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Tissue Culture Technologies and Their Applications

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Summary

Over the last century, plant tissue culture (PTC, in vitro technology) has evolved into a highly sophisticated biotechnological tool not only for understanding biochemical processes within the plant, but also in applied plant breeding, conservation and propagation. It is now the foundation in advanced biotechnologies. The most widespread application is micropropagation for producing clonal plant stocks with the market expected to grow to US$ 2.1 billion by 2030. Micropropagation is widely used in clonal propagation in horticulture, floriculture and forestry. PTC is also used in eradicating viral and bacterial diseases infecting clonal plant material to produce high-health planting material for agriculture, horticulture and forestry. In vitro technologies allow combining different methods such as meristem culture, thermotherapy, chemotherapy, electrotherapy and cryotherapy within one experiment to eradicate multiple diseases and/or to eradicate aggressive pathogens that cannot be eradicated using a single therapy. Also, in vitro technologies have a central role in the development and deployment of new cultivars to the industry

much faster and efficiently than traditional field-based plant breeding methods. This application encompasses an array of technologies to produce crop cultivars or even new man-made species with traits of interest. The approaches to cultivar improvement using PTC techniques include the induction of mutations and selection of desirable mutants with improved traits and in developing genetically modified crops using traditional transformation methods as well as gene editing techniques. PTC is the preferred pathway for developing interspecific and intergeneric hybrids that cannot be produced by hybridization in vivo due to various incompatibility issues. Ploidy manipulation, production of doubled haploids for hybrid development, increasing the proportion of hybrid seeds in apomictic species are some other applications in crop improvement. Because our plant genetic resources are not safe in the field due to climate change resulting in vagaries of weather as well as pests and diseases, ex situ conservation is becoming increasingly important. Again, PTC takes a central role in ex situ conservation, whether as tissue culture repositories or as cryopreserved collections. Finally, cell cultures are used in producing biopharmaceuticals, food ingredients, cosmetics, flavours, dietary supplements, fragrances, and biostimulants.

INTRODUCTION

Plant tissue culture (PTC) also called in vitro culture constitutes a critical component in plant biotechnology and refers to the culture of plant cells, tissues or organs on a nutrient medium under aseptic (sterile) conditions. Although the theoretical foundation of this technology was laid in early 1900s, considerable progress in practice was achieved after the discovery of the hormonal control of cell proliferation and organogenesis in vitro in the 1950s (Carra et al. 2024). Plant cell technology is now seen as the breakthrough technology that can help to meet the challenge in the next phase of plant breeding after the yield increases in Green Revolution have plateaued in the last two decades. Micropropagation – efficient mass propagation of clonal plants through tissue culture is the largest commercial application. Other plant biotechnologies that aid in developing new varieties and individual traits within existing plant varieties include cell and tissue manipulation, marker-assisted selection, transgenic technologies, genomics, and molecular breeding. Cell and tissue culture technologies provide a range of applications in the creation, conservation, and utilization of the genetic variability in crops, such as in vitro pollination and embryo rescue for distant hybridization, the production of haploids and doubled haploids, polyploid breeding, in vitro mutagenesis and selection of somaclones, in vitro selection, germplasm preservation (in vitro for medium-term and cryopreservation for long-term), protoplast fusion for producing somatic hybrids, and gene manipulation for producing transgenic crops or the newly emerging techniques that allow for the generation of gene-edited plants (Pathirana and Carimi, 2024).

Additionally, plant-based drugs have been used in all ancient civilisations and continue to be used in modern times. The market has been growing steadily for plant-based drugs and may reach USD 50

billion during the next five years (Bapat et al. 2023). As only about 1 % of the plant species are used in drugs, they are overexploited and threatened in their natural environments. Furthermore, extraction from plants is inefficient and laborious. Plant cell cultures present an alternate sustainable pathway to produce drugs. This approach has the advantage of homogeneity of cell suspensions, scalability, fast growth with no quiescence, less space requirement, and ease of handling and hence offers attractive options for the production of not only drugs but other bioactive secondary metabolites in the food and cosmetic industries.

During the growth in vitro, we control temperature, humidity, light intensity, light cycle and manipulate media components. The cultures are either maintained in highly controlled growth chambers or culture rooms in liquid or solid media. In this review, covering different applications of this technology, examples from author's own work will be presented.

Aseptic Culture

We use both physical and chemical methods to achieve sterile conditions. Equipment that uses physical methods include autoclaves (uses heat and pressure), laminar flow cabinets (filters including High Efficiency Particulate Air - HEPA filters), bead sterilisers or Bunsen burners (heat) and filter sterilising units used to sterilise media containing compounds that can get destroyed by heat. Chemical methods include surface sterilization of laminar hood, gloves and explants (part of plants introduced into culture) using ethanol or other sterilants, antibiotics used to prevent growth of bacteria in media etc.

In tissue culture we grow plant cells or tissues in artificial culture media, whose proportion and concentration of elements vary depending on the objective and plant species. We supply major and minor elements required for growth and development in the form of inorganic salts, for example, magnesium sulphate to supply magnesium and sulphur. There are many different media available commercially for different species and we need to test different media for less studied species.

Plants or cells growing in artificial media have none or very little photosynthesis. Even if leaves are present, their stomata are always open and non-functional. So, we need to supply carbon through the media as the plants in culture can't use CO_2 from the atmosphere of the culture vessel to fix carbon. The main carbon source is sucrose, but often we use other sugars such as maltose, fructose, glucose etc. Plant growth regulators are an important component in culture media, and we can drive the growth and development to our requirements by changing the proportions of auxins, cytokinins, abscisic acid, gibberellic acid etc. Additionally, we have to give support to plants by making media solid using gelling agents such as agar or gellan gum, unless we use liquid cultures.

Why Are Plant Cells and Tissues Amenable to Tissue Culture?

The plants grow and develop throughout their life and for this they have dividing meristematic cells that we can always use to initiate cultures (**Fig. 1A**). In addition to the meristematic tissue in apices and axillary buds, vascular cambium (a layer of cells between primary xylem and phloem) and cork cambium (a major portion of the bark of woody plants) also have meristematic tissue.

Dividing meristematic cells are easier to manipulate than differentiated cells. However, unlike most of animal cells, plant cells have totipotency and pluripotency. Totipotency is the ability of any living plant cell to revert to a meristematic (dividing) state, given the right environmental cues and then produce a complete plant. Pluripotency is the ability to regenerate an organ or tissue from a living cell. Thus, a differentiated plant cell can return to a meristematic state and then differentiate to produce tissues and organs that have specialised cells. This latter process is called de-differentiation.

For an example, we can get an anther to produce a haploid plant (plant with a single set of chromosomes) directly using low auxin and high cytokinin in media (**Fig. 1B**) and this is an example of direct regeneration. When we produce plants from cells through an intervening callus phase by manipulating plant growth regulators (**Fig, 1C and D**), the process is called indirect organogenesis This is generally a two-step process. For clonal propagation direct organogenesis is preferred because when going through a callus phase, there are chances of mutation induction, resulting in somaclonal variation.

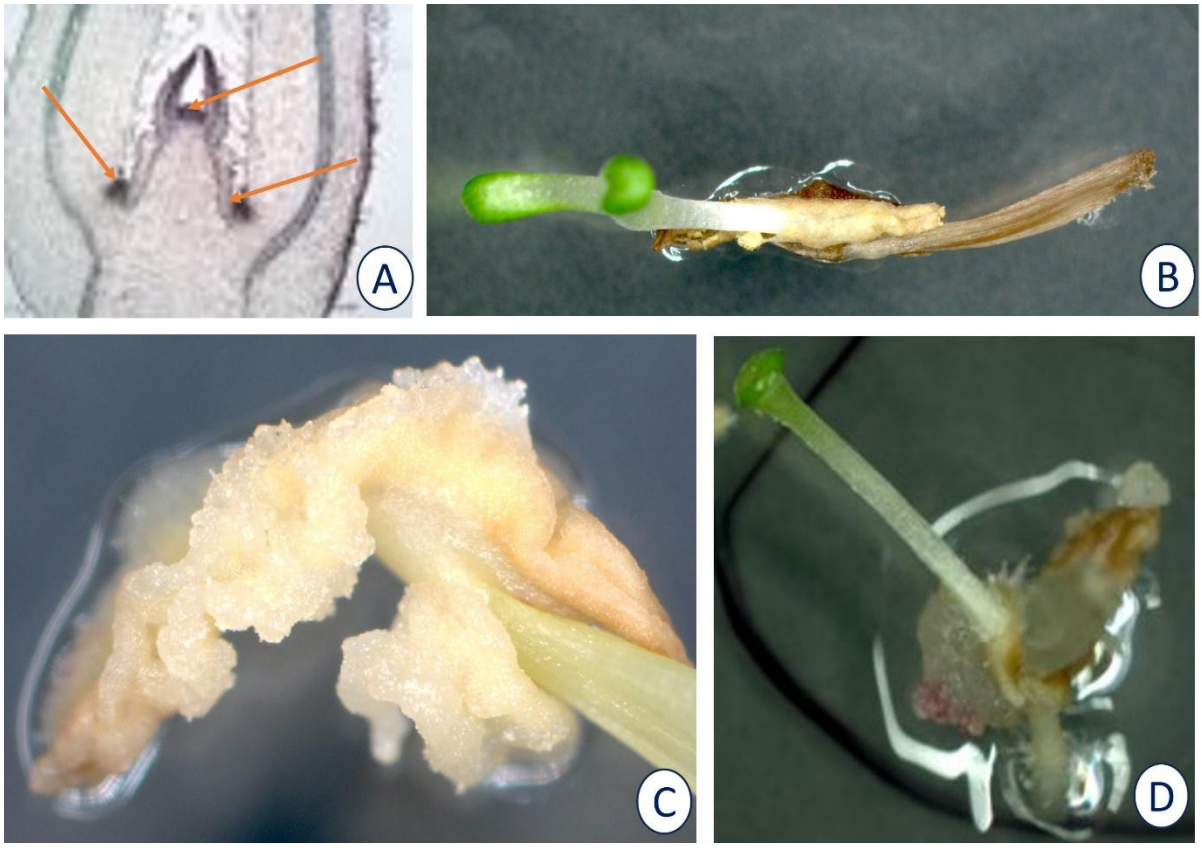

Figure 1. **A**) As plants continue to grow during their life, meristematic tissue (red arrows) is present for easy manipulation – meristems of shoot tip and axillary nodes in *Hibiscus rosa-sinensis;* **B**) Direct regeneration of a haploid plant from an anther of *Gentiana triflora;* **D**) Indirect organogenesis in the same species through an intervening callus phase **C**). (From Pathirana et al. 2011. Plant Cell Reports, 30, 1055–1065).

The ratio of auxins to cytokinins invariably determines structural organisation in vitro, a concept published as early as 1957 (Skoog and Miller, 1957). Since then, we have learnt much more about organogenesis in plants.

The differentiation of cells into organs or undifferentiated callus is guided by this ratio as well as concentrations and types of growth regulators used in media. This is illustrated in **Fig. 2.**

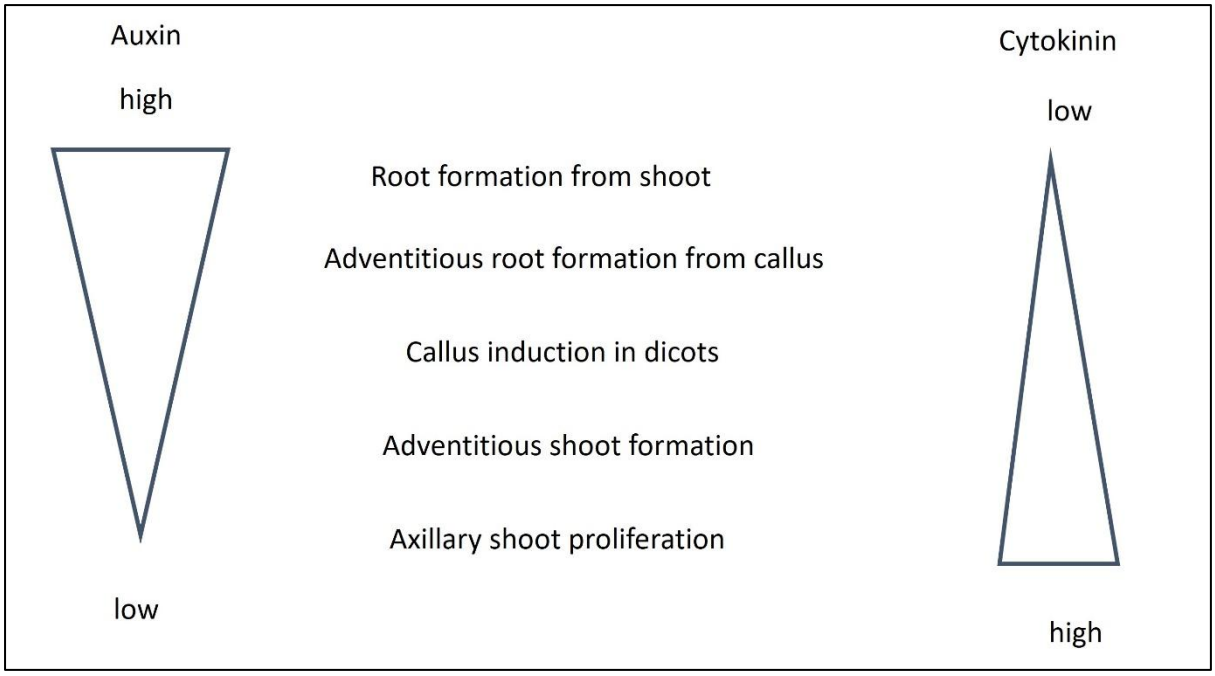

Figure 2. Determination of structural organisation of plants in vitro as affected by auxin/cytokinin ratio.

Clonal Propagation Through Tissue Culture – Micropropagation

The market

Clonal propagation in tissue culture, technically called micropropagation is the most widely used and widely known application among the many in vitro technologies. According to P&S Intelligence (2024), the global micropropagation market stood at US$ 1.28 billion in 2022 and is expected to grow to US$ 2.1 billion by 2030. Due to the growing interest in indoor plants, the largest driver of this growth will be the increased demand for orchids and other indoor plants. Additionally, the wide use of ornamental plants in decoration of commercial facilities such as hotels, airports, restaurants and office spaces add to this demand. The largest micropropagation facilities can be found in China, India, Thailand and South American countries where labor is relatively inexpensive as this is a labor-intensive process and automation is difficult and costly. Micropropagation is also used in large-scale propagation of banana, pineapples, potato, *Pinus radiata* (and many forest species) and many floricultural and horticultural crops, as well as for medicinal plants including medicinal cannabis (with high tetrahydrocannabinol content, which is the psychoactive chemical), with many countries legalizing its medicinal use.

The growth of the micropropagation industry is boosted by the demand exceeding the current production capacity. It is estimated that the global demand for healthy, clean and uniform planting material is about 16 trillion equating to US$ 4 trillion, whereas only 1.5 - 2 billion plants are produced through micropropagation (Wei et al. 1990). Undoubtedly micropropagation is the leading technology that can deliver quality plants round the year. Despite the tissue culture production facilities are located mainly in developing countries, major companies have already installed acclimation facilities for tissue cultured plants they buy, thus reducing costs.

Approaches to Micropropagation

Clonal multiplication in tissue culture is achieved through different pathways, and the main approaches are direct organogenesis as seen in our recent experiments with *Corymbia* spp. (**Fig. 3 A and B**) and kiwifruit (Saeiahagh et al. 2019), indirect organogenesis via a callus phase (**Fig, 1 C and D**), through somatic embryogenesis (SE) (**Fig. 3 C and D**) or using microtubers **(Fig. 4 and 5)**.

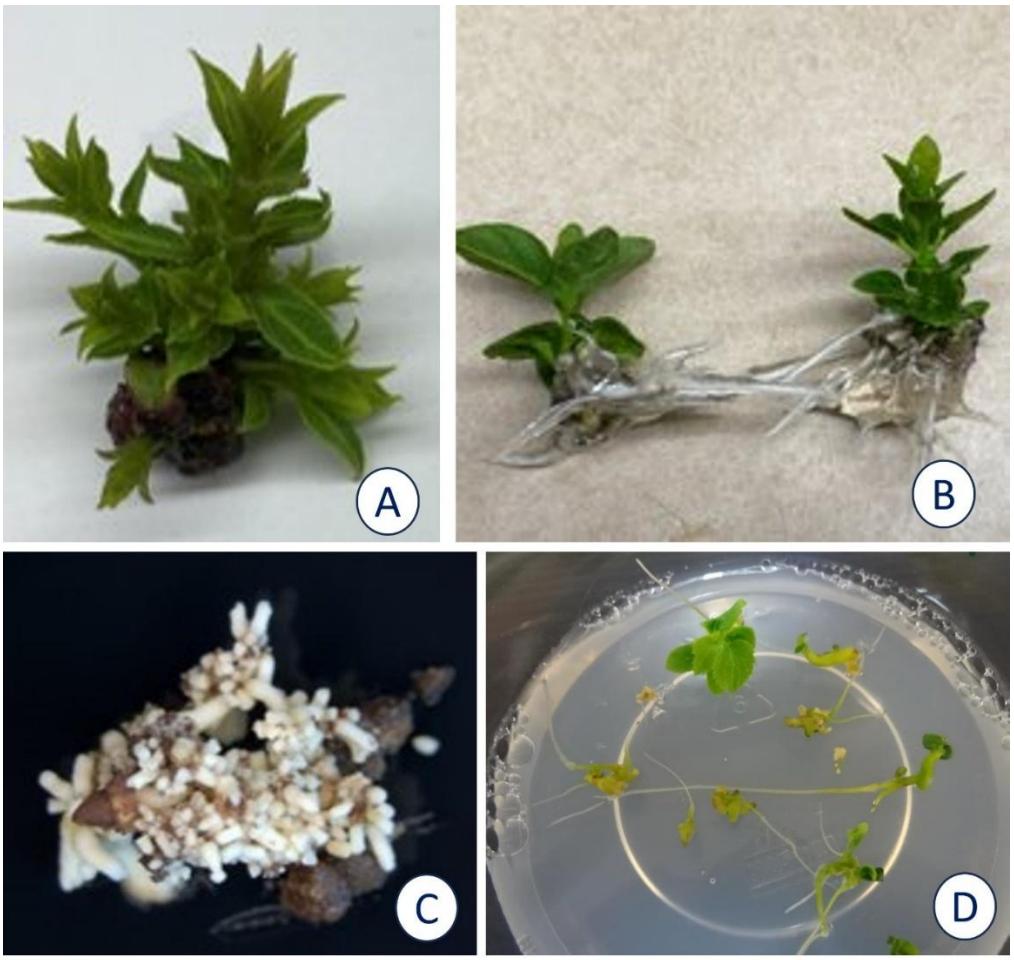

Figure 3. *Corymbia* micropropagation through direct organogenesis **A**) Multiple shoot formation from a shoot tip, **B**) rooting of separated microshoots. Production of somatic embryos of kiwifruit **C**) and their germination **D**). (C and D from Pathirana et al. 2016. Acta Hortic. 1127:17-222).

Organogenesis

Direct organogenesis involves the use of plant growth regulators, particularly cytokinins, to induce growth of existing shoot primordia in the shoot apex or in axillary buds, resulting in multiple shoots that can be separated and further multiplied. For indirect organogenesis, we first produce a callus tissue with undifferentiated cells from which large numbers of plantlets can be generated.

Micropropagation through somatic embryogenesis

Somatic embryogenesis involves producing bipolar structures resembling zygotic embryos from non-reproductive, somatic tissue such as leaves (**Fig. 3 C**), petioles, cotyledons or even roots, without a vascular connection with original tissue. These then go through a maturation process and can be germinated, usually by increasing the concentration of auxins in growth media or by culturing in hormone-free media (Carra et al. 2019) (**Fig. 3 B, 3 D**).

Somatic embryos differ from sexually produced zygotic embryos by the absence of a seed coat, although they have the embryonic axis with radicle and plumule including the cotyledons. The somatic embryos, unlike zygotic embryos, do not have tolerance to desiccation. Therefore, they cannot be handled like dry seeds. Zygotic seeds of many species can be dried to 6-8 % moisture content for storage and used for planting, retaining their viability at that low moisture content. However, somatic embryos cannot be dried. Studying the process of acquisition of desiccation tolerance in fertilized ovules of alfalfa (*Medicago sativa*), scientists at Guelph University in Canada managed to mimic this process in somatic embryos. Adding abscisic acid in correct concentration to media at the cotyledonary stage of embryo development was crucial (Senaratna et al. 1989). Thus, the possibility of imparting desiccation tolerance to somatic embryos exists, but the challenge is synchronizing the process of embryo development (to apply abscisic acid at cotyledonary stage) in tissue culture systems. Therefore, most of the somatic embryo-based systems of micropropagation rely on using alginate beads to encapsulate the embryos for short-term storge or directly germinating the embryos to produce plants. Synchronized production of somatic embryos in suspension cultures, maturing in solid media followed by drying and encapsulating in an inert substance such as clay with incorporation of more nutrients has the potential to revolutionize the clonal propagation industry.

Microtubers for propagation

The other popular method used in micropropagation is microtuber production in the case of tuber crops like potato (**Fig. 4**), ulluco (**Fig. 5**), yams etc. In major production areas of potato, microtubers are the preferred option for ‘seed potato’ production.

Microtubers can be produced from single nodal cuttings in media with high sucrose (6 – 9 %) supplemented with cytokinins, mainly kinetin. The cultures are maintained in the dark for tuber production. Potato and ulluco (*Ullucus tuberoses* - a South American tuber crop) microtuber production in our work in New Zealand is presented in **Figs. 4 and 5**, respectively. In potato, we wanted to understand if the microtubers can be used to screen potato germplasm for cold-induced sweetening (CIS), a problem encountered in processing potato (Pathirana et al. 2008). A rise in hex-

ose sugar levels during cold storage of potato tubers results in a brown, bitter tasting and unmarketable product. This is caused by invertase enzyme activity in cold storage and this activity and hexose sugar production is different in different potato cultivars. Comparison of field-grown potatoes and microtubers showed that there is a good correlation in CIS and hence microtubers are a good model for selecting potato genotypes for processing (Pathirana et al. 2008). In ulluco, our objective was to produce diverse mutants to adapt this new crop to New Zealand condition (Pathirana et al. 2011b). In vitro mutagenesis is an efficient way to produce large mutant populations and the ability of ulluco to produce microtubers is an advantage for easy transfer to field conditions for screening the mutant populations. Our work resulted in mutants with traits such as early maturity, altered tuber morphology and colour, less geosmin (a component in ulluco tubers that imparts an 'earthy' taste) and altered leaf colour.

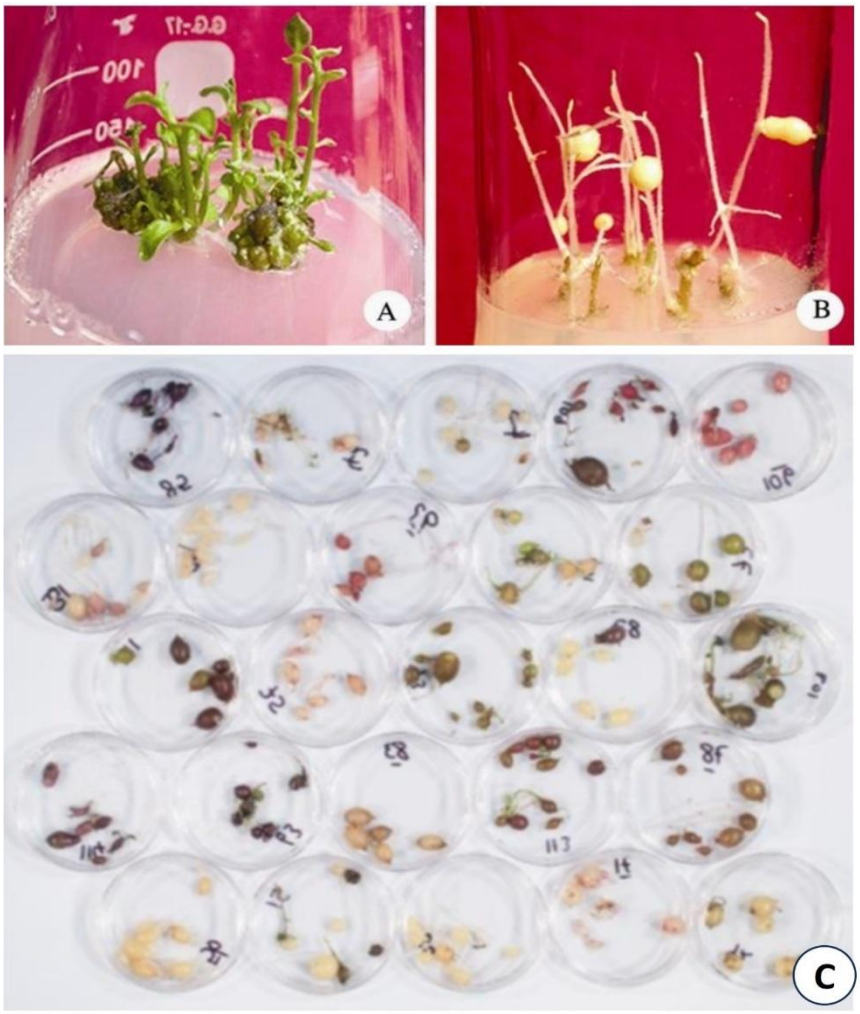

Figure 4. Stages of microtuber production in potato. **A**) Tissue cultured plantlets in culture media for microtuber production. **B**) Microtubers produced within 3-5 months. **C**) Morphological diversity of microtubers from different cultivars - they resemble their counterpart field-grown tubers not only in colour and shape, but also in cold-induced sweetening (From Pathirana et al. 2008. Post Harvest Biol Technol, 49; 180-184).

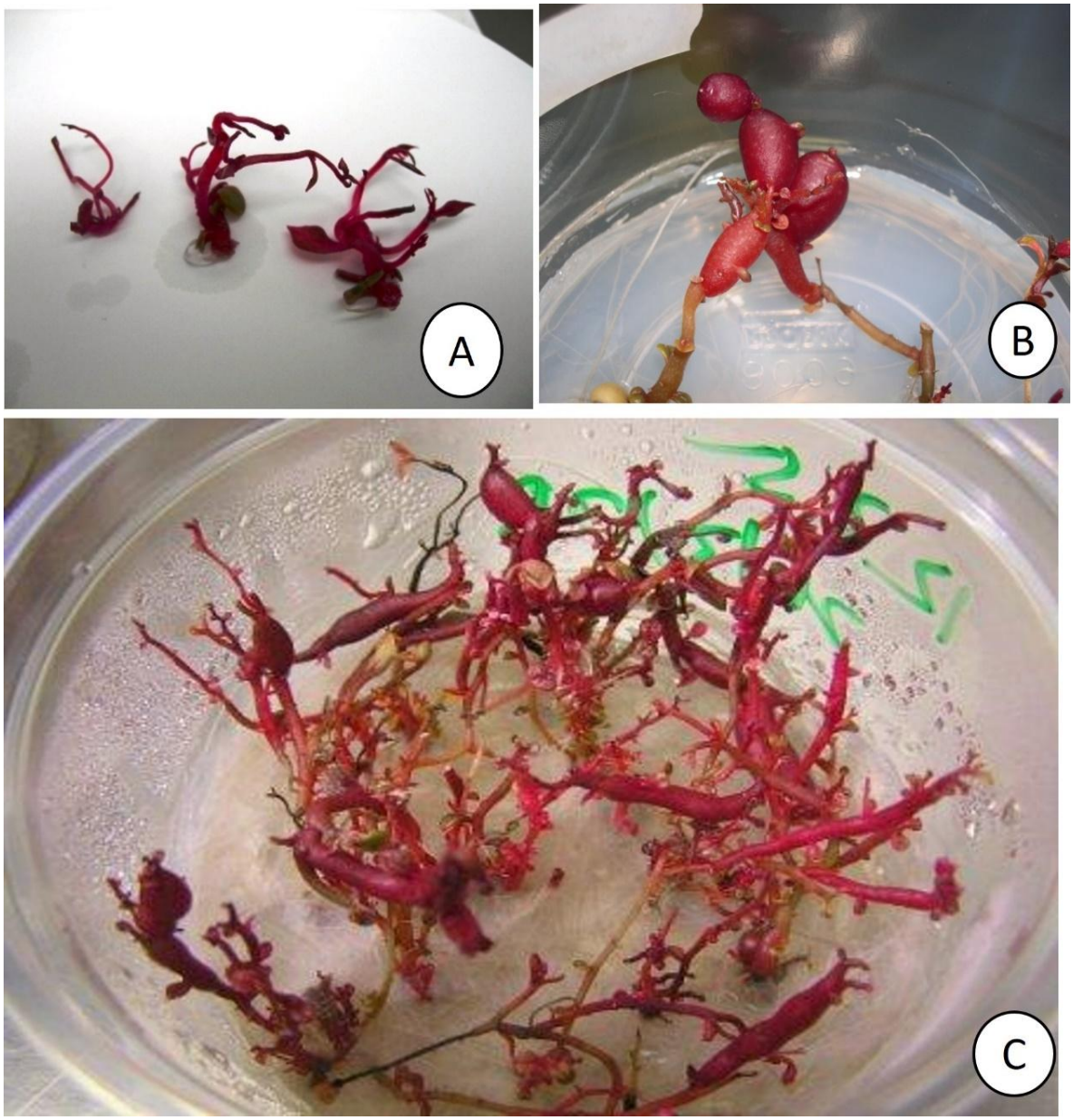

Figure 5. Production of microtubers of ulluco (*Ullucus tuberoses*) for a mutation breeding program. **A**) Microshoots in culture media with high sucrose and cytokinin for microtuber production, **B**) and **C**) Microtubers produced within three months.

Stages of micropropagation

Growing mother plants in a disease- and stress-free environment is important to initiate cultures for all the methods. Optimally they should be sourced from a greenhouse.

The stages of micropropagation through direct organogenesis include initiation of cultures on proliferation media, multiplication (Fig. 3 A), rooting of microshoots (Fig. 3 B), exflasking and acclimation. For micropropagation through direct organogenesis, only those parts of a plant that have meristematic tissue with shoot initials are used and cultured in media supplemented with higher proportion of cytokinins than auxins (Fig. 2). This proportion is reversed for rooting of microshoots. Sometimes the cytokinin used has an influence over subsequent rooting. Recently we have shown that the use of

meta-Topolin (*m*T - a natural cytokinin first found in poplar – *topola* is the Polish word for poplar) in place of benzylamino purine (BAP) or zeatin helps rooting of a 'difficult-to-root' red kiwifruit (Saeiahagh et al. 2019). Lower hyperhydricity (an anomaly often found in tissue cultures) and low residual effect of *m*T are some reasons for this success. To enhance rooting, supplementation of media with activated carbon, pulse treatment of microshoots with high concentration of auxins (1-50 g/L; 2 – 30 min), incubation for several days in the dark, reduced mineral and sucrose content in media as well as photoautotrophic micropropagation systems (systems mimicking natural growth conditions with higher light intensities, no sucrose supplementation and often using liquid cultures) have been used, and for each species, the methods need to be optimized. Some species like blueberry can be rooted in the greenhouse, without a rooting phase in tissue culture.

After the rooting phase, the plantlets are taken out from agar media, washed and transferred to specialized potting mixes. These potting mixes are often autoclaved to reduce the infection of tender and vulnerable plantlets in the early stages of acclimation.

Micropropagation through somatic embryogenesis has the potential for scaling up and automation of production of clonal plants. It is amenable to suspension cultures as shown in our work with kiwifruit (Pathirana et al. 2016) and grapevine (Pathirana and Carimi 2023). If the process can be well tested and established, mass production for nursery industry is a possibility as in the case of *Pinus radiata*, with scientists introducing machine learning algorithms to predict and select somatic embryos with high rate of germination success (Scion 2024). Our recent attempt for scaling up grapevine somatic embryo production (in this case for mutation induction for breeding purposes) is given in **Fig. 6** showing different stages and steps, starting with the establishment of cell cultures with proembryogenic masses. The proembryos then go through globular, heart, torpedo and cotyledonary stages in dicots. Abscisic acid is generally used for maturation, and they can be germinated on solid media (**Fig. 6**). Another advantage of somatic embryos is that they are easy to cryopreserve for long-term storage, a huge advantage for in vitro breeding and conservation as we have shown for kiwifruit (Pathirana et al. 2016) and for an endangered grapevine species (Carimi et al. 2016).

In conclusion, tissue culture has immense potential for mass production of clonal species in horticulture, floriculture and forestry and for industrial and medicinal crops species as well as for conservation.

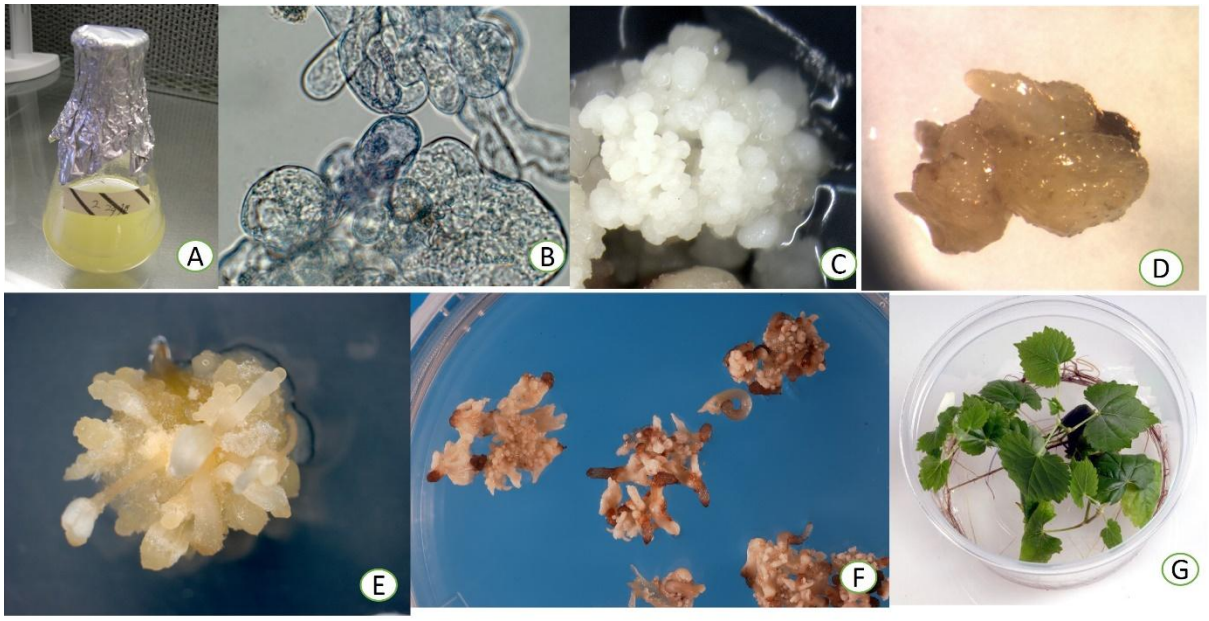

Figure 6. Somatic embryogenesis in grapevine. **A**) Embryogenic cultures in liquid media. **B**) Cells of embryogenic cultures under microscope (x20). **C**) Globular embryo formation. **D**) and **E**) Torpedo shaped embryos. **F**) Fully formed somatic embryos. **G**) Germinated somatic embryos.

Producing High-Health Plants Through Tissue Culture

Another application of PTC is the development and deployment of disease-free high-health plants for agriculture, horticulture and forestry. Often, we deal with clonally propagated crops and most of these are perennials. The vegetative propagation and exchange of budwood among regions and countries contribute to the spread of pathogens. This is true not only for fruit crops but also for root and tuber crops such as potato, cassava, yam and sweetpotato and for many ornamental crops such as orchids, anthuriums as well. The practice of vegetative propagation contributes to the spread of pathogens. Plant pathogens cause significant economic losses, for example in grapevine, fanleaf disease caused by a nepovirus (Grapevine fanleaf virus - GVFV) causes economic losses amounting to US$ 16,600 per ha, and in France where about 2/3rd of the vineyards is affected, it has an economic impact of at least US $1.5 billion per year. Another main viral disease affecting vineyards around the world is Grapevine leafroll-associated virus 3 (GLRaV 3) causing leafroll disease. It is estimated to cause losses from US $25,000 to US $ 226,000 per ha over a 25-year vineyard lifespan depending on the location and cultivar (Fuchs and Lemaire 2017).

In Australia, there are many virus diseases causing millions of dollar losses to the respective industries such as *Banana bunchy top virus* in banana – its exclusion will avoid AUD 15.9 - 27.0 million in annual losses for the banana industry (Cook et al. 2012), leafroll disease in grapevine – even more than 50% yield loss including reduced quality (Nicholas 2006), *Potato virus Y* in potato, *Strawberry mild yellow edge virus* in strawberry, *Cassava mosaic virus* in cassava, sweet potato virus disease in sweet potato, *Blueberry scorch virus* in blueberry

etc. In addition to transmission by the vectors, these diseases are transmitted through infected planting materials and cuttings. Furthermore, several undetected viruses and viroids keep reducing yield and quality of Australian crops such as *Potato virus X*, earlier thought to be due to 'degeneration' of cultivars. Therefore, establishment of orchards of horticultural crops and seed production of potato free of damaging diseases is a key control measure. Towards this many countries have sanitary selection programmes and certification of clonal stock. In Australia, potato seed is certified free from *Potato virus Y* by the Australian Seed Potato Certification Authority and grapevine clonal stock needs to be certified free from several viruses. However, once the stock is infected, it is important to have robust methods to eliminate the infecting viruses.

In general, the term eradication is used to describe the process of destroying all infected plant material after an incursion of a new disease. An excellent example comes from Australia in eradicating citrus canker, a disease caused by *Xanthomonas* bacteria. There have been several outbreaks; 1912, 1991, 1993 (in NT), 2018 (NT + WA), 1984 and 2004 (QLD) that were successfully eradicated. While strict quarantine, sourcing plant material from disease-free fields/orchards, inspection etc. are important, often we encounter situations where the need for eradicating infecting microorganisms from planting stock for reuse.

Traditional Practices Used to Eradicate Pathogens from Clonal Stock

Heat therapy is a traditional method often used for this and is successful in eradicating some pathogens from planting material. It consists of keeping plants, or a part of them, at temperatures between 35°C and 54°C, within the physiological tolerance limits of each plant species, for a predetermined period. The selected temperature should represent the best compromise between virus degradation and plant survival. Advantage is that the threshold of thermal sensitivity of some viruses is lower than that of plant cells and that the damage caused to plant tissues by the thermal stress can more easily be reversed than viral damage. Some examples of heat therapy for disease eradication from planting material include mint (*Mentha* sp.) rhizomes infected with mint rust (*Puccinia menthae*). The fungus can be eradicated by immersing in water at 44°C for 10 minutes and then transferring to cold water. This is effective for mint rust existing in the form of urediniospores. Hot water treatment (50°C for 2 h) is also used to control ratoon stunt bacterium (*Leifsonia xyli* subsp. *xyli*) in sugarcane planting material (setts) as well as to control nematode infections in bulb crops. Some seed borne diseases can also be controlled by hot water treatment (e.g. some fungal pathogens in wheat seeds and leaf spot of brassicas caused by *Alternaria brassicae* and black spot caused by *A. brassicicola*). Traditionally heat therapy has been used to reduce the viral load, but some viruses are heat stable. Although heat therapy is useful in reducing the incidence of virus diseases, when used alone it is often inadequate for clean stock certification programs.

Tissue Culture-Based Methods for Eradicating Diseases Infecting Vegetative Plant Material

PTC plays a significant role in eradication of microbial infections in clonal crops as different therapies can be applied alone or in combination when a single therapy is not

effective. Also, the methods can be applied anytime of the year and under highly controlled laboratory conditions, making them easily reproducible once protocols are established.

a) Meristem Culture

The simplest method is to use meristem tip culture because if extracted accurately, meristem is devoid of vascular tissue and consists only of actively dividing meristematic cells. Therefore, plants can be regenerated from the meristems without phloem limited bacteria and viruses such as Ampeloviruses causing leafroll disease in grapevine. The meristem is microscopic and is often less than 0.5 mm in most species. Shoot apical meristem (SAM) consists of an apical dome and one or two leaf primordia (Fig. 1A). Often meristem culture alone does not eradicate even phloem limited viruses. Moreover, there are many pathogens that are not limited to vascular tissue such as Nepoviruses (e.g. GFLV in grapevine), infecting the meristem and then therapies need to be combined to successfully eradicate the pathogens.

b) Combining Thermotherapy with Meristem Culture

Thermotherapy, when combined with meristem culture, is much more effective compared to the application of the two methods separately. Thermotherapy, as discussed above, either completely deactivates or partially kills the infecting virus. Thus, the chances of regenerating virus-free plantlets through meristem culture are greater when combined with thermotherapy. There are many examples such as eradicating GLRaV 1 and GLRaV 3 from infected 'Chancellor' grapevine by applying thermotherapy to in vitro plants followed by meristem culture (Dĩaz-Barrita et al. 2008), applying thermotherapy (37–40 °C for 4 weeks under hot air treatment) to shoots harvested from field-grown 'Oregon Spur–II" apple infected with Apple mosaic virus, Apple chlorotic leaf spot virus (ACLSV), Apple stem grooving virus, Apple stem pitting virus and Prunus necrotic ringspot virus followed by in vitro establishment and meristem culture (0.3 – 0.5 mm meristems effective for all viruses and 0.5- 0.6 mm effective for all except ACLSV – only 50% success for ACLSV) (Vivek and Modgil 2018) and applying thermotherapy (35 °C for 3 weeks) to potted nectarine (*Prunus persica* var *nectarina* Max) 'Arm King' infected with P*lum pox virus* (PPV) and *Prunus necrotic ringspot virus* (PNRSV) followed by meristem (1.3 – 2 mm) culture (Manganaris et al. 2003). As thermotherapy inactivates the virus, longer shoot tips that better survive in vitro culture can be used for culture initiation.

Thermotherapy (4 weeks with day/night conditions of 16/8 h and 40/36°C) of two-week-old cassava plants infected with African cassava mosaic virus and East African cassava mosaic Cameroon virus were used by Yéo et al. (2020) to establish tissue cultures from which meristems were isolated and cultured. They reported 88% of the regenerated plants free from both viruses. Combining thermotherapy with meristem culture for improved eradication of Onion yellow dwarf virus and Shallot latent virus from in vitro-cultured shallot shoots co-infected with both viruses (Wang et al. 2021) and *Bean yellow mosaic virus* from infected gladiolus (Sharifi Nezamabad et al. 2015) are among many other reports of successful use of this combined therapy.

c) In Vitro Chemotherapy

Since the first antiviral drug in humans was registered in the 1960s, many antiviral drugs have been developed. Antiviral agents targeting plant viruses are mainly derived from natural products and many products with varied control mechanisms have been developed. However, demonstration of the antiviral activity of a guanosine analogue agent, ribavirin (originally used as a drug for human hepatitis C virus) in tobacco cells infected with *Tomato spotted wilt virus* made this compound the most popular for virus eradication programs in tissue cultured plants. Its broad spectrum of activity against replication of both RNA and DNA viruses makes it the first choice in many virus eradication programs and is often combined with other therapies. Usually, the viricide is added in concentrations of 10 – 100 mg/L in media for growing infected plants and maintained for 2 – 6 weeks during which time thermotherapy regimes can also be applied. It is also used in combination with cryotherapy for viruses that are difficult to eradicate using a single therapy. Therefore, some examples of this method are discussed below under cryotherapy.

d) Cryotherapy

Cryotherapy involves treating the shoot tips of infected plants at ultra-low temperature, often in liquid nitrogen (LN) at -196 °C. The development of novel vitrification-based protocols has enabled cryotherapy to be applied once a protocol is developed. Unlike cryopreservation for conservation purposes, the recovery percentage of treated shoot tips need not be high for virus eradication. In fact, when optimized protocols are applied with high survival percentages, more cells of the meristem survive the treatment, reducing the chances of virus eradication. For example, we used droplet vitrification to test the suitability of cryotherapy for virus eradication of leafroll disease in grapevine. 'Chardonnay' and 'Lakemont Seedless' were infected with GLRaV-3, Pinot gris' and 'Sauvignon blanc 316' infected with GLRaV-2, and another clone of 'Sauvignon blanc' infected with both GLRaV-1 and GLRaV-3. All the plants regenerated after cryo-treatment (one hour in LN) tested negative for the viruses after six months in the greenhouse. The regeneration percentages were from 13 % (Chardonnay') to 30 % (Sauvignon blanc') (Pathirana et al. 2015a).

In New Zealand the potato germplasm collection of about 950 genotypes was maintained in the field until we could introduce cryopreservation. At the time, the accessions in the collection had many virus infections. Before cryopreserving the germplasm, it is important to clean up the collection. Virus eradication efficiency in potato infected with *Potato virus S, Potato virus A* and *Potato virus M* is variable (20 – 100%) when chemotherapy (2 weeks in 100 mg/L ribavirin) is used alone, whereas combining it with cryotherapy was more effective (80 – 100% efficiency). Cryotherapy and thermotherapy applied alone was also not effective (Bettoni et al. 2022) (**Fig. 7**). Now this method is routinely used to eradicate viruses before cryopreserving the potato germplasm for long-term conservation.

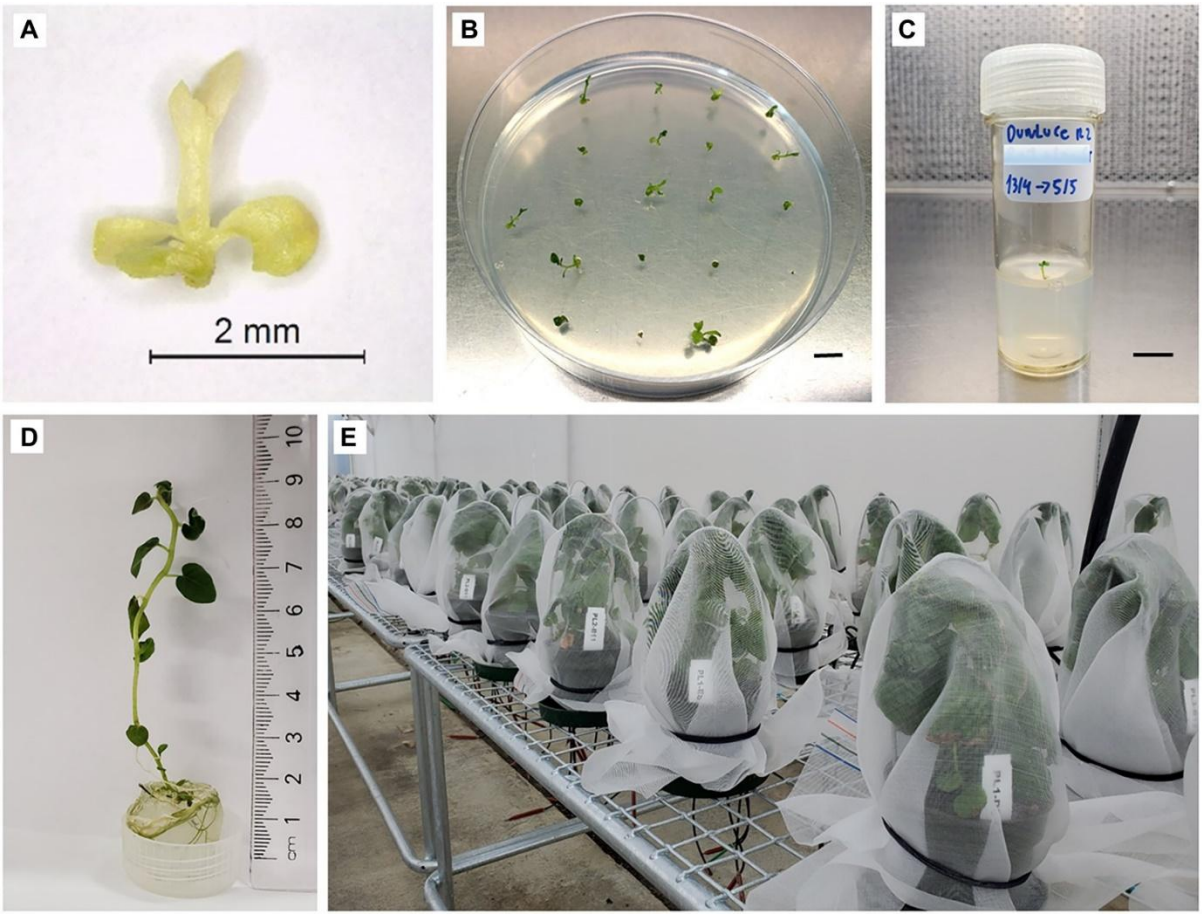

Figure 7. Shoot tip recovery process in potato "Dunluce" infected with *Potato virus S* following a combined chemotherapy + cryotherapy treatment. **A)** Shoot tip 1 week after combined chemotherapy + cryotherapy and **B)** 3 weeks recovery from cryoexposure. **C)** Shoot transferred to vial and **D)** grown for 3 months. **E)** Plants after 3 months of growth in the greenhouse. Bars = B 0.6 cm, C 0.7 cm. Reproduced from Bettoni et al. (2022).

Raspberry bushy dwarf virus (RBDV – a member of the species *Idaeovirus rubi* of the genus *Idaeovirus* in the family *Mayoviridae*) is one of just 17 horizontally transmitted viruses among over 1000 known plant viruses. Horizontally transmitted viruses are pollen-borne and after pollination and fertilization, get into the maternal tissue through the fertilized ovary. Thus, RBDV can spread rapidly within one flowering season and is most damaging and occurs in all raspberry growing regions in the world. Meristem culture, cryotherapy or thermotherapy alone are not effective on their own as it is aggressive and infects parts of the growing meristem as well. Therefore, we tested several combinations of chemotherapy, thermotherapy and cryotherapy. Chemotherapy (30 mg/L ribavirin) combined with thermotherapy (24°C for 8 h in dark and 39 °C for 16 h with light) for two weeks followed by cryotherapy was the most effective with 80 – 100% of plants regenerated after treatment testing virus-free in the greenhouse (Mathew et al. 2021). This work was conducted in New Zealand, and we cleaned up many clones of raspberry in our collection, so that the breeders can now use these genotypes as pollen or female parents in their breeding program without risking virus transmission to the progeny (**Fig. 8**).

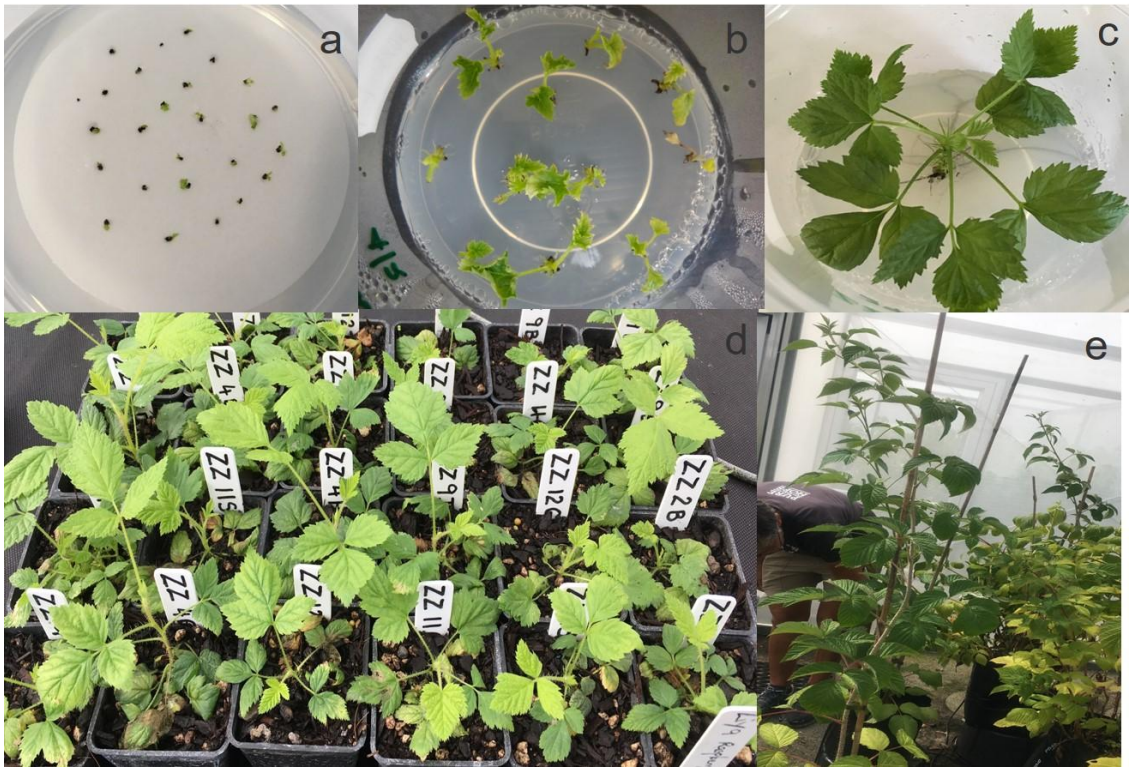

Figure 8. Different stages of thermotherapy + chemotherapy followed by cryotherapy of raspberry tissue cultures infected with *Raspberry bushy dwarf virus*. **A**) Cultured meristems on regeneration media after combined therapies. **B**) Regenerating plantlets after 4 weeks in culture, **C**) Shoot transferred to individual tubs after 4 weeks. **D**) Plants acclimatizing in the greenhouse after 6 weeks of exflasking. **E**) Plants in the greenhouse after 1 year from exflasking.

Often, cryotherapy is combined with other therapies for more aggressive viruses as already described for the eradication of three viruses in potato (Bettoni et al. 2022) and for RBDV in raspberry (Mathew et al. 2021). As only the meristematic cells with no vacuoles survive and the differentiated cells with vacuoles get destroyed due to ice crystallization, cryotherapy can be regarded as a very precise meristem culture (**Fig. 9**).

Wide Hybridisation to Create Interspecific And Intergeneric Crosses

Polyploidy (whole genome duplication events) is common in plant evolution, particularly among cultivated plants. Chromosome doubling following hybridisation between two related species produces an allopolyploid with chromosome compliments from both species and can be fertile. There are many examples such as wheat (Durum wheat - *Triticum durum* 2n = 4*x* = 28 tetraploid; bread wheat - *T. aestivum* 2*n* = 6*x* = 42 hexaploid), canola - *Brassica napus* (2n = 4x = 38, AACC) with genomes from *B. oleraceae* (2n = 18, CC genome) and *B. rapa* (2n = 20, AA genome) and upland cotton (*Gossypium hirsutum*, AADD, 2n = 52) (Pathirana and Carimi 2022). While this happens rarely in nature, with the technologies available, it is possible to produce polyploids experimentally.

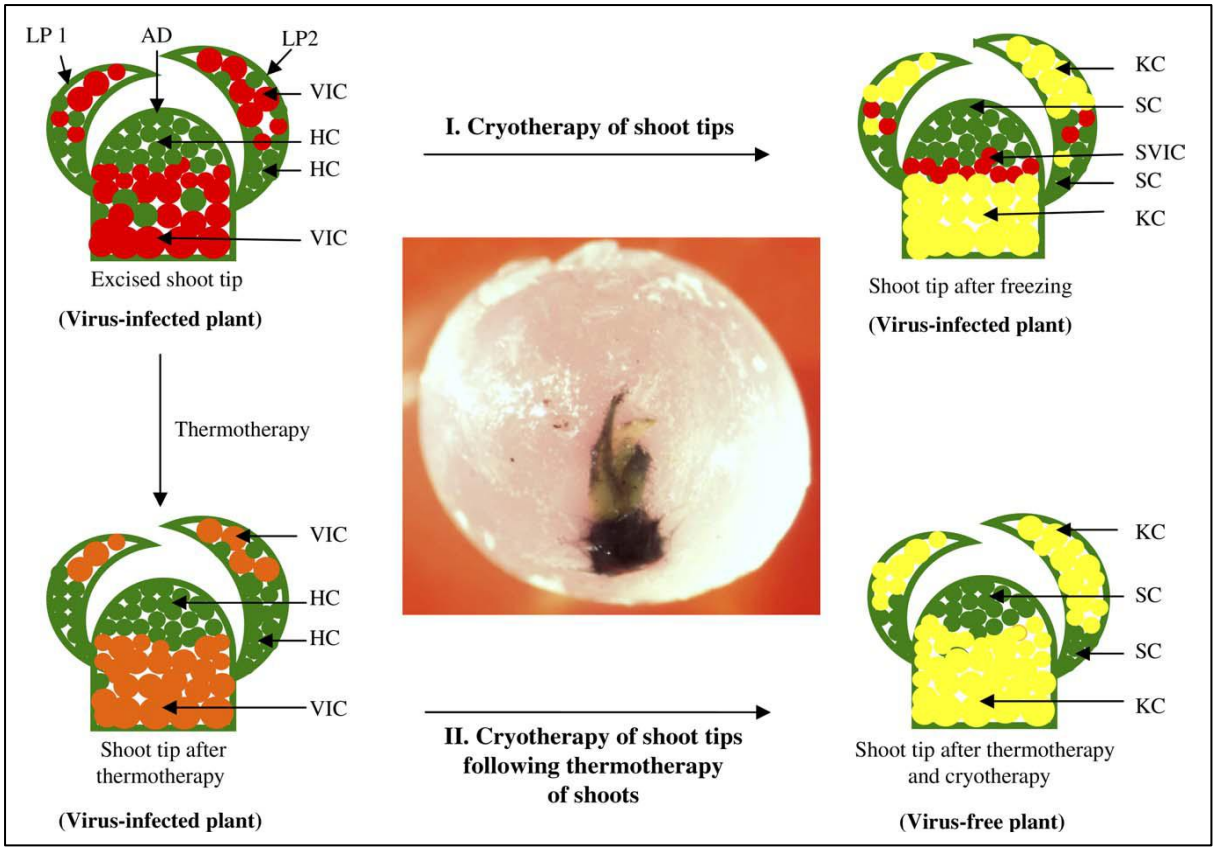

Figure 9. Schematic diagram depicting the effectiveness of the combination of thermotherapy and cryotherapy for enhanced elimination of viruses that can invade the meristematic cells efficiently. I) Most of the differentiated older infected cells are lethally injured whereas the youngest cells in the meristem survive the cryo-treatment. If the virus was not able to enter the meristem, the treatment would result in virus-free plants. However, in cases like here where the virus invades the meristem, shoots regenerated after cryo-treatment will remain infected. II) Additional suppression of virus and increased propensity of infected cells to be injured by cryo-treatment can be achieved by subjecting shoots to thermotherapy before excising shoot tips for treatment in liquid nitrogen. Thermotherapy causes stress and reduces survival of the cells and also accelerates degradation of viral RNA. An encapsulated raspberry shoot tip (1.5mm) used for cryotherapy is illustrated in the middle. AD, apical dome; HC, healthy cells; KC, killed cells; LP1, leaf primordium 1; LP2, leaf primordium 2; SC, surviving cells; SVIC, surviving, virus-infected cells; VIC, virus-infected cells. Reproduced from Wang et al. (2009).

Wide hybridisation is the term used to describe hybridisation between plants belonging to different species and genera. Species, by definition, is a group of plants or animals whose members are able to breed with each other, meaning individuals from different species cannot interbreed. Thus, it is generally difficult to obtain fertile progeny even if we are successful in producing seeds of interspecific hybrids. The reasons for failure can be before fertilization (pre-fertilisation barriers), for example the maternal species may have a longer style than the male species and the pollen tube may fail to reach the ovary even if it germinates on the stigma. An example of post-fertilization barrier is when the fer-

tilized ovary cannot divide by mitosis or endosperm fails to develop. We have solutions to these problems by manipulating floral parts in tissue culture and successfully producing interspecific and intergeneric hybrids.

Man-made Species

The first man made species is *Raphanobrassica*, developed by Karpachenko, a Soviet scientist, back in 1928. His objective was to have a cabbage (*Brassica oleraceae*) on top of a radish (*Raphanus sativa*) root. Although he produced the inter-generic hybrid, the phenotype was not as expected (**Fig. 10 A and B)** (Karpechenko 1932). This research is continuing even today and there are registered Raphanobrassica cultivars used as feed for farm animals in many countries, including Australia and are identified as better suited to lower rainfall livestock systems with the ability to maintain green leaf and withstand dry periods and more consistent production across different growing conditions (Watt et al. 2023).

Another well-known man-made species through intergeneric hybridisation is Triticale, and we must rescue the young embryo from the ovary of the mother plant after hybridisation and grow in tissue culture to produce new fertile hybrid. Triticale has the high yield of wheat (*Triticum*) combined with the resilience (cold, drought resistance) of rye (*Secale*) in one species and is popular mainly in central Europe – Poland, Belarus, Germany, Lithuania, Belarus etc. as well as in China, Australia and New Zealand (**Fig. 10 C and D**).

Interspecific Hybrids of *Vaccinium* (blueberry) – A practical example

In New Zealand, the breeders have been trying to combine the valuable characteristics of highbush, lowbush and rabbiteye blueberries but had difficulty getting fertile hybrids for breeding. We developed methods to successfully culture fertilized ovules and also rescue embryos from successful interspecific crosses and culture them and obtain fertile hybrids. In this research we rescued more than 200 hybrids from 14 different combinations constituting tetraploid, pentaploid and hexaploid blueberries for the breeding programme. As a result of this work, blueberry cultivation could be extended to areas where there were no suitable cultivars and also extend the period of availability of the fruit in the market as a result of release of early and late maturing varieties (Pathirana et al. 2015b). Different stages of the tissue culture of fertilised ovules are given in **Fig. 11**, and the numbers of hybrids produced in different combinations are described in Pathirana et al. (2015b).

Embryo Rescue to Increase Hybrid Seeds in Apomictic Species

Opuntia ficus-indica or prickly pear cactus is an important forage and food source in arid and semiarid ecosystems and is the most important cactus species in cultivation globally. This species is known for its high degree of apomixis, the phenomenon of asexual seed production that occurs without fertilization. While apomixis is useful for clonal propagation, it is a hindrance in plant breeding programs where genetic segregation is sought for the selection of superior genotypes.

In a collaborative project with Italian colleagues, we compared the mature seed-derived seedlings with those regenerated from *in vitro* embryo rescue at different post-anthesis days (PADs) in four Italian cultivars. The zygotic seedlings were discriminated from apomictic seedlings using molecular marker analysis based on inter-sequence single repeat (ISSR) primers. Ovules cultured at 35 PADs resulted in the highest percentage of zygotic seedlings, from 51% to 98% in the four cultivars.

Mature seeds harvested in the field produced much fewer seedlings per seed than in tissue culture-based embryo rescue and a lower percentage of zygotic seedlings (from 14% to 63%) (Carra et al. 2023). Therefore, breeders can now increase the availability of zygotic seedlings in prickly pear breeding programs through *in ovulo* embryo culture. This method may be applicable to breeding in other apomictic species such as apple (Bisognin et al. 2009), citrus (Yadav et al. 2023), mango (Yadav et al. 2023), mangosteen (Baskaware and Deodhar 2023), etc.

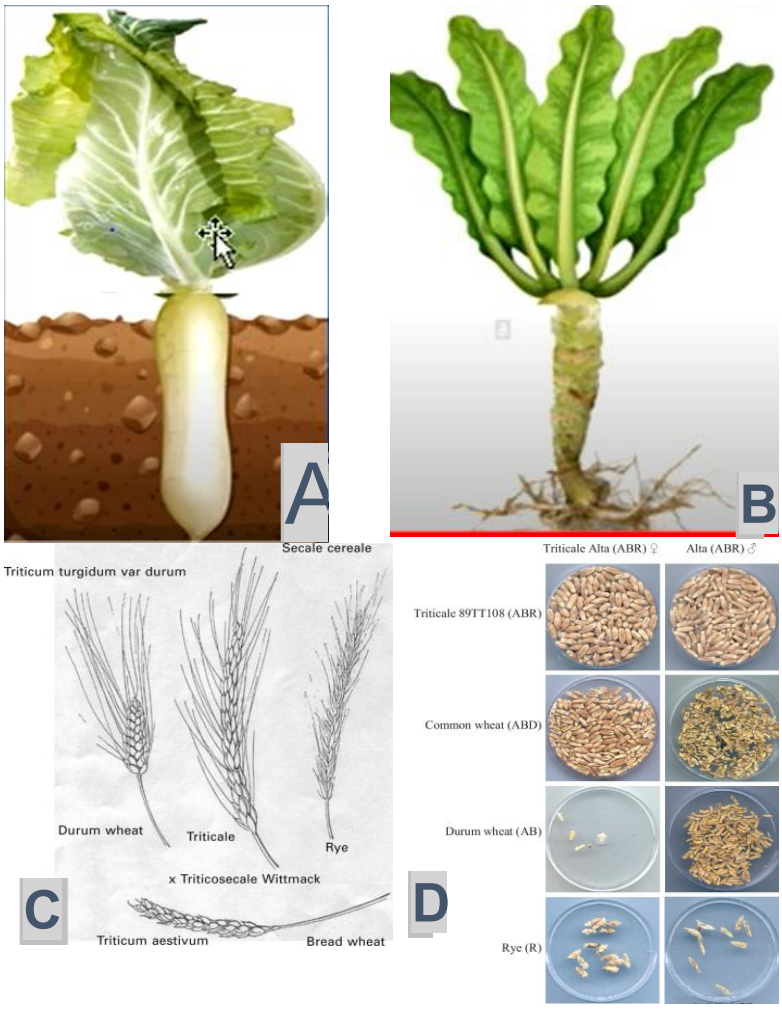

Figure 10. Man-made interspecific hybrids. Karpachenko wanted to produce a hybrid of cabbage with radish that has the root of radish and the top of cabbage (**A**) but what he obtained was something intermediate (**B**). **C**) Comparison of spikes and **D**) seeds of wheat, rye and triticale.

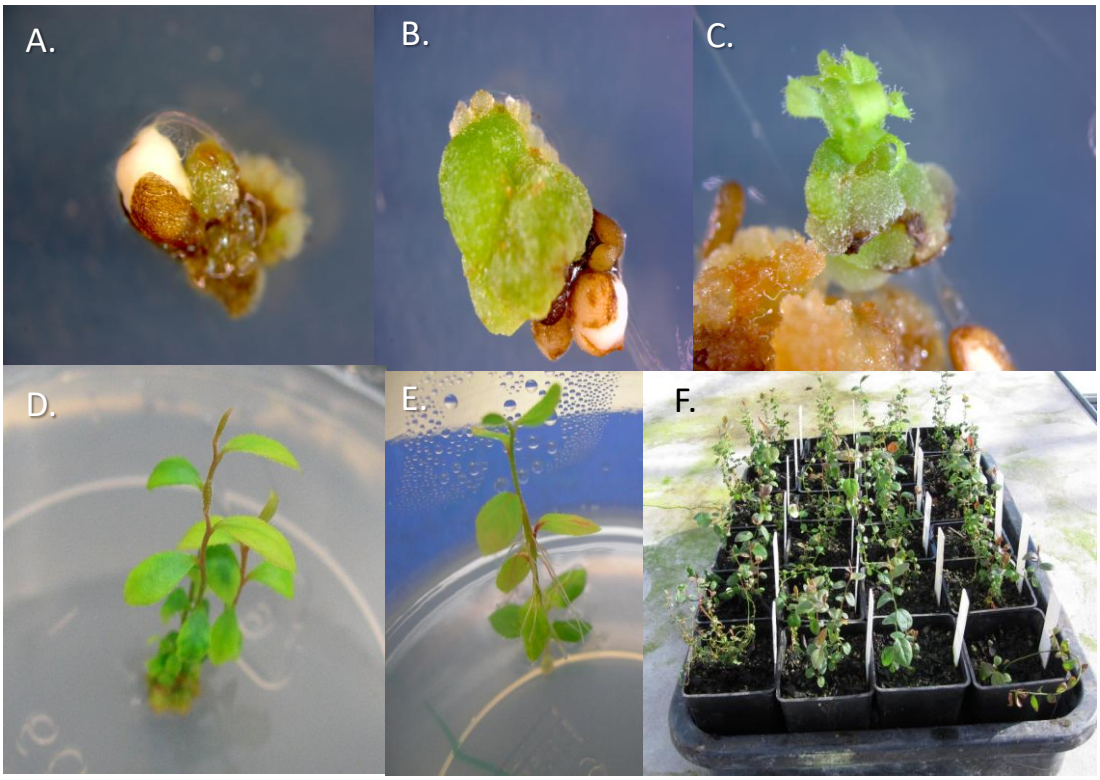

Figure 11. Stages of production of blueberry (*Vaccinium* spp.) interspecific hybrids through culture of fertilised ovules. **A**) Extracted ovules in culture. **B**) Expansion of ovules. **C**) Putative hybrid emerging from fertilised ovule callus, D) shoots growing from hybrid. **E**) rooted shoots of hybrid. **F**) Exflasked hybrids in the greenhouse after confirmation by SSR markers.

In Vitro Mutagenesis for Fast Tracking Crop Breeding

Traditional cross breeding takes about 7-8 years to develop elite material in annual crops and a further 3-4 years for field trials and their release. If back-crossing is involved, many more years will be required. Also, there is a limit to what we can achieve by cross breeding within the genome of the species.

Random mutation and selection

Induced mutagenesis allows to create new recombination through chromosome breakages and can also produce point (gene) mutations. Chromosome breakages that occur when ionising radiation is used as the mutagenic agent has the potential to break linkages of genes, creating novel genotypes. Chemical mutagens on the other hand produce more point mutations. Induced mutations are ideal for perennial crop improvement that have long turnover of generations, and most of the diversity we have in our fruit crops is due to sports or natural point mutations that have been selected and vegetatively propagated.

Traditionally, shoot tips or seeds are used to produce mutant populations, but large mutant populations need to be created for screening. This is expensive and difficult to achieve in the field, particularly for perennial crops. On the other hand, in vitro mutagenesis allows to handle large mutant populations in a small space; metaphorically in a Petri dish. Details of how to create such populations have been described in several reviews (Sharma et al. 2025;

Pathirana 2021; Pathirana 2011). After creating mutant population, we can even screen the population under in vitro conditions for different abiotic stress tolerance traits such as salt tolerance (Patade et al. 2008; Nikam et al. 2015), tolerance to toxic elements (Pathirana et al. 2002), heat tolerance (Das et al. 2000), cold tolerance (McClinchey and Kott 2008) etc. Many crop cultivars with herbicide tolerance have been developed through in vitro mutagenesis combined with in vitro selection, for example in sugarcane (Koch et al. 2012) and pepper (Venkataiah et al. 2005). Disease resistance screening of mutant populations in vitro can be achieved using bacterial suspensions; for example, we screened populations of gold kiwifruit for resistance to *Pseudomonas syringae* pv *actinidiae* – the most devastating pathogen for the New Zealand kiwifruit industry (Pathirana et al. 2016). We have also used a leaf disc method to screen a large mutant population of grapevine for *Botrytis* tolerance (Pathirana 2009).

In grapes, we first developed protocol for inducing embryogenic cultures (**Fig. 12a**) and then optimised the dose of gamma rays or chemical mutagen, ethyl methanesulfonate, and use the dose (dose = concentration of the mutagen x time of treatment) that results in 50% reduction in callus growth to treat a large population of callus cells (**Fig. 12b)** and then regenerate plants (**Fig.12 d & e**) (Carra et al. 2024; Pathirana and Carimi 2023). We can challenge the regenerating plants with the bacterium or its exudates. Alternately, we can have a large, chimera-free mutant population to screen in the field.

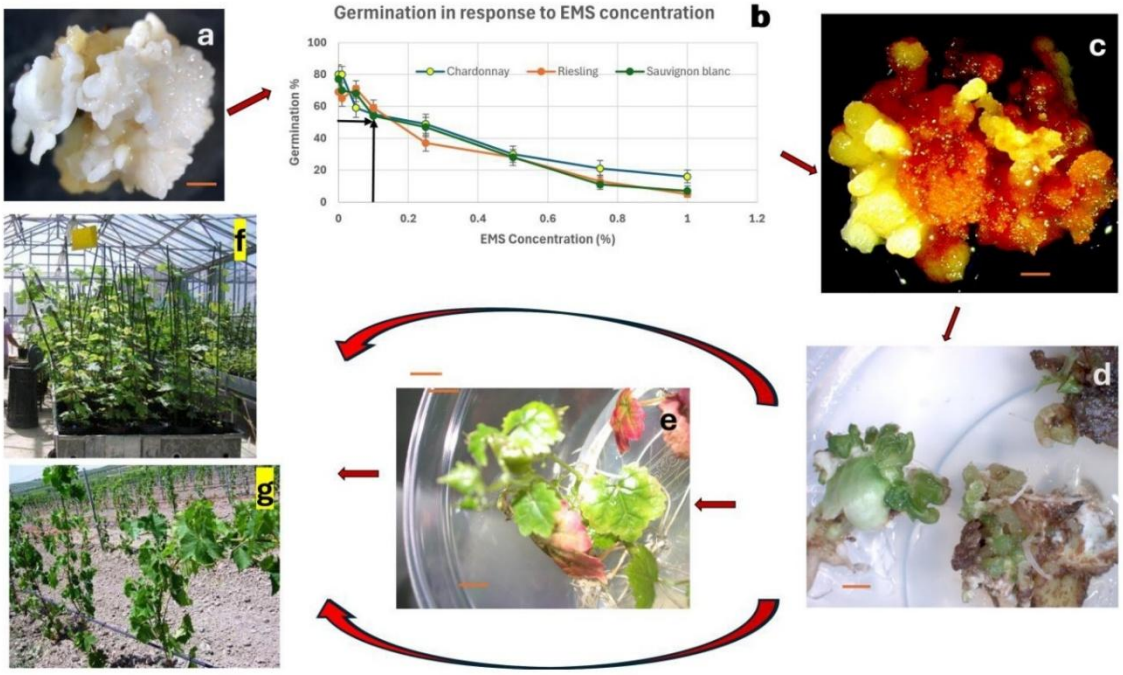

Figure 12. A scheme for using grapevine embryogenic cultures for mutation induction and screening. **A**) Embryogenic culture. **B**) Optimizing mutagen dose through growth reduction studies. **C**) Development of somatic embryos after treatment with optimized mutagen dose. **D**) Initial germination. **E**) Screening the germinated embryos for the trait of interest in vitro, **F**) in the greenhouse and **G**) under field conditions. Bars: (A, C, D) = 1 mm; (E) = 5 mm. Figure reproduced from Carra et al. (2024).

A protocol for plant regeneration from caryopses of *Indica* rice via a callus phase was developed and adopted to select regenerants in media simulating iron toxicity. Caryopses of three commercial Sri Lankan rice cultivars (RU 102, LD 355 and AT 353) were induced to produce callus on MS medium supplemented with either 2,4- dichlorophenoxyacetic acid (2,4-D) or chlorophenoxyacetic acid (CPA), and 6-benzylaminopurine (BAP). Plant regeneration was more efficient when 4-week-old calli induced using 9 µM 2,4-D and 0.5 µM BAP were transferred to MS medium supplemented with 2.2 µM BAP and 0.6 µM α-naphthaleneacetic acid (NAA). Three concentrations of Fe, three pH levels and three doses of γ irradiation (0, 100 and 150 Gy) were used in the selection experiment. Varieties LD 355 and AT 353 were more responsive than RU 102 to callus induction and plant regeneration. High iron concentration in combination with low pH significantly decreased callus induction and plant regeneration, but the few plants that were regenerated were more tolerant to iron toxicity and are being used in breeding programmes. The in vitro protocol developed for selecting rice mutants would be useful in supplementing the current breeding efforts to develop rice varieties tolerant to iron toxicity (Pathirana et al. 2002).

Use of In Vitro Culture Techniques in Plant Genetic Modification

Another well-known application of PTC is in plant transformation to produce genetically modified (GM) crops. Genetic transformation uses the natural process of gene transfer by a soil-borne bacterium in the genus *Agrobacterium* that produces crown-gall disease (by *A. tumefaciens*) and hairy root disease (by *A. rhizogenes*). These bacteria have small extrachromosomal circular DNA called plasmids. The tumour inducing plasmids (Ti plasmid) in these bacteria have a small region called transfer DNA (T-DNA) that gets transferred to the host plant and this natural process is used in experimental transfer of desired genes into crop plants. This process is exclusively done using in vitro cultures.

Hibiscus Transformation

In our experiments, we developed an efficient transformation and regeneration system for *Hibiscus rosa-sinensis* (**Fig. 13**) that will enable researchers to improve its quality in a number of ways (Trivellini et al. 2015). For example, by appropriate gene manipulation plants could be produced that have greater tolerance to frost or whose flowers have a much-extended display life.

Improved Selenium Accumulation in Solanaceous Species Through Transformation

Some plant species such as *Astragalus bisulcatus*, a legume, can tolerate growth on soils with high Se content. They are known as Se hyperaccumulators and can convert inorganic Se to methylselenocysteine (MeSeCys) by the enzyme selenocysteine methyltransferase (SMT). MeSeCys has been shown to have anticancer properties in mammals (Dong et al. 2001) and therefore more crop species that can accumulate MeSeCys would help in cancer prevention among populations, particularly those with low selenium intake. We used Agrobacterium transformation of two solanaceous species – tobacco (McKenzie et al. 2008) and tomato (Brummell et al. 2011) and in both species overexpression of *SMT*

transgene resulted in increased accumulation of MeSeCys. When transgenic tomato plants were fertilised with selenite or selenate at the stage of fruit development, liquid chromatography mass spectrometry showed that MeSeCys accumulated in the fruit but not in leaves. Also, MeSeCys was produced more effectively from selenite on a percentage conversion basis, but greater accumulation of MeSeCys could be achieved from selenate due to its better translocation from the roots (Brummell et al. 2011).

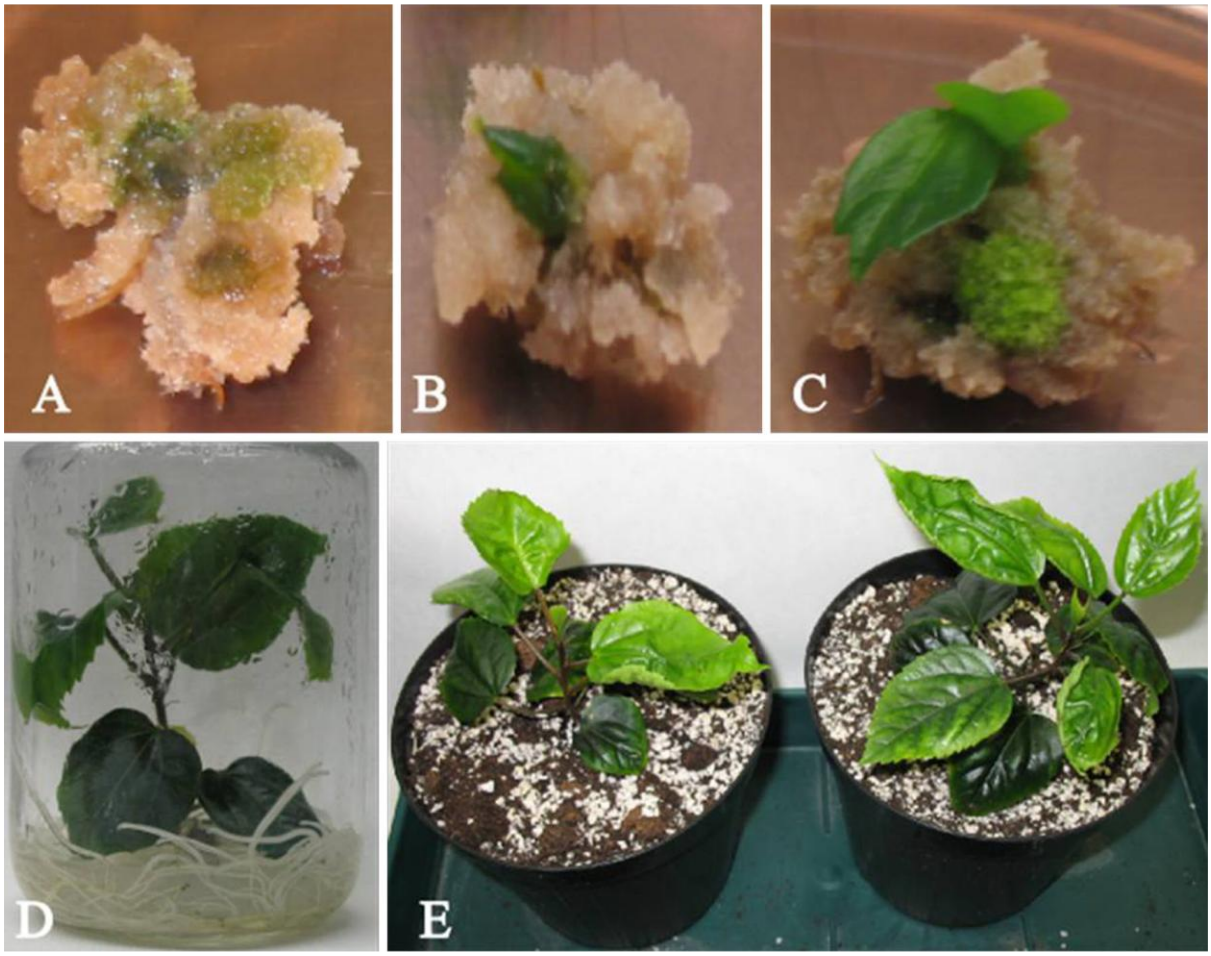

Figure 13. Plant regeneration from callus induced from axillary buds of *Hibiscus rosa-sinensis* and *Agrobacterium*-mediated transformation. **A**) Callus induced from axillary bud explants on basal medium supplemented with 6-benzylaminopurine and β-naphthoxyacetic acid after 3 weeks. **B**) Callus induced from an axillary bud on selective regeneration medium after 10 days of culture. **C**) A plantlet regenerated on selective regeneration medium after 4 weeks. **D**) A transgenic plantlet after 6 weeks on rooting medium. **E**) Transgenic plants transferred to soil in the containment greenhouse. Figure reproduced from Trivellini et al. (2015).

Golden Rice – Golden Opportunity

It is common knowledge that golden rice, developed through genetic modification allowing the grains to accumulate high amounts of beta carotene (23-fold compared to non-GM rice), can alleviate blindness due to vitamin A deficiency in malnourished children in Africa and Asia (Diaz and Fridovich-Keil 2025). Similar to all other GM crops, Golden Rice also had opposition, mainly due to misinformation. However, rigorous testing for safety for the growers, consumers and the environment resulted in Australia, Canada, United States and several other countries to designate cultivation of this GM crop safe. Philippines is the first country, in 2021, to approve commercial cultivation of golden rice. Unfortunately, in April 2024, in response to a petition filed by Greenpeace and another local group, Court of Appeals in the Philippines issued a cease-and-desist order on the commercial propagation of golden rice, citing a lack of "full scientific certainty" regarding their health and environmental impact. Nevertheless, by 2023 another 12 countries were in the final stages of this approval process including China, Bangladesh, India, South Africa and Vietnam (Diaz and Fridovich-Keil, 2025).

These examples show how transgenic plants produced using in vitro technologies can help not only to understand biological processes but also improve crop species for improved nutrition. Despite the opposition for the technology, many countries have approved growing of GM crops and in the last 15 years, GM crops have given an additional income of US$285 billion and annual incomes are increasing with more acceptance (Wijerathna-Yapa and Pathirana 2022).

In Vitro Culture for Targeted Genome Editing

The advent of targeted genome modification (TGM) technologies, which are based on the application of targeting modules, has helped researchers to work with individual plants rather than large populations when selecting in randomly generated mutant populations. In targeted genome editing, we are able to recognize and modify a particular DNA sequence. The three main targeting modules currently in use are zinc finger (ZF) DNA-binding domains, transcription activator-like effector (TALE) DNA-binding domains and clustered regularly interspaced short palindromic repeat (CRISPR) systems. These techniques offer targeted genome editing capabilities, allowing for precise modifications to the genome of the crop cultivar under modification. Although tissue culture-based systems are traditionally used, researchers are exploring methods to bypass tissue culture as plants of several families are difficult in tissue culture. These include TGM delivery through *de novo* meristem induction, virus mediated delivery and graft-mobile delivery, reviewed by Li et al. (2024). However, these methods are in their infancy and results have so far been achieved only in model plant species.

Development of Homozygous Plants for Heterosis Breeding – Doubled Haploids

Homozygous plants are used in breeding programmes for mining useful genes and for heterosis breeding, to produce high performing hybrids. In cross pollinating species like most of our horticultural and floricultural crops, producing homogeneous lines require backcrossing for 7-8 generations and in perennials such as coconut it can take the lifetime of a researcher. Instead,

we can use tissue culture to grow haploid plants from gamete cells found in the flower and diploidise them to produce 100% homozygous plants in one step.

Doubled haploid gentians

Gentians (*Gentiana triflora*) are one of the favourite Japanese flowers. In one of our collaborative projects, we produced doubled haploid lines in Gentians for our client, Hachimantai City in Japan and this program helped them to develop novel hybrids with even pink colour; gentians were known to have only purple flowers until then.

Anthers and ovaries from flowers at mid-late uninucleate stages of microspore development were cultured after treating them at 4°C for 48 h. In this work anthers and ovaries were cultured on a modified Nitsch and Nitsch (1969) medium supplemented with a combination of naphthoxyacetic acid (NOA) and BAP. The explants either regenerated new plantlets directly or produced callus that regenerated into plantlets upon transfer to basal media supplemented with BAP (**Fig. 14**). We used seven genotypes with different ploidy levels. Of the cultured ovaries 0–32.6% regenerated plants among the seven genotypes, whereas only 0–18.4% of cultured anthers produced plants. Nevertheless, all the seven genotypes responded either through ovary or anther culture. We used flow cytometry to assess the ploidy. There were more haploid (4.3 per 100 explants) and diploid plants (3.5 per 100 explants) than those with triploid (0.3 per 100 explants) or tetraploid (0.2 per 100 explants) relative nuclear DNA contents. All the diploid regenerants were shown to be gamete-derived (i.e. doubled haploids) by observing parental band loss using Randomly Amplified Polymorphic DNA (RAPD) markers (Pathirana et al. 2011a). Haploid plants were propagated on a proliferation medium and then treated with oryzalin for 4 weeks before transfer back to proliferation medium. Most of the resulting plants were diploids. Over 150 independently derived diploidised haploid plants were deflasked (Pathirana et al. 2011a).

Anther-derived fast-growing callus as a source for doubled haploid coconut

Coconut is a cross-pollinated perennial tree of immense importance in tropical countries. It can be propagated only by seeds, and crop improvement is through mass selection following mass, controlled or hand pollination. The current methods result in heterogeneous plantations and considering its perennial nature, there is a high demand for uniform, vigorous, elite planting materials for plantation establishment. Therefore, hybrid coconut will be high in demand if produced. Towards this objective, we investigated anther derived fast-growing callus (FGC) as a long-term source for doubled haploid production. After testing different media, sucrose concentrations and plant growth regulators, we found that the highest callogenesis and weight increase (growth) can be achieved with FGC cultured in solid Eeuwen's Y3 medium supplemented with 100 μM 2,4-dichlorophenoxyacetic acid with heat pretreatment at 38 ℃ for 3 days. Weight increase of FGC was negatively correlated with sucrose concentration. Incorporation of cytokinins facilitated the conversion of FGC to embryogenic callus. Presence of a cambium-like zone, a characteristic feature of embryogenic calli was confirmed by histological studies. Flow cytometry indicated that embryogenic calli derived from FGC originated from reduced

microspores (Perera et al. 2021). Our results indicate that anther-derived FGCs are a good source for long-term production of haploid embryogenic callus for developing doubled haploid coconut for heterosis breeding programs.

In Vitro Technologies for Biopharming and for Producing Bioactive Compounds

Biopharming is the use of a living system as a host for the manufacture of biological drugs. In recent years, plants have become increasingly more attractive and acceptable as an expression platform for the production of vaccines and there has been much progress. There are many reasons which make expression in plants a more attractive option.

One main reason is that plants cannot host human pathogens and the production from gene to product is much faster than any other eukaryotic system, very attractive in pandemic situations.

Although plant-based vaccines have been produced for a long time, mainly for vaccines used in animals, recently Health Canada has authorised the use of a plant-produced virus-like particle vaccine against COVID-19 in humans; the vaccine, COVIFENZ®, is made by Medicago Inc.in Québec. However, the association of the company with tobacco industry was a barrier for international recognition of the vaccine (Duong and Vogel 2022).

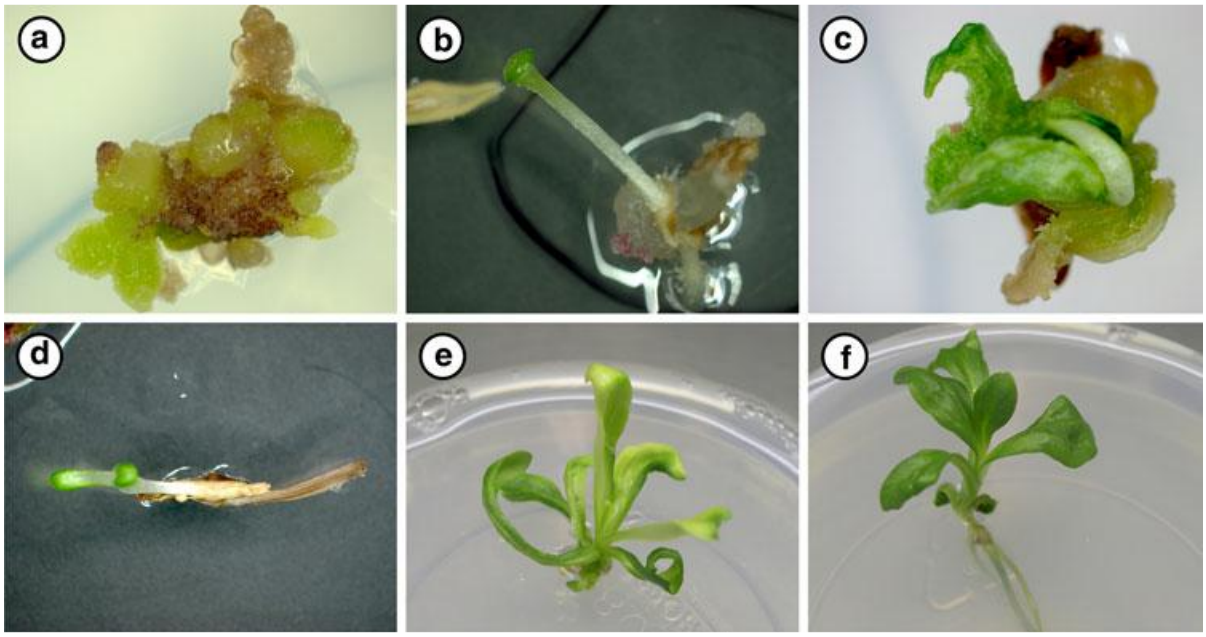

Figure 14. Typical response of *Gentiana triflora* anthers to culture on NOA/BAP supplemented media. **A**) Anther callus producing shoot initials in regeneration medium after 2 weeks under light. **B**) initial stage of plant regeneration from anther callus after 3 weeks on regeneration medium. **C**) Plant regeneration from anther-derived callus induced on NOA (1.4 mg l-1) and BA (1.8 mg l-1) medium after 6 weeks on regeneration medium. **D**) Direct plant regeneration from anther without callus formation in low NOA (0.7 mg l-1) and high BA (2.4 mg l-1) medium for 6 weeks. **E**) Regenerated shoot transferred to rooting medium. **F**) Rooting of shoots after 3 weeks on PGR-free medium.

In addition to the use in the pharmaceutical industry, in vitro plant cell cultures are used for production of natural flavors, agro-chemicals, colors, therapeutic proteins and bioactive compounds. There are many challenges in this area such as retention of the metabolic cues of natural plants in cell culture systems and scaling-up the production to create high yielding cell factories. These aspects have been reviewed in detail by Bapat et al. (2023).

Figure 15. The slow growth facility of kiwifruit at the Palmerston North site of the New Zealand Institute for Plant and Food Research Limited. Each accession is held in eight 35-mL plastic screw cap vials at 5°C after cold acclimation. Figure reproduced from Debenham and Pathirana (2021).

Application of In Vitro Techniques in Plant Conservation

Plant tissue culture repositories are an attractive option to conserve valuable clonal material and endangered species, away from the vagaries of a changing climate. They are much safer in the lab, and especially when duplicated in different labs. The plants from TC repositories are easily available for use, export and research.

Once established, cultures require constant attention and subculture when the plants start to senesce. Subculture not only increases labour costs but also increases the risk of contamination. Low temperature, low concentration of minerals and sucrose, low light intensity are used to reduce the frequency of subculture by slowing down metabolic processes. These repositories should have the backing of good database and IT capabilities so these can be searched for different characters and ordering. This author was involved in the development and methodological improvement of the in vitro repository of kiwifruit (*Actinidia* spp.) in New Zealand, currently holding over 900 accessions, each in six 35 ml screw-cap vials (**Fig. 15**). Before depositing in the cold culture room, kiwifruit tissue cultures are cold acclimated for 10 days at alternating temperatures with a short photoperiod (22°C with 5 μmol m^2 s^1 light for 10 h/2°C dark for 14 h). Thereafter in slow growth media, under low temperature and low light intensity, we can hold some genotypes for over 20 months without subculture (Debenham and Pathirana 2021). A total of 26 species, sub species and interspecific crosses are represented in this collection. Quality control of the collection is conducted regularly by comparing the SSR allele profiles of the parent plant in the field with those in tissue culture (Debenham and Pathirana 2021; Wiedow et al. 2017). The other more attractive and more secure option for conservation of clonal species or even seeds is cryopreservation. This author will elaborate on this method in a subsequent paper in IPPS Proceedings.

CONCLUSIONS

Plant tissue culture has become the foundation in advanced biotechnologies. The most widespread application is micropropagation for producing clonal plant stocks with the market expected to grow to US$ 2.1 billion by 2030. It is widely applied in plant propagation of horticulture, floriculture and forestry. Micropropagation can be achieved through direct and indirect organogenesis, somatic embryogenesis or microtubers. The method is also used in development and deployment of disease-free high-health plants for agriculture, horticulture and forestry. The advantage of tissue culture-based technologies for eradication of pathogens from planting materials is that different methods such as meristem culture, thermotherapy, chemotherapy, electrotherapy and cryotherapy can be combined when a single therapy is not effective for aggressive pathogens.

In vitro technologies have a central role in the deployment of new cultivars to the industry much faster and efficiently than traditional field-based methods. This application encompasses an array of technologies to produce crop cultivars or even new man-made species with traits of interest. Thus, in vitro technologies have a central role in ploidy manipulation, distant hybridization, doubled haploid production, increasing sexual seeds in apomictic species and also understanding biochemical pathways. In vitro technologies are more efficient for the development and deployment of mutants with improved traits and in developing genetically modified crops using traditional transformation methods as well as gene editing techniques. The market share of cell culture technologies is also increasing in the production of plant-based pharmaceuticals, food ingredients, cosmetics, flavours, dietary supplements, fragrances, and biostimulants. For biopharmaceuticals alone the market is expected to reach USD 50 billion during the next five years.

In vitro cultures are used in plant conservation, both in the form of tissue culture repositories for medium term conservation or cryopreserved collections for the long term.

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