must be taken into consideration:

Environmental growing conditions for the species that will be cultivated

Availability of electricity

Availability of water and drainage

Good all-round communication

## **Production Process**

The production process involves:

- The in vitro culture establishment stage
- The production stage

### **In vitro culture establishment stage**

It consists of taking plants for the test-tube from the field: for this, a clean lot is selected (free from pathogens) that guarantees the quality, uniformity, and strength of the material to be placed in the market when the production stage is finished.

The selected plants will be those with optimum growth, development, and phytosanitary conditions. These plants can go through the process of thermotherapy and meristem culture. The plantlets will be used as a source of explants for the production process. If pathogen cleaning is not necessary, entire buds are taken and placed in a temporary culture medium where they will be observed for one or two weeks and, if a bacterial infection is noticed, they are treated with antibiotics until the infection disappears.

### **Production stage**

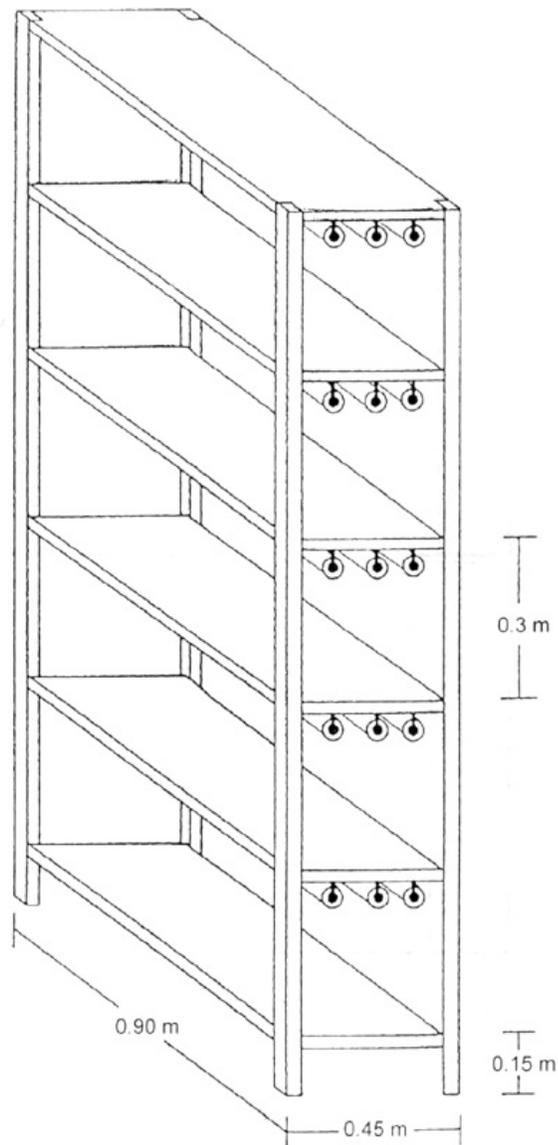
It consists of the massive propagation of explants and plantlets.

The propagation range depends on the species: the ranges commonly present in most of the micropropagated crops have been taken as a reference. In the same crop, the propagation range may vary according to the phytohormones in the culture medium.

The time of each propagation cycle depends on the species behavior, the culture medium, and the environmental conditions to which it is subjected: the average is between three or four weeks for each step.

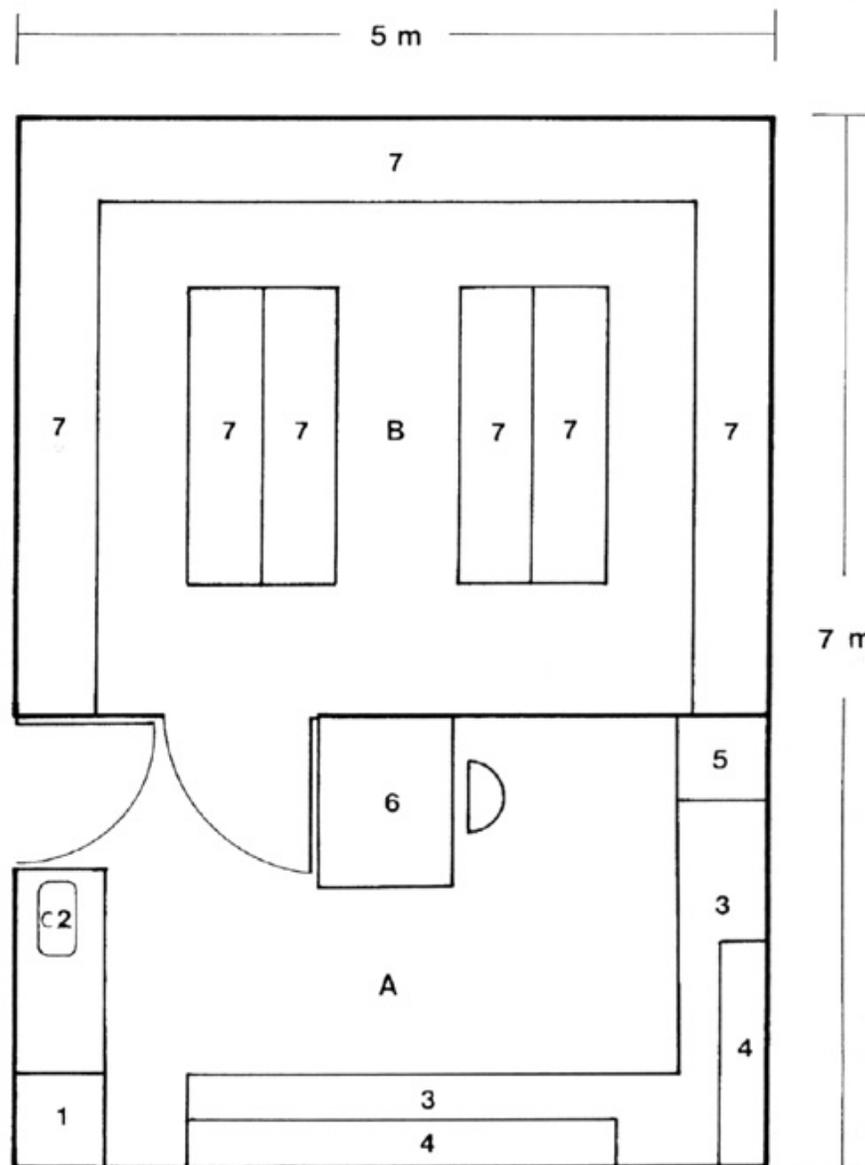
The Magenta plastic containers have been taken as standard, but any others can be prepared for this purpose. It is considered that 20 explants per container make a good plantlet development possible.

The graphics below show the design of the incubation shelves and of the micropropagation laboratory.



Nelson Espinoza R.

**Figure 1** Incubation shelf



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A Laboratory

B Incubation room

1 Autoclave

2 Washbasin

3 Working tables

4 Wall cabinet

5 Refrigerator

6 Transference chamber

7 Incubation shelves

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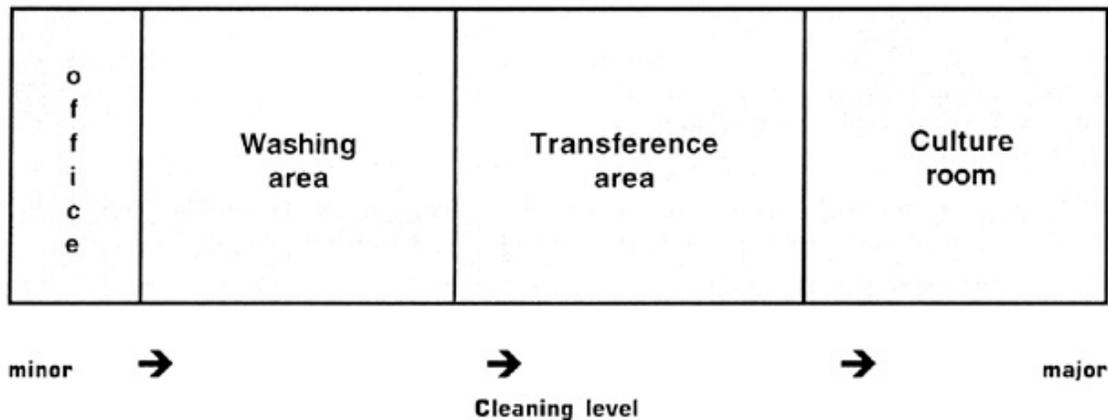
**Figure 2** Basic Small - Scale Micropropagation Laboratory

## Aseptic Conditions in the Laboratory

Asepsis in the tissue culture laboratory is one of the most important requirements. A good asepsis guarantees the plantlets' growth and the good use of the culture media.

## Asepsis in the Facilities

The tissue culture laboratory must have four basic environments: an office, a washing and media preparation room, a transference room, and a culture room, which require a minor to major grade of cleaning, as well as a minor to major restriction on personnel access.



The main purpose of asepsis is to maintain the rooms isolated from the external environment. Cleaning is carried out with more diligence on floors, furniture, and shelves by using disinfectants, and avoiding direct entrance from greenhouses or fields.

The entrance doors must have rugs continuously sprayed with acaricide powder

When entering, it is necessary to use lab coats to avoid dispersing dust from clothes.

## Asepsis in the Washing and Media Preparation Room

The wash room must be provided with the necessary materials for cleaning the used tubes, on covered shelves to avoid dust. Tables and shelves must be continuously cleaned.

All rejected material (tubes with old plants or tubes with used medium) must be immediately sterilized to avoid environmental contamination.

The media preparation area is the room where material is sterilized, so it requires a good cleaning to avoid media contamination before it is used. This area must be maintained in isolation to protect it from dust and other air-borne contaminants.

### **Asepsis in the Transference Room**

The transference room, where the laminar flow chambers are, is a place which must be kept very clean, since it is next to the culture room.

In this room a washstand is necessary where the personnel can wash their hands before they start propagation.

In this area, great care must be taken in the laminar flow chamber; the internal walls must be cleaned with alcohol 70<sup>0</sup>/o].

The filters and pre-filters (Hepa) within the chambers must be continuously revised to avoid contamination.

The tools within the chamber must be disinfected over a burner flame.

During propagation, hands and table surface will be continuously cleaned with a piece of cotton soaked in alcohol.

Only the material necessary for propagation will be permitted within the chamber. Other appliances (radios, books, etc.] are not permitted.

Never open a contaminated tube (or magenta] within the chamber; it must be sterilized immediately.

### **Asepsis in the Culture Room**

The disinfection of floors and shelves must be continuous. The tube racks must be cleaned with an acaricide/fungicide solution before they are put into the culture room for the first time.

The inside doors and walls must be frequently examined to avoid the preserve of fungi.

The air-conditioner must be regularly checked.

## **1.2 PREBASIC SEED PRODUCTION PLAN**

In seed production programs, propagation systems must be developed to make possible the maximum use of the places allocated for this purpose, Thus, greenhouses must be used economically for in vitro plantlets.

The diagram included here shows an example of a production plan developed in the INIA station at Huancayo, where three campaigns per year are carried out, and material is propagated for six greenhouses by using 3,850 plantlets per greenhouse.

During the first season, the in vitro plantlet propagation starts in August; they grow under the continuous observation of laboratory personnel. On November 2, the plantlets are taken to the greenhouses.

At the same time, soil-disinfecting activities and bed preparation will be carried out in the greenhouse to have it ready for the reception of material on November 2.

It is suggested that laboratory personnel take the plantlets to the greenhouse and participate in their transference to the beds, as well as in the washing of the magentas, before taking them back to the laboratory.

On March 3, (according to the diagram) plants will be ready to harvest and then the greenhouse is prepared for the following campaign. Note that harvesting and cleaning take one week (fumigation, renewal of beds, etc.)

The subsequent campaigns are similar Modifications may be carried out according to the availability of personnel and facilities.

**PRE-BASIC SEED PRODUCTION PLAN**

Variety: Perricholi

Place: INIA - Santa Ana Station, Huancayo

Conditions:

Two people in micropropagation

Three seasons per year

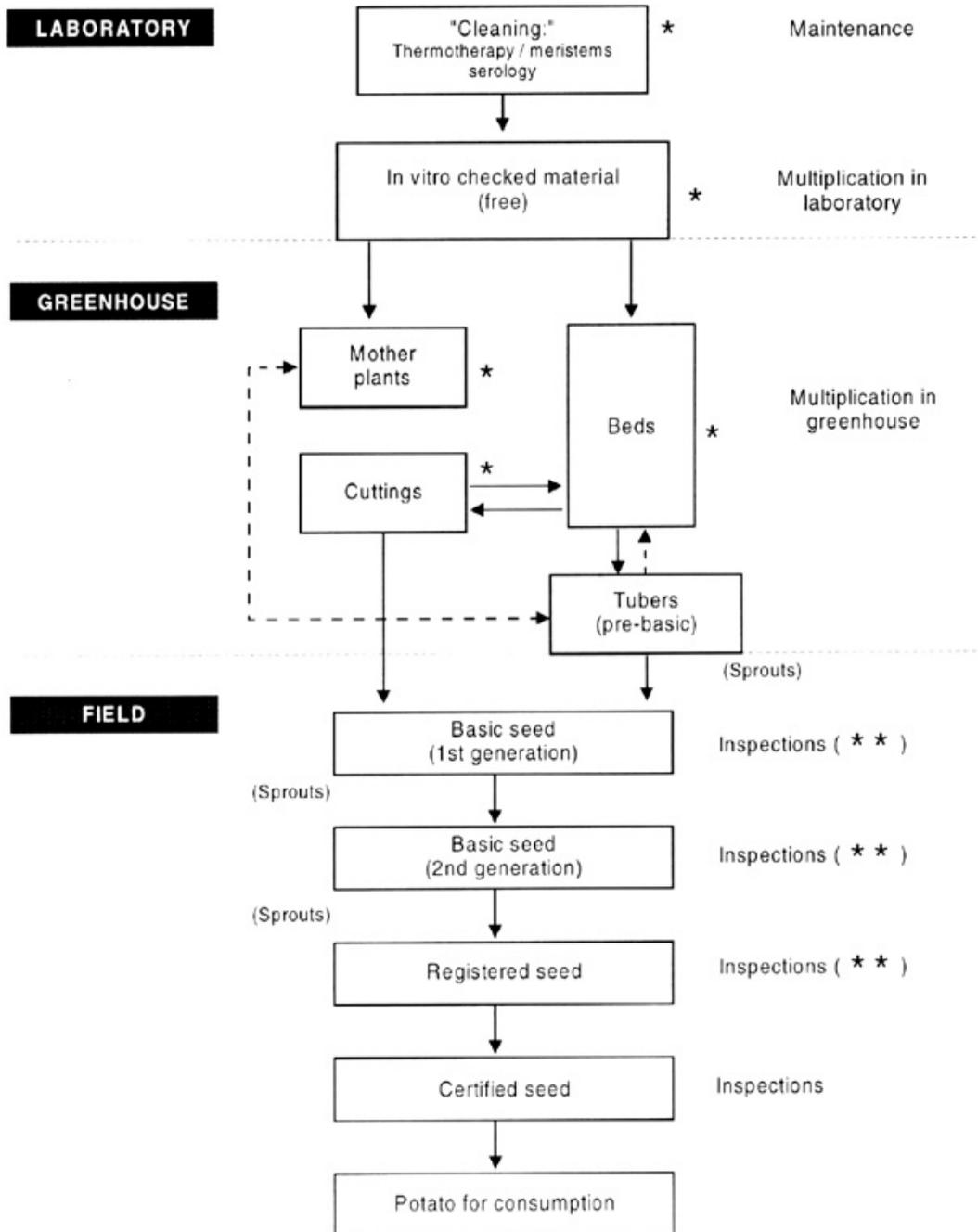
Six greenhouses

	Aug Sep Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug Sep Oct Nov
Tissue culture lab personnel Activities	<p>X →→→ 2</p> <p>X →→→ 7</p> <p>X →→→ 12</p>
Greenhouse personnel activities.	<p>2 → → → 3</p> <p>7 → → → 7</p> <p>12 → → → 13</p>

This program indicates a specific case in the coordination of in vitro propagation activities and the transference of plantlets to greenhouses.

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### GENERAL SCHEME OF POTATO TUBER-SEEDS PRODUCTION



- \* Quality control with ELISA
- \*\* ELISA is optional

Source: Dr. Oscar Hidalgo

### **1.3 MULTIPLICATION PLAN FOR IN VITRO PLANTLETS FOR GREENHOUSES**

The infrastructure of the tissue culture laboratories makes possible the propagation of large quantities of plantlets to provide material for one or several greenhouses.

The multiplication of 3,600 to 4,000 plantlets must be carried out under a defined program according to laboratory conditions.

The growing of the in vitro propagated plantlets will depend on the medium, the environmental conditions of the incubation room, and the variety. So, it is necessary to evaluate the plantlets in growing conditions in relation to time to establish an efficient system later, according to our requirements. Next, three propagation procedures, followed in different laboratories (different environmental conditions), are presented.

#### **Procedure 1 (Tissue culture laboratory, INIA-Huancayo)**

- Propagate nodes in 8 tubes (4 nodes per tube) to obtain 32 plantlets with 5 nodes each, in 2 1/2 weeks: the plantlets are cut, apical buds and roots are separated, and stems are planted in a liquid medium (5 stems of 4 nodes per erlenmeyer flask).
- Plant the apical buds (32 buds) in magentas (25 buds per magenta).
- Leave the stems growing in a liquid medium during 2 1/2 weeks to obtain 128 plantlets: bring them together with the plantlets growing in the magenta (32 plantlets) to obtain a total of 160 plantlets.
- Out the plantlets again (apicals and roots), and transfer stems only to a liquid medium (5 stems of 4 nodes per vial) for 2 1/2 weeks.
- Plant the apical buds (160 apicals in total) in magentas (25 buds per magenta). The 160 stems planted in a liquid medium produce 640 plantlets: if we add the 160 plantlets that are growing in magentas, a total of 800 plantlets with 5 nodes is obtained. That is 4,000 explants (nodes).
- Plant the nodes in magentas: after 3 1/2 weeks 4,000 plantlets will be ready for the greenhouse.

This procedure is carried out in 11 weeks under a solid-liquid-liquid-solid system: a total of 6,079 ml of culture medium is used.

#### **Procedure 2 (Private laboratory, Huancayo)**

- Put 5 stems in a herlemeyer flask (the apical bud and the root are removed). They will produce 30 plantlets in three weeks (5 nodes per plantlet, 150 explants).

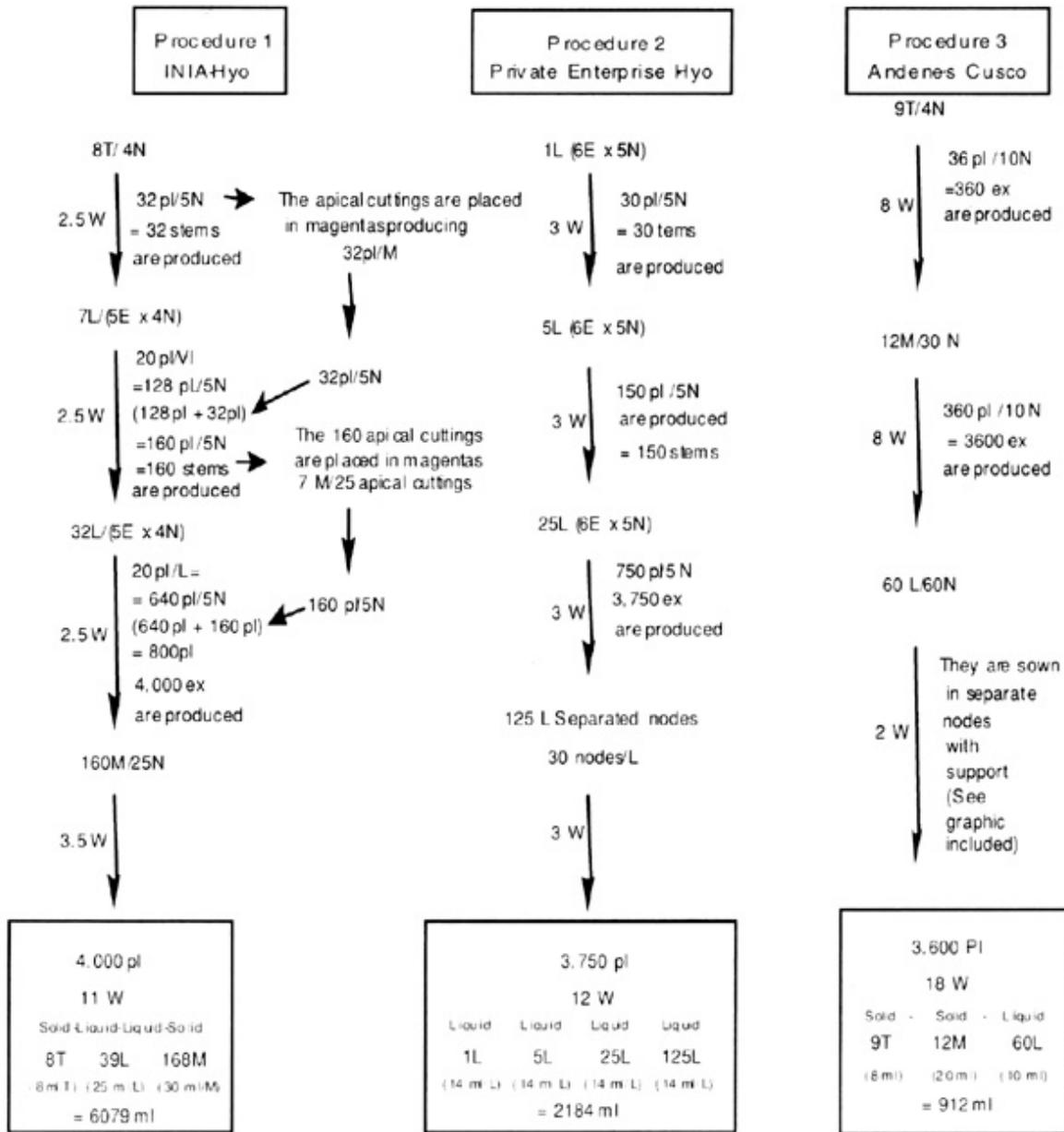
- Propagate the plantlets in 5 erlenmeyer flasks containing a liquid medium (6 stems of 5 nodes per flask]; after three weeks 150 plantlets are obtained (5 nodes each, 750 explants).
- Propagate the plantlets in 25 flasks with a liquid medium (6 stems of 5 nodes per vial]; after three weeks growth 3,750 nodes are obtained (5 nodes per plant] that will be propagated, as separated nodes, in flasks containing a liquid medium.

This procedure is carried out in 12 weeks under 4 steps of liquid media using 1.4 ml/each.

### **Procedure 3 (Laboratory Andenes, Cuzco)**

- Propagate plantlets in 9 tubes with 4 nodes each, and let them grow during 8 weeks until 10 nodes per plantlet (total: 380 nodes] are obtained.
- Propagate the plantlets in magentas (30 nodes per magenta], and 10 nodes per plant are obtained after 8 weeks' growth. This procedure produces 3800 explants which are propagated in 80 flasks containing a liquid medium (60 nodes per flask] (see graphic below]. After two weeks 3,600 plantlets will be ready to be transferred to the greenhouse.

This procedure is carried out in 18 weeks using a solid-solid-liquid system (9 tubes, 12 magentas and 60 liquid media: 912 ml of culture medium were used (the tubes contain 8 ml of medium, the magentas 20 ml and magentas with liquid media contain 10 ml).



T=tubes N=nodes L=erlenmeyer Flask C=cuttings W=weeks pl=plantlets M=magentas ex=explants

USE OF LIQUID MEDIUM IN PLANT PROPAGATION IN PROCEDURE 3

