General Requirements and Techniques

2

2.1 Introduction

A plant tissue culture laboratory, whether for research or for commercial purpose, should provide certain basic facilities, such as (i) washing and storage of glassware, plasticware and other labwares, (ii) preparation, sterilization and storage of nutrient media, (iii) aseptic manipulation of plant material, (iv) maintenance of cultures under controlled conditions of temperature, light and humidity, (v) observation of cultures and (vi) hardening of in vitro developed plants. The extent of sophistication in terms of equipment and facilities depends on the need and the funds available. Therefore, establishment of a new tissue culture facility requiring ingenuity and careful planning.

2.2 Requirements

2.2.1 Structure and Utilities

The construction of a laboratory from scratch is a costly affair but there is considerable scope for maneuverability with the design at the conceptional stage and in the selection of construction material. To begin with, a commercial laboratory is best housed in a pre-existing building with suitable modifications. After carefully examining the economic feasibility of the venture an independent facility may be erected. More often than not, for research work the tissue culture laboratory is carved out of the existing infrastructure, and several facilities/equipments are shared with other laboratories. A research facility should have at least four rooms: (i) *Washing Room,* for glassware washing, storage and autoclaving (ii) *Media Room*, for media preparation (iii) *Sterile Area*, for aseptic manipulation and (iv) G*rowth/Culture Room*, to maintain cultures under suitable environmental conditions. The culture room should also have a working table, a stereoscopic microscope and a good light source, preferably cool light (fiber optics), for observing cultures. The sterile transfer cabinets could be placed in the culture room or in a specially designed transfer room. In many research laboratories it is kept in an undisturbed area of a general lab.

In case the facility needs to be constructed, especially for a commercial setup, it would be desirable to locate the unit away from the city to avoid heavy pollution and vehicular vibrations. However, this may require transportation of the personnel. The location of the laboratory should not be near fields to avoid spurts of infection by the combines and threshers during the harvest seasons. The facility needs to be adequately protected from rains and winds as these carry spores, mites and thrips. Thermal insulation of the facility to conserve energy is another aspect requiring proper thought. One way is to have the transfer area and the growth rooms below ground level. In that case care must be taken to protect the lab from seepage and provide adequate ventilation. Alternately, these two rooms could have a double wall or built of hollow

S. S. Bhojwani and P. K. Dantu, *Plant Tissue Culture: An Introductory Text*,
DOI: 10.1007/978-81-322-1026-9_2, © Springer India 2013

bricks with air trapped in between, which could be vented during summers.

A tissue culture facility requires large quantities of good quality water. At the designing stage itself adequate attention should be paid to the source of water and waste-water disposal, especially where sewer facilities are not available, keeping in view the local municipal rules for health and environment.

A tissue culture unit must have power backup to save cultures from getting contaminated in the event of power failure or load-shedding from the mains during aseptic manipulations. Valuable cultures may be lost because of temperature shocks in the growth room during electricity breakdowns/shutdown. The generator may be fitted with a self-starting switch.

It is of paramount importance that a tissue culture laboratory is clean and movement of materials from one area to another occurs with minimal backtracking. These aspects should be the guiding principle while designing the layout plan of various rooms, pass-through windows, doors and hallways. It is necessary and desirable to isolate the 'clean area', comprising of transfer room and growth room from rest of the 'unclean area' and it should be treated as 'restricted area', out of bounds for visitors and outsiders. In the passage between these two areas, especially in a commercial set-up, one is required to wash hands and feet and wear sterilized overcoats and headgear before entering the 'restricted area'. Generally, high standards of sanitation need to be maintained and these have to be more stringent where dust, pollen and small insects abound in the environment. It is a good idea to have paved pathways and shrubs around. High levels of cleanliness and freedom from extraneous materials could be achieved by having positive air pressure, at least in the 'clean area'. Depending on the necessity, a Class 1,000 or Class 10,000 standard should be maintained for the clean room. For the movement of material in (sterilized medium, instruments, water, etc.) and out of (glassware, old and infected cultures, tissue culture produced plants for hardening, etc.) the 'clean area' a window with double door hatch should be provided to maintain high asepsis in the 'clean area'.

As far as possible indigenously available construction material, equipment, apparatus, and instruments should be used for cost effectiveness and ease of maintenance. Innovativeness and indigenous fabrication will go a long way in reducing the costs.

2.2.2 Washing Room

Depending on the availability of funds and space the washing and sterilization areas may be in separate rooms or in a common room. In either case, the washing area should have adequate supply of good quality hot and cold running water and an acid and alkali resistant big sink. Adequate steel or plastic buckets and tubs are required for soaking culture vials and other labwares used in medium preparation. Brushes of various sizes and shapes are essential for cleaning glassware, while it is optional to have a dish washing machine. For a commercial set-up an industrial dish washer is desirable. The media room should have a hot air cabinet to dry the washed labware, an oven for dry sterilization, and a dust proof cupboard for storage of plastic and glass-ware. When washing is done in media room a temporary partition can be erected between the two areas to prevent splashing of soap solution and any other interference in the two activities. Alternately, timings of the two activities could be staggered. Where the autoclave is to be housed within the media room an isolated area with provision for ventilation through an exhaust should be chosen.

Even if good quality water is available it cannot be used for final washing of labwares or for medium preparation as it contains impurities such as inorganic and organic compounds, dissolved gases, particulate debris and microorganisms. Water could be purified through distillation, deionization or reverse osmosis. Sometimes a combination of two or more is required. Water purity is measured in terms of resistivity (ohms cm^{-1}) or its reciprocal, i.e.,

conductivity (mhos cm^{-1}). Water for tissue culture should ideally have a conductivity of 5.0 μmhos cm^{-1} although a conductivity level up to 15 μmhos cm^{-1} is acceptable. Deionized water may be used for teaching laboratories or for rinsing labware but for research and commercial purposes, water distillation apparatus, a reverse osmosis unit or a Mili-Q purification system needs to be installed. The choice between the three is one of quality of final water, speed of production and cost. For a research laboratory a glass distillation unit with a handling capacity of 1.5 to 2 L h^{-1} of water should be sufficient. For a commercial set-up or where high purity water is required a Mili-Q purification system that can provide 90 L h^{-1} may be used. Proper storage tanks should be provided for the purified water.

2.2.3 Media Room

The media room is the kitchen of the tissue culture facility. The media room is provided with a working table in the centre and benches along the wall, the tops of which are either covered with granite or laminated board (Fig. 2.1). The tables and benches should be at a height suitable for working while standing and the space below them could be fitted with drawers and cupboards for storage purposes. The benches are required for keeping balances, pH meter, magnetic stirrers, hot plates etc. A top loading electronic balance with tare for weighing large quantities and an analytical balance for small quantities of chemicals must be provided. The balances should be isolated in a small chamber if the media room also houses the autoclave. In a large commercial laboratory it will be of help to have an automatic media dispenser.

For short term storage of certain chemicals, plant materials, and stock solutions a refrigerator and a deep freeze are required. These could be kept in the corridor if sufficient space in the room is not available. A single electrode pH meter that can read conductivity also should be provided. For filter sterilization of medium or solutions of thermolabile compounds an aspirator or vacuum pump may be required. For steam sterilization an autoclave or a domestic pressure cooker, depending on the quantity to be sterilized, is needed.

For emergencies a fire extinguisher and a first aid kit should be kept in this room.

Fig. 2.1 Media preparation

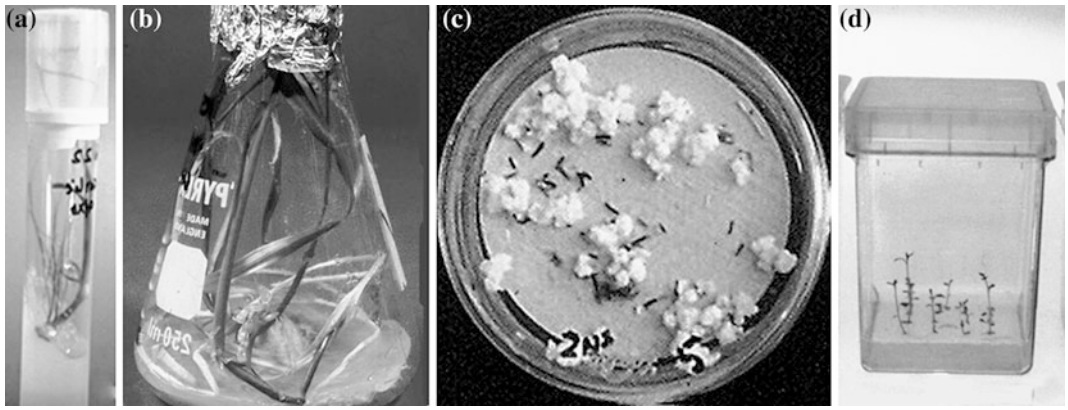

Fig. 2.2 Culture vials. **a** Culture tube with polypropylene cap. **b** Flask. **c** Petri plate. **d** Plastic jar (*Magenta box*)

2.2.4 Glassware/Plasticware

In a tissue culture laboratory culture vessels (Fig. 2.2) are required in bulk. Depending on the type of work, adequate supplies of these should be maintained. For standard tissue culture work rimless test tubes (25 × 150 mm) are widely used (Fig. 2.2a). The culture tubes are important for culture initiation and establishment even in a commercial set-up. For further mass multiplication larger containers such as jam bottles or other wide mouthed bottles are required. Erlenmeyer flasks have also been used as culture vessels (Fig. 2.2b). Only borosilicate or Pyrex glassware should be used.

Plastic culture vials, autoclavable and presterilized, have replaced glass culture vials to a large extent. A wide range of presterilized, disposable culture vials made of clear plastic, especially designed for protoplast, cell, tissue and organ culture, are available in the market under different brand names. The presterilized plastic Petri dishes (Fig. 2.2c), jars (Fig. 2.2d), screw cap bottles, and various cell culture plates come with their closures. For culture tubes and flasks, traditionally, non-absorbent cotton plugs wrapped in a single layer of cheesecloth have been used as a closure. Autoclavable, transparent polypropylene caps with a membrane built into the top are also available (KimKaps, Kimble, Division of Owens, IL). Cotton plugs provide excellent aeration but the medium dehydrates very fast. On the other hand, polypropylene caps reduce the rate of medium desiccation but increase moisture and gaseous accumulation within the container. However, it is important to ensure that the closure allows proper aseptic aeration and does not inhibit the growth of culture materials. In this regard it may be mentioned that Parafilm/Nescofilm, commonly used to seal Petri dishes, releases butylated hydroxytoluene, which is toxic to the cultured plant material (Selby et al. 1996). Alternately, one can use cling film for sealing, as 2-ethyl-1-hexanol released by it is not inhibitory to culture material.

Now it is possible to buy culture vessels made of different synthetic materials. Culture vessels made of polypropylene transmit 65 % light and those made of polycarbonate transmit almost 100 % light. Gas permeable fluorocarbonate vessels are available for use with plant materials that are sensitive to gas build up within the culture vials (Kozai 1991).

Besides culture vials, various other glass-/plasticware such as beakers, measuring cylinders, pipettes, etc. of various sizes are required for media preparation.

2.2.5 Transfer Room

In research laboratories the transfer hoods are placed in the growth/culture room or even in a quite corner of a general laboratory. However, in a commercial facility it is necessary to have separate transfer and growth room(s). There are

no special requirements for a transfer room except that a high degree of cleanliness and a worker friendly environment is to be maintained. Transfer hoods are placed in this room to carry out aseptic manipulations. Often the culture medium to be used is also stored here although, where possible, a separate store room within the 'clean area' is demarcated for this purpose. To transport culture medium or cultures in and out of the transfer room, trolleys with one or more shelves are helpful. These trolleys may also serve as side benches for the operator to hold extra culture medium, stock cultures, and/or freshly raised cultures until transferred to the growth room. Since fire/heat is constantly used in the transfer hoods it is advisable to keep a fire extinguisher in this area.

2.2.6 Growth Room

The inoculated culture vials are transferred for incubation to a growth room with controlled temperature and light conditions. It is of paramount importance to maintain cleanliness in this area. It can be achieved by having positive air pressure in the 'clean area' or an overhead air-curtain at the entry to remove surface dust. This room should have one door, and the windows should be avoided to prevent external light from interfering with the internal light cycle. Desirably, the junction of walls should be rounded rather than angular to prevent cobwebs. The wall paints should be of semi or high-gloss with a linoleum floor to withstand repeated cleaning. Tiller et al. (2002) reported that the polymer Hexyl-PVP when coated on a glass surface killed 99 % of harmful bacteria.

Growth rooms being closed units require devices to control temperature and light. Temperature is controlled by air conditioning. Tower air conditioning is an expensive proposition, but if that is the chosen option then adequate precautions should be taken to filter the cooled air to prevent dust, spores etc. from reaching the 'clean area'. With this type of air conditioning it is easy to maintain positive air pressure. In research laboratories generally window mounted or split air conditioners are used for cooling during summer and hot air blower for heating during winter. The air conditioners and heaters are hooked to temperature controllers to maintain the temperature at 25 ± 2 °C. For higher or lower temperature treatments, special incubators with built in fluorescent lights can be used. These incubators may be kept even outside the growth room with suitable measures to prevent anyone from tampering with the settings.

Generally, cool white fluorescent lamps with electronic ballasts are used for growth rooms because of the uniform light intensity they emit. Cultures are normally maintained in diffuse light (3000–5000 lux). The low light intensity can be achieved in 3.5 feet wide shelves by installing three tubes one foot above the surface of the shelf. A sheet of aluminium foil or coat of aluminium paint provided above the tubes maximizes light intensity below. Some provision should also be made for growing cultures in total darkness or under high light intensities. Automatic timers are used to regulate the photoperiod.

If the relative humidity in the growth room falls below 50 %, humidifiers are required to be used to prevent medium from drying rapidly. Dehumidifiers may be required when the humidity is very high, particularly during the rainy season, to prevent cotton plugs from becoming damp or condensing of water droplets, as both may increase chances of contamination of cultures.

Culture vials in the growth room are maintained on specially designed shelving unit that are either stationary or moveable. Stationary shelves may be fixed on walls of the room, or could be fitted into angular iron frames to form culture racks (Fig. 2.3) that are placed conveniently in the room. Alternately, the racks could be on roller coaster wheels that allows efficient use of space. Open shelves are generally preferred because of better air circulation. The shelves can be made of plywood board or rigid wire mesh. Each shelf is provided with a separate set of fluorescent tubes. In case the cultures are to be maintained under different photoperiod and temperature regimes it is advisable to have more than one growth room.

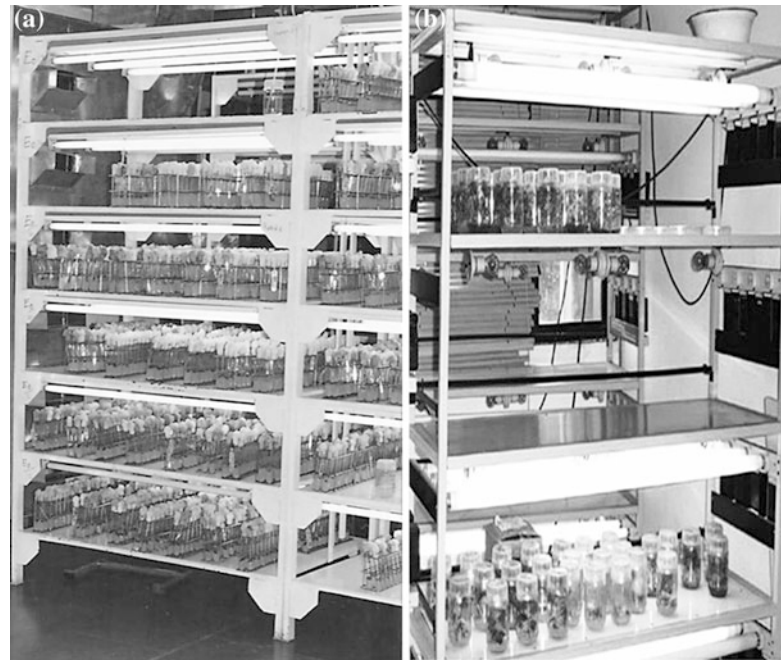

Fig. 2.3 **a** An illuminated culture trolley with six shelves holding culture tubes arranged in metallic racks. **b** An enlarged portion of a trolley as in (**a**), with cultures in screw cap bottles

Culture flasks, jars and Petri dishes can be placed directly on the shelves or in trays of suitable size, while culture tubes require some support such as a metallic wire rack (Fig. 2.3), which can hold 20–24 tubes. In commercial companies, where large quantities of culture vials have to be moved, it is not only convenient but also time saving to use autoclavable plastic or epoxy coated metallic trays for holding the culture vials. The culture vials and the trays holding them should be appropriately labeled to avoid mixing up. For transportation of culture vessels cart trolleys may be used.

The culture room should also have a shaking machine, either of the horizontal type or the rotary type, if cell suspension cultures are to be grown. Shakers with speed, temperature and light controls are also available.

2.2.7 Cold Storage

In a commercial setup it is necessary to have a cold storage maintained at 2–4 °C for temperate plants and 15 °C for tropical plants. These rooms are used to give treatment for breaking dormancy of some plant materials, storing of cultures to schedule workload, maintain 'mother' cultures and to hold harvested plants (Mageau 1991).

2.2.8 Greenhouse

In order to grow the mother plants and to acclimatize in vitro produced plants, a tissue culture laboratory should have a greenhouse made of glass, polythene or polycarbonate depending on the budgetary provisions. This facility should have a provision to maintain high humidity such as fan and pad system. It would be desirable to have a potting room adjacent to this facility. A separate autoclave might be required in this area if one wants to sterilize the potting mixture.

In a commercial laboratory provision for certain other rooms such as, a general storage, and employee's tea room, an administrative office and shipping and receiving centre should be made.

2.3 Techniques

This section deals with the basic techniques of maintaining cleanliness and asepsis in the laboratory and in the cultures.

2.3.1 Glassware and Plasticware Washing

All glassware and plasticware, except pre-sterilized ones, should be thoroughly washed when using for the first time. As a normal practice, the apparatus is soaked overnight in a standard laboratory detergent and scrubbed with a bottle brush manually or by a machine. These are then rinsed under tap water followed by a rinse in distilled water. Dried agar can be removed by heating. The contaminated glass and plastic culture vials should be autoclaved before opening for washing or discarding, respectively, in order to minimize the spreading of bacterial and fungal contaminants in the laboratory. The washed apparatus are placed in wire baskets or trays to allow maximum drainage and dried in a hot air cabinet at about 75 °C and stored in a dust proof cupboard. For transportation of washed labware from washing area suitable trays and mobile carts can be used.

2.3.2 Sterilization

Whether it is labware or culture medium, plant material or environment in the laboratory, instruments used for culture or the operator himself, all are sources of infection. The tissue culture medium being rich in sugar and other organic and inorganic nutrients supports good growth of microorganisms, such as fungi and bacteria. On reaching the medium the microorganisms may grow faster than the plant tissues, finally killing them. The microbes may also secrete toxic wastes into the medium inhibiting growth of cultured tissues. It is, therefore, absolutely essential to maintain a completely aseptic environment inside culture vessels. As a rule, plant tissue culture laboratory facilities should not be shared with microbiologists and pathologists, and contaminated vessels should be removed as soon as detected.

The various sources of contamination and measures to guard cultures against them are discussed in the following pages.

(i) *Glassware and Plasticware*. Culture vials are a major source of contamination, more so, if these have been in long use. The glass culture vials may be dry sterilized before pouring the medium to kill such bacteria, which might withstand autoclaving. Culture vials are generally sterilized together with the culture medium. For pre-sterilized medium the culture vials with proper closure may be sterilized by autoclaving or dry heating in an oven at 160–180 °C for 3 h. For dry heating the oven should have a fan mounted inside for better circulation of hot air. It is important that the oven is not over loaded. The glassware should be allowed to cool down before removing it from the oven. Otherwise, cool air sucked from outside may expose the load to bacterial contamination and also increase the risk of cracking.

Not all types of plastic labware can be heat sterilized. Only polypropylene, polymethylpentene, polyallomer, Tefzel ETFE and Teflon FEP may be repeatedly autoclaved at 121 °C. Sterilization cycle for polycarbonate vials should be limited to only 20 min as it shows loss of mechanical strength on repeated autoclaving. Polystyrene, polyvinyl chlorides, styrene acrylonitrile, are not autoclavable at all.

(ii) *Culture Medium.* The tissue culture medium, as stated earlier, is not only a source of contaminants but also supports their luxuriant growth. Therefore, it must be sterilized properly. Mostly, the culture medium is sterilized by autoclaving. Autoclave (Figs. 2.4, 2.5) is an apparatus in which sterilization is done by steam heating under pressure. The culture vials containing medium and closed with a suitable bacteria-proof closure are autoclaved at 15 psi and 121 °C for 15–40 min from the time the medium reaches the required temperature and pressure. Care should be taken to cover cotton plugs and other things that might get wet with aluminium foil before autoclaving. For sterilization of small quantities of medium a

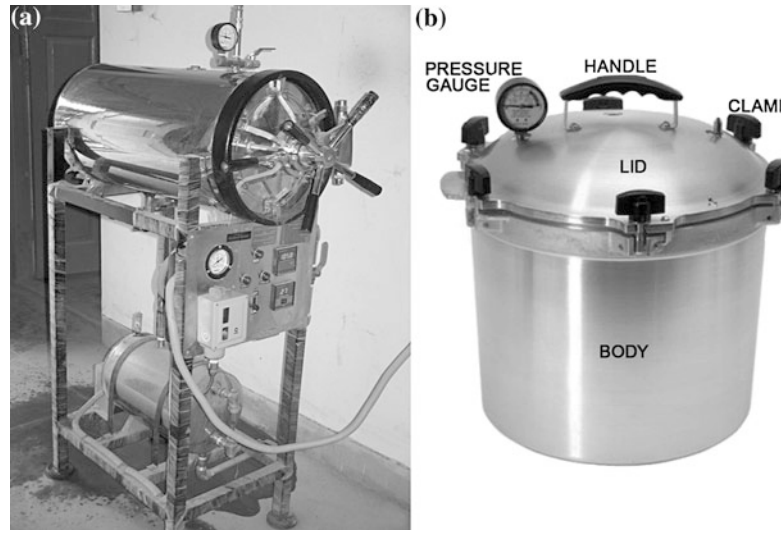

Fig. 2.4 A horizontal autoclave (**a**) and a small vertical autoclave (**b**)

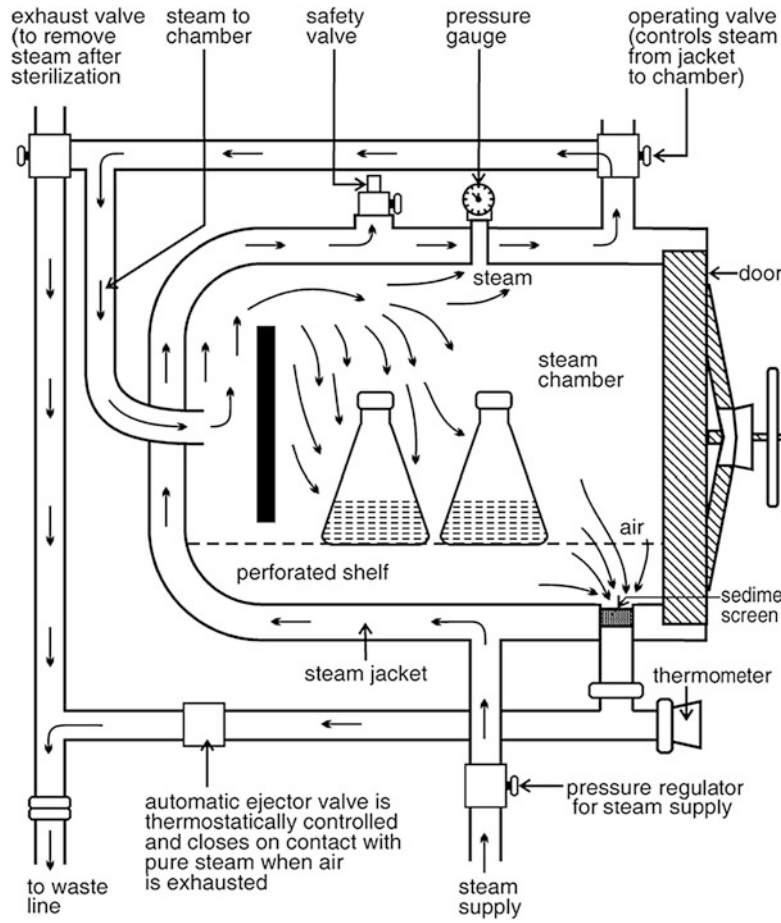

Fig. 2.5 Working diagramme of a horizontal autoclave

Table 2.1 Minimum time required for sterilization by autoclaving

Volume/container (ml)	Minimum sterilization time at 121 °C (min)
20–50	15
75	20
250–500	25
1,000	30
1,500	35
2,000	40

pressure cooker, which works on the same principle as an autoclave, may be used. The exposure time varies with the volume of the liquid to be sterilized (Table 2.1). Prolonged autoclaving may adversely affect gelling of the medium. Care must be taken not to open the pressure valve while autoclave is cooling and loosing pressure, as a rapid loss of pressure, will cause the medium to boil vigorously and overflow, wetting the vial closures. The pressure gauge should be at zero and temperature not more than 50 °C before the autoclave is opened.

It has been observed that 2–5 % of media get contaminated during manual pouring after autoclaving. Moreover, certain *Bacillus* bacteria survive even after autoclaving at 110–120 °C (Leifert et al. 1994). It is, therefore, advisable to incubate the sterilized culture medium at 30–35 °C for 24–48 h before use to ensure that it is free of contaminants.

Autoclaves are either horizontal (Figs. 2.4a, 2.5) or vertical (Fig. 2.4b) and are available in different sizes. The vertical types become cumbersome to use beyond a certain capacity because of the depth to be reached during loading and unloading. Horizontal autoclaves are easy to handle but are costlier. The decision on the type of autoclave to be bought depends on the funds and the objective. Horizontal autoclaves are available with single or double door facility. In the latter case the autoclave is so installed that its one door opens in the media room to load the medium and the other door opens directly into the 'clean area', for unloading the sterilized medium.

Autoclaving has some disadvantages such as change of medium pH and breakdown of some media constituents. The following components will be partly decomposed by autoclaving (Van Bragt et al. 1971):

(1) Sucrose breaks down into glucose, fructose, and some laevulose; the autoclaved medium with sucrose will contain several sugars,
(2) Gibberellic acid looses 90 % of its activity,
(3) Vitamin B_1 disintegrates into pyrimidine and thiazol,
(4) Zeatin, urea, vitamin C, colchicine and antibiotics are thermolabile.
(5) Plant extracts loose some of their effectiveness.

Therefore, thermolabile compounds cannot be autoclaved along with rest of the nutrient medium. These are, instead, filter-sterilized. The whole medium without the heat labile compound/s is autoclaved in a flask and kept in the sterilized hood to cool. The solution of the thermolabile compound is sterilized by membrane filtration and added to the autoclaved medium cooled to 50–40 °C in the case of semisolid medium or to room temperature when using a liquid medium. For filter-sterilization of a solution, bacteria-proof membranes of pore size 0.22–0.45 μm are used. The filter membrane is placed into filter holders (Fig. 2.6) of appropriate size and sterilized by autoclaving after wrapping in aluminium foil. Filters should not be sterilized at temperatures

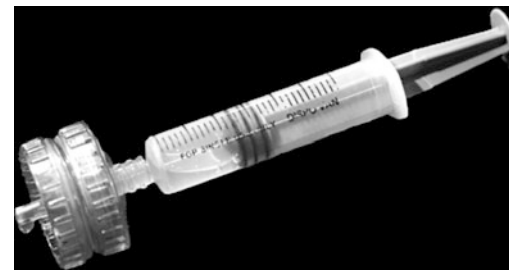

Fig. 2.6 A filter assembly with syringe

exceeding 121 °C. The thermolabile liquid taken in an unsterilized graduated syringe is gradually pushed through the sterilized filter assembly containing the membrane. The sterilized liquid dripping from the other end is added directly to the autoclaved medium. For large volumes, filter-sterilization can be carried out using a filtering set-up attached to a vacuum pump.

(iii) *Instruments*. The instruments for aseptic manipulation, such as forceps, scalpels, needles, and spatula, should be sterilized before use by wrapping in aluminium foil and autoclaving. Again, during aseptic manipulation the instruments are sterilized several times by dipping in 95 % ethanol and flaming and used after cooling. Alcohol should be regularly changed as some *Bacillus circulans* strains persist in alcohol for more than a week (Leifert and Waites 1990). Heat produced by Bunsen burner can generate eddy currents that could increase incidence of contamination during subculture. Several labs use glass bead sterilizers (Steripot), in which temperature rises to 250 °C within 5–20 min. Embedding the instruments in the heated beads for 5–7 min is adequate to sterilize them. Infrared sterilizers are also available for sterilizing instruments in the hood. The sterilized instruments are rested on a stand inside the laminar airflow cabinet slightly raised from the work table.

(iv) *Plant Material*. Plant surfaces harbour a wide range of microbial contaminants. This source of infection can be avoided by thorough surface sterilization of the plant material before planting it on the nutrient medium. Tissues with systemic fungal and bacterial infection are usually discarded in tissue culture work.

Plant tissues can be surface sterilized using various sterilants. The sterilant type, its concentration and the duration of treatment have to be determined empirically. Table 2.2 gives a guideline to get started.

Hypochlorite solutions (sodium or calcium) have proved to be effective in most cases. For example, 0.3–0.6 % sodium hypochlorite treatment for 15–30 min will decontaminate most tissues. Addition of a few drops of a surfactant (Triton-x or Tween 80) to the sterilant solution or rinsing the tissue for 30 s in ethyl alcohol before surface sterilization can enhance the efficiency of sterilization treatment. It is important to realize that a surface sterilant is also toxic to the plant tissues. Therefore, the concentration of the sterilant and the duration of treatment should be chosen to minimize tissue death. Periodic gentle shaking of the vial during sterilization is recommended. After the sterilization treatment the plant material is washed 2–3 times in sterilized distilled water in an aseptic area (laminar airflow chamber) to remove any traces of the toxic sterilant. To initiate cultures of delicate tissues, such as immature embryos, endosperm, nucellus and shoot tip,

Table 2.2 Effectiveness of some surface sterilizing agents[a]

Sterilizing agent	Concentration (%)	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

these are sterilized along with the surrounding tissues, and the explant is dissected out under aseptic conditions. Inoculation of the plant material on the medium is done in the laminar airflow cabinet (Fig. 2.7). The sterilized plant material or the plant material to be subcultured is placed on a presterilized ceramic tile, steel tray or Petri plate for cutting to proper size before inoculation.

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 plant material is either left exposed in the sterile hood until the alcohol evaporates or, if fairly hardy, flamed (Bhojwani 1980).

Several workers have used antibiotics and antifungal compounds to control explant contamination. Arbitrary use of antibiotics may be counterproductive as majority of the bacteria infecting plants are gram negative, which are less sensitive to commonly used antibiotics. Binomyl has been shown to reduce fungal infection when used with mercuric chloride.

(v) *Transfer Area and Growth room*. The chances of the cultures getting infected exist whenever the culture vials are opened to inoculate the sterilized plant tissue on the medium (inoculation) or for subculturing. To avoid this, all transfer operations are carried out under strictly aseptic conditions. Most laboratories use laminar airflow cabinets to carry out aseptic manipulations (Fig. 2.7). These are very convenient, as work can be stared within 10–15 min of switching on the airflow and can be continued for long hours.

A laminar airflow cabinet basically has drum type fans rotating at high speed to suck air from outside through a coarse filter which removes large particles, and the semi-clean air is thrown in the opposite direction. The dust-free air, which is under pressure, gets pushed through a fine filter, known as the "High Efficiency Particulate Air (HEPA)" filter. The HEPA filter prevents the entry of particles larger than 0.3 μm. The ultra clean air, free of fungal and bacterial contaminants, flows through the working area in the direction of the operator. The velocity of the air coming out of the fine filter is about 27 ± 3 m min^{-1} that is adequate for preventing the contamination of the working area by the worker sitting in front of it. All contaminants such as hair, salts, flakes, etc., get blown away, and a completely aseptic environment is maintained in the

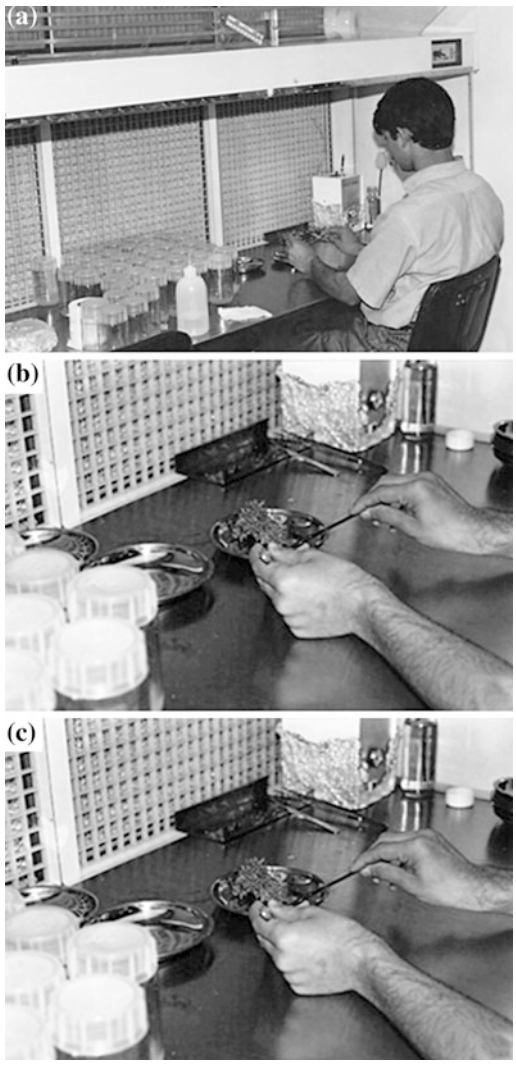

Fig. 2.7 **a–c** Aseptic manipulation in a laminar airflow cabinet

working area as long as the cabinet is on. The flow of air in no way hampers the use of a spirit lamp or a Bunsen burner attached to a LPG cylinder. The working bench is often fitted with germicidal UV lamp and fluorescent tubes for illumination. The airflow cabinet is provided with some power points to facilitate the use of microscope and other minor equipment during inoculation. The laminar airflow cabinet should not be kept facing frequently used doors and windows. It would be ideal, as stated earlier, to maintain these cabinets in the growth room or a separate transfer room in the 'clean area'.

The 'clean area' should, preferably, be under positive air pressure, as stated earlier, to maintain a particle count of 100–1000 ppm (parts per million = particles per m^3) and keep the area reasonably aseptic. Where this is not possible the rooms should be regularly cleaned with germicidal solutions such as ethyl alcohol. Germicidal UV lamps may be used but care should be taken to switch them off before starting the work as UV rays are harmful to eyes.

To check cleanliness in the transfer and growth rooms, a regular spore count should be carried out. This can be done by exposing Petri plates containing sterilized microbial culture medium overnight at different places and incubating them at 30–35 °C for 24–48 h. Such a measure can also be carried out to check for leaks in laminar airflow cabinet. Another measure to maintain cleanliness in the 'clean area' is periodic fumigation, preferably on weekends, with a germicidal gas (such as a mixture of 75 g potassium permanganate and 35 ml of 40 % formalin).

Small arthropods, such as thrips and mites, pose a very serious problem to the cultures. On entering the facility these spread very fast, especially in open shelf systems, thriving on the cultures and contaminating them with microorganisms. These insects may have an easy access with the persons working in the 'clean area', if they frequent lawns and bushes during breaks. So, care should be taken to prevent workers from sitting or strolling on lawns by providing a proper rest/recreation room within the facility. Frequent observation of the cultures and systematic housekeeping will go a long way in keeping contamination at bay.

2.4 Appendix I

A list of apparatus required for plant tissue culture work

1. Flasks (100, 250, 500 ml and 1, 2, 5 L)
2. Volumetric flasks (500 ml and 1, 2, 3 L)
3. Measuring cylinders (25, 50, 100, 500 ml and 1 L)
4. Graduated pipettes (1, 2, 5, 10 ml)/autopipettes of variable volumes
5. Pasteur pipettes and teats for them
6. Culture vials (culture tubes, screw-cap bottles of various sizes, petri dishes, etc.) with suitable closure
7. Plastic or steel buckets, to soak labware for washing
8. Washing machine, for washing labware
9. Hot-air cabinet, to dry washed labware
10. Oven, to dry washed labware and dry heat sterilization of glassware
11. Wire-mesh baskets, to autoclave media in small vials and for drying labware
12. Water distillation unit, demineralization unit, Milli Q unit or reverse osmosis unit, for water purification
13. Plastic carboys (10 and 20 L), to store high quality water
14. Analytical balance, to weigh small quantities and a top pan balance with tare facility, to weigh comparatively larger quantities
15. Hot plate-cum-magnetic stirrer, to dissolve chemicals
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 long periods, certain enzymes, coconut milk, etc.
19. Steamer or microwave oven, to dissolve agar and melt media
20. pH meter and conductivity, to adjust pH and conductivity 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. Exhaust pumps, to facilitate filter sterilization
24. Filter membranes and their holders, to filter sterilize solutions
25. Hypodermic syringes, for filter sterilization of solution
26. Medium dispenser, to pour medium
27. Trolley with suitable trays, to transport cultures, media and apparatus
28. Laminar airflow cabinet, for aseptic manipulations
29. Spirit lamp, burner, glass bead sterilizer or infra-red sterilizer, to sterilize instruments
30. LPG cylinder
31. Atomizer, to spray spirit in the inoculation chamber
32. Instrument stand, to keep sterilized instruments during aseptic manipulations
33. Large forceps with blunt ends, for inoculation and subcultures
34. Forceps with fine tips, to peel leaves
35. Fine needles, for dissections
36. Scalpel holder and surgical blades, for chopping of explants
37. Trays or ceramic tile, on which explants is chopped inside the hood
38. Stereoscopic microscope with cool light, for dissection of small explants
39. Digital camera with suitable attachment/s for macro and micro photography
40. Table-top centrifuge, to clean protoplast and isolated microspore preparations, etc.
41. Incubator shaker, for liquid cultures
42. Generator

2.5 Appendix II

A list of suppliers of equipment for setting up a tissue culture laboratory[a]

Equipment/apparatus	Manufacturer(s)
Glassware (culture tubes, flasks, beakers, pipettes, etc.)	*Borosil* Khanna Construction House, 44, Dr. R.G Thandani Marg, Worli, Mumbai 400018
Plasticware (beakers, test tube racks, desiccators, conical flasks, Petri plates)	*Tarsons* 856, Marshal House, 33/1, Netaji Subhash Road Kolkata 700001 *Polylab* AK Scientific Industries, 5531/9, Basti Harphool Singh, Sadar Thana Road, Delhi 110006 *Laxbro* Laxbro Manufacturing Co., W-53, MIDC, Baosari, Pune 411026
pH meter	*Labindia* Labtek Engineers Pvt. Ltd., Vandana House, 4th floor, L.B.S. Marg, Panchpakadi, Thane 400602 *Mettler* Metler Instruments AG, Ch-8606, Griefense, Switzerland

(continued)

(continued)

Equipment/apparatus	Manufacturer(s)
Balances, analytical and top pan	*Anamed* P.O. Box no. 8336, 31, Ujagar Industrial Estate, W.T.P. Road, Deonar, Mumbai 400088 *Mettler* Metler Instruments AG, Ch-8606, Griefense, Switzerland
Filter sterilization membranes	*Millipore (India) Pvt Ltd* 50A, 2nd House, Ring Road, Peewja, Bangalore *Tarsons* 856, Marshal House, 33/1, Netaji Subhash Road Kolkata 700001
Water distillation/purifiers units	*Bhanu units Infusil India* C-251, V Cross, Industrial Estate, Peenaya, Bangalore 560058 *National Physical Laboratory* Pusa Road, New Delhi 110022 *Millipore (India) Pvt Ltd* 50A, 2nd House, Ring Road, Peewja, Bangalore *Ion Exchange India Ltd.* 8, Block B, LSC, Naraina, New Delhi 110028
Laminar airflow cabinet	*Saveer Biotech Ltd.* 1442, Wazir Nagar, Kotla Mubarakpur, New Delhi 110003 *Thermadyne Pvt. Ltd.* 24th K.M. Mathura Road, Faridabad 121003 *Atlantis India Engineering Pvt. Ltd.* 4E/3, Jhandewalan Extension, New Delhi 110055
Autoclaves (horizontal and vertical)	*Nat Steel* Metal Chem Industries, 18, Crescent Industrial Estate, Kanjumarg (E), Mumbai 400042 *Yorco Sales Pvt. Ltd.* 11, Netaji Subhash Marg, Daryaganj, New Delhi 110002 *Hindustan Scientific Instruments Company,* Hindustan House, C-9, Vishal Enclave, New Delhi 110027
Oven, hot plates, magnetic stirrers, vortex	*Associated Scientific and Chemicals* 5531, Basti Harphool Singh, Sadar Thana Road, Delhi 110006 *Hindustan Scientific Instruments Company* Hindustan House, C-9, Vishal Enclave, New Delhi 110027
Shakers	*Hindustan Scientific Instruments Company* Hindustan House, C-9, Vishal Enclave, New Delhi 110027 *New Brunswick Scientific Co. Inc.* Ependorf India Ltd. First Floor, 24 Community Centre, East of Kailash New Delhi 110065
Trolleys for growth room, temperature controller, electronic timers, humidifiers and dehumidifiers	*Saveer Biotech Ltd.* 1442, Wazir Nagar, Kotla Mubarakpur, New Delhi 110003

(continued)

(continued)

Equipment/apparatus	Manufacturer(s)
Stereozoom microscope	*Nikon* Towa Optics (I) Pvt. Ltd., 223, Okhla Industrial Estate, Ph. II, New Delhi 11002
Air conditioners, central cooling system, window/split types	*Frick India Ltd.* Jeevan Vihar, 3 Sansad Marg, P.O. Box 118 New Delhi 110001 *Carrier Aircon Ltd.* Flatted Fact. Complex, Okhla, New Delhi 110021 *Voltas Ltd.* 7/1, Asaf Ali Road, New Delhi 110002
Chemicals	*Qualigen Fine Chemicals* Fisher Scientific Thermo Electron US India Pvt Ltd, A-255, TTC Idustrial Area, Navi Mumbai 400710 *Sigma Chemical Company* P.O. Box 14508, St. Louis, MO 63178, USA *HiMedia* 23, Vadhani Industrial Establishment LBS Marg, Mumbai 400086 *Sigma-Aldrich Chemicals Pvt. Ltd.* 12th Floor, B Block, 148 Statesman House Barakhamba Road, New Delhi 110001 *Merck Specialities Pvt Ltd* Shiv Sagar Estate A, Dr Annie Besant Road Worli, Mumbai 400018

[a] Manufacturers and suppliers mentioned here are for guidance only. Most of the dealers are from India. Every country has their own dealers and representatives of the major foreign companies

Suggested Further Reading

Bhojwani SS, Razdan MK (1996) Plant tissue culture: theory and practice, a revised edition. Elsevier, Amsterdam

Biondi S, Thorpe TA (1981) Requirements for a tissue culture facility. In: Thorpe TA (ed) Plant tissue culture methods and application in agriculture. Academic Press, New York

Cassells AC (ed) (1997) Pathogen and contamination management in micropropagation. Kluwer, Dordrecht

Cassells AC (2001) Contamination and its impact on tissue culture. Acta Hortic 560:353–359

Mageau OC (1991) Laboratory design. In: Debergh PC, Zimmerman RH (eds) Micropropagation: technology and application. Kluwer, Dordrecht

Pierik RLM (1987) In vitro culture of higher plants. Martinus Nijhoff, Dordrecht