

Unit 2: Different Tissue Culture Techniques

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2.1 Tissue culture techniques: the culture types

Plant tissue culture is the aseptic culture of plant cells, tissues and organs on a defined nutrient medium under controlled conditions of light, temperature and humidity, exploiting the totipotency of Unit 1. Every technique in this unit is a variation on one set up: an explant, the starting piece of plant, is surface sterilised, placed on or in a sterile medium supplying water, mineral salts, a carbon source, vitamins and growth regulators, and incubated while the scientist steers what it becomes. The medium most widely used as the general base is the Murashige and Skoog medium; the steering is done mainly by the auxin and cytokinin balance of Section 1.1. The culture types should be known as a family, because examination questions move between them.

Callus culture is the culture of an undifferentiated, disorganised mass of dividing cells, usually induced on solid medium with growth regulators, and subcultured by transferring pieces to fresh medium. Cell suspension culture takes callus, preferably friable callus that breaks apart easily, into agitated liquid medium, giving a dispersed population of single cells and small aggregates that can be sampled, scaled and treated uniformly like a microbial culture. Organ culture maintains a defined organ, a root tip, a shoot tip, an embryo, in vitro with its organisation intact. Protoplast culture strips the cell wall enzymatically to give naked cells that can fuse and can take up DNA. Haploid culture, from anthers or microspores, gives plants with a single set of chromosomes, valuable in breeding. Meristem culture uses the tiny, usually virus free shoot apex to recover clean plants from infected stock. The comparison below collects the family, and the diagram after it places the types on one map.

Culture type	Starting material	Organisation	Main uses in this subject
Callus	Explant on solid medium	Undifferentiated mass	Starting point for most work, metabolite studies
Suspension	Friable callus in liquid	Dispersed cells and aggregates	Scale up, bioreactors, metabolite production
Organ (root, shoot)	Excised organ	Organised, differentiated	Hairy roots, shoot cultures, metabolite production
Protoplast	Wall free isolated cells	Single naked cells	Fusion, hybridisation, direct gene transfer
Meristem and shoot tip	Apical meristem	Organised apex	Virus free plants, micropropagation
Anther and microspore	Pollen bearing organs	Haploid regenerants	Breeding, homozygous lines

The plant culture types

UNDIFFERENTIATED	ORGANISED	MAIN USES
Callus on solid medium	Root and hairy root culture	Production of metabolites
Suspension in liquid	Shoot and multiple shoots	Micropropagation
Protoplasts, wall free	Meristem and shoot tip	Fusion and gene transfer
Starting point for selection	Embryo culture	Virus free stock
Metabolite study systems	Keeps the organ chemistry	Breeding and conservation

Choose the culture whose organisation matches the organ that makes the drug

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Figure: The culture types of Unit 2. The undifferentiated cultures serve production and study; the organised cultures serve propagation and product profiles that need differentiated tissue.

2.2 Organogenesis

Organogenesis is the formation of organs, shoots or roots, from cultured cells or tissues. It proceeds by two routes that must be distinguished. In direct organogenesis the organ forms straight from the explant tissue without an intervening callus phase, as when a shoot tip or nodal segment produces shoots. In indirect organogenesis the explant first forms callus, and organs are then induced from the callus by changing the growth regulator balance. The steering rule is the classical one of Unit 1: a cytokinin rich balance drives shoot bud formation, an auxin rich balance drives root formation, and a regenerating culture is commonly moved through the two in sequence, shoots first on a cytokinin led medium, then excised shoots rooted on an auxin led medium. Organogenesis is the engine of micropropagation in Section 2.9 and of regeneration after gene transfer in Section 2.11, because a transferred gene is useless until the transformed cell can be grown back into a plant. Its risk is the shadow of Section 2.5: the longer cells spend as callus in the indirect route, the more variation accumulates, so propagation work prefers direct routes and short callus phases, while variation seeking work deliberately uses the indirect route.

2.3 Embryogenesis

Embryogenesis in culture is somatic embryogenesis: the formation, from somatic (non reproductive) cells, of embryo like structures that pass through recognisable stages and can germinate into whole plants, having both a shoot and a root pole in one body, unlike an organogenic shoot which must be rooted separately. Somatic embryos arise directly from explant cells or indirectly from callus or suspension cells, typically after exposure to an auxin led induction medium followed by transfer to conditions that allow embryo maturation. Their teaching value is threefold. They are single cell in origin in the well run systems, so a plant regenerated from one embryo is a true clone of one cell, which matters for transformation and selection work. They are produced in very large numbers in liquid culture, which suits scale. And they are the raw material of synthetic seed in the next section: an embryo is exactly the unit a seed contains. Somatic embryogenesis also serves as a model system for studying development and gene regulation, connecting this technique back to Unit 1.

2.4 Synthetic seed

A synthetic seed, also called an artificial seed, is a somatic embryo, or in extended use another propagule such as a shoot bud, encapsulated in a protective coating that lets it be handled, stored and sown like a true seed. The standard construction is a bead of hydrogel, classically formed from sodium alginate gelled

with a calcium salt solution, containing the embryo together with nutrients and protectants in the gel matrix, which acts as an artificial endosperm and seed coat in one. The advantages claimed for the technology should be listed with their conditions. It gives clonal propagules of plants that do not set seed, set seed poorly, or do not breed true from seed, which covers many medicinal hybrids and sterile clones. It allows direct sowing of elite genotypes and easier exchange and transport of germplasm in a small, clean package. Its limits are equally standard: somatic embryos must be of high quality and synchronised, the beads protect only briefly, storage life is short compared with true seed, and conversion of bead to plant in the field remains less reliable than in sterile culture. Examination answers should present synthetic seed as embryogenesis plus packaging: the biology is Section 2.3, and the bead is an engineering answer to handling that biology.

2.5 Somaclonal variation

Somaclonal variation is the variation, genetic and epigenetic, that appears among plants regenerated from culture, and among cultured cell lines themselves. Its causes follow from the culture process: the stress of dedifferentiation and rapid division, growth regulator exposure, and prolonged time in culture disturb chromosome number and structure, activate normally silent elements, and alter the chemical marking of DNA, so that regenerated plants may differ from the parent in growth, form, or constituent profile. The syllabus copies print this topic as "monoclonal variation", a misprint recorded in the verification file; the tissue culture term, used here, is somaclonal variation. The phenomenon is double edged and must be judged by purpose. For micropropagation and for production cultures it is a defect: the whole promise of clonal work is fidelity, and variation is managed down by direct regeneration routes, short culture periods, and verification of the product. For improvement work it is a resource: culture acts as a variation generator and a sieve at once, because selection can be applied to millions of cells in a dish, for salt or disease resistance, or, closest to this subject, for high producing cell lines whose raised constituent content can then be stabilised and scaled in Unit 3. A good answer therefore never calls somaclonal variation simply good or bad; it states the aim of the work and judges the variation against it.

2.6 Protoplast fusion

A protoplast is a plant cell from which the wall has been removed, classically by digestion with cellulase and pectinase type enzymes in an osmoticum that stops the naked cell bursting. Freed of the wall, protoplasts of different plants can be fused, by chemical fusogens such as polyethylene glycol or by an electric pulse method, to give a hybrid cell carrying the nuclei and cytoplasms of both parents: somatic hybridisation. Its applications define its place in the syllabus. It crosses barriers that sexual crossing cannot: unrelated species, sterile partners and combinations where pollen or seed fail, so new combinations of traits, including constituent pathways, can be assembled. It transfers cytoplasmic traits, such as cytoplasmic male sterility and some resistances carried in chloroplasts or mitochondria, in ways breeding cannot. Asymmetric fusion and cybrid production refine the tool, keeping most of one genome with a sample of the other. The honest limits: regeneration from fused protoplasts is difficult in many species, hybrids may be sterile or unstable, and the technique is demanding. Protoplasts also serve gene transfer, since DNA crosses a naked membrane more readily than a walled one, which links this section to Section 2.11.

2.7 Hairy root cultures and their applications

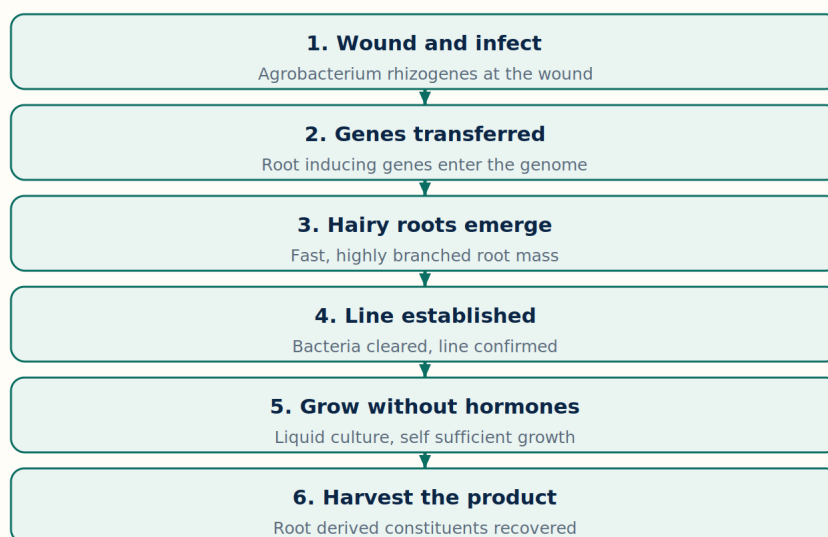
Hairy root culture begins with a natural genetic engineering event. The soil bacterium *Agrobacterium rhizogenes* infects wounded plant tissue and transfers part of its plasmid into the plant genome; the transferred genes redirect the tissue to produce masses of fast growing, highly branched roots, the hairy root phenotype, and to synthesise opine compounds the bacterium uses. In culture, roots induced this way are excised and grown in liquid medium without growth regulators, because the transferred genes make

them self sufficient in growth control. Their properties read like a specification written for secondary metabolite production, which is why the syllabus gives them prominence. They grow fast for plant tissue, often faster than suspension cells. They are genetically and biochemically stable over long periods, where suspensions drift. They are differentiated organs, so they keep the root specific pathways that callus switches off, answering the regulation problem of Section 1.7. And they are hormone independent, simplifying medium and scale up. Their applications follow: production of root derived medicinal constituents, study of root metabolism and biosynthesis, biotransformation, and, with foreign genes added through the same bacterial route, production of improved or novel profiles. Scale up needs bioreactors adapted to a tangled root mass rather than free cells, the design problem of Unit 4. The path below states the production sequence.

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Wound the plant tissue and infect with Agrobacterium rhizogenes
|
Transferred genes induce hairy roots at the wound site
|
Excise roots; clear the bacteria; confirm the transformed line
|
Grow the root line in liquid medium, no growth regulators
|
Select and maintain the stable, high producing line
|
Harvest roots or medium for the root derived constituents
  
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The hairy root culture path



Differentiated and stable: the root keeps the chemistry callus loses

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Figure: The hairy root path. The bacterium does the gene transfer once; the resulting root line then grows fast, without added hormones, and keeps the root chemistry that undifferentiated cultures lose.

2.8 Multiple shoot cultures and their applications

A multiple shoot culture is an organised shoot culture in which a shoot tip or nodal explant, under a cytokinin led medium, produces not one lengthening shoot but a multiplying cluster of shoots, each of which can be separated and subcultured to repeat the cycle. Shoot cultures sit at the differentiated end of the culture family: like hairy roots they keep organised tissue and therefore keep constituent pathways that belong to shoots and leaves, and they are genetically steadier than callus. Their applications are twofold. In propagation they are the working system of micropropagation, Section 2.9, because each cycle multiplies plant numbers geometrically from verified mother stock. In production they serve as a culture system for

shoot and leaf derived constituents, grown in liquid or temporary immersion systems at scale, with the differentiated profile a suspension cannot offer. Together with hairy roots they teach the unit principle: choose the culture whose organisation matches the organ that makes the drug, root cultures for root drugs, shoot cultures for aerial part drugs, and undifferentiated cells where the pathway survives dedifferentiation or where cells alone are the experimental object.

2.9 Micropropagation of medicinal and aromatic plants

Micropropagation is the rapid clonal multiplication of plants in vitro, and for medicinal and aromatic plants it answers concrete supply problems: elite high yielding mother plants can be multiplied without limit of season; species that are seed shy, sterile, slow or over collected from the wild can be raised in numbers for cultivation instead of collection; and planting material can be delivered disease free, including virus free where meristem culture is used to clean infected stock. The standard stages should be known as a numbered sequence, because examination questions ask for the stages by name. Stage one, establishment: a suitable explant, commonly a shoot tip or nodal segment from a verified mother plant, is surface sterilised and established in sterile culture. Stage two, multiplication: shoots are multiplied, usually as multiple shoot clusters of Section 2.8, through repeated subculture cycles. Stage three, rooting: separated shoots are rooted, on an auxin led medium in vitro or, in some systems, ex vitro during acclimatisation. Stage four, acclimatisation or hardening: plantlets, raised on sugar in humid sterile air, are weaned to soil, normal humidity and photosynthesis in graded steps, the stage at which losses are highest if it is rushed. Quality control runs across the stages: mother stock is authenticated and indexed for disease, and clonal fidelity is respected by favouring direct shoot routes over callus routes, per Section 2.5. The stages are drawn below.

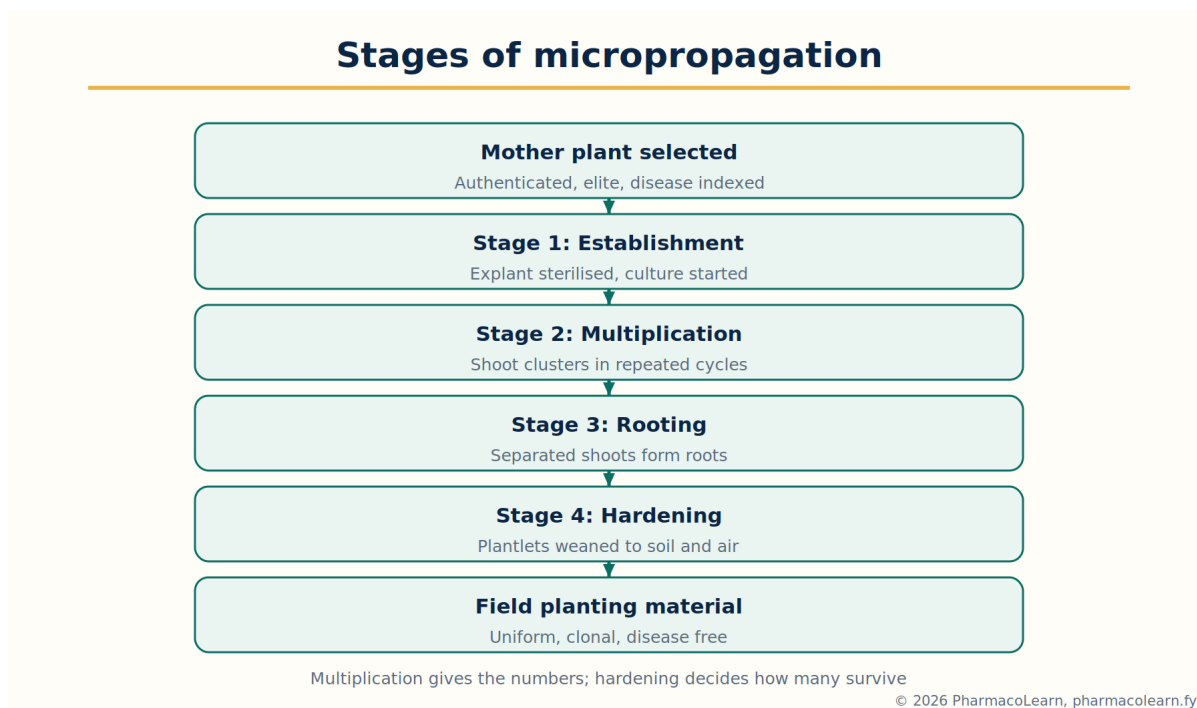


Figure: The four stages of micropropagation. Multiplication gives the numbers; acclimatisation decides how many of those numbers survive as plants.

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Select and authenticate the elite mother plant; index for disease
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Stage 1: surface sterilise the explant and establish it in culture
|
Stage 2: multiply shoots in repeated subculture cycles
|
Stage 3: separate shoots and root them
|
Stage 4: harden the plantlets and transfer them to soil
|
Field planting material: uniform, clonal and disease free
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2.10 Sterilization methods involved in tissue culture

Sterility is the working condition of everything in this unit, because plant cells grow slowly and rich media grow microbes fast: one contaminated vessel is lost, and contamination in a production culture is a batch failure. Sterilization methods divide by what is being sterilised, and the division is the examinable structure. Instruments and vessels are sterilised by dry heat in a hot air oven or by moist heat in an autoclave, steam under pressure, the workhorse method for media, water and heat stable equipment. Culture media are autoclaved after preparation, with heat sensitive components, some growth regulators, vitamins and antibiotics, instead sterilised by membrane filtration and added aseptically to the cooled sterile base medium. Work surfaces and transfer areas are disinfected with alcohol and by ultraviolet light in laminar airflow cabinets, which protect the work with a continuous flow of filtered air; the air itself is sterilised by filtration. The explant, which arrives carrying a microbial load on its surface, is surface sterilised, classically by a sequence of detergent washing, alcohol, and a chemical sterilant such as sodium hypochlorite or mercuric chloride solution, followed by repeated rinses in sterile water, timed to kill surface microbes without killing the tissue. The operator is part of the system: aseptic technique, flaming of instrument tips, minimal exposure of open vessels, and quarantine and inspection of new cultures complete the method. Each method has its failure mode, and examination answers gain by naming one: autoclaving degrades heat labile ingredients, surface sterilants damage delicate explants at excess exposure, and filtration protects only what passes through it sterile technique, which is why the methods are used in combination, not alone.

2.11 Gene transfer in plants and their applications

Gene transfer is the delivery of chosen DNA into plant cells followed by regeneration of plants carrying and expressing it, the applied summit of Unit 1 and the entry to transgenesis in Unit 4. The methods divide into indirect and direct. The indirect, biological method uses *Agrobacterium tumefaciens*: the disarmed tumour inducing plasmid, its disease genes removed, carries the gene of interest between its transfer DNA borders, the bacterium transfers that segment into the plant cell, and it integrates into the plant genome. It is efficient, tends to give clean, low copy insertions, and works best in dicotyledonous plants, though its range has been widened by technique. Direct methods deliver DNA physically or chemically: particle bombardment, the gene gun, shoots microscopic particles coated with DNA into cells and works across species and into chloroplasts; electroporation pulses protoplasts so DNA enters through temporary pores; microinjection places DNA directly; and polyethylene glycol mediated uptake serves protoplast systems. Whichever the route, the workflow after delivery is common: the transferred DNA carries a selectable marker, transformed cells are selected, shoots are regenerated by organogenesis or embryogenesis, and the resulting plants are verified for the presence, copy state and expression of the gene, the verification methods of Unit 4. Applications in medicinal plants should be stated in the syllabus terms: raising the yield of a phytopharmaceutical by adding or strengthening pathway genes, suppressing a competing branch or an unwanted constituent by silencing, conferring disease and stress resistance that stabilises field yield,

and creating production systems, including hairy roots carrying added genes, that make what the unmodified plant cannot. The comparison of the two biological and physical leads is tabulated below.

Feature	Agrobacterium mediated transfer	Particle bombardment
Delivery agent	Living bacterium with a disarmed plasmid	DNA coated microscopic particles
Insertion pattern	Usually low copy, defined borders	Often multiple copies, random sites
Host range	Best in dicots, widened by technique	Very wide, including monocots
Special strength	Clean events, large DNA segments	Chloroplast transformation, no host dependence
Main limits	Host range and bacterial clearing	Copy number control, tissue damage

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