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Cryopreservation: Preservation in a Frozen State

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ARTICLE ID: 12

Introduction:

Cryopreservation, from the Greek word Kryos (meaning frost), refers to the process of preserving biological material by freezing it at extremely low temperatures. This method is widely used to store plant cells, tissues, and organs, keeping them viable for future use by halting all metabolic activities.

Common Storage Temperatures:

- Solid carbon dioxide: –79°C
- Low-temperature deep freezers: –80°C or below
- Vapor phase of nitrogen: –150°C
- Liquid nitrogen: –196°C (commonly used for plant material)

At these temperatures, cellular activity stops completely, preserving the biological material in an inactive state.

Importance Of Cryopreservation:

- **Conservation of Genetic Material:** Cryopreservation is extensively used for the long-term storage of plant genetic resources. It allows the preservation of rare, endangered, or valuable plant species without risking genetic degradation over time.
- **Freeze Storage of Cell Cultures:** Cell cultures can be cryopreserved for extended periods, maintaining their viability and functionality for future research, breeding, or biotechnological applications. This is particularly useful for plant breeding programs and genetic research.
- **Maintenance of Disease-Free Stocks:** Cryopreservation helps in storing plant tissues free from pathogens, ensuring that disease-free plants can be regenerated whenever needed.
- **Cold Acclimation and Frost Resistance:** Cryopreservation techniques are also employed in studying cold acclimation and enhancing frost resistance in plants. By exposing plant

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tissues to controlled freezing environments, researchers can develop cold-hardy varieties that can better withstand harsh climates.

Key Considerations for Cryopreservation:

- **Freeze Tolerance:** The plant's ability to survive freezing temperatures, varies by genotype.
- **Ice Crystal Formation:** The potential damage caused by ice crystals forming within the cells.

Steps Involved in Cryopreservation:

- **Raising Sterile Tissue Cultures**: Various types of plant tissues can be used for cryopreservation, including apical and lateral meristems, organs such as embryos, endosperm, ovules, and anthers or pollen, as well as seeds, cultured cells, somatic embryos, protoplasts, and calluses. Callus tissues, particularly in tropical species, tend to be more resistant to freezing damage, making them an ideal choice. The best time for cryopreservation is during the rapid growth phase of the callus, typically one or two weeks after transferring to a growth medium. The water content in cells or tissues must be low, as this helps them tolerate extremely low temperatures by minimizing the amount of freezable water.
- **Addition of Cryoprotectants and Pretreatment:** Cryoprotectants are substances that protect plant tissues from damage caused by freezing. The following are some pretreatment methods used for desiccation before freezing:
  - **Air Desiccation-Freezing:** Seeds are dried in desiccators with silica gel for 4 to 48 hours before freezing in liquid nitrogen.
  - **Pre-growth Desiccation:** Explants like zygotic and somatic embryos are pre-grown on cryoprotectant media to enhance desiccation tolerance.
  - **Encapsulation-Dehydration:** Explants are suspended in a 3–5% sodium alginate solution and encapsulated in a 100 mM calcium chloride solution.
  - **Vitrification:** Tissues are treated with cryoprotectants in a vitrification solution (e.g., PVS2) and rapidly frozen.
  - **Addition of Cryoprotectants:** Tissues are treated with DMSO (5–10%) and glycerol (10–20%) for freezing at –196°C; combinations may be used for improved survival.

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Freezing:

The type of ice crystals formed within plant cells during freezing is crucial for the survival of the tissue. To address this, three different freezing techniques have been developed:

- **Rapid Freezing:** Plant tissues are placed in vials and quickly immersed in liquid nitrogen, cooling at rates of –300°C to –1000°C per minute. This straightforward method has been effective for cryopreserving shoot tips of carnation, potato, strawberry, Brassica napus, and somatic embryos.
- **Slow Freezing:** Tissues are gradually cooled at rates of 0.1°C to 10°C per minute from 0°C to –100°C before liquid nitrogen immersion. This method reduces intracellular ice formation through slow dehydration and is often used with computer-controlled freezers. Successful examples include meristems from peas, strawberries, potatoes, and cassava.
- **Stepwise Freezing:** This technique combines rapid and slow freezing by initially cooling tissues to –20°C to –40°C, holding for 30 minutes, and then rapidly freezing to –196°C in liquid nitrogen. This approach allows for cellular dehydration and minimizes large ice crystal formation, yielding excellent results with strawberries and suspension cultures.

Storage:

Once frozen, plant cells or tissues are stored at temperatures ranging from –70°C to –196°C. The ideal storage conditions include liquid nitrogen freezers operating at –150°C (vapor phase) or –196°C (liquid phase). For long-term storage, –196°C is optimal, as it completely halts all metabolic activity and prevents biochemical damage. As long as there is a steady supply of liquid nitrogen, the frozen material can be maintained with minimal care. Freezing or storage injuries can occur if cells are not stored at sufficiently low temperatures.

Thawing:

Thawing is the process of bringing cryopreserved material back to its original state. Typically, this is done by placing the ampoules containing the samples in a warm water bath (35°C to 45°C). The ampoules are swirled gently in the warm water until the ice has melted, but care must be taken not to leave the samples in the bath for too long to avoid damaging the delicate thawed cells. Minimizing handling during the pre-growth phase is critical to preserving the integrity of the cells or tissues.

Determination of Survival/Viability:

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The only definitive test for determining the survival of cryopreserved plant materials is observing the re-growth of plants from the stored tissues or cells. However, cell viability can be assessed at various stages through different techniques. Fluorescein diacetate (FDA) staining, along with growth measurements based on parameters like cell count, packed cell volume, fresh and dry weight, and mitotic index, can be used. Additional staining methods include:

- TTC Staining: In this method, cell survival is determined by the amount of formazan produced during the reduction of triphenyl tetrazolium chloride (TTC), which produces a pink colour as an indicator of viability.
- Evan's Blue Staining: A drop of 0.1% Evan's blue solution is added to a drop of cell suspension on a slide and examined under a microscope. Living cells remain unstained, while dead cells take on a blue colour.

Plant Growth and Regeneration:

Certain additives in the growth media can improve the survival and regeneration of frozen tissues. For instance, gibberellic acid (GA3) has been found to enhance the survival of freeze-preserved shoot tips of tomatoes, while activated charcoal has shown beneficial effects for carrot regeneration.

Limitations of Cryopreservation:

- **High Skill Requirement:** Specialized expertise is needed to handle and maintain cultures effectively.
- **Sophisticated Facilities Needed:** Advanced, expensive equipment like liquid nitrogen freezers and sterile environments are often necessary.
- **Potential Genetic Instability:** Cryopreservation may lead to genetic instability, affecting the quality of regenerated material.
- **Cell/Tissue Damage:** Freezing, thawing, and storage can damage cells or tissues, primarily due to ice crystal formation and dehydration.
- **Large Storage Space Requirement:** Significant space is needed for slow-growth cultures, which can hinder large-scale projects.

Conclusion:

Cryopreservation is a technique used to preserve biological materials at extremely low temperatures, stopping all metabolic activity. It is essential for conserving genetic material, maintaining disease-free stocks, and supporting research in plant breeding and biotechnology.

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Despite its benefits, it requires specialized skills and equipment, with challenges like ice crystal formation and genetic instability. When managed well, it ensures the long-term viability of plant material.