MATERIALS AND METHODS

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Source of Seed Tubers

Virus-free certified seed tubers of potato (*Solanum tuberosum* L) were collected from the Tuber Crop Research Center, Jaydevpur, Bangladesh. Two varieties were selected for current research studies, var. Dheera, which is a late maturing variety, and var. Heera, is an early maturing one (Plate:1). Details of the varieties are given in Table 1.

Table 1: Performance of two varieties (Source: Tuber Crop Research Center, BARI, Jaydevpur, Bangladesh)

Variety	Alu-12 (Dheera)	Bari Alu-1 (Heera)
Released by	Int. CIP, Lima, Peru (1987) Rel. 1993	Int. CIP, Lima, Peru (1983) Rel 1990
Yield range (t/ha)	20-35	25-40
Field reaction to disease	Field tolerant to virus diseases; degenerates slowly	Tolerant to PLRV and fungal foliar disease; degenerates slowly
Days to sprouting	60-70	50-60
% of Wt. Loss	8.6	10 2
% rottage loss at 90 days	0 0	5.0
Other Character	First growing, stem many, medium sized tuber, oval in shape, light yellow colour, smooth skin, shallow eyes Tolerant to heat Matures within 90-95 days	Tuber somewhat big flat round, light yellow in colour, light yellow flesh, skin smooth, foliage first growing, shallow eye Mature very early (80 days) but may be harvested earlier, high degree of stress tolerance, particularly wet soil and can be planted very early or very late

Glassware

All the glassware used in the present studies were either Borosil or Corning make. Erlenmeyer flasks (150 or 250 ml) and test tubes (25 x 150 m, 15 x 125 mm) were used as culture vessels. Non-absorbent cotton plugs were used to seal the culture vessels. All the glassware were dipped in chromic acid (prepared by dissolving 100 gm of potassium dichromate in 500 ml of concentrated sulphuric acid and diluted to 2:1 with water) before using, followed by thorough wash with tap water. They were finally rinsed with distilled water and dried in hot air oven at 60°-80°C.

Plate 1. Certified potato varieties kept on acid washed sand for sprouting:

a. var Dheera (DR)
b var Heera (HR)


DHEERA
a
JHEERA
b

Chemicals

All the chemicals used in the present studies were of analytical grade. Only sucrose, used in the preparation of culture media, was pure-tissue culture grade obtained from Sisco Research Laboratories (SRL). Other chemicals were obtained from E-merk, British Drug House (BDH), Loba, Qualigens, Ranchem (Ranbaxy), Sulab. All the plant growth regulators like auxins, and cytokinins were obtained from Sigma Chemical Company, USA.

Culture Media

MS medium (Murashige and Skoog, 1962) was used throughout the course of the study supplemented with various additives and growth regulators. L.S. medium (Linsmeir and Skoog, 1965) was also tried initially for shoot multiplication. According to the requirement, the medium was prepared either as a liquid or semi-solid matrix. 0.7-0.8% Difco bacto agar (Qualigens) was used as gelling agent for the semi-solid medium. In the case of liquid stationary culture, Filter Paper Bridge was used to support the plantlet.

Carbon Source

Sucrose was used as a carbon and energy source. According to the requirement 3-12% sucrose was used in the culture medium.

Medium Composition or Preparation

Four different stocks were prepared for MS medium (Murashige and Skoog, 1962) - macronutrients, micronutrients, vitamins and iron stocks. They were prepared in double distilled water and stored in plastic or amber coloured bottle in the refrigerator. KH_2PO_4 (Potassium dihydrogen phosphate), Myoinositol and sucrose were added at the time of mixing of the stocks (Table: 2). Various concentration and combination of plant growth regulators were used depending on the aim of the experiments.

Table 2: Chemical composition of Murashige & Skoog medium (1962)

(a) Macro nutrients

Chemicals	Original conc. (mg l^{-1})	Conc. of stock solution (mg l^{-1})	Volume of stock per litre of medium
$NH_4 NO_3$	1650	33,000	
KNO_3	1900	38,000	
$CaCl_2 2H_2O$	440	8,800	50 ml
$MgSO_4 .7H_2O$	370	74,00	
KH_2PO_4	170	added directly	

(b) Micro nutrients

H_3BO_3	6.2	620	
$MnSO_4 . H_2O$	16.9	1690	
$ZnSO_4 . 7H_2O$	8.6	860	
KI	0.83	25	10 ml
$Na_2MoO_4.2H_2O$	0.25	83	
$CuSO_4.5H_2O$	0.025	2 5	
$CoCl_2.6H_2O$	0 025	2.5	

(c) Organic constituents

Myo-inositol	100	(added directly)	
Nicotinic acid	0.5	50	
Pyridoxine-HCl	0.5	50	10 ml
Thiamine-HCl	0.1	10	
Glycine	2	200	

(d) Iron source*

$FeSO_4 \ 7H_2O$	27.8	5560	5 ml
Na_2 EDTA. $2H_2O$	37 3	7460	

(e) pH 5.8

(f) Sucrose 30g / l to 120 g/l

* The $FeSO_4.7H_2O$ and Na_2 EDTA.$2H_2O$ were dissolved separately in approximately 200 ml of double distilled water. Both the solutions were heated until it dissolved completely. The $FeSO_4$ was added to the warm EDTA solution with continuous stirring. The solution will have a slight yellowish tinge. The volume was then adjusted to 1000 ml with double distilled water The Fe-EDTA solution prepared by this method could be stored up to 2 months without precipitation, in amber coloured bottle, under refrigeration.

The pH of the medium was adjusted to 5.8 ± 2 with freshly prepared 1N HCl or 1N KOH. In the case of semi solid medium agar was added after adjusting the pH and boiled with constant stirring. Liquid media was dispensed directly after adjusting the pH, in tubes (10-15 ml) or flasks (15/50 ml). Vessels were then plugged with non-absorbent cotton plugs. Media and other inoculation instruments such as forceps, scalpel, scissors, petridishes etc. were wrapped with paper and autoclaved at 121°C and 15 lbs pressure for 20 minutes.

Aseptic Conditions

All tissue culture operations were carried out aseptically in laminar airflow hood (Klenzaids, India). Before any operation, the working bench was wiped with alcohol. Culture vessels and other equipments were exposed to UV irradiation for 30 min. All the instruments used for inoculation (forceps, scalpel, scissors, needles, etc.) were frequently dipped in alcohol and flame sterilized.

Culture conditions

For the establishment of meristems and maintaining the shoot multiplication, cultures were maintained in a culture room with controlled conditions. Temperature was maintained at 23° ± 2°C and photoperiod 14/10 h. (day/night). Liquid cultures were kept on a rotary shaker (120 rpm). Subculturing was done every 21 days. For tuberization, cultures were kept in a BOD incubator at 15° ± 2° C in continuous dark.

Source of the explants

Whole potatoes were washed with water, dipped in Bavistin and $HgCl_2$ solution and then kept on acid washed moistened sand under 16 h. photoperiod of light for sprouting. Within 10-15 days healthy sprouts were seen from the eyes. The sprouts were collected for the initiation of cultures.

Surface sterilization of explants

Tips from the sprouts were collected and treated with 0.2% chloramphenicol, 0.2% Bavistin and 0.1% activated charcoal in a rotary shaker. Further treatments were

carried out under sterile conditions inside the laminar air flow hood. Treatment with various concentrations of $HgCl_2$ was given for various durations and washed with sterile distilled water for 3-4 times to remove the traces of mercuric chloride

Inoculation procedures

a) Initiation and maintenance of shoots *in vitro*

0.2-0.4 cm long shoot apices were dissected carefully from surface sterilized sprouts and were inoculated on MS medium solidified with 0.8% agar. Apical bud grows out into an elongated shoot. Single nodes with dormant buds were subcultured to multiply them after every 21 days. At every subculture a plantlet yields at least 4-6 nodal cuttings. Usually 5 single node cuttings were cultured per culture tube containing 10-15 ml of solid medium and tubes were sealed with cotton plugs.

b) Liquid culture for multiplication:

Liquid medium was prepared similarly as the solid but was devoid of agar. For liquid stationary cultures the media was prepared in test tubes (15 ml) and the shoots were supported with filter paper bridges. For liquid agitated cultures 5 pieces of microshoots, having 3 single nodes each, were inoculated in 150 ml erlenmeyer flasks containing 15-20 ml of liquid propagation medium and kept on a rotary shaker for agitation.

c) Tuberization media

Three kinds of tuberization media was employed for induction of microtubers

- Solid medium: single node cuttings were inoculated on the semisolid tuberization medium
- Bilayer medium: shoots were grown on solid propagation medium and liquid tuberization medium was poured over the semisolid propagation medium
- Liquid medium: Shoots were grown on liquid propagation medium and after 21 days the propagation medium was replaced with liquid tuberization medium.

At tuberization stage, different concentration of sucrose (3-12%) along with several combinations of growth regulator and growth inhibitors were tried for optimizing the tuberization.

Storage Condition

Microtubers were harvested after 45-60 days. After harvesting they were dipped in a dilute solution of fungicide (Bavistin,0.1%) for 5-10 min., washed in sterile distilled water, dried on filter paper and kept in glass petridishes. Petridishes were sealed with para-film and stored in a desiccator at 4°C.

Germination Studies

Microtubers were taken out from the refrigerator after an interval of 30days and used for the germination studies. When brought to the room temperature, microtubers started sprouting within 10-15 days. Sprouted microtubers were planted directly to the seedbeds during 1st week of Dec. 2000, at a spacing of 10 x 20 cm (10 cm between the microtubers and 20 cm between the rows). Only Farm Yard Manure (FYM) was applied before plantation. Microtubers were planted in a randomized block design with 4 replicates. Plots were irrigated by furrow irrigation throughout the growing period according to evapotranspiration requirements to avoid drought stress during crop growth. Minitubers were harvested after 100 days.

Encapsulation of Single node potato cuttings

5 mm long single node cuttings were dissected from micropropagated plantlets and were dipped in MS medium supplemented with 3% Na-alginate. Along with alginate solution, microcuttings were than dropped into 60 mM $CaCl_2$ solution with the help of a sterile pipette. Encapsulated microcuttings were kept in the same solution for half an hour and later washed with sterile distilled water to remove excess $CaCl_2$. These encapsulated single node cuttings are small bead like structures with a diameter of 5 mm. They were stored on moist filter paper in sterile petridishes, sealed with parafilm and kept in a refrigerator at 4°C. Germination percentage of these encapsulated nodes was studied after 10, 20 and 30 days. Germination were carried out *in vitro* in MS basal medium and *in vivo* in soil-peat mixture (1:1).

Developmental Studies of Microtuber

Shoots derived from propagation medium, agitated in a rotary shaker, were used for the anatomical studies. Single node cuttings were collected from 1st node to the 5th node after the addition of tuberization medium on 0, 1st, 2nd, 3rd, 4th, 5th, 10th and 15th days of incubation.

Microtechniques

a) Fixation and preparation of the block

To study the anatomical changes during microtuberization, 0.5 cm long single node segments were fixed in FAA (FAA-Formation: Alcohol: Acetic Acid-5:90:5) They were dehydrated in Tertiary butyl alcohol series and embedded in paraffin wax with ceresin, m.p. 58-60° C (Johansen, 1940).

b) Microtomy

Transverse and longitudinal sections (10-12 μm) were carried out on a rotary microtome (Leica, West Germany). Ribbons were spreaded on glass slides using 4% formalin and Haupt's adhesive (Berlyn and Mikchle, 1976). They were deparaffinised in xylene series and brought down to water and stained.

c) Staining

Stains, histochemical reagents and techniques were used as follows:

- General staining by Toludine blue and Borax (Vreugdenhil *et al.*, 1999)
- Starch and insoluble polysaccharides staining by periodic acid-schiffs reagent (PAS reaction) according to Mecully (1966), Feder and O'Brien (1968).
- Starch localization by I_2KI, according to Johansen, (1940).

Statistical Analysis

All the experiments were repeated thrice. Values are represented as mean ± standard error (S.E. of the replicates). The data were analysed by Duncan's multiple range test (Duncan, 1952). All the computations and analysis were carried out using SPSS statistical package.