Chapter II: In Vitro Vegetative Propagation

In vitro vegetative propagation, or micropropagation, is an artificial method of asexual reproduction and offers several advantages over conventional propagation methods. This technique has made it possible to propagate species with scarce seeds, germination difficulties, and/or for which cutting or grafting techniques are inapplicable, leading to a greater diversity of commercialized plants.

Similarly, several other techniques, all derived from in vitro culture, play an important role in improving the agronomic or horticultural performance of cultivated plants.

Micropropagation is used for the purpose of mass propagation, as it allows for the production, starting from a single individual (plant), of a considerable number of plants that are genetically identical to the mother plant.

The reproduced plants are not only true-to-type but also exhibit great uniformity.

Furthermore, the use of this technique requires little space and can be scheduled independently of the seasons. The technique therefore undoubtedly represents a powerful tool with significant industrial and economic prospects.

I. In Vitro Culture: Definition and History

In vitro culture: whole plants or plant fragments (explants) placed outside their natural environment under sterile conditions on a nutrient medium (originally using laboratory glassware).

As early as 1902, Haberlandt, a German biologist, observed the natural potential of vegetative propagation (cuttings). Following his work, he stated the first major principle that would pave the way for plant micropropagation: the principle of cellular totipotency – "every plant cell is capable of regenerating another individual identical to the one from which it originated."

In 1934, White successfully cultured tomato roots on a medium containing water, mineral salts, a yeast extract, sugar, and a plant hormone, the only one known at the time: auxin.

In 1939, Gautheret obtained a mass of undifferentiated cells from carrot tissue: a callus. This callus can be cultured indefinitely. With this, in vitro culture ("in glass") truly began.

In 1962, Murashige and Skoog studied the vegetative propagation of tobacco and developed the first basic medium for in vitro culture. This medium contains mineral salts, B-group vitamins, sugars, auxins, and cytokinins.

Table 1: Composition of two common culture media: MS (Murashige and Skoog) and B5 (Gamborg, Miller, and Ojima

	MS	B_5
Macroéléments	Mg/l	Mg/l
NH_4NO_3	1650	2500
KNO_3	1900	150

$CaCL_2.2H_2O$	440	134
$MgSO4.7H_2O$	370	250
KH_2PO4	170	150
Micro-éléments	Mg/l	Mg/l
$MnSO_4.H_2O$	22.3	10
$ZnSO_4.7H_2O$	8.6	2
H_3BO_3	6.2	3
KI	0.83	0.75
$Na_2MoO_4.2H_2O$	0.25	0.25
$CuSO_4.5H_2O$	0.025	0.025
$CoCl_2.6H_2O$	0.025	0.0125
Fe - EDTA	Mg/l	Mg/l
Na_2-EDTA	37.3	37.3
$FeSO_4.7H_2O$	27.8	27.8
Substances organiques	Mg/100ml	Mg/100ml
Glycine	0.2	1
Acide Nicotiniq	0.5	1
Pyridoxine.HCl	0.5	10
Thiamine HCl	0.1	100
Myo-inositol	100	1
Saccharose	30000mg	30000mg
Agar	08 g/l	08g/l
pH	5.7	5.5

Table 2: Types of growth regulators and their solubility.

Growth Regulators	**Solvents**
Auxin (IAA)	EtOH or NaOH
Cytokinin (Kinetin)	1N NaOH
Gibberellin (GA3)	EtOH

Organogenesis

Organogenesis is the fundamental basis of vegetative propagation, which always relies on the formation of new meristems. Starting from an explant, it leads to the formation of a new individual through the development of buds (caulogenesis) and roots (rhizogenesis).

Caulogenesis II.1.1. Definition

Caulogenesis refers to both the initiation and development of terminal, axillary, adventitious, or newly formed buds on a callus.

- Terminal buds derive from the plumule of the embryo.
- Axillary buds are generally produced by the two or three superficial cell layers of the stem.
- Adventitious buds are formed in unusual locations. They are formed from differentiated organs of the plant (between nodes, tubers, roots, etc.).
- Newly formed in-vitro buds can appear on the initial explant or on a callus; they can be considered a special case of adventitious buds.

Origine des Bourgeons (Études Cytologiques)

- **Tissus compétents :** La capacité à former de nouveaux bourgeons provient principalement de tissus spécifiques contenus dans l'explant :
  - **Très forte :** Cambium.
  - **Élevée :** Parenchyme vasculaire ou libérien (phloème, xylème).
  - **Très faible ou nulle :** Parenchyme cortical ou modulaire (moelle).
- **Différences entre espèces :**

Chez les Conifères (ex: Pin) : L'origine est souvent **unicellulaire**, provenant des couches subépidermiques du mésophylle.

Chez les Angiospermes : L'origine est souvent **pluricellulaire**, pouvant provenir de cellules épidermiques, de tissus palissadiques, du mésophylle spongieux ou de la gaine périvasculaire.

2. Régulation Hormonale de la Caulogenèse

- Le déclenchement de la formation des bourgeons est principalement contrôlé par l'équilibre entre deux hormones :

Cytokinines

- **Auxines**

Le concept de balance hormonale (Skoog & Miller) : Le devenir des tissus en culture est déterminé par le rapport auxine/cytokinine.

Auxine seule : Survie des tissus.

Auxine majoritaire : Stimule la **rhizogenèse** (formation de racines).

- **Cytokinine majoritaire :** Stimule la **caulogenèse** (formation de bourgeons).

Équilibre entre les deux : Favorise la **callogenèse** (formation d'un amas de cellules indifférenciées, le cal).

Title: Origin of Buds and Hormonal Regulation of Caulogenesis

The text explains the origin and hormonal control of bud formation (caulogenesis) in *in vitro* culture.

1. Origin of Buds (Cytological Studies)

Competent Tissues: The ability to form new buds originates from specific tissues within the explant:

Very high: Cambium.

High: Vascular or phloem parenchyma (xylem, phloem).

Very low or none: Cortical or pith parenchyma.

- **Differences between species:**
  - **In Conifers (e.g., Pine):** The origin is often **unicellular**, arising from the sub-epidermal layers of the mesophyll.
  - **In Angiosperms:** The origin is often **multicellular**, with meristems able to form from epidermal cells, palisade tissues, spongy mesophyll, or the perivascular sheath of cultured explants.

II.2. Rhizogenesis
II.2.1. Definition

Rhizogenesis refers to the formation and growth of roots. Root meristems are categorized into several types based on their origins.

Lateral roots form spontaneously on the main root under natural conditions.

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Adventitious roots are produced by various organs, either spontaneously, accidentally following an injury, or induced under conditions of cutting and layering.

Neoformed roots within a callus, in in-vitro culture, can be considered a specific case of adventitious meristems.

Origin of Neoformed Roots

Rhizogenesis is a complex phenomenon involving different phases: dedifferentiation, the formation of clusters of meristematic cells, and the differentiation and organization of these meristematic clusters into a root primordium which will develop into young roots.

The origin of the cells involved in healing depends on the plant species. However, in all cases, it is the vascular cambium (generating phloem and xylem tissues) that gives rise to tissues with good callus-forming ability.

The callus is primarily formed from secondary meristematic-type cells, which incorporate some neighboring parenchyma cells. The meristematic cells subsequently differentiate and organize to give rise to a new root.

Environmental Factors Affecting Rhizogenesis

Oxygenation of the medium has always been considered favorable for rooting. The choice of substrate is guided by the dual necessity of ensuring both moisture and drainage.

A high temperature (20 to 25°C) promotes rhizogenesis, hence the benefit of heating cutting trays.

The optimal pH depends on the species. It has long been known that some plants are acidophilic (Azaleas, etc.). Conversely, Coleus cuttings tolerate a wide range of pH.

Supplementary lighting promotes the rooting of leafy cuttings in some instances, although it is not clearly known whether this is due to a photosynthetic effect or an action on the synthesis of endogenous growth regulators through photoperiodism.

Watering by "mist" or artificial fog often gives excellent results, particularly with herbaceous cuttings. The "mist" has several effects: reduction of water loss, decrease in temperature at the foliage level, reduction of evapotranspiration. It simultaneously allows the cutting to retain a larger leaf area, leading to better

Factors Specific to the Explant

Genetic factors certainly play a decisive role. Within the same species, some cultivars root easily, while others do so with difficulty.

The reserves provided by the cutting (particularly carbohydrates) promote rhizogenesis.

The age of the mother plant is a well-known factor. Cuttings taken from young plants generally have a better ability to form roots than those taken from older plants. In woody plants, it is common for rooting of cuttings to be possible only from young seedlings.

Gradients in rhizogenic ability have often been observed depending on the position of the cutting on the plant. It is common for axillary shoots taken near the base of the stem to exhibit good rhizogenic ability. In many herbaceous plants, cuttings with a vegetative bud taken from a flowering stem only root if they come from the basal part of the plant, farthest from the flowering regions (example: Beetroot). However, there is no precise rule regarding these gradients.

III. Technical Conditions for In Vitro Culture

The technique of in vitro culture of organs or tissues of higher plants can be considered an extension of microbiology methods.

In vitro culture must be aseptic, which implies the sterilization of conditions allowing the maintenance of cultures protected from microbial or fungal contamination.

In vitro culture also raises the problem of the climatic environment of the culture chambers: humidity, temperature, light intensity, photoperiod.

An essential problem is the choice of the composition of the culture medium: liquid support or agar, mineral macro and micro-elements, addition of various organic compounds, combinations of growth regulators.

The explant must find in the culture medium everything it needs to survive, multiply, and regenerate a new individual. For this, it must find everything that the plant provides under natural conditions (Fig. 23).

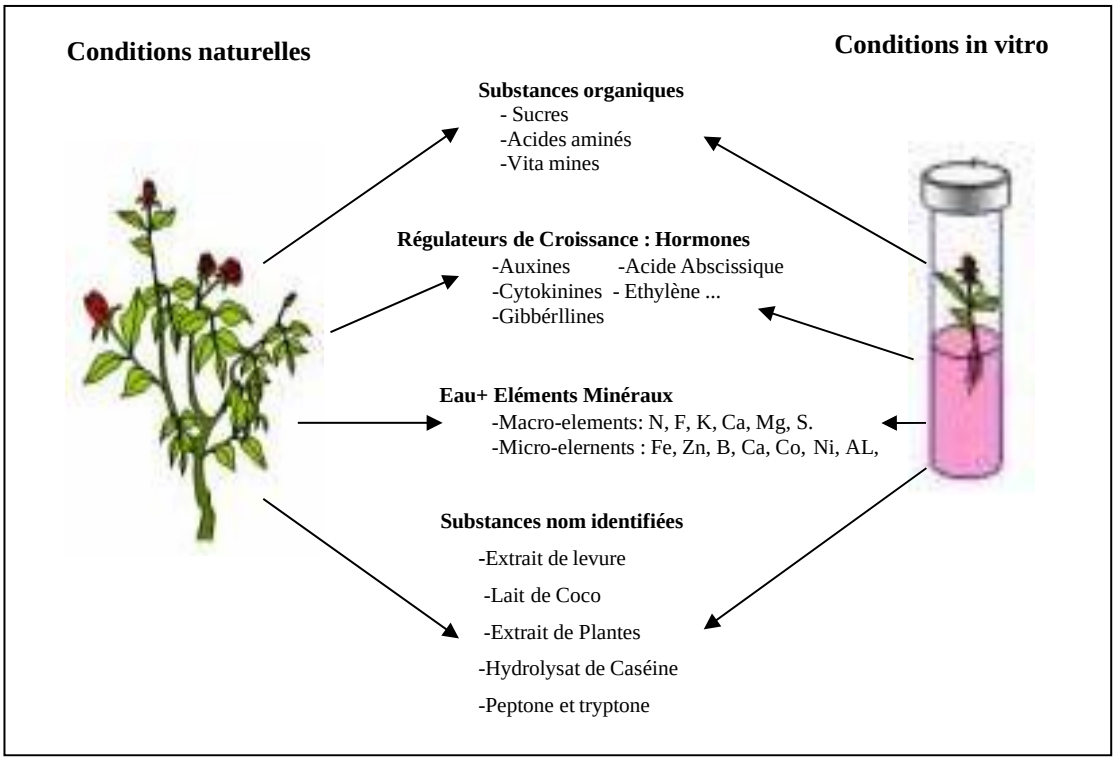

Figure 23: Culture Medium

Containers

Cultures on a medium solidified with agar are often carried out in glass tubes but also in a variety of other containers: cylindrical flasks (called Erlenmeyer flasks) or cylindrical-conical ones, Petri dishes, bottles, preserving jars, etc.

The dimensions of the containers are essentially a function of the size and number of explants. For example, meristems or shoot apices are usually initially seeded in small tubes. Later, during successive transplantations, they can be placed in larger tubes. The growth of bud clusters on media supplemented with cytokinin is often maintained in large containers; conical flasks or jars.

Experience with separated cell cultures shows that it is necessary to accompany the cells with nurse tissues or to seed the surface of the medium with a sufficient quantity of cells. It is therefore essential to adjust the amount of medium well to the dimensions of the tissues.

Asepsis

Asepsis Conditions

The aseptic subculturing of uncontaminated material requires only minimal precautions. On the other hand, during the initial seeding of the explant, achieving asepsis remains one of the major difficulties of in vitro culture.

Asepsis does not present particular difficulties when the plant is lightly contaminated and the tissues of the explant are well protected. This is often the case when seeding meristems or shoot apices, which are naturally protected by leaf primordia.

Glassware is sterilized by moist heat in an autoclave (for example, at 120°C for 45 minutes). Large instruments (forceps, scalpels, etc.) are flamed using a Meker burner after immersion in alcohol. Small instruments (needles, razor blades, etc.) are usually sterilized by immersion in alcohol followed by rinsing with sterile water. Culture media are sterilized by autoclaving (for example, at 110°C for 20 minutes). One of the serious drawbacks of autoclaving is that it decomposes and inactivates, at least partially, some heat-unstable compounds (vitamins, etc.).

It may be essential, when it is not a matter of routine subculturing but of rigorous experiments, to sterilize heat-unstable substances separately by microfiltration and to add them to the basal medium after autoclaving.

Sterilization of Plant Material

Disinfecting material contaminated by soil bacteria is always difficult and uncertain. It is better to operate under aseptic conditions or limit contamination whenever possible. For example, in the case of tuberized roots of chicory, beetroot, and carrot, coming directly from the field, establishing an aseptic culture is often very difficult. But we have observed that a preliminary stay of the roots in vermiculite, a substrate unfavorable to the proliferation of microorganisms, allows for obtaining relatively healthy material that can then be sterilized by usual methods.

For seeds, the tissue culture method involves immersing the explants for a few moments in alcohol, then in a calcium hypochlorite solution, and rinsing them several times in sterile water.

In difficult cases, the use of mercuric chloride ($HgCl_2$ at 0.25‰ for 6 hours or at 0.5‰ for 1 to 3 hours) has often given us better results than calcium or sodium hypochlorite.

Immersion of the plant material in an antibiotic solution (penicillin and streptomycin at 1 mg/L in combination) for 2 hours after treatment with hypochlorite has sometimes shown some effectiveness but with inconsistent results.

Culture Medium

3.1. Support

1- Agar/

The success and development of in vitro cultures have been linked to the use of agar, which solidifies the medium.

However, agar has some disadvantages. The main one is that it provides insufficient aeration, which then inhibits the growth of certain tissues. Furthermore, the composition of agar is variable and poorly defined. It is also possible that agar may provide organic or mineral elements, perhaps beneficial for growth, as micro-elements.

2- Mineral Medium

Among the elements necessary for plant life, macroelements and microelements are generally distinguished.

Carbon, oxygen, and hydrogen constitute nearly 95% of the dry matter, and the six other essential macroelements are: nitrogen, phosphorus, sulfur, potassium, magnesium, and calcium. The first three are

fundamental constituents of plant tissues (proteins, nucleic acids...). The last three are involved particularly in maintaining the balance between cations and anions in the plant.
Microelements play an essential role in enzymatic mechanisms as activators or constituents of coenzymes. The main ones are iron, copper, zinc, manganese, molybdenum, and boron.

3.3.1. Sugars

Tissues in in vitro culture are largely heterotrophic for carbon due to the absence or insufficiency of chlorophyllous assimilation. Therefore, it is generally essential to add carbohydrates to the culture medium. The two most commonly used sugars are: glucose and sucrose.
The optimal sugar concentration for the growth of tissue strains is not always easy to determine; the optimal sucrose concentration varies from 2% to 8% depending on whether growth is being evaluated.

3.3.2. Vitamins

Various vitamins promote the growth of tissues in culture; and it is not excluded that the lack of certain ones could be a limiting factor for organogenesis phenomena.

3.3.3. Amino Acids and Various Protein Extracts

Mixtures of amino acids thus appear to have synergistic effects, strongly stimulating the proliferation of calli and organogenesis in various examples. However, the effect of amino acid supplementation appears very variable depending on the species and the type of morphogenesis studied.:

4- Growth Regulators

Their influence on cell divisions and tissue development occurs at very precise concentrations, with the slightest variation (on the order of 10^{-2} mg/L) being able to alter the obtained results. These substances are divided into five groups: auxins, cytokinins, gibberellins, abscisic acid, and ethylene (Fig. 2.8). Cells generally respond to combinations of at least two of these hormones, and tissue development is a function of the concentration ratio between them.
Among auxins, β-Indolylacetic Acid (IAA) is the only natural substance used; it can be favorably replaced by synthetic auxins like Indole-3-butyric acid (IBA), α-Naphthaleneacetic acid (NAA), and 2,4-Dichlorophenoxyacetic acid (2,4-D), which is effective due to its callus-inducing potency but toxic at high concentrations.
Cytokinins have different actions depending on the concentrations at which they are used, and their effectiveness varies according to the plant material (maintaining survival, stimulating divisions, cell differentiation). The most frequently used are kinetin (or 6-furfurylaminopurine) and Benzyladenine (BA = 6-Benzylaminopurine), which are obtained by synthesis, and zeatin and Isopentenyladenine (2iP), which are natural substances.

There are many gibberellins: Gibberellic Acid A3 (GA3) is the most commonly used. Alone, it is not very effective but exerts a synergistic effect with auxins and cytokinins; it is often used with auxin to accelerate the growth of already formed organs.
Finally, complex natural substances of poorly defined composition, such as yeast extract, casein hydrolysate, coconut milk, and various fruit juices, are generally added to the culture medium. Coconut milk - very commonly used - is a mixture of vitamins, amino acids, various carbohydrates, and growth regulators. In combination with 2,4-D, it has proven particularly effective for establishing tissue strains from recalcitrant materials.

Culture Chamber: The main physical factors of the climatic environment for in vitro cultures are: humidity, temperature, and light.

Humidity/

Generally, sealing the culture containers ensures sufficient humidity in the ambient atmosphere. It is therefore not necessary to provide devices for controlling humidity within the culture chamber itself.

Temperature.

The temperature of many culture chambers is constant, around 22 to 25°C. It can be higher for tropical plants (27° to 28°C) or lower for certain species (18° to 20°C).

Light /

Light Intensity

The lighting of culture chambers is generally provided by fluorescent tubes. The triggering of organogenesis phenomena (Rhizogenesis, Caulogenesis) is generally observed with light intensities of 2000 to 5000 Lux, sometimes lower.

Photoperiod

Long photoperiods equal to 16 hours are used to ensure the growth of calli or organogenesis.

Acclimatization

After the multiplication phase, the use of a culture medium with a different composition allowed the initiation of roots.

The plants, which were in sterile pots, are now transplanted into a natural environment: in a greenhouse, on