Chapter 2

Laboratory Requirements And General Techniques

2.1. INTRODUCTION

The size of a tissue culture set-up and the extent to which it is equipped are governed by the nature of the project undertaken and the funds available. However, a standard tissue-culture laboratory should provide facilities for: (a) washing and storage of glassware, plasticware and other labwares, (b) preparation, sterilization and storage of nutrient media, (c) aseptic manipulation of plant material, (d) maintenance of cultures under controlled conditions of temperature, light and, if possible, humidity, (e) observation of cultures, and (f) acclimatization of in vitro developed plants. For research work at least two separate laboratories or rooms should be available; one for glassware washing and storage, and media preparation (media room), and a second (growth/culture room) to store cultures. The culture room should contain a culture observation table provided with binoculars and an adequate light source. Depending on the local conditions, the sterile transfer cabinets may be housed in the culture room, in a quiet corner of an ordinary research laboratory, or a specially designed transfer room. A separate balance room may be shared with other laboratories. For a commercial set-up, a more elaborate set-up is required.

For other reviews on the subject, see De Fossard (1976), Biondi and Thorpe (1981), Bridgen and Bartok (1987), Pierik (1987), Torres (1989) and Mageau (1991).

2.2. REQUIREMENTS

2.2.1. Structures and utilities

Very often, a research or commercial laboratory is required to be set up in already existing structures; few can construct facilities from the ground up. In either case certain basic guidelines should be followed. If a new laboratory is being constructed, its location should preferably be away from the city or otherwise adequate precautions should be taken to

protect the facility from heavy pollution and vehicular vibrations. Care should also be taken to locate it away from fields where combines or threshers are used in order to cut down contamination spurts during the harvest season. Preferably, the facility should be protected from any onslaught of heavy winds and rain which are carriers of spores, mites and thrips. The growth room and the transfer room should be adequately insulated to conserve energy. This has been achieved in some cases by trapping air between a double wall construction. During a hot season, advantage could be had by venting the air between the two walls.

A tissue culture facility requires large quantities of good quality water and provision for waste water disposal. This aspect requires special consideration where public water and sewer facilities are not available. Disposal of any waste is also governed by local municipal codes for health and the environment.

A generator back up should be provided, at least to the transfer room, growth room and other essential equipment to prevent shut-down of transfer hoods during the operation and an abrupt change in temperature in the growth room due to power failures, which could happen even where a reliable source of electricity is available.

The organization for a commercial tissue culture set-up has been described, with diagrams, by several authors (Torres, 1989; Mageau, 1991). These should be treated as guidelines because the size and design of a facility would be governed by the shape and size of the land available and the proposed capacity of the company.

A single level structure, providing easy access to various work areas, is preferable to facilitate the frequent movement of materials between areas. The layout of the rooms, their pass-through windows, doors and hallways must allow a work flow pattern that maintains maximum cleanliness and promotes minimal backtracking. A clearcut demarcation of the unclean (washing room, medium preparation room, autoclaving room, general store, offices) and clean area (transfer room(s) and growth room(s)) should be made. Entry into the clean area should be restricted and generally through a passage where the workers must take-off shoes, wash hands and feet, change outer clothes, and wear headgear and slippers provided inside. Commercial laboratories should, as a rule, maintain a positive air pressure, if not in the whole building, at least in the clean area. These precautions are mandatory to deter the introduction of microorganisms into the culture vessels. Movement of material in (sterilized medium, instruments, water, etc.) and out (glassware and other things for washing and sterilization, tissue culture produced plants for hardening, etc.) of the clean areas through double door hatches should help in maintaining higher asepsis in the clean area.

2.2.2. Media room

The washing area in the media room should be provided with brushes of various sizes and shapes, a washing machine (if possible), a large sink (preferably lead-lined to resist acids and alkalis) and running hot and cold water. It should also have steel or plastic buckets to soak the labware to be washed, ovens or a hot-air cabinet to dry the washed labware and a dust-proof cupboard to store them. When the preparation of the medium and washing of the labware are done in the same room, as in many research laboratories, a temporary partition can be erected between the two areas to guard against the danger of soap solution splashing into the medium and any other interference in the two activities. If this is not possible, the washing time should be so arranged that it does not overlap with media preparation. An industrial dishwasher may be useful for a commercial set-up.

A good supply of water is a must for media preparation and final washing of glassware. Since tap water cannot be used for preparing medium, provision must be made to purify water. De-ionized water may be used for teaching laboratories but for research and commercial purposes, water distillation apparatus, a reverse osmosis unit or milli-Q water purification systems need to be installed. For a research laboratory, a glass distillation unit with a handling capacity of 1.5–2 l h^{-1} of water should be sufficient. For commercial houses, a Milli-Q purification system (Millipore Co., USA), which can provide 90 l h^{-1} of purified water, free of organic impurities, ionic contaminants, colloids, pyrogens, and traces of particles and micro-organism, may be used. Proper storage tanks should be installed to store purified water. For further details on water purification and storage refer to Gabler et al. (1983) and Callaghan (1988).

The usual facilities required for the preparation of culture medium include: (a) benches at a height suitable to work while standing, (b) a deep freeze for storing the stock solutions, enzyme solutions, coconut milk, etc., (c) a refrigerator to store various chemicals, plant materials, short-term storage of stock solutions, etc. (d) plastic carboys for storing distilled water, (e) weighing balance(s), (f) a hot plate-cum-magnetic stirrer for dissolving chemicals, (g) a pH meter, (h) an aspirator or vacuum pump to facilitate filter-sterilization, (i) a steamer for melting agar, and (j) an autoclave or a domestic pressure cooker for media sterilization. Of these items, a refrigerator and deep freeze may be kept in a corridor or another laboratory close to the media room. Use of weighing balances in the media room should be avoided. Alternatively, a small weighing chamber may be provided in a comparatively dry corner of the media room.

2.2.3. Culture vessels

Different types of vessels have been used to culture plant materials. While in some cases the choice of culture vials is dictated by the nature of the experiment, in others it has been guided mainly by the convenience and preference of the worker. For standard tissue and organ culture work, glass test tubes have been widely employed. Wide-mouth glass bottles of different sizes and sometimes even milk bottles have been used, especially for micropropagation. In tissue culture work only borosilicate or Pyrex glassware should be used. Soda glass may be toxic to some tissues, especially with repeated use (De Fossard, 1976).

In many laboratories the glass culture vials and other labware required for media preparation have been largely replaced by suitable plasticware. Some of the plastics are autoclavable. A wide range of presterilized, disposable culture vials (made of clear plastic), especially designed for protoplast, cell, tissue and organ culture work are now available in the market under different brands. These are becoming increasingly popular with those who can afford them.

Disposable plastic culture vials (petri-dishes, jars, bottles, various cell culture plates) and screw-cap glass bottles are supplied with suitable closures. For culture tubes and flasks, traditionally cotton plugs, sometimes wrapped in cheese-cloth, have been used. However, if the use of such stoppers is found time consuming and inconvenient, a wide choice of alternative closures exists. A number of plastic (polypropylene) and metallic (aluminium and stainless steel) cap closures are available. Transparent, autoclavable, polypropylene caps with a membrane built into the top, produced by KimKaps (Kimble, Division of Ownes, IL), are claimed to be very effective in preventing moisture loss from tubes. Local availability and cost influence the selection of a culture tube closure. However, it is important to ensure that the closure does not inhibit the growth of the cultured plant materials.

With a better understanding of the role a culture vessel plays in the growth and developmental behaviour of plant tissues enclosed in them has resulted in the development of culture vessels made of different synthetic materials. It is possible to buy vessels made of polypropylene which transmits about 65% light and those made of polycarbonate which transmits almost 100% light. Gas permeable fluorocarbonate vessels have been used in experiments with plant materials sensitive to gaseous build up within the culture vials (Kozai, 1991a). Osmotek Ltd., Israel, has introduced repeatedly autoclavable, polypropylene 'liferafts', provided with interfacial membrane and floats to culture plant materials in liquid medium without submerging them. The membrane is treated with

a surfactant to make it hydrophilic. The surfactant is removed during cleaning, and must be reapplied prior to the next use. These rafts are available in different sizes to fit culture tubes, magenta boxes and round jars. Osmotek Ltd. is also producing vented polypropylene lids which ensures better gas exchange in plant tissue cultures, thereby reducing the hyperhydration problem. The vent is covered with a membrane with 0.3 μm pores.

2.2.4. Growth room

The room for incubating cultures is maintained at a controlled temperature. Usually air-conditioners and heaters, attached to a temperature controller, are used to maintain the temperature around 25 ± 2°C. For higher or lower temperature treatments, special incubators with built-in fluorescent lights can be used. These may be installed even outside the culture room, in the corridor or in any other laboratory. However, when kept in the corridor, precautions must be taken to avoid the risk of people tampering with the adjustment knobs. In commercial companies which have more than one growth room, it may be possible to maintain different growth conditions in different rooms. Since cleanliness is paramount in this area, enough care should be taken to prevent any direct contact with the outside. The paint on the walls and the flooring should be able to withstand repeated cleaning. Desirably, the junction of the walls should be rounded rather than angular to prevent cob webs.

Cultures are generally grown in diffuse light (less than 1 klx). Some provision should also be made for maintaining cultures under higher light intensities (5–10 klx), and total darkness. Diurnal control of illumination of the lamps (fluorescent tubes) can be achieved by using automatic time-clocks.

If the relative humidity in the culture room falls below 50%, provision to increase humidity should be made to prevent the medium from drying rapidly. With very high humidity, cotton plugs become damp and the chances of contamination of cultures increase.

The culture room should be provided with specially designed shelves to store cultures (see Figs. 2.1 and 2.2). While some laboratories have shelves on the wall along the sides of the room, others have them fitted onto angular iron frames (culture racks) placed conveniently in the room. The culture racks may be provided with wheels for more efficient utilization of space. The shelves can be made of glass or rigid wire mesh. Each shelf is provided with a separate set of fluorescent tubes. Insulation between the lamps and the shelf above ensures a more even temperature around the cultures. To prevent a build-up of hot air in the shelves due to

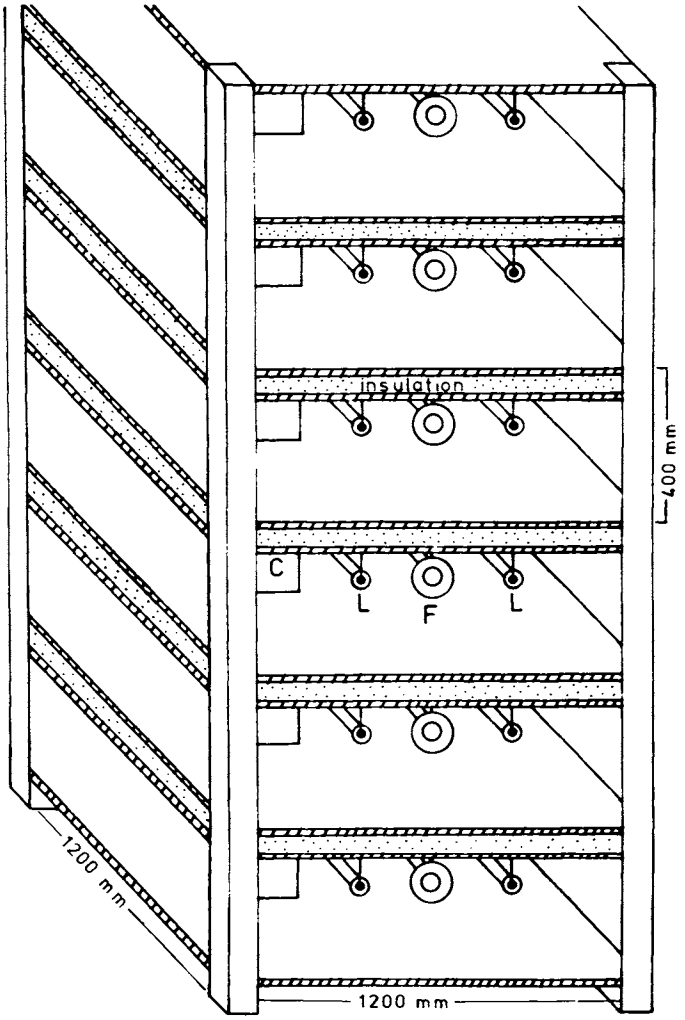

Fig. 2.1. Diagram of a shelving unit especially designed for storing cultures. C, control panel; F, fan; L, light source.

the lamps, ventilation of the individual shelves can be provided by fitting a small fan at one end of the shelf and blowing air through a plastic pipe running the length of shelf. Holes are drilled on the sides of the pipe at appropriate distances to allow even air flow along the length of the pipe. Another point to consider is the heat generated by the ballast of the fluorescent tubes. This could be obviated by mounting all the ballast on a panel outside the room and having flexible wiring. Alternatively, electronic ballast may be used, which are expensive initially but will save on the wiring. They are also energy efficient and do not heat up much.

Fig. 2.2. Illuminated shelves of culture racks with culture jars held in plastic trays (courtesy of Dr Vibha Dhawan, TERI, New Delhi).

While flasks, jars and petri-dishes can be placed directly on the shelf or trays of suitable sizes, culture tubes require some sort of support. Metallic wire racks, each with a holding capacity of 20 or 24 tubes, are suitable for this purpose. In commercial companies, the handling of culture jars can also be made convenient by using autoclavable plastic/ metallic trays (Fig. 2.2). On one face of the culture tube racks and trays, there should be a label giving details of the experimental or production details (e.g. name of the plant, explant, medium, date of culture, name of operator).

The culture room should also have a shaking machine, either of the horizontal type or the rotatory type if cell suspensions are grown. Shakers with temperature and light controls are also available.

It is desirable to have emergency power points attached to a generator, to maintain both light and temperature in the culture room, and also to eliminate the risk of suspension cultures dying due to stoppage of the shakers in the event of a major power breakdown at the mains. Such a catastrophe may ruin important experiments. Some temperature-sensitive strains of tissues may even die.

2.2.5. Greenhouse

In order to grow the mother plants and to acclimatize in vitro produced plants, the tissue culture laboratory should invariably have a green house/glass house/plastic house attached to it. The sophistication of this facility will depend on the funds available. However, minimum facilities for maintaining high humidity by fogging, misting or a fan and pad system, reduced light, cooling system for summers and heating system for winters must be provided. It would be desirable to have a potting room adjacent to this facility.

2.3. TECHNIQUES

This section deals with techniques other than media preparation which is discussed in Chapter 3. Techniques specific to various other areas of cell, tissue and organ culture have been described in the respective chapters.

2.3.1. Glassware and plasticware washing

Detergents especially designed for washing laboratory glassware and plasticware are available. After soaking in detergent solution for a suitable period (preferably overnight) the apparatus is thoroughly rinsed first in tap water and then in distilled water. If the glassware used has dried agar sticking to the sides of the tubes or jars, it would be better to melt it by autoclaving at low temperature. To recycle glassware that had contaminated tissues or media, it is extremely important to autoclave them without opening the closure so that all the microbial contaminants are destroyed. Even the disposable culture vials should be autoclaved prior to discarding them, in order to minimize the spread of bacteria and fungi in the laboratory. The washed apparatus is placed in wire baskets or trays to allow maximum drainage and dried in an oven or hot-air cabinet at about 75°C and stored in a dust-proof cupboard. Half of one or more shelves in the oven or hot-air cabinet may be lined with filter paper on which instruments and more fragile and small objects (e.g. filter holders, sieves, etc.) can be laid out. Glassware washing can also be done using domestic or industrial dishwashers.

2.3.2. Sterilization

Plant tissue culture media, which is rich in sucrose and other organic nutrients, support the growth of many micro-organisms (like bacteria

and fungi). On reaching the medium these microbes generally grow much faster than the cultured tissue and finally kill it. The contaminants may also give out metabolic wastes which are toxic to plant tissues. It is, therefore, absolutely essential to maintain a completely aseptic environment inside the culture vessels. For this, two obvious general precautions are: (1) not to share the plant tissue culture working area with microbiologists and pathologists, and (2) to remove contaminated cultures from the culture area as soon as detected.

There are several possible sources of contamination of the medium: (a) the culture vessel, (b) the medium itself, (c) the explant, (d) the environment of the transfer area, (e) instruments used to handle plant material during inoculation and subculture, (f) the environment of the culture room, and (g) the operator. In the following few pages some measures taken to guard the cultures against contamination from any of these sources are discussed. The reader should refer to the excellent reviews by Cassells (1991), Leifert and Waites (1990), and Leifert et al. (1994) for a detailed exposition on contamination in cultures.

(i) *Medium.* The microbial contaminants are normally present in the medium right from the start. To destroy them, the mouth of the culture vial containing the medium is properly closed with a suitable bacteria-proof closure and the vial is autoclaved (steam heating under pressure) at 1.06 kg cm^{-2} (121°C) for 15–40 min from the time the medium reaches the required temperature. If an autoclave is not available, a domestic pressure cooker may be used. Sterilization depends on the temperature and not directly on the pressure. The exposure time varies with the volume of the liquid to be sterilized (see Table 2.1). Monnier (1976) reported that heating at 120°C decreased the nutritive value of the culture medium for young *Capsella* embryos. Best results were obtained when the medium was autoclaved at 100°C for 20 min. Care must be taken while cooling the solution. A rapid loss of pressure, exceeding the rate of reduction in temperature will make the liquid boil vigorously. The pressure gauge of the autoclave should be at zero (temperature not higher than 50°C) before the autoclave is opened.

It has been observed that 2–5% of media are contaminated during manual pouring after autoclaving (Leifert et al., 1994). Moreover, certain *Bacillus* species have been shown to survive even after autoclaving of the medium at 110–120°C for 20 min. It is, therefore, advisable to store the medium for about 7 days before use.

Some of the plant growth regulators (e.g. GA_3, zeatin, ABA) urea, certain vitamins, pantothenic acid, antibiotics, colchicine, plant extracts and enzymes used in tissue culture are thermolabile. These compounds

TABLE 2.1

Minimum time necessary for steam sterilization of media as suggested by Biondi and Thorpe (1981)

Volume/container (ml)	Minimum sterilization time at 121°C (min)
20–50	15
75	20
250–500	25
1000	30
1500	35
2000	40

should not be autoclaved. When using such a compound the whole medium minus the heat-labile compound is autoclaved in a flask and kept in the sterilized hood to cool down. The solution of the thermolabile compound is sterilized by membrane filtration and added to the autoclaved medium when the latter has cooled to around 40°C in the case of a semi-solid medium (just before the setting of agar) or to room temperature when using a liquid medium. For filter sterilization of the solutions, bacteria-proof filter membranes of pore size 0.45 μm or less are used. The membranes are fitted into filter holders of appropriate size and autoclaved after wrapping in aluminium foil, or enclosed in screwcap glass jars of a convenient size. Sterilization temperature for filters is critical; it should not exceed 121°C. A graduated syringe (need not be sterilized) carrying the liquid is fixed to one end of the sterilized filter assembly (see Fig. 2.3) and the solution is gradually pushed through the membrane present in the middle of the assembly. The sterilized solution dripping out from the other end of the assembly is added to the medium or collected in a sterilized jar and added to the medium using a sterilized, graduated pipette. Large filter assemblies are also available for filter sterilization. The solution to be filter-sterilized should first be clarified by passing through a No. 3 porosity sintered glass filter. This facilitates filter-sterilization by reducing the plugging of membrane filter pores.

(ii) *Glassware and plasticware.* Glass culture vials are mostly sterilized together with the medium. For pre-sterilized nutrient medium the glassware (culture vessels and other labware) may be sterilized by autoclaving or dry-heating in an oven at 160–180°C for 3 h (De Fossard, 1976). Disadvantages of dry-heat sterilization are poor circulation and slow penetration. Therefore, proper loading of the oven is essential. The glassware is allowed to cool before removal from the oven. If removed before suffi-

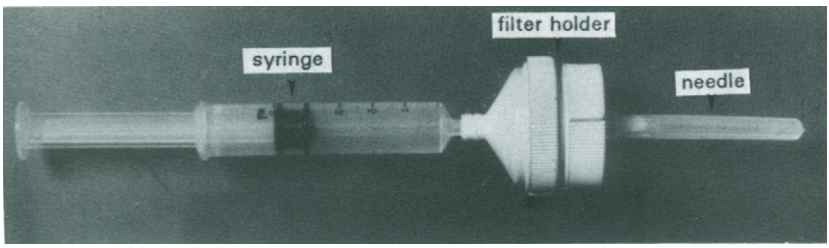

Fig. 2.3. 'Swinnex' Millipore filter assembly for sterilizing small volumes of liquids. The needle is not always required.

cient cooling has taken place, cool air from the exterior may be sucked into the oven, exposing the load to bacterial contamination and the risk of cracking.

Certain types of plastic labware can also be heat sterilized. Polypropylene, polymethylpentene, polyallomer, Tfzel ETFE and Teflon FEP may be repeatedly autoclaved at 121°C (Biondi and Thorpe, 1981). Of these, only Teflon FEP may be dry-heat sterilized. Polycarbonate shows some loss of mechanical strength with repeated autoclaving, and sterilization cycles for it should be limited to 20 min. A large variety of presterilized culture vessels are also available which could be directly used to pour autoclaved media.

(iii) *Instruments*. The instruments used for aseptic manipulations, such as forceps, scalpels, needles, and spatula, are normally sterilized by dipping in 95% ethanol followed by flaming and cooling. This is done at the start of the transfer work and several times during the operation. De Fossard (1976) has suggested the use of 70% alcohol because 95% and 100% alcohol can harbour bacterial spores without killing them. However, for flame sterilization of instruments 95% alcohol has been found entirely satisfactory. The alcohol should be regularly changed as *Bacillus circulans* strains persist in alcohol for more than a week (Leifert and Waites, 1990).

Effective sterilization of instruments can be achieved by flaming in a Bunsen burner. However, the heat liberated by a Bunsen burner is enormous, and the air currents generated could increase incidence of contamination during sub-culture. In recent times the glass bead sterilizer (steripot) and infra-red sterilizer have become available for sterilizing the instruments. In the glass bead sterilizer (Fig. 2.4) a high watt element heats up the glass beads contained in a brass crucible at the centre of the box. The temperature of the beads is raised to 250°C in 15–20 min. Sterilization of instruments is effected by pushing them into the beads for 5–7 s. A regulator maintains the temperature at 250°C by a 15 s cut off and

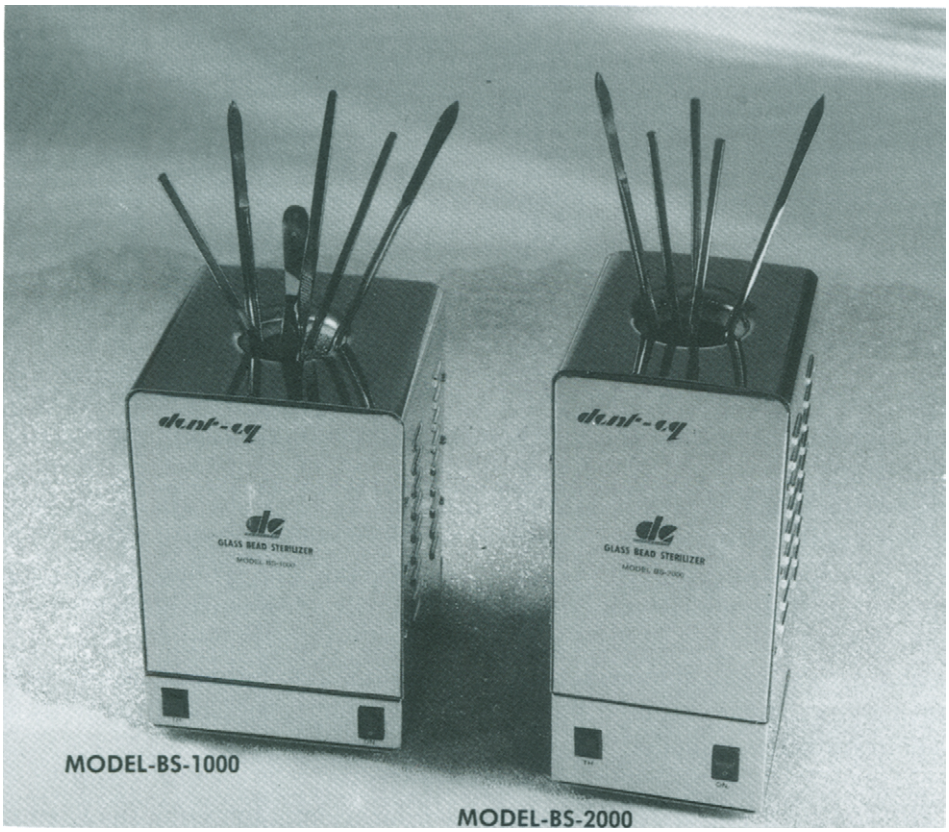

Fig. 2.4. Glass bead sterilizers (courtesy of Mrs Nanda Prasad, Dent-eq, Bangalore).

an indicator light or a dial thermometer indicates the temperature. Glass beads need to be replaced when they turn black. The infrared sterilizer has a cavity where temperature rises to almost 700°C. Here sterilization is effected by a 2–5 s exposure at this temperature. Being well insulated, these sterilizers do not spill out large quantities of heat. These instruments are also safe compared to a Bunsen burner which could cause heat burns and may also be a fire hazard.

(iv) *Plant material*. Surfaces of plant parts carry a wide range of microbial contaminants. To avoid this source of infection the tissue must be thoroughly surface sterilized before planting it on the nutrient medium; tissues with systemic fungal or bacterial infection are usually discarded in tissue culture studies.

To disinfect plant tissues various sterilizing agents have been used (see Table 2.2). Hypochlorite solutions (sodium or calcium) have proved to

TABLE 2.2

Effectiveness of some surface sterilizing agents[a]

Sterilizing agent	Concentration used (%)	Duration (min)	Effectiveness
Calcium hypochlorite	9–10	5–30	Very good
Sodium hypochlorite	2[b]	5–30	Very good
Hydrogen peroxide	10–12	5–15	Good
Bromine water	1–2	2–10	Very good
Silver nitrate	1	5–30	Good
Mercuric chloride	0.1–1	2–10	Satisfactory
Antibiotics	4–50 mg l^{-1}	30–60	Fairly good

[a]After Yeoman and Macleod (1977).
[b]20% (v/v) of a commercial solution.

be effective in most cases. For example, 0.3–0.6% sodium hypochlorite treatment for 15–30 min will decontaminate most tissues. It is important to realize that a surface sterilant is also toxic to the plant tissue. Therefore, the concentration of the sterilizing agent and the duration of treatment should be chosen to minimize tissue death.

Ethyl and isopropyl alcohol have also been used to surface sterilize some plant tissues (methanol should never be used). After rinsing in ethanol for a few seconds the material is either left exposed in the sterile hood until the alcohol evaporates (Kao and Michayluk, 1980) or, if fairly hardy, flamed (Bhojwani, 1980a).

In general, if the explant is fairly hard and large enough to be easily handled, the inoculum can be directly treated with the disinfectant. For example, in the culture of mature seeds, mature endosperm of euphorbiaceous plants or nodal explants, whole seeds, decoated seeds or stem pieces, respectively, are surface sterilized. However, when immature ovules, embryos or endosperm are to be cultured, the customary method is to surface sterilize the ovary or the ovule, as the case may be, and dissect out the explant under aseptic conditions so that the soft tissues of the inoculum are protected from the toxic effects of the sterilizing agent. Similarly, for raising cultures of delicate shoot apices and pollen grains, shoot buds or flower buds, respectively, are surface sterilized and the explant excised aseptically. Such explants are usually free of microbial contaminants. A 30 s rinse of the plant material in 70% ethanol before treatment with the sterilant or the addition of a few drops of surfactant, such as Triton-X and Tween-80, to the sterilization solution enhances its efficiency. A careful dissection of shoot apices may give high frequency of healthy cultures even without a surface sterilization treatment (Quak,

1977). However, dissection of wet material should be avoided. After surface sterilization treatment (not applicable when using alcohol), the plant material must be rinsed three or four times in sterile, distilled water to remove all traces of the sterilizing agent.

Treating wheat seeds in a 1% (v/v) solution of cetavlon (Cetrimide, ICA) for 2 min before hypochlorite treatment was found very effective in reducing bacterial contamination of cultures (Bhojwani and Hayward, 1977). In cases where the explant carries a heavy load of micro-organisms on its surface it may pay to wash it in running tap water for an hour or more. Often aseptic seedlings are raised through seed culture and their various parts (roots, stem pieces, leaves, etc.) are utilized for initiating cultures.

Antibiotics and antifungal compounds have been used by several workers to control explant contamination. Arbitrary use of antibiotics might not yield any useful results as the majority of the bacteria infecting plant materials are gram-negative, which are less sensitive to the commonly used antibiotics (Leifert et al., 1994). The mode of action and effectivity of the antimicrobial agents should be fully understood before use (Table 2.3). Micro-organisms can be accurately identified by fatty acid profile, pattern of carbon compound utilization, and nucleic acid studies (Buckley et al., 1995). However, if these procedures are found expensive the classical method of using liquid medium or an enriched agar medium may be employed. Reed et al. (1995) reported that streptomycin at 1000 g l^{-1} for a period of 10 days was effective against endophytic bacteria and less phytotoxic to *mentha* spp. than gentamicin, neomycin and rifampicin. However, antibiotics have been shown to restrict rooting, general growth and multiplication in plant cultures (Leifert et al., 1994). Antifungals, such as binomyl has been shown to reduce fungal infection when used with mercuric chloride (Mederos and Lopez, 1991).

Interestingly, Attree and Scheffield (1986) found that it was physically possible to separate micro-organisms from plant cells and protoplasts by using a sucrose gradient centrifugation. This could be combined with dilute hypochlorite and/or antibiotic treatment of cells before or after centrifugation (Bradley, 1988; Finner et al., 1991).

(v) *Transfer area.* Finally, it is very essential that all precautions are taken to prevent the entry into the culture vial of any contaminant when its mouth is opened either for subculture or for planting fresh tissues (inoculation). To achieve this, all transfer operations are carried out under strictly aseptic conditions.

In most laboratories laminar air-flow cabinets (see Fig. 2.5), which are available in various shapes and sizes are used for aseptic manipulation.

TABLE 2.3

Mode of action of some antimicrobial agents[a]

Antimicrobial compound	Mode of action	Comments
Aminoglycosides		Bactericidal
Streptomycin		
Kanamycin	Inhibit protein synthesis	
Neomycin	by interaction with 30S	
Gentamicin	50S ribosomes	
Tobramycin		
Amikacin		
Spectinomycin		(an aminocyclitol)
Quinolones		Bactericidal
Nalidixic acid		
Ofloxacin	Interfere with DNA	
Norfloxacin	replication by inhibition	
Enoxacin	of DNA gyrase	
Ciprofloxacin		
β-Lactams		Bactericidal
Penicillin		
Ampicillin		
Carbenicillin		
Cephradine		
Cephamandole	Inhibit bacterial cell	
Cefuroxime	wall synthesis	
Ceftazidime		
Sublactam		(sulphone)
Imipenem		(carbapenem)
Aztreonam		(monobactam)
Tetracyclines	Inhibit protein synthesis by acting on 30S ribosome	Bacteriostatic
Trimethoprim and suplhanamides	Inhibit synthesis of tetrahydrofolate (at different sites)	Bacteriostatic
Chloramphenicol	Inhibit protein synthesis by acting on 50S ribosome	Bacteriostatic
Macrolides and lincosamides		Bacteriostatic
Erythromycin	Inhibit protein synthesis	
Lincomycin	by acting on 50S ribosome	
Glycopeptides		
Vancomycin	Interferes with bacterial cell wall synthesis	Bactericidal for Gram +ves only

TABLE 2.3 (*continued*)

Antimicrobial compound	Mode of action	Comments
Polymixins		Bactericidal
Polymixin B Polymixin E	Attach to cell membrane and modify ion flux, resulting in cell lysis	for Gram –ves esp. *Pseudomonas* (*Proteus* resistant)
Rifampicin	Interferes with mRNA formation by binding to RNA polymerase	Resistance emerges readily

[a]After Falkiner (1990). According to the author, the agents which act specifically on bacterial cell walls would be more suitable to control infection in plant tissue cultures.

Work can be started within 10–15 min of switching on the air flow, and one can work uninterrupted for long hours.

Essentially, a laminar air-flow cabinet has a small motor to blow air which first passes through a coarse filter, where it loses large particles, and subsequently through a fine filter. The latter, known as the 'high efficiency particulate air (HEPA)' filter, removes particles larger than 0.3 μm, and the ultraclean air (free of fungal and bacterial contaminants) flows through the working area. The velocity of the air coming out of the fine filter is about 27 ± 3 m min^{-1} which is adequate for preventing the contamination of the working area by the worker sitting in front of it. All contaminants such as hairs, salts, flakes, etc., are blown away by the ultraclean air flow, and a completely aseptic environment is maintained in the working area as long as the cabinet is kept on. The flow of air does not in any way hamper the use of a spirit lamp or a Bunsen burner.

In temperate countries, air-flow cabinets are used in ordinary laboratories. However, in tropical and sub-tropical countries, where atmospheric dust is very high, it would be better to house the cabinet in a culture room fitted with double doors in order to prolong the effective life of the filters. Under no circumstances should the hood be kept opposite a door or a window which is frequently used.

Maximum chances of cultures getting infected occur when the culture vials are opened during initiation of fresh cultures or subculture of established cultures. Therefore, most of the commercial tissue culture companies have a separate 'transfer room' in the clean area and maximum cleanliness and least air turbulence is maintained in this room. The movement of people in this area should be restricted. In this room the walls should be made smooth so that dust does not settle. The walls can

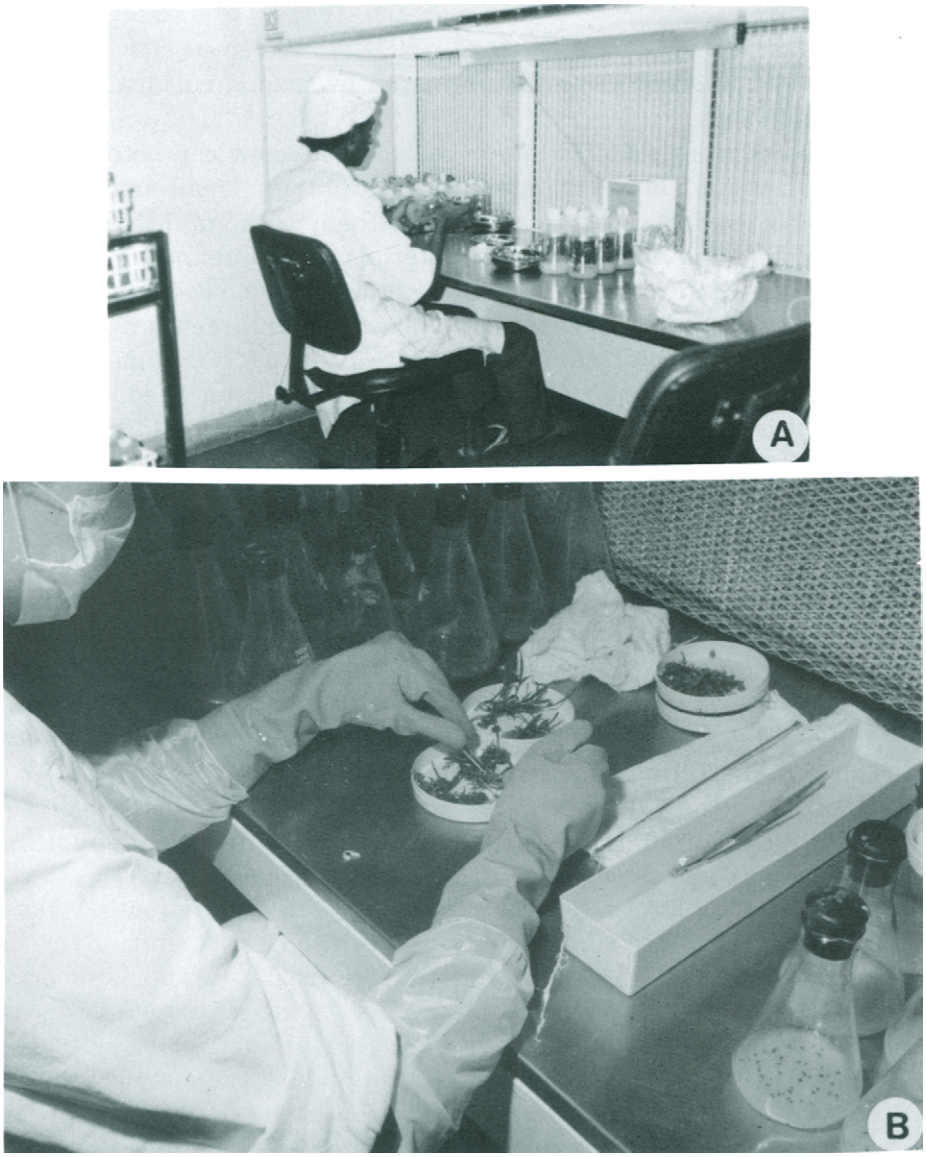

Fig. 2.5. Laminar air-flow cabinets in use. (A) Courtesy of Dr Vibha Dhawan, TERI, New Delhi; (B) courtesy of South Pacific Orchids Ltd., New Zealand.

be painted with high gloss and water tolerant paint to withstand repeated cleaning. A one-piece floor covering of linoleum extending a few centimetres up the walls is easier to clean. The transfer hoods, light and air-conditioners in the transfer room should be connected to an emergency source of power.

APPENDIX 2.I

The sequence of steps commonly involved in aseptic culture of plant tissues[1] is as follows:

(a) Pieces of plant material are collected in a screw-cap bottle and a dilute solution of the disinfectant, containing a small amount of a suitable surfactant, is poured onto them. The liquid should be enough to fully immerse the material. After putting on the closure, the bottle is taken to the aseptic transfer hood. During the sterilization period the bottle is shaken two to three times.

(b) After sterilization treatment, the cap of the bottle is removed and the liquid poured out. An adequate quantity of sterilized, distilled water is poured onto the material and the cap replaced. After shaking a few times, the water is discarded. Such washings with sterile distilled water are repeated three to four times.

(c) The material is then transferred to a pre-sterilized petri-dish.

(d) While the plant material is being treated for disinfection the instruments required are sterilized by dipping them in 95% ethanol and flaming, and allowed to cool. It may be necessary to sterilize the instruments each time after handling tissue.

(e) Suitable explants are prepared from the surface sterilized material using sterilized instruments (scalpels, needles, cork-borer, forceps, dissecting microscope, etc.).

(f) Closure of the culture vial is removed, the inoculum transferred onto the medium, the neck of the vial flamed (in the case of glass vials only), and the closure replaced in quick succession.

[1] From step (b) onwards all the operations are performed under aseptic conditions. Precise methods for raising aseptic cultures of various plant tissues and organs are described at appropriate places in subsequent chapters.

APPENDIX 2.II

A list of apparatus required for tissue culture work.

1. flasks (100 ml, 250 ml, 500 ml, 1 l, 5 l);
2. volumetric flasks (500 ml, 1 l, 2 l, 3 l);
3. measuring cylinders (25 ml, 50 ml, 100 ml, 500 ml, 1 l);
4. graduated pipettes (1 ml, 2 ml, 5 ml, 10 ml);
5. Pasteur pipettes and teats for them;
6. culture vials (culture tubes, screw-cap bottles of various sizes, petri-dishes, nipple flasks, etc.) with suitable closure;

} glassware or plasticware for media preparation

7. plastic or steel buckets, to soak labware for washing;
8. hot-air cabinet, to dry washed labware;
9. oven, to dry washed labware, and dry-heat sterilization of glassware;
10. wire-mesh baskets, to autoclave media in small vials and for drying labware;
11. water distillation unit, demineralization unit, Milli Q unit or reverse osmosis unit for water purification;
12. plastic carboys (10 l and 20 l), to store high quality water;
13. balances, one to weigh small quantities and the other to weigh comparatively larger quantities;
14. hot plate-cum-magnetic stirrer, to dissolve chemicals;
15. exhaust pump, to facilitate filter sterilization;
16. plastic bottles of different sizes, to store and deep-freeze solutions;
17. refrigerator, to store chemicals, stock solutions of media, plant materials, etc.;
18. deep freeze, to store stock solutions of media for longer periods, certain enzymes, coconut milk, etc.;
19. steamer or microwave oven to dissolve agar and melt media;
20. pH meter, to adjust pH of media and solutions;
21. autoclave or domestic pressure cooker, for steam sterilization of media and apparatus;
22. heat-regulated hot plate or gas stove for steam sterilization in domestic pressure cooker;
23. filter membranes and their holders, to filter sterilize solutions;
24. hypodermic syringes, for filter sterilization of solution;
25. medium dispenser, to pour medium;
26. trolley with suitable trays, to transport cultures, media and apparatus;

27. laminar air-flow cabinet, for aseptic manipulations;
28. spirit lamp, burner, glass bead sterilizer or infra-red sterilizer to sterilize instruments;
29. atomizer, to spray spirit in the inoculation chamber;
30. screw-cap bottles, to sterilize plant material;
31. instrument stand, to keep sterilized instruments during aseptic manipulations;
32. large forceps with blunt ends, for inoculation and subcultures;
33. forceps with fine tips, to peel leaves;
34. fine needles, for dissections;
35. stereoscopic microscope with cool light, for dissection of small explants;
36. table-top centrifuge, to clean protoplast and isolated microspore preparations, etc.;
37. incubator shaker, for liquid cultures.