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SOP.12. *IN VITRO* CONSERVATION OF PLANT GENETIC RESOURCES

1. Purpose

The application of *in vitro* culture methodologies for the conservation of vegetatively propagated species.

2. Field of application

Plant Genetic Resources Bank of Suceava.

3. Definitions, Abbreviations, and Terms - Selection

- **In vitro conservation:** Methods of preserving the integrity, vitality, and viability of biological material under aseptic conditions and artificial environments for short or medium durations, adhering to specific processing and storage conditions.
- **Aseptic (sterile or axenic):** Free of microorganisms (bacteria, fungi, viruses, mycoplasmas).
- **Explant:** A fragment from an organism (apex, organ, or organ fragment) detached from its original location, serving as the piece from which a culture will be initiated.
- **Inoculum:** Explant introduced into a culture medium prepared for this purpose.
- **Inoculation:** The operation of placing an organism, fragment, callus, cells, or portion of a culture into or on a nutrient medium.
- **In vitro:** ("in glass") Refers to processes occurring under sterile culture conditions using glass or plastic containers.
- **In vivo:** ("in life") Refers to processes occurring in an organism under normal living conditions.
- **Micropropagation:** A procedure for asexual or vegetative multiplication of plants *in vitro*.
- **Sterilization:** The process of eliminating microorganisms.
- **Subculture:** A procedure where the inoculum, or its fragments, is placed onto a fresh culture medium under aseptic conditions.
- **Vitroplantlet:** A plantlet generated and grown *in vitro*.

4. Procedure Description


a. Sterilization of Culture Vessels

Pre-sterilization of glassware is performed in dry heat ovens at 160°C for a minimum of 3 hours.

Glass objects and instruments are wrapped in paper or aluminum foil and placed on oven racks.

To prevent post-sterilization infections, proper handling of containers is ensured during transport from the autoclave to the sterile hood.

Asepsis for equipment placed in the sterile hood and operator's hands is ensured using 70% ethanol.

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b. Preparation of Culture Media

Culture media consist of:

- Inorganic substances: Macronutrients, micronutrients, and chelated iron (FeEDTA).
 - Organic substances: Vitamins, amino acids, carbohydrates, hormones, and a gelling agent.
- Two methods are used to prepare culture media:

- Using a powdered mixture of Murashige-Skoog medium (containing macro and micronutrients and vitamins) supplied by specialized companies, with the addition of other necessary components according to the recipe.
- Using stock solutions of concentrated media components (macro/micronutrients, FeEDTA, vitamins, growth regulators) diluted as needed according to the selected recipe.

General rules for stock solution preparation:

- Dissolve substances in double-distilled water.
- Dissolve each inorganic compound in a proportional amount of water, ensuring optical homogeneity, before mixing solutions in the order specified in the recipe.
- For substances with low solubility, gentle heating on a steam bath may be applied.
- Growth regulators are dissolved individually, following the manufacturer's instructions.
- Stored refrigerated or frozen.

c. Sterilization of Media and Water

Media are portioned into culture containers, sealed with aluminum foil, and sterilized to prevent fermentation.

Sterile distilled water is prepared in Erlenmeyer flasks, flat-bottom flasks, or jars with lids.

Media and distilled water are sterilized via autoclaving at 120°C (1 atmosphere pressure) for a minimum of 15 minutes.

Inoculations are performed only after the culture media have cooled and solidified.

d. Aseptic Treatment of Plant Material

Plant material used for explants is aseptically treated with various substances, based on the biological material's fragility and the disinfectant's penetration depth.

Tween 80 is added to reduce surface tension.

Disinfectants must be strong enough to destroy surface microorganisms but easily removable with sterile distilled water.

Routine disinfectants ensure surface sterilization, but deeper tissues may still harbour bacteria, viruses, or mycoplasmas. Rigorous phytopathological control of the source material is conducted to reduce such risks.

For potato, pre-sprouted tuber buds are disinfected with 70% ethanol for 1 minute, followed by 2–3 rinses with sterile distilled water.

e. Inoculations and Subcultures in the Hood

Inoculation involves placing aseptic biological material into sterile containers with culture media using sterilized instruments.

Instruments (e.g., forceps, spatulated needles, scalpels) are stainless steel and long enough to access the culture medium easily.

The laminar flow hood is sterilized with UV light for 20–30 minutes before use and then ventilated for another 20–30 minutes to ensure aseptic conditions.

Instruments are sterilized after each use in a glass bead sterilizer (250–300°C, for 2–3 minutes).

Inoculation Process:

The shoot tip, plantlet, or explant is held with sterilized forceps, and meristems, micro-cuttings, or other inocula are introduced into the medium.

The container is sealed with sterile polyethylene film and secured with rubber bands.

The success of the culture is determined by the ability to perform subcultures.

Subculture

Secondary cultures are initiated by transferring fragments from an old culture onto fresh medium.

Subcultures are performed to maintain division, regeneration, and organogenesis or to restore these capabilities if cells enter senescence.

The frequency of subcultures depends on inoculum development, growth conditions, and medium composition.

- f. Vial labelling includes:** inoculation date, access number, subculture number, and medium type.
- g. Transfer of cultures to the growth chamber:** After inoculation, the vitrocultures are transferred to the climate-controlled growth chamber to resume the growth and development processes. The vials are placed on shelves, illuminated in a preset, adjustable and automated mode, at a light intensity of 2500 lx, a period of 16 hours of light/24 hours, temperature of 20 – 22°C, optimal conditions necessary for the good development of the cultures.
- h. Monitoring and Conservation**

Cultures are monitored daily during the first week and every 3–5 days afterward for contamination or necrosis.

Plantlets are transferred to a conservation chamber for *slow-growth* storage, with reduced temperature (6°C in darkness and 12°C in light), and light intensity (1000–1500 lx), generated by LED tubes.
- i. Acclimatization**

Vitroplantlets are transitioned to *in vivo* conditions gradually to overcome environmental shocks.

For potatoes, mini-tubers are transplanted directly into greenhouse soil. Garlic bulbils or plantlets are carefully washed to remove agar medium and replanted in biodegradable pots filled with a soil-perlite mix (3:1).