European Online Journal of Natural and Social Sciences 2018;
Vol.7, No 2 pp. 424-443
ISSN 1805-3602
www.european-science.com

Elimination of Chhirkey and Foorkey viruses from meristem culture of large Cardamom (*Amomum subulatum* Roxb.)

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Received for publication: 14 February 2018.
Accepted for publication: 10 May 2018.

Abstract

Large cardamom is one of the important cash crops in the mid hills of Nepal. *Amomum subulatum* has 0.91 – 1.22m leafy stem. The leaves are 0.30m-0.61m ×0.08-0.10m in size. They are green and glabrous on both surfaces. Of many diseases and pests, two viral diseases namely 'Foorkey' and 'Chhirkey' are the main problems for large cardamom cultivation. Production has been declining due to these diseases. Viruses can be eliminated either by thermo therapy or meristem culture or both. Heat therapy has been proved to be a highly successful method for inactivation or elimination of viruses from perennial crops in order to obtain virus- free stock material. The aim of the study is detection of 'Foorkey' and 'chhirkey' virus with PRSV antibody by Double Antibody Sandwich – Enzyme Linked Immunsorbent Assay (DAS- ELISA), Regenerate shoots from meristems of two cultivars Ramsahi and Golsahi, Multipy virus free shoots of cultivars Golsahi and Dambarsahi by tissue culture.

Both Chhirkey and Foorkey viruses were tested with DAS ELISA using antibodies of papaya ring spot virus (PRSV) obtained. Then applied in tissue culture. Maximum shoot growth and root number were observed on MS +0.1 mg/l BA +0.1 mg/l NAA in both liquid and solid media. The rooted planted of Golsahi and Dambarsahi were successfully acclimatized in the screen house is strongly recommended that specific antibodies of Chhirkey and Foorkey should be prepared for indexing of these viruses.

Keywords: Chirkey and Foorkey viruses, DAS ELISA, Regenerate, Screen house, Shoot growth

Introduction

Nepal is located between latitudes 26 ^{0} 22' N- 30 2 N and longitudes 80 ^{0} 4' E – 88° 12 E. It has diverse topography and wide elevation ranges from the 60m – 8848m above sea level. The climatic conditions also vary from the tropic to the artic with the average rainfall of 1600mm/year. These conditions favor successful growth of different types of plant. So, Nepal is rich in biodiversity. The estimated number of flowering plants is estimated at 6500 species (Shrestha, 2001)

Large Cardamom (*Amomum subulatum* Roxb.) is one of the major flowering plants cultivated by the farmers of the middle hills in the eastern development region of Nepal. It is indigenous to the eastern Himalayan region probably of Nepal and hence also known synonymously as Nepal cardamom. It is cultivated in an altitude range of 700-2000m above sea level. It thrives well in the shadowy, moist and sloppy lands where the cultivation of the other crops is virtually impossible. It is usually cultivated under the shade of *Alnus nepalensis* and *Schima wallichii*.

Three commercial cultivars of large cardamom namely Ramsahi, Golsahi and Dambarsahi exist in Nepal. Ramsahi grows well above 1600m whereas Golsahi grows in altitude between 1200-1600m. Dambarsahi is the low altitude loving cultivar that thrives well below 1200m (De Waard and Ranjit, 1976).

Large cardamom is one of the important cash crops in the mid hills of Nepal. It improves the socio-economic situation of rural people where other crops are difficult to grow. Farmer gets timber, wood and fodder from the area under large cardamom cultivation. It is also an important source of earning foreign currency.

Amomum subulatum has 0.91 – 1.22m leafy stem. The leaves are 0.30m-0.61m ×0.08-0.10m in size. They are green and glabrous on both surfaces. The spike is very dense. The peduncle is short about 0.05-0.07m. The bract is red brown in colour. The outer bract is ovate and inner one is obtuse. The calyx and corolla are 2.5cm long. The segments are obtuse and shorter than the tube. The upper side is cuspidate. The lip is yellowish white. The filament is very short. The anther is crest and entire. The capsule is globosely red and densely echinate.

Seeds of the large Cardamom are mainly used for spices, culinary purposes, and Ayurveda medicines. Essential oil from seeds is used in perfumery and pharmacological product. The seeds are also used for medical purposes.

So far, large cardamom is propagated by both vegetative and by seeds. Vegetative propagation by rhizome is generally used for small scale propagation while seed is used for large –scale propagation. Although vegetative propagation gives cloned plant, it is responsible for spreading viral as well as fungal diseases. The traditional method for plant propagation has been doing by seeds, so far. It produces diseases free plants but there are many problems. Seedling may differ from the mother plants, as they are cross-pollinated .High seed dormancy and low germination percentages are other problems. So, alternate method of propagation must be used to produce diseases free cardamom plants.

Of many diseases and pests, two viral diseases namely ‘Foorkey’ and ‘Chhirkey’ are the main problems for large cardamom cultivation. Production has been declining due to these diseases. Infected plants give less number of flower resulting in decline in production. Up to 80% loss of the production has been recorded due to these diseases. These diseases cannot be controlled completely through chemical means although their vectors can be controlled to some extent. For the eradication of the diseases, the infected plants should be removed and virus-free cloned plantlets should be planted.

Biotechnological tool can be applied for producing virus-free clonal plants. Meristem culture is one such tool. Isolation of meristem, aseptic culture in artificial nutrient medium and regeneration of plants has been proved successful for this purpose. Such plants also help increase production and productivity. Virus indexing is necessary for virus elimination and production of virus free plants. In Nepal, antibodies specific for ‘Foorkey’ and ‘Chhirkey’ viruses are not yet available. So far indexing plants Papaya Ringspot virus PRSV antibody as tested positive by Gonsalves *et al*., (1986) for cardamom mosaic virus of Hara *et al*., 1978 reported 11 genera and 35 species of plants belonging to the family Zingiberaceae in Nepal. But, Press *et al*., 2000 have put these figure at 11 genera and 45 specie**s.** In Nepal, 3 species of Genus *Amomum* of Zingiberaceae have been reported (Hara *et al*., 1978). They are *Amomom aromaticum* Roxb., *A. dealbalatum* Roxb. And A. *subulatum* Roxb respectively. It is a rhizomatous shade loving, perennial herb. It is commonly called large cardamom or ‘Alaiche’ in Nepali. It is sometimes called Nepal cardamom or Great Indian Cardamom. The species is believed to be the native of moist, subtropical, deciduous and evergreen

forest of sub Himalayan region at latitude 30^oN. It is a native of the Nepal's eastern Himalayan region (Purseglove *et al*., 1981).

Meristem is the central portion of a rapidly growing shoot, bud or other part of plant where cell division is taking place. The viruses are found to be heterogeneously disturbed in the host plants. The apical and root meristem of plants are reported to be frequently devoid of viruses and contain very low concentrations. The plant viruses can be eliminated by culturing meristem and regenerating plants from them. In 1952, Morel and Martin had come with the brilliant idea of isolating in vitro the apical meristem of dahlias infected with the viruses, from which they could obtain virus free plants. They were first to obtain virus free dahlias and potatoes with the help of meristem culture. Since then, many viruses have been eliminated from many plants including crops by meristem culture.

Viruses can be eliminated either by thermo therapy or meristem culture or both. Heat therapy has been proved to be a highly successful method for inactivation or elimination of viruses from perennial crops in order to obtain virus- free stock material (Quak, 1977). The fact that heat treatment is sometimes ineffective can be due to the plant itself being too sensitive to heat for unknown reasons (Pierik,1997).However,the use of small tips avoided the need for preliminary heat therapy , and the appropriate combination of growth factors allowed the use for single medium for production of complete plant within a short time (Alconero *et al*., 1975).As heat treatment may impede the metabolism and reduce growth of the meristems, trials are needed to find out how long meristem can be kept at high temperatures and still develop in to virus-free plants (Quak, 1977).

A quick review of meristem culture, regeneration and *L.perenne* virus elimination is presented below. Dale (1977a) regenerate plant of *Lolium multiflorum* from meristem of size 0.2-1.1mm on MS + 1mg/l 2,4-D.The plants regenerated in culture were free from viral particles. Dale (1977b) regenerated plants from meristem tips of grass species *Lolim multiflorum*,, *Festuca pratensis F.arundinacea,Phleum* sp and *Dactylis glomerata* on medium with 0.01mg/l 2,4-D and 0.2 mg/l kinetin. They also found that survival rate was highly correlated with the size of the tips cultured.

Popov (1977) observed that addition of 6- BAP to culture medium stimulated the meristem to form numerous axillary buds in strawberries. Rooting of buds was planted in a medium with auxin and without cytokinin.

Hussey (1976) found that in *Lilium* sp.branching was promoted by concentration of 2-8 mg/l BAP when 1 to 5 laterals were formed. In *Fritillaria* and *Hippeastrum*, branches formed at 2mg/l (one to three branches per plant). In case of *Narcissus* and *Hyacinthus*, it was found that 2 mg/l BAP promoted branching but distortion occurred at higher concentrations. He found that root was inhibited in all species by concentration of BAP that caused branching.

Gonsalves *et al*., (1986) found that four viruses (zucchini yellow mosaic, papaya ring spot types p and w, cowpea aphid borne viruses and severe strain of (NL-8) of bean common mosaic) give positive reaction in indirect ELISA) which were not positively reacted with antiserum with CarMV in direct ELISA.

Gupta, (1986) eradiated mosaic disease for three cultivars of banana (*Musa acuminate*) and plantain (*M. auminata x M. Kalbissiana*) by heat therapy (38-40 C for 14 days) and meristem tip (1.5-2.0mm) cultured MS +1mg/l Thiamine HCL+0.5mg/l Nicotine acid +0.7mg/l BA+0.7mg/l Kin N.

Perez *et al*., (1987) regenerated shoot from root meristem of *Digitalis obscure* K. on MS+1.0PPM BA+0.1 or 0.5ppm NAA.

Bhagyalakshmi and Singh (1988) induced shoot from meristems of ginger with or without leaf primodia on third quarter strength of MS+ sucrose 6% +Coconut milk (CM) 20%+100mg/l

Ascorbic acid (AA) 400 mg/l Glutamic acid +250mg/l Activated Charcoal (AC) +0.5mg /l BAP+0.4mg/l BA and Agar 0.8% Multiplication of shoots were obtained by meristem derived shoots on three – quarter MS+ 3% sucrose +10mg/l AA + 100mg/l AC+4-5mg/l BAP and 0.8% agar . Liquid media (gained or static) were less effective than solid medium for micro propagation. Kinetin and NAA incorporated at various levels (0.01-0.08mg/l). With or without added BA+IBA, neither improved plantlet formation nor enhanced shoot multiplication.

Nagasawa and Finer (1988) obtained callus for suspension culture from meristem of garlic (*Allium sativum* L.) on liquid medium containing MS salt + B5 vitamins + 3% sucrose +1ppm NAA+2ppm BA.

Shrestha and Rajbhandari(1988) observed swelling of meristem of *Cymbidium gigantum* Wall ex.Lindl after 3 weeks of inoculation on MS media +5 mg/l BAP+ 1mg/l NAA. The number of Plantlets was 6-10 in initial culture however from the 2nd subculture the number increased up to 10-15. Complete seedlings were developed 6-8 months after initial excision of meristem. They also observed that 0.1 mm was difficult to establish in culture whereas 0.5mm long piece survived.

Camara *et al*., (1989) regenerated plantlet on MS medium supplemented with all possible combinations of NAA and BAP at the concentration of 0.05 and 0.50 mg/l from 0.5mm long meristem with one leaf primodium of garlic (*Allium satium* L.) They also observed that plantlet grew at the lower concentration and callus was formed at highest (5mg/l) concentration of the growth promoters tested.

Ohkoshi (1991) regenerated virus free plantlets by culturing root meristem of wilson onion and sweet potato in medium with Hyponex 0.3% (w/v) +NAA (0.5 mg/l) +kin (2mg/l), +Agar (0.8%) incubated under 2000 lux for 16rs/day at 25 c. The plantlets were regenerated from meristem after 3 months. Multiplication was obtained in MS+0.5mg/l NAA+1.5 mg/ 1BA after 4 months. Rooting occurred on divided shoot in Hyponex +0.5 mg/lNAA = 2.0mg/l Kin.in 1 month. Tanaka *et al*., (1991) obtained numerous plantlets of *Lilium japonium* after transplantation of shoot primodia in 0.02 ppm NAA at pH 5.8 which is obtained from MS liquid + 2 ppm NAA + 0.2ppm BAP or 4.0 ppm NAA and 2.0 ppm BAP after 3 months at 22°C under 10000 lux.. Shakya (1993) obtained plantlets from meristem of *Solanum tuberosum* Cv. Cardinal in MS medium supplemented with 1.0mg/l BAP and 0.1 mg/l NAA. She also found that survivability of meristem was related to the size of meristem. Shrestha and Rajbhandari (1993a) obtained protocorms on MS+BAP 2.2 mg/l + NAA 1.8 mg/l and 10% coconut milk from meristem of *Cymbidium grandniflorum*. Shoots obtained by sub culturing protocorm on the same medium. Shrestha and Rajbhandari (1993b) obtained protocorm from shoot tips of *Dendrobium densiflorum* Lindl on MS+ BAP 2.5 mg/l + NAA 1mg/l+15% coconut milk +1g/l casein hydrolysate. Shoots were obtained by culturing protocorms in MS+0.25 mg/l BAP +0.25 mg/ 1 BAP + 0.01 mg/l NAA + 20 mg/l adenine sulphate and 10% coconut milk. Sago and Takami (1993) eliminated onion yellow dwarf virus (OYDV) and garlic leaf virus (GLV) viruses from welsh onion culturing meristem tips. They found that when virus-free explants were cultured on MS+0.5mg/l NAA +10-20 mg/ 1 BAP, numerous shoots were produced. The multiple shoots were divided into many pieces and shoots which were subculture on MS + 2.0mg/l BAP multiplied at a higher rate. Roots were induced from shoots subculture two times on the MS medium excluding BAP. After transplanting into sandy loam soil, more than 90% of the plantlets easily grew. Dunbar et *al*., (1993) obtained rooting of shoot of inter specific *Arachis* sp. in MS and B5 in basal medium. Yasogi *et al*., (1994) obtained the greatest number of shoots in *Dendrobium* sp. on a medium containing 0.1mg/l NAA and 0.1mg /l BA. In both parts of segment culture a maximum of 5 multiple shoots were induced in the same medium after 8 weeks in MS medium +2% sucrose +0.8% agar at pH 5.8. Multiple shoots were not obtained in NAA and BA

free medium but roots were developed. Shrestha and Rajbhandari (1993b) obtained protocorm like bodies from shoot apical meristems of *Cymbidium Longifolium* in MS supplemented with BAP 2.0 mg/l, 1 mg/l NAA +10% coconut milk +3% sucrose. Shoots were obtained in subculture of the protectors. They also showed green swelling of meristem after 4 weeks. The number of shoots was 6-8 on initial culture rapid multiplication of shoot occurred in MS media containing 1 mg/|BAP, 1.5mg/l kinetin and 10mg/l adenine sulphate.

Verbeek *et al*., (1995) eliminated the four garlic (*Allium sativum*) virus viz. garlic commom latent virus (GCLV), Garlic strain of leek yellow stripe virus (LYSV-G), onion yellow dwarf virus (OYDV-G),onion mite borne latent virus (OMBLV-G taxonomically unassigned virus)by 0.15-1.00mm meristem tips cultured on MS medium. Smaller size seemed to favor virus elimination but smaller than 0.4 led to increasing failure to regeneration. Sagimura (1995) produced virus-free patchouli (*Pogostemon cablin*) by isolating and culturing meristem tips on medium supplemented with 0-2 ppm BAP. Multiple shoot proliferation was initiated during culture. Complete plantlets were regenerated by transferring multiple shoots to a medium devoid of phytohormones. Plantlets thus produced were transplanted to the soil with a high rate of a survival and tested by ELISA to check elimination of Patchouli mild mosaic virus. Pant *et al*., (1996) obtained multiple shoots from apical domes of shoot tips of *Cnidium officinale* Makino (Apiaceae) by culturing them on MS solid with 10^{-6} M BAP. Average numbers were 5.3 shoots per segment. Shoot tip either developed into plantlets or formed multiple shoots without formation of intermediate callus.

Robert *et al*., (1998) obtained multiple shoot formation when meristems of *Allium sativum* were grown on induction medium supplemented with 1 μM IAA and 1 μM BA and were transferred to the multiplication medium with 5 μM jasmonic acid and 5 μM 2 isopentyl adenine. On average, 6-7 shoots were obtained from one meristem without callus formation. Pre-treatment of plants with thermo therapy did not interfere with initial shoot development but drastically reduced the multiplication of shoot. Elimination of onion yellow dwarf virus (OYDV) was 90-100% as tested by ELISA.

Luciani *et al*., (2001) obtained highest multiplication rates of 140,542,743 shoots per split shoots after 3 subcultures in 140 days for three clones of garlic namely Espanol Seleccion Ascasubi (ESA), E.S. Mednos (ESM) and I 50 of garlic respectively on MS 5 μM NAA +10 μM BAP.

Ranjit (2002) eliminated citrus tristeza virus by meristem culture in different species of *Citrus* sp. collected from different part of Nepal.

Shah (2002) found best rooting of shot tips of *Asparagus racemosous* willed. In MS+0.5ppm, 1ppm NAA and 1ppm IAA.mall cardamom (*Ellettaria cardamomum* Maton) may also be used against chhirkey and Foorkey viruses.

The aim of the study is detection of ‘Foorkey’ and ‘chhirkey’ virus with PRSV antibody by Double Antibody Sandwich – Enzyme Linked Immunsorbent Assay (DAS- ELISA), Regenerate shoots from meristems of two cultivars Ramsahi and Golsahi, Multipy virus free shoots of cultivars Golsahi and Dambarsahi by tissue culture

Methodology

The present work as carried out in Green Research and Technology (GREAT) laboratory at Old Baneshwor , Kathmandu,Nepal which has well developed facilities for viral disease testing tissue culture. The materials used in the work were three cultivars of *Amomum subulatum* Roxb. (Large cardamom) namely ‘Golsahi’, ‘Ramsahi’ and ‘Dambarsahi’**.**

The cultivar Golsahi was collected from Pandam Ilam (Ca.1400m) and Ramsahi was collected from Pakhribas , Dhankuta (Ca.1800 m). These cultivars were either infected with streak

mosaic virus (Chhirkey) or stunty mosaic virus (Foorkey). The cultivar Dambersahi was established in vitro. It was originally collected from Taglichowk, Gorkha (Ca.1000m).

The plants of Golsahi collected from Ilam were infected with stunty mosaic virus showing stunted growth, extremely smaller leaves, and large number of vegetative shoots arising from the bases and devoid of flowers.

The plants of Ramsahi which showed Chhirkey disease symptoms, had streak mosaic on leaves. The infected plants were generally shorter with less flower than normal ones.

The methods used for viral diagnosis and micropropagation are as follows:

DAS-ELISA

Both Chhirkey and Foorkey viruses were tested with DAS ELISA (Clark and Adams, 1977) using antibodies of papaya ring spot virus (PRSV) obtained from Bioreba, Switzerland.

Leaves of plants severely infected by 'Chhirkey' and 'Foorkey' were used for ELISA test. Eighteen samples each of Foorkey and Chhirkey infected leaves of Golsahi and leaves of Ramsahi were used. Two papaya leaf infected with papaya ring spot virus leaves were collected from Rampur, Chitwan,Nepal were also used. Each sample had two replicates.

Table 1. DAS ELISA reagents

1	Coating buffer(pH9.6)	Amount gm/I
	Sodium Carbonate (Na_2CO_3) Sodium Bicarbonate ($NaHCO_3$)	1.59gm 2.93gm
2	Phosphate Buffer Saline (PBS) at pH 7.4	
	Sodium Chloride (NaCL) Potassium Dihydrogen Phosphate(KH_2PO_4) Sodium Orthophosphate ($Na_2HPO_4.12\ H_2O$) Potassium Chloride (KCL)	8.00gm 0.20gm 2.90gm 0.20gm
3	Washing Buffer	
	PBS Tween 20 (Polyoxyethylene Sorbitan Manolaurate)	1.00 Litre 0.50 ml
4	Extraction Buffer at pH 7.4	For 1 Litre
	PBS PVP 40 (Polyvinylpyrrolidene M.W. 40000)	1.00 Litre 20gm
5	Conjugate Buffer	
	PBS Tween PVP 40 OVA or BSA Ovalbumin or Bovine Serum Albumin	1.00 Litre 20.00 gm 2.00gm
6	Substrate Buffer	For 1 Litre
	Diethnolamine Distilled water	200ml 800ml
7	Reaction stopping solution	
	Sodium hydroxide (NaOH)	120gm

Coating of antibody

20 μl Antibody of PRSV was first diluted in a 20 ml coating buffer at the rate of 1:1000. The wells of ELISA plates were coated with 200 μl of antibody by micropipette and incubated at 37℃ for 4 hours. Then the wells of the plate were washed 3 times with washing buffer.

Loading of sap

0.5gm young leaf samples of both Chhirkey and Foorkey and PRSV infected plants were homogenized in 5 ml of extraction buffer separately. The scissor and knife was flame sterilized each time. The homogenized sap were centrifuged at 2000rpm for 3 minutes and the supernatant was loaded into the ELISA plate at the rate of 200 μl per well. The plate was incubated at 37℃ for 4 hours. After incubation plate was again washed carefully for 3 minutes with washing buffer. Similar process was done for DAS_ELISA on in vitro plants.

Coating with conjugated antibody

20 μl of the conjugated antibody was linked to enzyme called p-nitro phenyl (alkaline) phosphates was diluted at 1:1000.200 μl conjugated antibody solution were loaded in each well and incubated at 37℃

For 4 hours. After incubation, each well was washed 3 times with washing buffer.

Addition of substrate

20mg of p-nitro phenyl phosphate substrate was dissolved in 20ml of substrate buffer. 200 μl for substrate solution was added to each well and incubated at room temperature. Optical density reading was taken at 60 minutes at 405nm using Humareader (ELISA plate reader)

After reading, reaction was stopped by adding 50 μl of 3 M NaOH.

Tissue culture

Preparation of stock solution

The based medium of Murashige and Skoog (MS) (1962) was prepared by working one liter which contains the following compounds as given in Table 2

Stock solutions were prepared by making 20 times concentration of MS-A, and 200 times concentration of MS-B, C and D solutions as shown in Table 2. All stock solutions were kept in clear brown bottles and kept in refrigerator at 4℃.

Media preparation

Twenty gram of sucrose were dissolved in 400ml of distilled water in 1000ml conical flask. Fifty ml of macro elements (MS-A), five ml each of MS-B, MS-C, MS-D were added. Hundred mg of myo-inositol was added and dissolved completely by using magnetic stirrer. The final volume was adjusted to one liiter and pH was adjusted to 5.7 by diluting NaOH or HCI after the plant hormones were added.

Hormone preparation

Two hormones namely naphthalene acetic acid (NAA) and benzyl adenine (BA) were used.

Fifty ml of 100 ppm NAA was prepared by dissolving 5 mg of NAA first in few ml of ethanol and then final volume was adjusted with distilled water.

Fifty ml of 1000 ppm BA were prepared by dissolving 50 mg of BA first in few ml of NaOH and then the final volume was adjusted with distilled water.

Different concentration of BA and NAA were prepared by using the formula.

$S_1V_1=S_2V_2$

Where,

S_1= Concentration of NAA or BA in stock solution

V_1=Volume of NAA or BA in stock solution.

S_2= Strength of NAA or BA require in medium

V_2= Volume of Medium

For liquid medium 10 ml of medium was poured to 25x150 mm size culture tubes with or without M shaped Filter Paper Bridge. The tubes were capped with lids. The tubes were autoclaved at 121℃ /15-lb/sq inch for 20 minutes.

For solid medium, the agar at the rate of 6.5 gm. /I was added and heated to boil. Fifty ml of solution were dispensed to jam bottles and capped with lids and autoclaved for 121℃ /15-lb/sq inch for 20 minutes.

Table 2: Constituents of basal MS medium

A	**Macro elements**	Amount mg/I
1	Ammonium Nitrate (NH_4NO_3)	1650
2	Potassium Nitrate (KNO_3)	1900
3	Calcium Chloride ($CaCl_2.2H2O$)	440
4	Magnesium Sulphate ($MgSO_4.7H_2O$)	370
5	Potassium Dihydrogen Phosphate(KH_2PO_4)	170
B	**Microelements**	Amount mg/I
1	Boric Acid (H_3BO_3)	6.2
2	Maganese Sulphate ($MnSO_4.4H_2O$)	22.3
3	Zinc Sulphate ($ZnSO_4.4H_2O$)	8.6
4	Potassium lodide (KI)	0.83
5	Sodium Molybdate($Na_2MoO_4.2H_2O$)	0.25
6	Copper Sulphate ($CuSO_4.5H_2O$)	0.025
7	Cobalt Chloride ($CoCl_2.6H_2O$)	0.025
C.	Iron Source	Amount mg/I
1	Sodium EDTA(Na_2.EDTA)	37.3
2	Ferrous Sulphate($FeSO_4.7H_2O$)	27.8
D	Organic Constituents	Amount mg/I
1	Nicotinic acid	0.5
2	Pyridoxic HCI	0.5
3	Thiamin HCI	0.1
4	Glycine	2.0
E	Myo-inositol	100mg/I
F	Carbon source Sucrose	20gm/I
G.	Solidifying agent Agar	6.5gm/I

Sterilization

Buds with some tissues were excised and kept in teepol solution for 10 minutes with shaking. Then they were washed in running water for 20 minutes. They were surfaced sterilized with 0.1% (W/V) mercuric chloride for 5 minutes and 1%(v/v) sodium hypochlorite solution for 10 minutes. Then they were rinsed 3 times with sterile distilled water. The surface sterilization was carried out under laminar air flow.

Instruments used for inoculation such as forceps, needles, scalpels, Petridis's, beakers, bottles were sterilized in autoclave. The cabinet of laminar air flow and stereomicroscope were first wiped with spirit before and after inoculation and was exposed to UV light for 1 hour. Hands were disinfected with spirit.

***Meristem Inoculation**:*

Meristems were excised from buds. Scalpels were used to remove the outer scales. After removing 8-10 scales, the white dome shaped meristem was exposed. Meristems of different sizes namely up to 0.1mm in length without leaf primodium ,0.11 mm to 0.20 mm, 0.21 to 0.30mm, 0.31 to 0.40mm,0.41 to 0.5mm with one to three leaf primodia were excised with the help of blade tip, scalpel and needle. The isolated meristem was inoculated in different tubes on Filter Paper Bridge. The size of meristem was measured by micrometer. Meristems were established in MS media supplemented with 1ppm BA+0.1 ppm NAA.

Survibility of meristem was calculated) as Survival rate (%) =

$$\frac{\text{No. of shoot meristems survived}}{\text{No. of shoot meristems plated-no. of shoot meristem contaminated}} \text{x100}$$

Incubation

The culture tubes containing inoculate were kept in incubation room at 25 ℃ + 2℃, with light intensity of 2000 lux, for 14 hour photo periods.

Subculture

An observation of inoculation was made of weekly intervals. Subcultures were done at interval of 3-4 weeks. Subcultures of meristem were done on the solid medium having same concentration of hormone until 2-4 shoots cm long were regenerated.

Multiplication

Two cultivars of large cardamom namely Golsahi and Dambarsahi were used for multiplication of the shoots. Shoots about 2-4 cm were inoculated on medium with different concentrations of BA and 0.1 ppm of NAA. Both liquid and solid media were used.

Rooting

Uprooted shoots were inoculated for rooting. Five different concentrations of NAA were used. Each treatment contained 10 replicates.

Acclimatization

Plantlets with well – developed roots were acclimatized. Sand, soil, and compost manure were mixed in 2:2:1 proportion for acclimatization purpose. Each plantlet was planted on plastic pot having soil mixture under the screen house. Moisture level was controlled by covering them by plastic pot having soil mixture under the screen house. Moisture level was controlled by covering them by plastic for a week. After a week plastic was removed for 1 hrs/day. The time periods gradually increased to 2, 3, 5,7,10 hrs for 10 days. Then they were completely acclimatized under screen house condition.

DAS ELISA of *in vitro* plants regenerated from meristem

Two cultivars namely Golsahi and Ramsahi regenerated from meristems were tested for the presence of Chhirkey and Foorkey using PRSV antibodies. Golsahi was infected with Foorkey while Ramsahi was infected with Chhirkey. Twenty five in vitro plants of Golsahi and three in vitro plants of Ramsahi regenerated from meristems of different sizes were tested by DAS-ELISA using PRSV antibodies. Compared with the positive A_{405} value of 0.523 and blank A_{405} 0.040, none of the samples tested were positive for Chhirkey and Foorkey. The three in vitro samples for Chhirkey (C2-C4) registered A_{405} value of 0.145, 0.097, and 0.135 respectively. These values were all less

than that A_{405} value of 0.200. Among the 25 in vitro samples for foorkey tested , only 4 samples namely A9,A10,B2 and C1 were found to register A_{405} value of 0.218,0.207,0.247, and 0.265 that were slightly more than 0.200. The remaining 21 in vitro samples were all tested negative for PRSV which might be reflected the absence of Foorkey disease on these regenerated in vitro plants.

Results

DAS-ELISA of Chhireky and Foorkey viruses was done on field samples of large cardamom leaves of two cultivars namely Ramsahi and Golsahi by using PRSV antibodies. Eighteen samples were tested for each virus. The result of the test showed that seven samples out of eighteen with visible symptoms of Foorkey on Golsahi were positive whereas six samples out of eighteen with visible symptoms of Chhirkey on Ramsahi were found to be positive. Mean absorption values of each virus were given in 3.

The absorption values for Chhirkey were found in the range of 0.076 to 0.350. Out of 18 samples tested, 6 (33%) were above the range of 0.2 whereas 12 (67%) was below the 0.200 value. The three samples with highest A_{405}values for Chhirkey were A8 (0.350), A5 (0.332) and A1 (0.299) respectively. Meristems were excised from these for later experiments for virus elimination. Yellow colour reaction could be seen in case of each sample which has >0.30 absorption values. Positive live samples of PRSV in papaya leaves had A_{405} values of 0.369 and 0.613 respectively. Whereas the healthy samples and blank reading had A_{405} values of 0.044 and 0.103 respectively.

The absorption values for foorkey C (1-12) and D (1-6) were found in the range of 0.110 to 0.298. Out of 18 samples tested, 7 (39%) were above the range of 0.200 values whereas 11 (61%) were below the 0.200 values. The three samples with highest A_{405} values for foorkey were C1 (0.298), D6 (0.286) and C2 (0.244) respectively.

Table 3: Mean absorption values (A_{405}) from large cardamom leaf samples for the diagnosis of Chhirkey (streak mosaic virus) and Foorkey (stunty mosaic virus) in DAS-ELISA using PRSV antibodies.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.299	0.278	0.134	0.185	0.332	0.180	0.174	0.350	0.162	0.086	0.099	0.203
B	0.215	0.093	0.169	0.076	0.161	0.153	0.062[a]	0.080[a]	0.369[b]	0.044[C]	0.103[d]	0.613[b]
C	0.298	0.244	0.168	0.238	0.213	0.203	0.181	0.199	0.150	0.158	0.160	0.155
D	0.110	0.157	0.195	0.230	0.173	0.286	-	-	-	-	-	-

A (1-12), b (1-16) for Chhireky (Streak mosaic virus of large cardamom)
C (1-12), D (1-6) Foorkey (Stunty mosaic virus of large cardamom)
a=Healthy plant reading of large cardamom
b= PRSV+ ve sample
c= Papaya healthy reading
d= Blank Reading
∗DAS-ELISA condition: Leaf tissue extract dilution 1/l0, dilution of gammaglobulin antibody (1:1000), and coating of gammaglobulin antibody: 200 μl/ well
Absorption (405nm), Reading after 1 hour of incubation.

Table 4: Serological reaction of antigen to PRSV antibody in DAS-ELISA

Antigen	Mean Absorption value of A_{405}±S.D.	Host/Tissue
Streak mosaic virus	0.280^{a} ±0.054	Large cardamom leaf (Chhirkey)
Stunty mosaic virus	0.244^{b} ±0.020	Large cardamom leaf (Foorkey)
PRSV	0.491^{c}± 0.122	Papaya leaf (PRSV infected)

DAS ELISA condition∗ - Tissue extract dilution 1/l0
Dilution of Gamma Globulin antibody 200 μl / well
a=Mean of 6 positive samples
b=Mean of 7 positive samples
c=Mean of 2 positive samples
S.D. = Standard Deviation:

The serological reactions of large cardamom antigens of Chhirkey and Foorkey to Papaya Ring Spot Virus (PRSV) antibodies were shown in Table 4.1b. The mean positive value for Chhirkey was slightly greater than that for Foorkey. Meaning that the reactions of Chhirkey antigens to PRSV antibodies were slightly greater than that of Foorkey antigens.

All meristems excised from the buds did not survive. Many of them died. Survival of meristem was observed up to 8 weeks after inoculation. Small sized (<0.2) meristems could be observed as living within two weeks. Blackening or browning of meristem indicated death. Larger than 0.2 mm sized meristems could be observed as living up to 8 weeks. Survival of meristem depended upon the size and was different in two cultivars. Small meristem (up to 0.1mm) had low survival rate. Survival of meristem of Cultivars Golsahi and Ramsahi were given in Table 3 and 4.

Table 5.Effect of size of meristem on survival rate of Golsahi

S.N.	Meristem size group	No. of meristem cultured	Contamination	Dead	Survived	S.R. (%)
1	Upto 0.1 mm	13	3	9	1	10
2	0.11 to0.2mm	17	-	14	3	18
3	0.21 to 030	15	-	12	3	20
4	0.31 to 0.40mm	18	5	8	5	38
5	0.41 to 0.50mm	13	4	5	4	44

Table 6: Effect of size of meristem on survival rate of Ramsahi.

S.N	Meristem size group	No. of meristem cultured	Contamination	Dead	Survived	S.R. (%)
1	Upto 0.1 mm	12	2	8	2	20
2	0.11 to 0.2mm	23	5	14	4	22
3	0.21 to 030	18	4	10	4	28
4	0.31 to 0.4mm	16	5	6	5	45
5	0.41 to 0.50 mm	14	4	5	5	50

Meristem of Cv. Ramsahi had higher survival rates both in the smallest and the highest meristem size categories than Cv. Golsahi. Some were contaminated by both fungi and bacteria. The survival rates of Golsahi meristems ranged from 10 to 44 percentages whereas there of ramsahi ranged from 20 to 50 percentages. Generally, it has been observed that bigger the meristem sizes

Meristems of both cultivars were established in liquid MS supplemented with 1.0 mg/l BA and 0.1mg/l NAA and 2% sucrose. Meristems were subcultured on solid medium having similar concentrations of hormones at every 3 to 4 weeks. Five subcultures were done until upto 4 cm shoot were regenerated.

Meristems of Golsahi became green after two weeks of inoculation (Plate no.5). Meristems swelled and size of the meristem increased at every subculture (Plate no.6). The swollen meristem was differentiated into shoot primodium after 5 sub cultures in 15 weeks. About 1 cm shoots were regenerated after average of 21 weeks of culture. Larger meristem (< 0.30mm) meristem regenerated earlier (18 weeks) while smaller regenerated after 24 weeks.The number of microshoot increased as the number of subcultures increased. Micro shoots proliferated from the meristems ranged from2-13 in number. Cent percent regeneration occured from survived meristems.

Response of meristem of Ramsahi was similar to Golsahi but larger meristems (>0.3mm) regenerated into microshoots after 14 weeks of inoculation while it took 18 weeks for the smaller ones (<0.20mm). Number of microshoot ranged from 2 to 4.

Habit of large cardamom,Large cardamom plant infected with Chhireky, Large cardamom plant infected with Foorkey,Plate of DAS-ELISA of Chhirkey,Meristem of Golsahi after two weeks of inoculation MS+1ppm BA+ 0.1ppm NAA,Swollen meristem of Golsahi after 12 weeks of inoculation MS+1ppm BA+0.1ppm NAA,

Shoot regenerated from 0.2mm sized meristem og Golsahi after 24 weeks of inoculation.MS+1ppm BA+0.1ppm NAA.Two shoots regenerated from 0.2mm sized meristem of Golsahi after 24 weeks of inoculation MS+ 1ppm BA+0.1ppm NAAMultiple shoot regenerated from meristems after 20 weeks of inoculation MS+1ppm BA + 0.1ppm NAA.

Table7. Indexing of viruses by DAS-ELISA on plants regenerated from meristem

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.153	0.136	0.113	0.115	0.169	0.160	0.119	0.140	0.218	0.207	0.092	0.114
B	0.113	0.247	0.147	0.157	0.135	0.132	0.156	0.170	0.118	0.136	0.156	0.150
C	0.265	0.145	0.097	0.135	0.523[a]	0.040[b]						

A (1-12), B (1-12), C1=In vitro plant regenerated from Foorkey infected plants

C (2-4) = In vitro plant regenerated from Chhirkey infected plants

a=PRSV +ve sample

b= blank reading

ELISA condition: leaf tissue extract dilution 1/l0, 4005nm, Gamma globulin antibody dilution 1:1000 coating antibody: 20 μl well, reading taken after 1 day.

For multiplication, 2-4 cm. long individually separated shoots were used. Observation was taken after 8 weeks of inoculation. Multiplication along with shoot growth and root induced was also recorded.

Table 8: Effect of hormones on shoot multiplication and growth and root numbers on solid medium.

Parameters	Hormone combination BA(ppm)+NAA (ppm)				
	0+0	0.1+0.1	0.5+0.1	1.0+0.1	2.0+0.1
Proliferation (Shoots/Shoot tip)	1±0	1±0	1.54±0.74	1.75±0.59	2.5±0.73
Shoot growth (cm)	1.34±0.38	3.14±1.00	2.61±0.74	2.27±0.37	2.1±0.89
No. of Roots	2.37±0.60	9.5±1.66	3.0±1.03	2.08±1.11	1.5±1.29

Table 9. **Effect hormones on shoot multiplications growth and number of roots on liquid medium**

Parameters	Hormone combination BA(ppm)+NAA(ppm)				
	0+0	0.1+0.1	0.5+0.1	1.0+0.1	2+0.1
Shoot proliferation (Shoots/ Shoot tip)	1.0±0	1.71±1.03	3.5±0.5	4.17±1.34	5.67±0.47
Shoot growth (Cm)	1.62±0.28	3.79±0.92	2.87±0.60	2.50±0.76	2.25±0.83
No. of roots	3.0±0.86	7.86±0.64	6.62±1.11	4.33±1.70	3.5±0.87

Shoot response was better in liquid medium that in solid medium. In basal medium, no proliferation of adventitious shoots occurred but in other media supplemented with phyto-hormones, result was better not only in shoot proliferation and shoot length but also in root induction per shoot. High number of shoot proliferation (5.67 shoots/ shoot tip) was found in MS+2 ppm BA+0.1 ppm,NAA was best for Cv. Dambarsahi

Multiplication of Cv. Golsahi

Shoot response of Golsahi was little different from that of Dambarsahi.

Table 10. Effect of hormones on shoot multiplication, shoot growth and rooting of Cv. Golsahi and solid MS medium.

Parameters	Hormone combination BA (ppm)+NAA(ppm)				
	0+0	0.1+0.1	0.5+0.1	1.0+0.1	2.0+0.1
No. of shoot proliferation (Shoots/ shoot tip)	1±0	2±0.57	3.83±1.06	2.5±0.5	3±0.81
Shoot growth (cm)	2.11±1.07	2.25±0.65	1.14±0.44	1.33±0.74	2.1±0.73
No. of roots	6.33±1.24	7.85±2.10	4.16±0.89	8.8±1.16	7.16±2.06

In solid MS+0.1 ppm BA+0.1ppm NAA, shoot proliferation was found, shoot growth was Maximum and roots were induced. The highest number of shoot proliferation was Maximum in MS +0.5 ppm BA+0.1ppm NAA.

But, the highest numbers of roots were induced in MS+1.0ppm BA+0.1 ppm NAA.

In liquid MS basal medium, no adventitious shoot proliferation occurred but shoot growth and rooting were observed. Maximum shoot proliferation (7.25 shoots/ shoot tip) was found in MS medium supplemented with 2 ppm BA and 0.1 ppm NAA (Plate No. 24) Growth of the shoot and root induction were best in MS supplemented 0.1 ppm BA and 0.1 ppm NAA with values of 4.5 cm and 6.5 roots respectively.

Table 11. Effect of hormones on shoot multiplication, shoot growth and rooting of Cv. Golsahi on liquid MS medium.

Parameters	Hormone combination BA(ppm)+NAA (ppm)				
No. of shoot proliferation	0+0	0.1+0.1	0.5+0.1	1.0+0.1	2.0+0.1
	1.0±0	2±0	3.5±0.5	4.33±0.47	7.25±0.33
Shoot growth (cm)	1.77±0.19	4.5±0.5	3.0±0	2.25±0.83	2±0.70
No. of roots	3.25±0.83	6.5±0.5	6.0±1.22	3.33±0.94	3.25±1.09
Culture condition: Liquid media, 25+2℃, 8 weeks, 8 replicates					

On MS medium without any hormones, no proliferations of shoot occurred by shoot length increased by 1.5 to 2 cm and root numbers increased from 2-4.

Two shoots were proliferated per shoot. Four to 5 cm long shoots growth was found. Two numbers of roots proliferated were 6 -7 in the MS medium supplemented with 0.1 ppm BA+0.1 ppm NAA.

Two to three shoots were proliferated per shoot tip in MS medium supplemented with 0.5 ppm BA+ 0.1 ppm NAA. Three cm long shoot where found and root induced in the medium was in the range of 5-8 (Plate No. 22).

On MS medium supplemented with 1.0 ppm BA and 0.1 ppm NAA, 3-4 adventitious shoots were proliferated per shoot. Shoot length was increased in the range of 2-3cm. Two to four adventitious roots were proliferated from shoot.

Six to 7 shoots and buds were proliferated from the shoot which was reared on MS medium supplemented with 2.0 ppm BA+0.1 ppm NAA. Little shoot growth was found which was in the range of 1 to 3 cm. Two to five adventitious roots were proliferated on this medium. Six to 7 shoots and buds were proliferated from the shoot which was reared on MS medium supplemented with 2.0 ppm BA+0.1 ppm NAA. Little shoot growth was found which was in the range of 1 to 3 cm. Two to five adventitious roots were proliferated on this medium

Shoots of Dambarsahi which are not rooted in multiplication medium were used in rooting. MS medium without cytokinin and different concentrations of NAA were used. The mean number of roots induced was given in Table 12.

Table 12: Effect of NAA on rooting of Shoots tips of Dambarsahi:

NAA concentration (ppm)	0	0.1	0.5	1	2
No. of roots	2.37±0.60	5.1±0.83	6.1±2.55	7.4±3.07	6.3±2.1

Culture condition: Solid medium, 25± 2℃, 3 weeks, 10 replicate

Adventitious roots were induced in solid MS basal medium. Two to four roots were induced on hormone – free medium. The mean number of roots increased up to 7.4 in 1 ppm NAA but it decreased at 2 ppm. The length of the root was in the range of 1.5-2 cm.

The number of adventitious roots induced on MS+0.1 ppm NAA was in the range of 4-6. Aerial root hairs were also found. Two to 12 roots were induced in MS+0.5 ppm NAA while it increases from six to 12 in MS+2 ppm NAA which was 6

The rooted planted of Golsahi and Dambarsahi were successfully acclimatized in the screen house. The percentage success of Acclimatization with golsahi was plants were 85.7 under screen house condition in both cases

Discussion

Seeds of the large cardamom are widely used as spices. They are also used in Ayurvedic medicines. Dry capsules are used for flavoring curries, rice pudding, pulau, cakes, and bread and other culinary purposes (Dhakal, 2001). The volatile oil extracted from its seeds is used in perfumery and pharmacological products and also for flavoring liquors and other beverages.

The seeds are used for tonic of heart and liver. The outside covering is good for headache and toothache and heals stomatitis. In combination with seeds of melons, it is used as diuretic in case of stone of kidney. The seeds are used as antidote to either snake venom or scorpion venom. The oil extracted from them is applied to eyelids to allay inflammation. It is an aromatic stimulant and stomachic. The seeds are used in gonorrhea and as an aphrodisiac (Pruthi, 1976).In the present investigation detection of two viruses Chhirkey and Foorkey was partial by DAS-ELISA .Not all but 39% of Foorkey and 33% of Chhirkey samples were found to have above A_{405} values 0.2. In case of Chhireky some sample there is moderate positive reaction which were near the low positive value of PRSV. The symptom of the cardamom mosaic virus was similar to those of Chhirkey (Streak on leaves) and potyvirus as observed by electron microscope. The Chhirkey may be a type of potyvirus and have serological relationship with another potyvirus antibody PRSV. So, some samples have given positive reaction. This result differ from that of Gonsalves *et al*.,in that they had found the serological relationship between CarMV and PRSV in indirect ELISA but the present result was found by DAS- ELISA.

In case of Foorkey none of reading above 0.200 couldn't be seen by nacked eyes but these values were measured by absorption photometer. Alhwat *et al*., 1981 described Foorkey was isometric virus with 37 nm diameters. There was no literature found to describe such serological relationship between isometric virus and potyvirus antibody. So, the positive reaction of samples witn Foorkey symptoms with PRSV antibodies may be an artifact.The survival meristems in both the Ramsahi and Golsahi was in similar i.e. bigger the size of the meristem, higher was the survival rate. But, survival of the particular size meristem in both cultivars was different. The relationship between size of the meristem and survival rate can be explained by cell number and amount of endogenous hormones. In smaller sized meristem, cell numbers are less than large sized meristem. So, exposure to dry air in laminar air flow could be killed from earlier than larger sized meristems. Another reason may be due to the amount of hormones in smaller meristems. There was less amounts of hormones which was insufficient for cell metabolism. The relationship between size and survival of meristem was also observed by Dale, 1977b, Shrestha and Rajbhandari 1993b, and Shakya, 19930.

Dale (1977b) found that survival rate of meristem tips of *Lolium multiflorum* ,*L.perenne* ,*Festuca pratensis* ,*F. aroundinacea* and *Phelum* sp. and *Dactylis glorerala* was highly correlated with The size of tips cultured on the medium with 0.01 mg/l2,4-D and 0.2 mg/ 1 kinetin.Shrestha and Rajbhandari (1988) observed that 0.1 mm meristem of *Cymbidium gigateum* Wall. Ex lindli was difficult to establish in culture where as 0.5 mm long piece survived.

The meristems were cultured on MS medium with 1.0 mg/l BA and 0.1 mg/l NAA. Meristems become green due to chlorophyll formation in cells. The greening of the meristem was also observed by Shrestha and Rajbhandari,(1994) in meristem of *Cymbidium longifolum*. The swelling of the meristem was due to the cell division in meristem stimulated by both endogenous as well as exogenous hormones. The size of the meristem was increased at every subculture due to

availability of exogenous hormones provided in the medium. Similar result was observed by Shrestha and Rajbhandari (1988) in *Cymbidium longlifolium*.

In the present investigations, the larger sized meristem regenerated earlier than the smaller ones. This can be explained by the presence of higher amount of endogenous hormones in the larger meristems.

Multiple shoots were obtained after sub- cultured of green swollen meristems in same medium in this study. Similar result was obtained by Shrestha and Rajbhandari in 1988 in *Cymbidium gigatum*, Shrestha and Rajbhandari (1983) in *Cymbidium grandiflorum* and *Dendrobium densiflorum* and Shrestha and Rajbhandari in 1994 in *Cymbidium longifolium*,They obtained shoots by subculture of green swollen meristems or protocorm like bodies in same medium.

In the present investigation, Shoots were regenerated in MS+1.0 mg/l 1 BA and 0.1 mg/l NAA which coincided with Perez *et al*., 1987 and Shakya 1993. Perez *et al*., obtained this result in *Digitalis obscura* L. from root meristem. Shakya also obtained this result in *Solanum tuberosum* Cv. Cardinal.

Pant *et al*., (1996) obtained shoots from apical dome of the shoot tips of *Cnidium officinale* Makino by culturing them on MS solid medium 10^{-6} M .BAP without formation of Intermediate callus. This is also similar to the result of the present work.Gupta (1986) regenerated the plant of banana (*Musa acuminata*) and plantain.(*M.acuminata* x *M. balbissiana*) cultured on MS+1 mg /l Thiamine HCL+0.5 mg/ 1 Nicotinic acid +0.7 mg/ 1 BA +0.7 mg/ 1 Kinetin.Plants which were regenerated from meristem culture were indexed by DAS-ELISA. It was found that 4 out of 25 in vitro plants regenerated from meristem of Golsahi were still weakly infected with Foorkey as indicated by their A_{405} Values of slightly more than 0.200. All other plants were completely free form Foorkey.

The virus free plants were acclimatized in screen house (Plate No.31) although serological relationship between Foorkey and PRSV couldn't be explained; A_{405} values in vitro plants were lower than A_{405} values from Foorkey infected plants. There were no characteristics symptoms of Foorkey in the acclimatized plants. Rhis might be due to the absence of causal agent of Foorkey in the acclimatized plants. In case of Chhirkey highest A_{405} value (0.135) was very less than that of mean A_{405} from Chhirkey infected plants (0.280) whose buds were used for excision of meristem. This different in A_{405} values was due to absence of streak mosaic virus in such plants which was regenerated from meristem.This showed that meristem culture could be used to eliminate Chhirkey virus from cardamom plants and the elimination of the virus could be confirmed by the use of PRSV antibodies in DAS-ELISA.Two cultivars of large cardamom Golsahi and Dambarsahi were used in both solid and liquid media.

In this investigation basal medium alone was not effective for proliferation of axiliary shoots. Similar result was obtained by the Yagosi *et al*., (1994) in *Dendrobium* sp.

Increasing exogenous cytokinin (BA) in medium increase the number of axillary shoots in both cultivars. This was similar for solid as well as liquid media. Highest number shoots were obtained in MS+2 ppm BA+ 0.1 ppm NAA except in 0.5 ppm BA and 0.1 ppm NAA in solid media for Golsahi. Increasing number of axillary shoot with the increasing cytokinin concentration was due to the suppression of apical dominance phenomenon. High cytokinin (BA) content in both liquid and solid medium promoted the axillary shoot proliferation. This result was coincided with Hussy (1976), Popov (1977), and Sako and Takami (1993).Popov (1977) observed that addition of BAP to culture medium stimulated to form numerous axillary buds in strawberries, Hussey (1976) found that branching of *Lilium* sp. was promoted by concentration at 2-8 mg/l. He also found that at 2 mg/l BAP one to three branches were formed in *Fritillaria* , *Narcissus* and *Hyacinthus* (Monocotyledons)

where strong apical dominance was observed. But, result of present investigation was different from Hussey (1976) who observed those roots were inhibited in all species by concentration of BAP that caused branching. In the present investigation, rooting was observed in all plants having branching except that some shoots were devoid of root in Cv. Dambarsahi in MS solid +2ppm and 1 ppm BA+0.1 ppm NAA. Sako and Takami (1993) obtained multiplication in Welsh onion at higher rate in individually separated shoots on MS+2.0mg/l BAP.Multiplication of shoots were obtained at high BA (2ppm) and NAA (0.1 ppm). This result was similar to that of Hussey (1976) but contrast to that of Shoyama and Nishioka (1994) and Lucioni *et al*., (2001). Shayama and Nikhioka (9194)found that addition of 4μM BAP and 11.4 μM IAA to MS +2.2 μM BAP + 2.9 μM IAA promoted the axilary shoot proliferation from single shoot in *Swertia japonica*. The present result was coincided with Husseys(1976) result that one to three branches per plant were obtained in *Fritillaria* and *Hippeastrum* of monocotyledons which was similar to the Cv. Dambarsahi in solid medium at MS+2.0 mg/l BAP +0.1 mg/l NAA. The number of shoots multiplication were less than the number of shoots obtained by Luciani *et al*., (2001) in garlic. They obtained 140 shoots per split shoots in 140 days on MS medium.

Maximum shoot growth and root number were observed on MS +0.1 mg/l BA +0.1 mg/l NAA in both liquid and solid media. This was similar to Shoyama and Nishioka (1994) in *Swertia japonica* (L) Makino. But, this was in contrast with the result of Choi and Kim (1991) in ginger where optimal growth was obtained in MS+5.0 ppm BA+0.5 ppm NAA; with the increase of cytokinin (BA) concentration in both liquid and solid media, shoot growth and root numbers decreased and became the lowest at MS+2 ppm BA +0.1 ppm NAA. This trend was observed in Cv Dambarsahi .Similar effect was observed in liquid media for Golsahi but in solid media highest rooting found in MS+1.0ppm BA+0.1 ppm NAA.

Liquid medium was better for axilliary shoot proliferation, shoot growth and root induction than solid medium for Cv. Dambarsahi and Golsahi. This result was contrasted to the Bhagyalakshmi and Singh(1988) who obtained better multiplication result in solid medium in ginger shoot, derived from meristems than in liquid medium Thus shoots were multiplied at MS+2 ppm BA+0.1 ppm NAA; liquid medium.

In vitro rooting of Cv. Dambarsahi was also obtained from the MS basal medium also. Basal medium was sufficient for rooting in Vitro. This result was coincided with au Popov, 1977, Dunber *et al*., 1993. Yasogi *et al*., 1994 and Sagimura, 1995 obtain rooting of strawberries on the medium planted in a medium without auxin and cytokinin. Dunber *et al*.,(1993) observed that rooting of shoot derived from meristem, on MS B5 medium in Interspecific *Arachis* hybrid. Yasogi *et al*.,(1994) also observed that roots were developed in MS basal medium in *Dendrobium* sp. Sagimera (1995) obtain plantlets from the shoot on medium which was devoid of phytohormones in *Pogostemon cablin*.

Rooting was well developed on all the media supplemented with auxin only. Roots were well developed on MS media supplemented with 0.1, 0.5, 1 and 2 ppm of NAA but number of roots was reduced in MS+2 ppm NAA. These results were coincided with Lloyd and Mario (1975), Sake and Takami (1993), Mujib and Jana (1994) and Shah (2002). Lloyd and Mario (1974) observed that roots were induced on Knudsons medium +20 gm/ 1sucrose+1 μg/ml NAA in banana. Sako and Takami (1993), obtain root from shoot of welsh onion derived from the meristem, on the MS medium which contain no BAP which was similar to the present study. Mujib and Jara (1994) observed good in vitro rooting in shoot of *Dendrobium* sp. with MS + 0.1 mg/l NAA which coincided with the present investigation.

Shah (2002) observed that in vitro rooting in shoot tips were induced in the MS medium supplemented with 0.5, 1.0 mg/l NAA and 1mg/l IAA in *Asparagus racemosous* which was similar to the present investigation.

High auxin showed inhibitory effect in Cv. Dambarsahi. In vitro rooting was easily made on MS alone but numbers of roots were highest at MS+1 ppm NAA. In the present investigation both Cv. had high acclimatization potential to acclimatize under screen house condition

Conclusion

In general, it is strongly recommended that specific antibodies of Chhirkey and Foorkey should be prepared for indexing of these viruses. Plants of large cardamom which have been producing by seeds should be replaced by the plants regenerated from meristem which help to increase production and productivity. Public awareness to viral disease and disease free planting material is necessary. Research on disease of such cash crop should be concentrate by NAST, NARC, CDC, PAC and other institutions/organizations

Acknowledgment

Authors are grateful for Central Department of Botany.

Abbreviation

BA: Benzyl Adenine; BAP: Benzyl Amino-Purine; CarMV: Cardmom Mosic Virus; CDC: Cardamom Development center; Cv: Cultivar; DAS-ELISA: Double Antibody Sandwitch-Enzyme Linked Immonesortbent Assay; EDTA: Ethylene Diamino Tetra Acetate; GDP: Gross Domestic Production; IBA: Indole butyric Acid; ICAR: Indian Council Of Agricultural Research; IgG: Immunoglobin type G; IgM: Immunoglobin type M; MS: Murashine and Skoog; NAA: Napthalene Acetic Acid; PRSV; Papaya Ring Spot Virus

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