Materials and Methods

3. MATERIALS AND METHODS

3.1 Materials selected for micropropagation and sources of explants:

The total number of orchids according to the survey list was found to be 218 species available in Darjeeling and Sikkim Himalayas, out of which 20 different species have been taken into consideration for their micropropagation in the present study. The selection was based on their horticultural or medicinal importance. All the 20 species along with their status have been shown in Table-8 and the places of collection have been shown in the Map (Fig. 1).

Table 8: Orchid species used for micropropagation in the present study.

Names	Status
Bulbophyllum ornaticimum (Rchb.f.) J.J.Sm.	Ornamental and Horticultural
Coelogyne corymbosa Lindl.	Medicinal and Ornamental
Coelogyne cristata Lindl.	Medicinal and Ornamental
Coelogyne elata Lindley.	Ornamental and Horticultural
Coelogyne flavida Wall. Ex. Lindl.	Ornamental and Horticultural
Coelogyne nitida (Wall.) Lindley.	Ornamental and Horticultural
Coelogyne ovalis Lindley.	Medicinal and Ornamental
Cymbidium elegans Lindley.	Ornamental and Rare sp.
Dendrobium chrysanthum Lindl ExWall.	Medicinal and Ornamental
Dendrobium crepidatum Lindl.	Ornamental and Horticultural
Dendrobium densiflorum Lindl. Ex wall.	Medicinal and Ornamental
Dendrobium fimbriatum Lindl.	Rare and Endangered sp.
Dendrobium longicornu, Lindley	Medicinal and Ornamental
Dendrobium nobile Lindl.	Medicinal and Ornamental
Dendrobium primulinum Lindl.	Ornamental and Horticultural
Pleione humilis (Smith) D.Don	Medicinal and Ornamental
Pleione maculate (Lindl.) Lindl.	Medicinal and Ornamental
Pleione praecox (Smith) D.Don.	Ornamental and Horticultural
Rhynchostylis retusa Bl.	Medicinal and Rare sp.
Thunia alba Reichenbach fil.	Ornamental and Horticultural

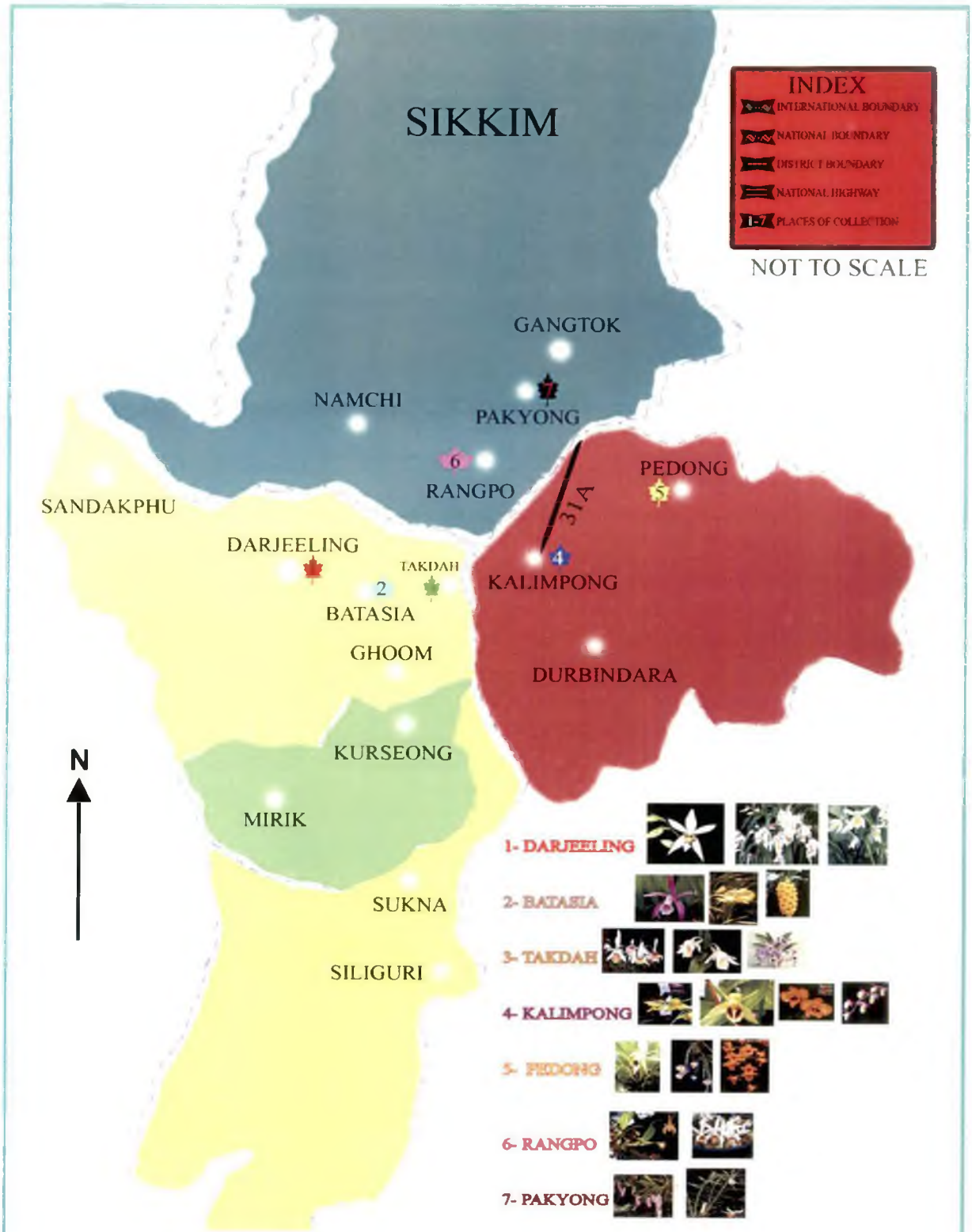

Fig.1: Map showing the places of collection.

Sources of explant: The source of explant was undehised fruits of the selected plant. Collection of undehised fruit or seed pod started from the natural habitat without damaging the mother plant on the basis of flowering and fruiting calendar. The total area covered for the collection of explant for tissue culture in the laboratory is being shown on the map (Fig.1). Main area includes different forest locations of Batasia, Ghoom, 6th Mile, Darjeeling (Latitude 26°31′ - 27°13′ N, Longitude 87°59′ - 88°53′ E), Kalimpong (Latitude 27.073610 N, Longitude 88.477859 E) and Sikkim (Latitude 27.20 N, Longitude 88.40). Approximate harvesting period of the pods has been shown in the Table-9.

Table 9: Seed harvesting time of orchids used in the present study.

Names of the orchids	Time of harvesting (Days)
Bulbophyllum ornatissimum (Rchb.f.) J.J.Sm	120
Coelogyne corymbosa Lindl.	170
Coelogyne cristata Lindl.	270
Coelogyne elata Lindley	150
Coelogyne flavida Wall. Ex. Lindl.	170
Coelogyne nitida (Wall.) Lindley	240
Coelogyne ovalis Lindley	300
Cymbidium elegans Lindley	210
Dendrobium chrysanthum Lindl. Ex Wall.	275
Dendrobium crepidatum Lindl.	215
Dendrobium densiflorum Lindl. Ex wall.	221
Dendrobium fimbriatum Lindl.	120
Dendrobium longicornu, Lindley	201
Dendrobium nobile Lindl.	108
Dendrobium primulinum Lindl.	217
Pleione humilis (Smith) D.Don	248
Pleione maculate (Lindl.) Lindl.	270
Pleione praecox (Smith) D.Don	247
Rhynchostylis retusa Bl.	300
Thunia alba Reichenbach fil.	218

3.2 Seed Culture Technique:

The seed culture technique helps in germination of plants where the normal growth is difficult. This technique ensures much better germination particularly in the genera where germination is not possible due to some abnormality within the seeds (Arditti *et al.*, 1982; Linden, 1980; McIntyre *et al.*, 1972; Pathak, 1989; Pathak *et al.*, 1992; Vij and Pathak, 1988; Vij *et al.*, 1995; Yam and Weatherhead, 1988). The orchid seeds are minute and exhibit very poor level of differentiation. The development of the embryo is suppressed as such that the embryo is arrested at a bulbous stage. In spite of being insubstantial embryo,

they can germinate in nature if infected with a suitable fungus (Bernard, 1904). However, the germination can bypass the fungal requirement by supplying an appropriate sugar rich nutrient medium *in vitro* (Knudson, 1925). Significantly, the orchid ovules are also capable of development *in vitro*. Prior to being fully ripe the capacity to germinate drops progressively (Arditti, 1967; Withner, 1943).

3.3 Micropropagation Technique:

The technique of orchid micropropagation has been originated since many years, its origin lies in several lines of research and came from the work of many well known scientists, (Arditti and Krikorian, 1996). Method for the *in vitro* culture of isolated plant cells, leaves and organs or seeds are not difficult or complex but they do require some equipment and certain skills and knowledge. More than one hundred years ago the method of propagating *Phalaenopsis* was viewed by simply placing an explant on a medium and cultured until it produced a plantlet or died.

The modern history or orchid micropropagation started when a new (*in vitro*), simple and practical method for vegetative (clonal) propagation of *Phalaenopsis* was developed (Rotor, 1949). The nutrient medium used to culture the *Phalaenopsis* nodes was Knudson "C" (KnC) formulated for the asymbiotic germination of orchid seeds by Lewis Knudson. The solution was improved and published as Knudson C, "KC" reasonably a good medium for orchid seed germination (Knudson, 1946). This medium is used very widely for orchid seed germination (Arditti *et al.*, 1982) and the propagation of some orchids.

3.4 Media Components:

The germination response of seeds, multiplication of shoots tips directly or indirectly and their subsequent development into seedling has been tested in a variety of chemically defined medium. Among them Vacin & Went (1949) [VW], Knudson 'C' (1946) [KnC], Murashige & Skoog 1962 [MS], Orchimax and orchid maintenance media were selected and used for the present work. The detail compositions of the media are given in Table-10.

Table 10: Showing the basic components of media

COMPONENTS	Vacin & Went (1949)	Knudson C (1946)	MS (1962)	Orchimax	OMM
Macronutrients (mg/lt)					
$Cacl_2$	-	-	332.02	166	166.08
$Ca(NO_3)_2$	-	241.30	-	-	-
$Ca_3(PO4)_2$	200	-	-	-	-
KCl	-	250	-	-	-
KH_2PO_4	250	250	170	85	85.00
KNO_3	525	-	1900	950	950.00
$MgSO_4$	122	122.15	180.54	90.35	90.34
NH_4NO_3	-	500	1650	825	825.00
$(NH_4)_2SO_4$	500	500	-	-	-
Micronutrients (mg/lt)					
$COCl_2.6H_2O$	-	-	0.025	0.0125	0.0125
$CuSO_4.5H_2O$	-	-	0.025	0.0125	0.0125
$Fe_2(C_4H_4O_6)_3$	23.13	-	-	-	-
FeNaEDTA	-	-	36.70	36.7	37.30
$FeSO_4.7H_2O$	-	25	-	-	27.80
H_3BO_3	-	-	6.20	3.1	3.10
KI	-	-	0.83	0.415	0.42
$MnSO_4.H_2O$	5.68	5.68	16.90	8.45	8.45
$Na_2MO_4.2H_2O$	-	-	0.25	0.125	0.12
$ZnSO_4.7H_2O$	-	-	8.60	5.3	5.30
Vitamins (mg/lt)					
myo-inositol	-	-	100	100	100
Glycine	-	-	2	-	-
Nicotinic acid	-	-	0.50	1.00	1.00
Pyridoxin HCl	-	-	0.50	1.00	1.00
Thiamine HCL	-	-	0.10	10.00	10.00
Buffers (mg/lt)					
MES(Morpholino ethane sulfonic acid)	-	-	500	1000	1000
Organics (gm/lt)					
Sucrose	20	20	-	20	20
Tryptone	-	-	-	2	2
Activated Charcoal	-	-	-	2	2

In the orchid tissue culture media different macro and micro elements were mixed together. All the chemicals were stored in accordance with instructions on the package or in the refrigerator. Some chemicals may absorb water from air and either solidify or liquefy. Such chemicals were stored under vacuum in refrigerator.

Macro elements:

Macro elements are the major elements or nutrients that are needed in relatively larger amounts. They include calcium (Ca), magnesium (Mg), nitrogen (N), phosphorus (P), potassium (K) and sulfur(S). Depending on the medium used, several salts were used to supply each mineral in that medium.

Stock solution of macro elements (except those containing nitrogen) could be stored at room temperature (usually about 22°C). Nitrogen containing stock solutions may be contaminated even in the refrigerator and therefore, stored in a freezer, within a container that is capped tightly to prevent evaporation. After mixing in the medium, all macro element salts were sterilized by autoclaving.

Micro elements:

Culture media vary widely in the use and content of micro- or minor elements or nutrients. Several micro elements were used in the present study. Micro element stock solution was stored in the freezer. Microelements were mixed in the medium, as and when required. Finally the medium was sterilized by autoclaving.

Iron:

Many iron salts are mixed in the medium. Some are soluble initially, but in solution the iron is oxidized to an insoluble or sparingly soluble form. Ferric chloride ($FeCl_3$), ferrous sulfate ($FeSO_4$), and a number of other can be used in the preparation of media. Instead of using iron compounds directly, certain alternative compounds may be used. These are ethylene diamine tetracetic acid (EDTA,MW 292.25), disodium salt (Na_2EDTA,MW 336.02) and disodium dehydrate ($Na_2EDTA.2H_2O$,MW272.24), these compounds are used widely in the culture media including Murashige-Skoog (MS) medium (Murashige and Skoog, 1962).

3.5 Plant hormones and propagation additives:

Plant hormones are the substances of critical importance to *in vitro* culture of the plant cells, tissues as well as to the differentiation of cultured plantlets.

A century ago the existences of plant hormones was only being suggested but at present, the plant hormones are included in the culture media for tissue culture, to control development and also in differentiation during *in vitro* propagation.

Auxin:

Gottlieb Haberlandt, was the first to propose the existence of plant hormones by stating that pollen tubes affect ovary growth through the release of substances called as *Wuchsenzyme* ("growth enzymes") (Haberlandt, 1902; Arditti and Krikorian, 1996; Laimer and Röcker,2003). Hans Fitting for the first time showed, after working with *Phalaenopsis* pollinia, the pollen tubes do indeed release a substance which brings about post-pollination phenomena and ovule development in orchids (Fitting, 1909a, 1909b, 1910, 1911, 1921). He named the substance as *Pollenhormon*.

The first intimation that *Pollenhormon* contains auxin was by Fredrich Laibach and reported that the active substances can be extracted with diethyl ether (Laibach, 1932).

Auxin was discovered in Holland by Frits W. Went (Went 1926, 1990; Went and Thimann, 1937) before Laibach extracted it from *Pollenhormon*. It was identified as indole-3-acetic acid (IAA) in 1934 (Went and Thimann, 1937; Haagen-Smith, 1951) and made successful use in tissue culture (Gautheret, 1935, 1937, 1983, 1985; Loo 1945a, 1945b). At present IAA and a number of synthetic auxins are used in orchid micropropagation.

The most commonly used auxins in orchid tissue culture media are the naturally occurring auxins, indole acetic acid (IAA), and the synthetics naphthalene acetic acid (NAA), indole butyric acid (IBA) and 2,4-dichlorophenoxyacetic acid (2,4-D). Other auxins and occasionally auxin-amino acid conjugates are also used in some media. The result of auxin may differ from each other qualitatively and quantitatively i.e., the effect of one auxin on a certain species may be different from those of other auxins, and may differ with the orchid species. The effects of different concentrations of the same auxin may differ in respect to one species and may not be the same with another orchid species.

Most auxins are not destroyed by autoclaving at 110-120°C for 50-60 minutes especially if the pH is non-acidic. However, autoclaving at low pH and in the presence of other factors may destroy IAA (Posthumus, 1973). It has been confirmed in a study that IBA is more stable than IAA (Nissen and Sutter, 1990). In the present study different concentrations of NAA were used.

Cytokinin:

The effects of cytokinin differ like those of other hormones. The synthetic kinetin (6-furfuryl aminopurine), benzyladenine (N^6-benzylaminopurine, N^6-benzyladenine, BA, BAP) dimethylaminopurine (DMAP), thidiazuron (TDZ), and the naturally occurring zeatin are used most commonly in orchid culture media. Experiments with aqueous solution of kinetin, zeatin and isopentenyladenosine have shown that they are not broken down when autoclaved for 1h at 120°C (Dekhuijzen, 1971). Thidiazuron (N-phenyl-N'-1,2,3-thiadiazol-5-ylurea, TDZ) is a later addition to the list of cytokinin which are used in orchids, specially *Phalaenopsis* (Ernst,1994). It has been proved to be very potent cytokinin (Mok *et al.*, 1982; Mok and Mok 1985). BAP in different concentrations were used in the present study.

Gibberellins:

Gibberellins are used very seldom in the culture media for orchids. When used GA_3 is the most common form. Autoclaving reduces gibberellin's activity by more than 90% (Van Bragt and Pierik, 1971). Therefore, gibberellins must be cold-sterilized through filtration or by dissolving them in ethanol. Gibberellin was not used in the present study in any form.

Amino acids:

The most commonly used amino acid in orchid culture media is glycine because it is a component of the MS medium (Murashige and Skoog, 1962). Other amino acids are also used in some media.

Polyols:

The only polyols used in orchid culture media are *myo*-inositol, *meso*-inositol, *i*-inositol, inositol, cyclohexitol, inosite, meat sugar and bios I; chemically it is hexahydroxycyclohexane ($C_6H_{12}O_6$) and it is a part of the MS medium (Murashige and Skoog 1962). Hexitols in coconut water have also been shown to have growth promoting effects on plant embryos (Van Overbeek *et al.*, 1941; Shantz and Steward, 1952, 1955). However these substances were not added to culture media other than as a part of coconut water.

Coconut water:

Haberlandt suggested the utilization of embryo sac fluids (Haberlandt, 1902; Krikorian and Berquam, 1969; Laimer and Rücker, 2003). Then there was the use of liquid endosperm of coconut i.e., coconut water as an additive to the medium. Consequently, at present coconut water is widely used in micropropagation of many plants.

The substances like cytokinin, zeatin was isolated from coconut water (Letham, 1968) some suggested that the presence of such agents explained the reasons for its activity (Galston, 1969; Skoog, 1994).

F. Mariat may have been the first to publish the use of coconut water also referring to it as milk and copra extract as additive to media employed for orchid seed germination. When added at concentration of 2% it did not inhibit germination and development but the seedlings were yellowish green. At higher levels coconut water was inhibitory to germination and development and the seedlings died (Mariat, 1951; Arditti and Ernst, 1984). Experiments with *Phalaenopsis* seedlings showed that coconut water can induce proliferation of protocorms (Ernst, 1967b). In the present study coconut water was used in different concentrations.

Hydrolysates and Autolysates:

Peptone, tryptone (both hydrolysates), yeast extracts (an autolysate), and a number of other complex additives are used in some orchid tissue culture and micropropagation media. In the present study yeast extract was used.

Charcoal:

Activated charcoal or carbon is extensively used for the absorption of substances. Both animal and vegetable charcoals are available, but the later are preferable for culture media.

The first attempt to darken a culture medium used for orchid seed germination was made in an effort to germinate American *Cypripedium* sp. (Curtis, 1947). Lampblack had no effect of any kind. The addition of animal charcoal to a culture medium used for *Cymbidium* plantlets improved differentiation and plantlet growth but reduced the proliferation of protocorm-like bodies (PLBs) and the formation of aerial roots (Werkmeister, 1970a,

1970b, 1971). Werkmeister was the first to use charcoal to darken orchid culture media. He did it to study the effect of dark media on root growth and development.

Activated vegetable charcoal improved seedling growth of the terrestrial *Paphiopedilum* (Ernst, 1974) and the epiphytic *Phalaenopsis* (Ernst, 1975, 1976) orchids. One possible explanation of the effects of charcoal on orchid seedlings or tissue culture derived plantlets is that it improves aeration. Growth similar to that on charcoal containing media was also observed when seedlings of *Paphiopedilum* and *Phalaenopsis ambionensis* were grown on Pyrex glass wool alone or in combination with Nuchar C vegetable charcoal (Ernst, 1974, 1975, 1976; Arditti, 1979; Arditti and Ernst, 1984). One possible explanation of the effects of charcoal on orchid seedlings or tissue cultured derived plantlets is that it improves aeration. A second possibility is that charcoal adsorbs ethylene (Ernst, 1975) and may also adsorb plant hormones and vitamins and this may explain the fact that it can also be inhibitory to growth (Fridborg and Eriksson, 1975; Weatherhead *et al.*, 1979).

3.6 Solvents:

Distilled water has been used as a solvent in the preparation of culture media. Ethanol (ethyl alcohol, 70% in distilled water) has been used as a sterilizing solvent for substances that cannot withstand autoclaving.

3.7 pH:

The term pH is indicative of the alkalinity of acidity (i.e., hydrogen ion concentration) of a medium. It is defined as the logarithm of the reciprocal of the hydrogen ion concentration. The pH is important because it affects the availability of nutrients.

The pH of culture media has been adjusted to 5. Growth may be inhibited if the pH is lower or higher than the appropriate for the plants under culture.

3.8 Solidifiers:

Agar was the major solidifier used for orchid seed germination and micropropagation media for several decades and hence has been used in these experiments also. It is widely used where it is added slowly with continuous stirring to the medium and then to bring the mixture to boil. Once agar gets dissolved, medium has to be autoclaved after dispensing into culture vessels.

Gellan gum (phytagel, Gelrite) is an agar substitute consisting of glucuronic acid, rhamnose and glucose produced through fermentation by *Pseudomonas elodea*. It was discovered by Kelco Division of Merck and Co., in San Diego, California. The gum produces a high strength, colorless and clear gel. While using gellan gum, there should be continuous stirring to avoid the formation of lumps in the media.

3.9 Media preparation:

The media preparation was required immediately after the collection was made and that required very careful and systematic steps. It can be sequenced as follows:

1. The required amount of the desired media was dissolved in little amount of the distilled water, dissolved properly and carefully.
2. All the additives and desired hormones were incorporated carefully and slowly.
3. The volume was made up to the required total volume by the addition of distilled water.
4. pH was adjusted prior to autoclave as 5.00. If the pH of the media indicates below 5.00, it was balanced with the help of few drops of 1(N) NaOH and if it exceeds 5.00, then was balanced with few drops of 1(N) HCl.
5. To solidify the medium required amount of agar-agar powder (8gms/L) was added and brought to boil with continuous stirring so as to avoid the formation of lumps in the medium.
6. The proper amount of the medium was dispensed in the culture tubes, vessels and Erlenmeyer flasks.
7. Tubes, flasks and vessels were adequately capped and covered with the help of brown paper and rubber bands.
8. The prepared culture medium was sterilized under high temperature and pressure in an autoclave maintaining the pressure at 121°C and 15 lb psi for 15minutes.
9. After sterilization, they were taken out from the autoclave, cooled at room temperature and kept at the working table of laminar air flow and further sterilized by spraying 70% ethanol.

3.10 Sterilization procedure:

The capsule or the green pod collected from the experimental garden is usually contaminated with micro organisms and other contaminants, thus needs surface sterilization.

The pod was first cleaned thoroughly with the help of a soft brush, scraping slowly and washing under running tap water followed by submerging with 5% Tween 20 (liquid detergent) for 10 minutes and subsequently rinsed with distilled water until the forth is totally washed away. Further, the pod was taken to the laminar air flow and on that table once again it was surface sterilized by freshly prepared 0.1% mercuric chloride solution for 15 minutes with occasional agitation and was subsequently rinsed three times with autoclaved distilled water. Now, the explant was ready for the inoculation.

3.11 Inoculation:

After surface sterilization, the explants were taken in a sterile petridish containing filter paper to soak the surface water of the fruit or capsule. The capsules were cut longitudinally with the help of sterilized surgical blade and forceps. Mass of tiny seeds was inoculated on the slant surface of the desired media in the culture tubes.

3.12 Culture conditions:

The culture conditions under which the explants, seeds, PLBs and callus masses are maintained can determine the success and failure of culture and further affects the growth and development of the *in vitro* plantlets. The seeds are placed on a solid medium establishing a good contact between them and the medium with even distribution. The seeds are not totally buried in the agar solidified medium in order to prevent death from improper gas exchange.

After inoculation of the seeds, the culture tubes were kept at 22 ±1°C under 16 hours photoperiod from cool white light giving 2659 lux.