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Medicinal plant conservation by tissue culture: *Ex-situ* & *in-situ* techniques

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Abstract
The use of medicinal plants has surged in recent times, with their roots in traditional medicine practices across various countries. However, the heightened demand for these plants, coupled with increased human activities, has led to the endangerment of species and the destruction of their habitats. This situation necessitates urgent conservation efforts for endangered medicinal plants. Among the various conservation methods, biotechnology stands out as an effective approach that doesn't compromise the plants' primary traits. Tissue culture, a key technique in biotechnology, plays a significant role in this endeavour. Unlike animals, plants have the ability to regenerate from cells and produce a complete organism. Consequently, tissue culture can generate entire plants from small plant parts, such as leaves, buds, or stems, while maintaining their genetic makeup. This method allows for the rapid propagation of a large number of uniform plants, making it a valuable tool for conserving native or endangered species and preserving precious germplasm resources.

Keywords: Biotechnology, Maintaining genotype, Maintaining species.

Introduction

Medicinal plants (MPs), also called medicinal herbs, can be defined as any plant that is used for therapeutic purposes, contains secondary metabolites, and can also be applied as a precursor for the production of new medicines (Castillo-Pérez *et al*. 2021) [11]. These species have a pivotal and significant role as sources of valuable chemicals and drug-lead compounds in the health of both plants and humans (Singh 2018; Gusain *et al*. 2021 [28]; Ur Rehman *et al*. 2021). Various parts of herbs and plants are used for curative as well as therapeutic aids, such as fruits, leaves, barks, seeds, flowers, resins, rhizomes, roots, and stems (Ur Rehman *et al*. 2021; Alqethami *et al*. 2017) [4], which have a special and distinct physiological impact on the human body (Kumar and Satapathy 2011: Borokini and Omotayo 2012) [8]. In addition to being used as medicine to treat human and plant diseases, MPs are also an important source of substances that are useful for maintaining the health and condition of the body. About two-thirds of the world's people use MPs for safety maintenance (Ur Rehman *et al*. 2021).

Currently, population growth has increased the demand for herbal drugs and natural medicines. The application of MPs is developing quickly over the universe, which has resulted in the loss of MP species 100–1000 times the natural overthrow rate. Although plants have been in danger of extinction for decades, species extinction and habitat destruction are now on the rise around the world due to the high demand (Gusain *et al*. 2021) [28].

According to a report by the International Union for Conservation of Nature and the World Wildlife Fund, there are about 50,000–80,000 floral species used in therapeutic applications worldwide. It is estimated that nearly 15,000 of these species are endangered due to overharvesting and habitat destruction (Barboza *et al*. 2009) [6]. At present, almost 20% of the wild resources of MPs are depleted by enhancing population and plant utilization. Therefore, the cultivation and propagation of different species of MPs are the most efficient way to bridge the gap between supply and demand (Wang *et al*. 2020).

Due to the importance of MPs in human health and the growing need for these plants, the preservation and sustainable utilization of these plants is an important issue now that there are different methods for this work. The use of biotechnological methods such as tissue culture is a suitable and efficient way to reproduce and preserve important medical and economic plants, synthesize bioactive compounds for use in pharmaceuticals, and produce important secondary metabolites for use in medical activities. This chapter highlights the application of tissue culture for *in vitro* regeneration of rare endangered or threatened medicinal plants.

Importance of Medicinal Plants and Their Conservation –

Evaluation of the multiple publications on MPs shows an increasing desire for research in this field. Research on medicinal plants has been on the rise since 1960, and in general, from 1960 to 2019, more than 110,000 studies on medicinal plants have been published. Three periods have been identified:

a. From 1960 to 2001, more than 1300 studies were published, and there was a steady increase.
b. It grows faster from 2002 to 2011 when it reaches a maximum of more than 6200 releases.
c. From 2011 to 2019, publications stabilized at around 5000 per year (Salmerón- Manzano *et al*. 2020).

The importance of using MPs to maintain human health is well known. Currently, it is known that two-thirds of the world's plant species have medicinal value (Krishnaiah *et al*. 2011). The greater availability, cost-effectiveness, and nontoxic nature of medicinal plants have led to the utilization of these drugs as a good source of therapeutic agents (Agbor and Ngogang 2005) [1]. According to the WHO,1 traditional medicine meets the basic healthcare requirements of 80% of the world's population, and the use of plant extracts and their active ingredients for treatment is more prevalent (World Health Organization 1993).

Medicinal plants are considered an essential resource in the treatment/inhibition of different types of sickness (Rakotoarivelo *et al*. 2015) [50]. Because medicinal plants have several components with therapeutic value, therefore, these plants can be used to treat various human diseases in the form of drugs or formulations (Joshi *et al*. 2011) [35]. In the past, medicinal plants were applied by our ancient ancestors to preserve and flavour food, relieve pain, treat headaches, and even prevent a variety of diseases. Knowledge on the healing properties of different plants has been passed down from generation to generation over the centuries. The biological attributes of plant species are due to the active compounds synthesized in secondary metabolism, which are used all over the world for several aims, such as the treatment of infected sickness (Mohammed 2019) [44].

Different parts of MPs are applied, including various kinds of seeds, roots, leaves, fruits, bark, flowers, or even the whole plant. In most ingredients of MPs, there are active compounds that are directly or indirectly involved in the treatment of diseases and are used as herbal medicines in medicine. Active compounds (substances) are synthesized and stored in different parts of these plants, which can have different uses by having different physiological effects on living organisms (Jamshidi-Kia *et al*. 2018) [33].

It has been found that one of the causes of some diseases such as cancer, dementia, arthritis, asthma, mongolism, and Parkinson's disease is the presence of free radicals and ROS including hydrogen peroxide, superoxide radicals, and hydroxyl radicals. These compounds are produced through aerobic respiration or from exogenous sources in the human body (Halliwell and Gutteridge 1990) [29]. Free radicals are harmful in higher concentrations. The reaction of ROS with several biological molecules, including lipids, proteins, and deoxyribonucleic acids (DNA), causes an imbalance between oxidants and antioxidants. Herbs have a strong antioxidant activity due to their flavonoids, terpenoids, alkaloids, diterpenes, tan- nins, glycosides, and other phenolic compounds; they can scavenge these free radicals and, by guarding cells against oxidative damage, can protect human health against several diseases (Singh *et al*. 2016).

Medicinal plants are not uniformly distributed throughout the world, and the number of species of these plants varies from country to country or region to region. China, India, Colombia, South Africa, the USA, Paraguay, Mexico, Thailand, Vietnam, Pakistan, Malaysia, Korea, France, Nepal, the Philippines, Bulgaria, Sri Lanka, and Chile have the highest number of plant species, respectively (Chen *et al*. 2016) [15]. Improper use of natural resources for medicinal plants causes the extinction of these species, which is one of the challenges facing these important plants. About 15,000 species of 50,000–80,000 flowering plant species around the world are endangered due to overharvesting and habitat destruction (Barboza *et al*. 2009) [6], and due to the growth of the human population and excessive consumption of plants, 20% of their wildlife resources are declining.

Generally, with population growth, increasing demand, and inadequate supply of plant medicines, the use of plant materials as a source of medicine is developing quickly all over the world (Cole *et al*. 2007) [16]. Considering that the abundance of medicinal plants in nature is low, and also that these plants grow slowly, overharvesting these plants from nature causes the destruction and loss of their species and leads to the depletion of resources and even extinction of species (Baker *et al*. 2007) [5].

Different parts of the plant have different sensitivities to harvest; for example, root and whole plant harvesting is more destructive to medicinal plants (such as plants, shrubs, and trees) than the harvesting of leaves and flowers or their buds. Several biological characteristics are associated with the risk of extinction, such as habitat characteristics, distribution range, population size, species diversity, growth rate, and reproductive system (Chen *et al*. 2016) [15]. Therefore, various strategies must be adopted to preserve and promote agricultural biodiversity.

Strategies for conversation

The emergence of new challenges to human health has increased the need for medicinal plants. Studying plants and fungi to find medicinal properties or other uses is a useful way to determine the importance and value of these plants and makes more efforts to preserve valuable species of these plants around the world. Recently, in 2019, 1955, and 1886, new plant and fungal species were reported, respectively (Cheek *et al*. 2020 #31) [14]. Some may produce beneficial compounds for humanity (Cheek *et al*. 2018 #32) [13].

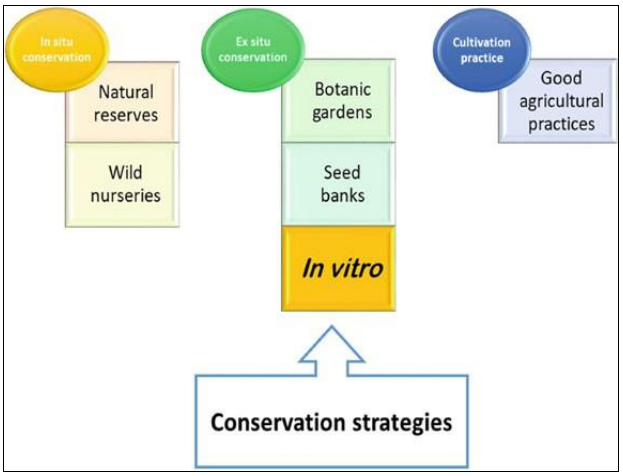

Fig 1: Several conservation strategies for medicinal plants (Kadam and Pawar 2020)

Preservation of endangered medicinal plants and fungi in endemic regions is more significant than focusing on globally endangered species. Therefore, considering plant habitats and conserving them are better ways than preserving individual species.

In Situ Conservation

This type of conservation includes areas such as natural reserves and national parks (collectively referred to as "protected areas") as well as the preservation of wild plants in managed habitats such as farms and plantation forests (World Health Organization 1993). On the other hand, preservation of ecosystems and natural habitats is defined as in situ conservation. In this method, the genetic resources present in their natural environment, as well as the conservation of domesticated or cultivated forms, in an environment where they have developed distinctive characteristics. In most regions, medicinal plants are native species of the region, and the secondary metabolites present in their different parts, which have medicinal proper- ties, may not be expressed under cultural conditions and respond only to cultivation in natural environments (Coley 1983) [17].

Ex Situ Conservation

When different species are protected in areas outside their natural habitat and the genetic material is transferred to an area far from the place where it is found, this conservation method is called ex situ conservation This method facilitates access to germplasm for evaluation and use (Salgotra and Chauhan 2023) [52].

One of the simplest methods to maintain more plant germplasm is seed storage. In this method, seeds are reserved as active (medium-term) or base (long-term) collec- tions. In an active collection, the seeds remain above 65% for 10–20 years, while in a base collection, the seeds are stored at -20 ° C and can survive for a long time (more than 50 years) (Salgotra and Chauhan 2023) [52].

Another method of ex situ conservation is botanical gardens. Botanical gardens contain live plants that grow in greenhouses and gardens. They are used for growing and showing plants generally for scientific and educational aims. Currently, there are more than 2000 botanical gardens that keep 80,000 plant species in their living collections (Brütting *et al*. 2013) [9]. In botanical gardens, plants can grow in relatively improved environmental media (intensive cultivation, respectively high fertility, and high levels of disturbance) (Brütting *et al*. 2013) [9].

Effective conservation requires the use of different strategies; also, the appropriate conservation method varies from species to species. Therefore, in addition to the method of seed bank and botanical garden, the use of new methods such as *in vitro* culture is also necessary for conservation. It is an effective method for the ex-situ preservation of species of medicinal and economically important plants (Fay 1994) [23]. Tissues such as meristems, axillary buds, and zygote embryos are used for tissue culture. Adventitious buds and somatic embryos derived from leaves, stems, roots, or calluses are also used, but these undifferentiated tissues should be conserved with caution because of their potential for soma clonal diversity (Engels *et al*. 2006) [22]. *In vitro* methods for the conservation and use of the plant germplasm can have various advantages and disadvantages.

Plant Tissue Culture

One of the newest and most efficient methods for the reproduction and preservation of important plant species is the use of plant tissue culture. In addition to being used as research tools, these methods have been used industrially in recent years for plant propagation, production of disease-free plants, plant breeding, and synthesis of secondary metabolite. A major advantage of plant tissue culture is that hundreds and many plants are produced from one plant in a continuous process using small pieces of tissue (called explants). Also, a single explant can be propagated out of season and regardless of the weather throughout the year in a short period and placed under controlled conditions (Idowu *et al*. 2009) [32].

In vitro culture is a very effective instrumentation for long-term germplasm preservation in controlled and contaminated conditions (Hussain *et al*. 2012) [31]. The tissue culture method is used successfully to conserve endangered species, especially for plants that are not easily propagated by conventional propagation methods. Using plant tissue culture methods, genotypes with high reproduction rates can be multiplied by creating a controlled growth environment. Because in this system the explants are small, it is possible to reduce the space requirement and thus reduce labour costs to maintain germplasm.

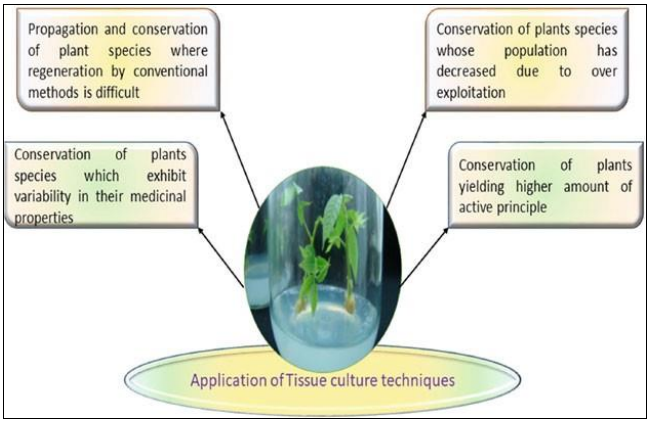

Fig 2: Different utilizations of tissue culture in medicinal plant conservation (Kadam and Pawar 2020)

Plant materials can be conserved in the *in vitro* conditions using slow-growing media at normal room temperature for a longer duration or by cryopreservation methods (Sudhersan *et al*. 2003). Due to the high propagation coefficient and low need for the number of primary plants and space, so far, many species of endangered plants have been easily grown and conserved via micropropagation (Oseni *et al*. 2018) [46]. Tissue culture techniques are used for various plants (Fig. 2). Various factors affect the success of tissue culture in plants (Fig. 2). One of the effective factors in tissue culture is the culture medium. The presence of all nutrients required for the natural growth and development of plants, including macronutrients, micronutrients, vitamins, other organic compounds, carbon sources, plant growth regulators, and some gelling agents (in the solid media), is required in the plant culture medium. The most widely used medium for vegetative micropropagation of most plant species is Murashige and Skoog (MS). Another important factor is the pH of the medium, which affects plant growth. Also, the activity of plant growth regulators is usually adjusted from 5.4 to 5.8. There are two types of culture medium: solid culture medium and liquid culture medium. The composition of the medium, especially plant growth regulators and the nitrogen source, has deep impacts on the initial explant reaction (Oseni *et al*. 2018) [46]. Plant growth regulators or phytohormones of culture medium are one of the main agents in regulating the growth of cells and plant tissues. The most common growth regulators include auxins, cytokinins, and gibberellins.

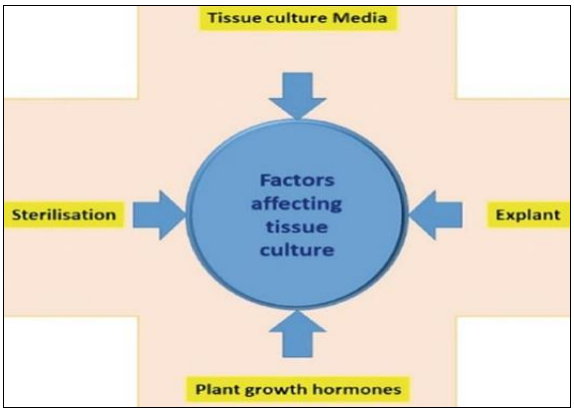

Fig 3: Various factors affecting plant tissue culture

Medicinal Plant Conservation by Tissue Culture

Medium-Term Conservation Through Slow Growth Method

The slow-growing storage method is used commercially in laboratories to preserve storage cultures at low temperatures (Moriguchi and Yamaki 1988) [45]. Slow-growing storage (also known as medium-term protection) can be a storage method by reducing metabolic activity. In this method, the growth rate of laboratory cultures is reduced by keeping them in a modified growth medium, and they can be stored in this condition for a long time, from a few months to 1 year or more, depending on the species (Lambardi and Ozudogru 2011) [41].

Generally, there is an observed decrease in the growth of *in vitro* cultures when the culture medium and/or culture conditions were changed (Engelmann 1998, 2004) [20]. Lowering the temperature is one of the most widely used methods of changing the culture medium, which can usually be accompanied by a reduction in light intensity (Engelmann 2011) [21].

Also, reducing the growth of *in vitro* cultures through the use of minimal media and growth retardant, resulting in longer distances between subcultures, can lead to medium-term plant protection. A variety of plant species, such as endangered plants, of tropical as well as temperate origin can be protected through medium-term conservation strategies (Chauhan *et al*. 2019). The use of osmotic regulators, such as sucrose and mannitol, by causing osmotic stress to the substances under protec- tion, reduces the water potential, limits the access of water to the explants, and acts as an inhibitory factor for plant growth. In addition, growth regulators are also com- monly applied to preserve laboratory germplasm, of which ABA is one of the most widely utilized (Oseni *et al*. 2018) [46].

Currently, slow-growing cultivation is used for a variety of commercial purposes including crop production, storing plant material for future needs, and when there is a shortage of labor for transplanting and limited space (Kozai *et al*. 1997). This method is also used for conservation projects, where the storage of seed-derived plants ensures the preservation of the genetic variability of the species (Pence 2010) [47]. For the protection of endangered species, this method is more economical and valuable due to the reduction of the number of crops and their long-term storage (de Carvalho *et al*. 2014) [10].

The results showed that Bacopa monnieri (Thakur *et al*.) Pennell stem tips, nodal sections, and multiple-shoot clumps could be grown on MS media with 20 g l-1 sucrose and free of growth regulator, in culture tubes with polypropylene caps, or in culture bottles conserved for more than 20 months. After storage, the plants were propagated well in MS media containing 0.5 mg l-1 N6-benzyladenine. Using MS medium without a growth regulator, stem growth and rooting were achieved, and rooted plants with 100% establishment were successfully transferred to the soil. A comparison of chemical profiles of mother plants and protected plants *in vitro* (after field establishment) using HPLC showed the similarities of chemical profiles (George *et al*. 2007) [24]. In another research, the conservation of B. monnieri under tissue culture conditions was performed using a single-node explanton MS media with 0.2 mg l-1 BA. Regenerated shoots were maintained for 12 months with up to a 100% survival rate. These plants did not show any morphological changes compared to the native plants after transfer to soil (Sharma *et al*. 2007).

The lack of carbon source in the culture medium reduced the cell and shoot proliferation of Capparis spinosa which was able to survive 17 weeks in the control treatment. After 17 weeks, the survival of C. spinosa seedlings with 3% sorbitol was 100%, if stored in light conditions (Al-Mahmood *et al*. 2012) [3]. An effective protocol for maintaining the shoot culture of B. monnieri under slow-growth conditions was presented. The results showed that multiple-shoot clumps survived up to 20 months in half-strength MS medium with 20 g l-1 sucrose in polypropylene cap culture bottles with 90% viability. Also, these plants were successfully planted in the soil (George *et al*. 2007) [24].

Recently, in research on B. monnieri, explants of shoot tips and nodal segments, in combination with a mineral oil coating, were used for *in vitro* regeneration and effective medium-term preservation. For medium-term protection, a standard propagation medium MS containing 0.2 mg l-1 benzyladenine (BA) was used. The results showed that mineral oil coating led to a very significant reduction in growth and increased survival of cultures. After 12 months of protection *in vitro*, nodal segment explants encapsulated in combination with mineral oil coating showed the highest survival rate (91.6 ± 5.7%) and the number of branches (21.4 ± 1.6) (Sharma *et al*. 2020).

In vitro plantlets and *in vitro* plantlets without tips of C. spinosa were success- fully stored using an MS culture medium with an appropriate concentration of osmoticum (sucrose, sorbitol, mannitol, or glucose) at different concentrations (0, 3, 6, 9, or 12%) at 24 ± 2 °C, for up to 17 weeks. In addition, conservation under light was found to be more beneficial than in dark (Hana *et al*. 2012) [30]. In another research, the basic medium dilution method was used to protect Hypericum perforatum L. In this experiment, sterile shoots were cultivated in MS media with reduced macronutrients. Investigating the growth rate of the plant after 3 months indicated that the use of dilution of the base media affects the growth of H. perforatum. The application of ¼ MS + 0.1 mg l-1 BA combination can suppress the growth of the culture without showing symptoms of necrosis up to 3 months after storage (Syahid 2021).

Slow-growth conditions conserved the nodal parts of Nyctanthes arbor-tristis L. for up to 180 days in one-eighth strength MS medium with 0.5% sucrose with a satisfactory survival percentage (40.28 ± 2.04%). It was also found that subsequent adding of 0.5 mg l-1 of ABA had a positive effect on maintenance and increased the viability of nodal parts by 40.36 ± 1.01% (Mishra *et al*. 2022) [43]. The explant of Ruta graveolens was conserved without serious damage on MS media by various levels of sucrose, glucose, fructose, or sorbitol at a temperature of 24 ± 2 °C for up to 16 weeks (Al Shhab *et al*. 2021). Stevia rebaudiana microshoots were successfully stored for 16 weeks on MS medium containing sucrose, sorbitol, or mannitol, at a temperature of 24 ± 2 °C, with a survival rate of 93.6% after 32 weeks, and 89.3% were able to grow again (Shatnawi *et al*. 2011).

Stem tips and nodal segments obtained from *in vitro* propagated shoots of Silene schimperiana were encapsulated in calcium alginate beads for medium-term pres- ervation. The encapsulated plants were kept for 5 months at 4 °C with 100% survival. The most efficient gel complex to form uniform beads was 3% Na-alginate and 100 mM

CaCl2·2H2O. About 90% of regenerated seedlings were successfully adapted to greenhouse conditions (Ghareb 2021) [27]. Utilization of man- nitol or sorbitol as osmoticum and reducing sucrose to 1.5% (w/v) in 1/2 MS media for tissue cultures of Asparagus racemosus shoots conserved these plants for 6 months at 25 ± 2 °C without subculture. Surviving shoots from slow-growing cultures were regenerated with 100% efficiency (Thakur *et al*. 2015). 1/2 MS culture medium with 3% sorbitol and 2% sucrose was selected as the best medium for storage with slow growth of Arnica montana cultures, which effectively delayed the growth of seedlings, thus conserved 6 months without subculture (Petrova *et al*. 2021) [48]. Using nodule explants cultured on MS media with mannitol, sorbitol, and ABA and on 1.2 MS stored at 15 ± 2 and 26 ± 2°, Spilanthes acmella grows slowly under *in vitro* conditions. The maximum percentage of survival was obtained in MS + 2% mannitol at a temperature of 15 ± 2 °C at the end of 8 months. Mannitol 2% and sorbitol 2% were effective in slowing the growth of the cultures in terms of the number and length of the stem (Joshi and Jadhav 2013) [35]. Other researchers showed that 20 g l-1 of mannitol was suitable for maintaining the slow growth of Glycyrrhiza glabra with only one subculture per year. Shoots showed 100% survival and full recovery after protection. Modified 1/2 MS media contain 0.25 mg l-1 BA, 1.0 mg l-1 IAA, and 10 mg l-1 B-9 along with 10 mg l-1 AdS for shoot growth and leaf growth as well as root growth (Srivastava *et al*. 2013).

Long-Term Conservation Through Cryopreservation

For long-term preservation of plant species, cryopreservation (storing in liquid nitrogen at -196 °C) is usually used. It is a conservation method for the long-term preservation of plant genetic resources safely and cost-effectively. It was previously thought that tropical plant tissues could not be cryopreserved, but using these cryopreservation methods, these tissues were also successfully conserved in liquid nitrogen (LN) (Oseni *et al*. 2018) [46].

This method enables the indefinite storage of endangered plants and ensures the survival of specimens after thawing. In the cryopreservation method, all freezable water is removed from tissues using physical or osmotic dehydration followed by ultrafast freezing. The metabolic and biochemical reactions of the cell are stopped due to a large decrease in temperature, thus preventing the formation of intracellular ice crystals that can cause cell death and destruction of cell organelles during the freezing process.

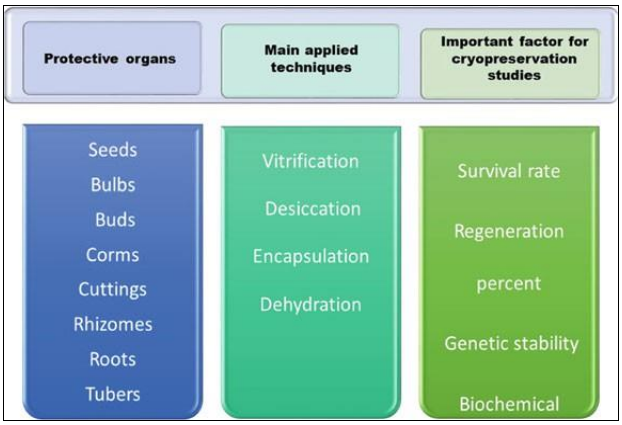

Fig 4: Cryopreservation protective organ, techniques, and evaluation factors

Currently, one of the most innovative and cost-effective biotech- nological approaches is the use of cryopreservation, which allows long-term safe protection of plant biodiversity without the risk of genetic changes (Benelli 2021) [7]. Since the 1970s, research on cryopreservation techniques using various plant organs, tissues, and cells has begun, and so far, various cryopreservation methods have been introduced and developed (e.g., slow pre-freeze method, vitrification method, dehydration method) (Oseni *et al*. 2018) [46]. The following figure briefly shows the plant organs and the methods used and the attributes investigated in preservation by the freezing cryopreservation (Fig.).

In research, the tips of Picrorhiza kurroa were cryopreserved. Shoot tips from 4-week-old propagated cultures were pre-cultured on MS medium before dehydrating using PVS2 solution at 0 °C. Dehydrated shoot tips were immediately swallowed in liquid nitrogen (LN). The average survival in terms of normal stem formation after 4 weeks of plating was about 20% without callus formation (Sharma and Sharma 2003). *In vitro*, cryopreservation of shoot tips of Dioscorea deltoidea was done success- fully. Organ regeneration after being placed in liquid nitrogen (LN) up to 83%, and by encapsulation-dehydration, the most regeneration frequency recorded was 76% (Mandal and Dixit-Sharma 2007) [41]. In another study, Hypericum perforatum L. middle and basal root sections successfully cryopreserved and froze at 10 days after isolation indicate the highest plant regrowth after freezing (Yang *et al*. 2019). A cryopreservation protocol was developed for Valeriana jatamansi Jones, a medicinal plant of high commercial value in the Himalayan region. Cryopreserva- tion of shoot tips with PVS2 at 0 °C for 110 min showed the highest shoot recovery (91.6%) after transfer to LN (Sharma *et al*. 2021). The shoot tips of Dioscorea deltoidea were frozen using the vitrification and encapsulation-dehydration method. The results of HPLC analysis showed that the diosgenin content of regenerated plants from frozen shoot tips was similar to control plants (Dixit-Sharma *et al*. 2005) [18]. Overnight pre-culturing of isolated Kaempferia galanga L. shoot tips in MS media with 0.4 M sucrose, osmotic protection for 20 min, and dehydration with PVS2 for 20 min at 0 °C was optimal. Cryoprotected shoots gave 50–60% survival and 30–40% regeneration rates after flash freezing in LN followed by rapid thawing. The incorporation of GA3 (gibberellic acid) into the culture medium after thawing was necessary to induce shoot greening in the early phase (Preetha *et al*. 2013) [49]. After cryopreservation of Atractylodes macrocephala, survival and growth rates of 76% and 62% were reported (Zhang *et al*. 2015). Seeds of Satureja bachtiarica were treated at -196 °C and transferred to LN and stored for 90 days. According to the results, growth indices (germination percentage, germination rate) were not affected by liquid nitrogen (Ghaffarzadeh-Namazi *et al*. 2015) [25]. Also, cryopreservation with PVS3 is recommended as the most appropriate protocol for Satureja spicigera callus freezing (Ghaffarzadeh-Namazi *et al*. 2017) [26]. Embryogenic calli from *in vitro* grown tillers of Anemarrhena asphodeloides Bunge were successfully cryopreserved by the cryo-encapsulation method. Survival of frozen embryogenic calli, including after storage, reached 80%. The plants obtained from control and frozen embryogenic callus did not show any significant difference in morphological growth (Sen-Rong and Ming-Hua 2012).

Encapsulation-vitrification is a combination of two methods of encapsulation- dehydration and vitrification. Explants are encapsulated in alginate beads and then incubated in LS and/or PVS and exposed to liquid nitrogen (Gantait *et al*. 2017). This system has gained popularity because it is a rapid method that is physically and chemically harmless. This method is more effective than conventional cryopreservation systems and has a larger utilization for germplasm preservation because it is effective for smaller shoot tips and protocorm-like bodies of medicinal orchids (small embryo-like tissues that can potentially develop into whole plants) (Kundu *et al*. 2018).

As a result, the utilization of tissue culture techniques in the protection of rare and threatened medicinal plant species can lead to the preservation of natural germplasms and protection against natural calamities that cause the loss of wild species and thus reduce biodiversity and affect the ecosystem (Oseni *et al*. 2018) [46].

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