Acta Scientific Biotechnology

Volume 3 Issue 6 December 2022

Research Article

Diagnosis of *Citrus tristeza* Virus (CTV) From Mother Plant and Seedling of *Poncirus trifoliata* (Trifoliate Orange) and *Citrus reticulata* (Mandarin Orange) and Production of Virus Free Plants Through Tissue Culture

Amrit Jnawali, Binju K.C, Biplab Adhikari, Sudha Acharya and Prof Dr Mukunda Ranjit *

Department of Biotechnology, Kantipur Valley College- Purbanchal University , Nepal

***Corresponding Author:** Prof Dr Mukunda Ranjit , Department of Biotechnology, Kantipur Valley College- Purbanchal University, Nepal.

Received: September 23, 2022

Published: November 10, 2022

Abstract

Citrus fruits are one of the most delicious fruits belonging to the family *Rutaceae*. One of the major problems of *Citrus* in Nepal is *Citrus tristeza* Virus (CTV). CTV is the severe disease of sub-tropical crops of Nepal. CTV was diagnosed in different mother plants and seedlings of *Poncirus trifoliata* and *Citrus reticulata.* CTV was not transferred from infected mother plants of Trifoliate orange and Mandarin orange to their seedlings if the seeds are grown in *in-vitro* culture and *ex-vivo* (in insect proof screen house). DAS-ELISA techniques were used for the diagnosis of CTV from the *Citrus* plants. The values of <0.2 on ELISA plate reader was considered a negative reference for CTV. CTV infected Trifoliate orange and Mandarin orange seeds were grown in -vitro culture in MS Medium and the media optimization was done for the seedlings obtained from them. The best shoot multiplication, shoot length was found better in 1 mg/l BAP+0.1 mg/l NAA media and similarly best root number and root length was found better on 1 mg/l NAA media.

Keywords: *Citrus tristeza* Virus (CTV); DAS-ELISA; *Poncirus trifoliata; Citrus reticulata*

Abbreviations

Ab: Antibody; Ag: Antigen; BAP: 6-benzylaminopurine; CTV: *Citrus tristeza* Virus; DAS-ELISA: Double Antibody Sandwich-Enzyme Immuno Sorbent Assay; GA: Gibberellic Acid; Ha: Hectares; IBA: Indole,3-butyric Acid; Min: Minute; MS: Medium Murashige and Skoog Medium; $NaClO_2$: Sodium Hypochlorite; PBS: Phosphate Buffer Saline; PNP: p-nitrophenyl Phosphate; PVP: Polyvinylpyrrolidone; RT: Room Temperature

Introduction

Nepal, country of Asia, lying along the southern slope of the Himalayan Mountain ranges. It's territory extends roughly 500miles (800 km) from east to west and 90 to 150 miles from north to south.. Its total area is 1,47,181 sq.km. In Nepal, 65.5% of the population are engaged in agriculture and it contributes to national GDP of about nearly one third. (Sanjay and Thapa, 2017) Citrus is the important cash crop for the hill farmers of Nepal. The citrus in Nepal is cultivated in small as well as commercial scale at an altitude of 650-1400 m above sea level. The Eastern and Western and mid -hills are the major citrus producing area of Nepal with altitude ranging from 1000-2000 [1].

Citrus is the genus of flowering tree and shrubs belonging to the Aurantioideae Rutaceae family. It grows well in the temperature range of 15-30 degree Celsius with well distributed annual rainfall of 1250-1850 mm. At present, citrus is being cultivated in about 60 districts of Nepal. It is grown in an area of 46,328 ha with only 26,759ha productive area which accounts for a total production

of 2,39,773 mt and the productivity is 8.96 mt/ha. Citrus fruit contribute 22.3% of total fruit production in Nepal. The export share of citrus fruit in Nepal is about 3% by volume and share on the export by values are insignificant [2].

Citrus tristeza Disease is caused by *Citrus tristeza* virus (CTV) which belongs to the closterovirus group which have thread like, flexuous virions, insect vector, cause cytopathological structure (insulin bodies) in infected phloem tissue and have positive sense, single stranded Rna genome of upto 20kb [3]. The virus particles are located in the phloem of the host plant and distribute its transportation system. CTV is transmitted by several species of aphids, also some species of dodder. However, it is not transmitted by seed and soil. The virus causes vein clearing, stem pitting, yellowing, slow and quick decline, leaf falling, root decaying etc. CTV can be detected by DAS-ELISA. It involves capture of a virus to a specific antibody in an ELISA, then the virus is further captured by another antibody conjugate with an enzyme and a detection step uses a specific substrate of the used enzyme. The developed color is finally read by an ELISA reader. For a virus free plant no color development occurs [4].

Tissue culture is the *in-vitro* aseptic culture of cells, tissue, organs or whole plants under controlled nutritional and environmental conditions often to produce the clones of plants. It is clear *in-vitro* culture is an essential of plants -biotechnology offers innovative approaches in several research areas. Micro propagation is an important asexual method that can be used for production of virus free plant as well as virus free rootstock. As all of us know, meristem tissue culture, *in-vitro* micro grafting and cryotherapy of shoot tips, followed by shoot tips tissue culture or *in-vitro* micro grafting are the basic tissue culture method for virus elimination in plants such as citrus [5].

History and description of CTV

Citrus tristeza virus (CTV) is assumed to have originated in China quite a long time ago. Tristeza, also known as quick decline in the United States, is one of the destructive diseases of citrus especially in lime and sweet orange grafted on sour orange and has a worldwide distribution. The climatic condition of the mid hills of Nepal favors the cultivation of high quality. CTV belongs to the closterovirus group. The causal virus is filamentous, non-envelop, and usually flexuous, with a clear modal length of 2000nm and 12nm wide. Its genome consists of single stranded RNA and has total genome size of 20kb [6].

Natural spread of CTV is through propagation of infected bud woods and by aphids. Temperature and humidity is found to be a major factor for population fluctuation of citrus aphids. Pick population was reported when the temperature was 25˚c and population was found least when temperature was below 20˚c [7].

The first record of *Citrus tristeza* Virus (CTV) Nepal was in 1971. According to the (*Regmi et al* 2001) among two strains of CTV virus found in Nepal. First strain is endemic and cause stem pitting and vein clearing symptoms only on lime and do not show symptoms in sweet orange and mandarin while second strain shows symptoms on sweet orange and mandarin [8].

Depending on the viral strain and species or scion-rootstock combination, the virus may cause no symptoms or one of three symptoms: Tristeza, stem pitting or seedling yellow. Tristeza disease is decline syndrome caused by CTV infection of different citrus species.

Stem pitting disease is probably initiated by interrupting meristematic activity of limited areas of the cambium that results in irregular radial growth with local depression at the inactivated point.

Seedling yellow is characterized by stunting, production of small pale or yellow leaves, or reduced root system and sometimes a complete cessation of growth of sour orange, grapefruit, or seedlings [9].

CTV can be detected by DAS-ELISA. It involves a capture step of a virus to a specific antibody in an ELISA, then the virus is further captured by another antibody conjugated with an enzyme and a detection step uses a specific substrate of the used enzyme. The developed color is finally read by an ELISA reader. For a virus free plant no color development occurs (Ranjit, 1997; Ranjit et al, 1998; Panthi and Ranjit, 2012).

Measures to control CTV damage include quarantine and budwood certification programs, elimination of infected trees, use of tristeza tolerance rootstock or cross protection with mild, isolate, depending on CTV incidence and on the virus strain and host varieties pre dominant in each region [9].

Description of DAS-ELISA

Enzyme linked immunosorbent assay (ELISA)

Enzyme Linked Immunosorbent assay that is considered the gold standard of immune assay. This immunological test is very sensitive and is used to detect and qualify substances including antibodies, antigen, proteins, glycoproteins and hormones. The detection of these products is accomplished by complexing antibodies and antigens to produce some measurable results [10].

Double antibody sandwich - enzyme linked immunosorbent assay (DAS-ELISA)

The DAS-ELISA procedure described by *Garnsey and Combra* in 1991 was used with commercial CTV specific coating and alkaline phosphatase- conjugated polyclonal antibodies for detection of CTV. Absorbance values at 405 nm (O.D405) were measured on polystyrene microplate containing 96 wells. An O.D reading of 2.5 times greater than that of negative control was recorded was positive [11].

DAS-ELISA provides qualitative measures of virus titer in infected plants, this test has been used to determine the relative titer of mild and severe CTV isolates in different citrus hosts for research purposes. Due to following reasons DAS-ELISA for plant virus detection is preferred more;

- It is highly sensitive (1-10 ng/ml purified virus)
- It requires minimal amount of antiserum
- It is readily adapted for large scale use
- It is qualitative and
- Whole test can be performed within 24 hrs [12].

Principle of DAS-ELISA

It involves a capture step of a virus to a specific antibody in an ELISA, then the virus is further captured by another antibody conjugated with an enzyme and a detection step uses a specific substrate of the used enzyme. The developed color is finally read by an ELISA reader. For a virus free plant no color development occurs. (Panthi and Ranjit, 2012).

Plant tissue culture

Plant tissue culture is the aseptic culture of cells, tissue, organs and their components under defined physical and chemical conditions *in vitro*. The theoretical basis for plant tissue culture was proposed by Gottlieb Haberlandt in his address to the German academy of science in 1902 on his experiment on the culture of single cells [13]. Micro propagation is one of the most useful aspects of plant tissue culture technique. It has found the widest practical application. The process of micro propagation involves the following four distinct stages [14]. The first stage is culture initiation which depends on explant type or the donor plant at the time of excision. Explants from actively growing shoots are generally used for mass scale multiplication. The second stage is shoot multiplication which is crucial and achieved by using plant growth regulators (PGRs) generally, auxins and cytokinins. In the third stage, elongated shoots are subsequently rooted either ex vitro or *in vitro*. The fourth stage is acclimatization of *in vitro* grown plants, which is an important step in micro propagation [15].

Application of tissue culture

The purposes of plant tissue culture are the following;

- Production of improved crop varieties
- Production of disease free plant (virus)
- Genetic transformation
- Production of secondary metabolites
- Production of varieties tolerant to salinity, drought and heat stress [16].

Citrus tissue culture

Tissue culture is the best option to produce disease free seedlings of the fruit crop rapidly. Micro propagation and use of *in-vitro* grafting is very helpful for production of virus free planting materials in citrus. Different levels of *in vitro* hormones affect the success of callusing, shooting, and plant regeneration in citrus. Shoot bud, flower bud and *in-vitro* seedlings epicotyl was use as explant to study the hormonal effect of citrus micro propagation [17].

In-vitro propagation of citrus plant usually done in MS media and different concentration of NAA and BAP with optimal PH range of 5.6-5.8, light intensity 2000 lux and temperature at 25 ± 2 ˚c (Panthi and Ranjit, 2012). For the rapid propagation of disease free citrus cultivar, the protocol has been developed which consists of MS supplement with 1 mg/l BAP and 0.1 mg/l NAA and rooting medium MS supplement with 1 mg/l NAA [18].

Cytokines (BAP) is involved in various growth process and growth of shoots, at the cellular level controlling many gene

expression and secondary metabolites synthesis that triggers increase cell division, while auxin play a role in cell enlargement and triggering proton pump to increase the amount of H+ into the cell so that the cell cytoplasm become more acid that causes the loosening of polysaccharide bond on the cell wall [19].

Materials and Methods

The materials used for different aspects of micro propagation of virus free citrus are given below.

DAS-ELISA

The following materials were used for DAS-ELISA.

Chemicals

Carbonate coating buffer (1 litre) Sodium carbonate (Na_2CO_3) Sodium hydrogen carbonate ($NaHCO_3$) pH = 9.6	 1.59g 2.93g
Phosphate buffer salIne (PBS) (1 litre) Sodium chloride (NaCl) Disodium Hydrogen orthophosphate (Na_2HPO_4) Potassium chloride (KCl) PH = 7.4	 8.0g 0.2g 2.9g 0.2g
Washing buffer (PBS + TWEEN-20) Phosphate buffered saline(PBS) Tween-20 pH = 7.4	 1 litre 0.5 ml
Extraction buffer PBS Polyvinylpyrrolidone (PVP) pH = 7.4	 1 litre 0.5 ml
Conjugate buffer PBS PVP40 BSA PH = 7.4	 1 litre 20g 2.0g
Substrate buffer (1 litre) PNP substrate Diethanolamine pH = 9.8	 1 g/ml 8 ml
Reaction Stopping Solution (1 litre) NaOH	 120g(3M)
Antibody (gamma-globulin) Conjugate antibody (alkaline phosphatase) Alkaline phosphate (substrate) Standard positive and negative reference Ethanol	

Table a

Apparatus

The equipment and apparatus used for DAS-ELISA are given below.

- ELISA plate
- ELISA plate reader (Huma reader)
- Micropipette
- Incubator
- Refrigerator
- Pipette tips
- Mortar and pestle
- Glasswares

Culture media for *Citrus reticulate* and *Poncirus trifoliata*

MS media

Constituent	Final concentration (g/l)
Macronutrients (MS A) 10X	
NH_4NO_3	16.50
KNO_3	19.00
$CaCl_2 7H_2O$	4.40
$MgSO_4 7H_2O$	3.70
KH_2PO_4	1.70
Micro Nutrients (MS B)100X	Final Concentration (g/100ml)
KI	0.0083
H_3BO_3	0.062
$MnSO_4 7H_2O$	0.223
$ZnSO_4 7H_2O$	0.086
$NaMoO_4 2H_2O$	0.0025
$CuSO_4 5H_2O$	0.00025
$CoCl_2 6H_2O$	0.00025
Iron Sources (MS C) 100X	Final Concentration (g/100ml)
$FeSO_4 7H_2O$	0.278
Na-EDTA $2H_2O$	0.373
Vitamins (MS D) 100X	Final Concentration (g/100ml)
Glycine	0.02
Nicotinic Acid	0.005
Pyridoxin HCL	0.005
Thiamin HCL	0.001

Table 1: Composition of MS Media.

- Cytokinin (BAP)
- Auxin (IBA)
- GA_3
- Disinfectant: detergent, ethanol, $NaClO_2$
- Autoclaved distilled water
- Sugar
- Agar
- Myoinositol

Apparatus

- Dissecting microscope
- Culture jar
- Parafilm tape
- Cotton
- Scalpel, scissor and forceps
- Laminar hood
- Filter paper
- Autoclave
- Growth chamber with light intensity (2000 lux)
- Sand
- Compost
- Soil
- Tray

Screen house.

Methods

Sample collection

The fruits of different citrus species (Trifoliate orange and mandarin orange) were collected from Kathmandu valley. The fruits were from the CTV infected mother plant, the fruit were collected from two different mandarin trees and a single trifoliate tree. The seeds were extracted from each fruit of mandarin and trifoliate orange. The seeds of mother trifoliate orange were named as "T1", seeds of first mandarin orange were named as: M1" and seeds of second mandarin orange were named as "M2". Total of 20 seeds were extracted from trifoliate "T1", 50 from mandarin "M1" and 10 from Mandarin "M2".

Culture of trifoliate and mandarin orange seeds

In-vitro culture

Surface sterilization of seeds

The collected seeds were placed on the water to check their viability, floated seeds are discarded as they can consider as non-viable and only seeds which are sunk in the water are preferred for culture and they are washed with 2, 3 drops of tween-20 mixing with tap water with continuous shaking of beakers for 10 min. Then the seeds were washed with distilled water for several times until the foam completely disappeared. Then they were dipped into $Hgcl_2$ and kept for 4 min under the laminar hood in aseptic condition. Finally they were washed with autoclaved distilled water for 3-4 times.

Preparation of media

Preparation of stock solution of MS-medium

Each stock solution was prepared as in the given concentration shown in the materials required by completely dissolving its components in the accurate amount in the given volume of distilled water. Each stock solution was kept in sterilized volumetric flask, labelled it and preserved in the refrigerator.

preparation of hormone stock solution

The auxin (NAA), cytokinin (BAP) and GA3 were used for the investigation. NAA, BAP and GA3 were prepared in the concentration of o.5 mg/ml, 1 mg/ml and 0.1 mg/ml respectively.

The stock solution was prepared by dissolving 0.05 gm of NAA. 0.1 gm. of BAP and o.o1 gm. of GA3 in NaOH first and sterilized distilled water was added to make final volume 100 ml each.

Preparation of working solution of MS-medium

The working solution of MS-medium was prepared from the stock solution of MS-medium by using the formula

$V1S1 = V2S2$

Where,

V1= Initial volume of solution to be added

S1= Strength of the stock solution

V2= Final volume of the medium

S2= Required strength of the medium.

To prepare 1lt of MS-medium, 100 ml of macronutrient (MS A), 10 ml of micronutrients (MS B), 10 ml of iron EDTA (MS C) and 10 ml of vitamins (MS D) were taken serially from the respective stock solution in the sterile conical flask. The NAA (0.5 mg/ml), BAP (1 mg/ml) and GA3 (0.1 mg/ml) were also added. Then 3% of sugar i,e 30 gm each weighed dissolved properly. 10 ml of inositol and cacl2 was added. Then volume was then made 900ml by adding sterile distilled water. Then the PH was adjusted to 5.6-5.8 using 0.1N NaOH and 0.1 N HCL, After adjusting the PH the volume was finally made 1000 ml. 0.65% agar i,e. 6.5 gm. was added to the medium and allowed to dissolve by heating in the Bunsen burner. 20 ml of medium was poured in each culture jar and covered by lead with labelling. The media was then autoclaved at 121˚c for 14-20 min 15lb/sq. inch. The required apparatus such as beaker, forceps, scissors, razor blade and petri plates were also autoclaved.

Inoculation

Before inoculation, the laminar hood was cleaned properly with ethanol. The required autoclaved apparatus and media were also cleaned with ethanol and exposed to the UV light for 15-20 minutes. After the period, the UV was closed and the air blower and fluorescent light was opened and ready for use.

The seeds were surface sterilized as described above. The seeds were inoculated on the prepared media in the culture jar. Then the jars were incubated in the plant growth chamber where temperature was maintained at 25-28˚c.

Ex-vivo culture

For *ex-vivo* culture, sand, soil and compost were mixed in the ratio of 1:1:1 in the plastic tray. Then the extracted seeds from fruits 9(i,e. only viable) were mixed in the charcoal and then they were cultured in the prepared substrate in the tray. Then they were incubated in the skin house.

DAS-ELISA technique to detect virus

Procedure

Preparation of sample

- Remove leaves from plantlets which was done in laminar flow hood by maintaining sterile condition.
- Cut mid libs of leaves (0.3g) and homogenize it with an extraction buffer (3 ml).
- Centrifuge at 1000 rpm for 1 or 2 min and take the clear supernatant as a sample and store the sample at room temperature until it is ready to use.

Coating with antibody

- Dilute the coating antibody (7 µl) in a coating buffer (7 ml) at the rate of 1:1000 dilution.
- Coat each well of the ELISA plate with 100µl of diluted antibody solution.
- Incubate it for 2hrs at 37°c in the incubator.
- Empty the plate by inverting over a sink and shaking.
- Fill the wells with a washing buffer and shake inverted.
- Repeat the washing 2 times more.

Loading the virus sample

- Add 100 µl of prepared sample to each of the wells and store at room temperature overnight.
- Next day incubate the plate for 20 min at 37°c and wash 3 times with a washing buffer.

Adding conjugated antibody

- Dilute the conjugate antibody (7 µl, same antibody used for coating but it was linked with an enzyme called p-phenyl phosphates with conjugate buffer (7 ml) at the rate of 1:1000 dilution.
- Add conjugate antibody (100 µl) in each well of ELISA plate and incubate at 37°c for 2hrs.
- After incubation, the plate was again washed very carefully with a washing buffer for 3 times.

Adding substrate

- Prepare the substrate solution by adding 679 µl of diethanolamine +3ml of distilled water whose pH was maintained 9.8 with 14 mg 0f PNP substrate.
- Load substrate (100 µl) in each well and incubate it at 37°c for 30 min.
- Wait until a strong yellow color develops in positive control, which takes 30 to 60 minutes.
- The ELISA plate was read at 405 nm with a spectrophotometer. The positive control OD should be above 0.38.
- After the complete result, the reaction was stopped by adding (100 µl) of 3M NaOH in each well.

In-vitro media optimization

Media optimization

Media optimization for both shoot and root multiplication was done only for the Negative sample (O.D <0.20) which were confirmed after the DAS-ELISA.

Media optimization for shoot multiplication

For shoot multiplication, three different concentrations of (BAP+NAA) were prepared i,e. 0.5 mg/lt, 1 mg/lt and 2 mg/lt. Then the negative *in-vitro* sample was sub-cultured in three replicates. Then they are incubated in the incubation room at a temperature of 25-28 degree Celsius, light intensity of 2000-2500 lux, photoperiod of 16 hrs. light and 8 hrs. dark. After each 3 weeks, shoot length and shoot multiplication was observed in each replication.

Media optimization for rooting

For root multiplication, three different concentrations of NAA were prepared i,e. 0.25 mg/lt, 0.5 mg/lt and 1 mg/lt. Then the negative *in-vitro* sample was sub-cultured in three replicates. Then they are incubated in an incubation room at a temperature of 25-28 degree Celsius, light intensity of 2000-2500 lux, photoperiod of 16 hrs. Light and 8 hrs. Dark. After each 3 weeks, shoot length and shoot multiplication was observed in each replicates

Sand rooting

Hardening/Acclimatization

For about 2 weeks, *in-vitro* plantlets were kept for hardening, outside the incubation room, at normal temperature. After 1week of hardening outside, the lids of the culture bottle were loosened, so as to acclimatize the plantlets inside the *in-vitro* condition to outside condition. After 2 weeks of acclimatization, the plant bottles were ready for transfer into sand.

Washing and cutting

The plantlets were taken out from the bottles using the forceps. Then the plantlets were washed properly to remove the media. Then, it was cut and divided into appropriate sizes.

Washing of sand and dry

The sand was taken and it was washed properly to remove the mud from sand, after washing for sometimes the sand was dried in the hot air oven for sterilization.

Transfer the sand into the tray

Take a tray and make hole at corner and center of the bottom of the sand tray with the help of driller to allow the flow of excess water from the tray. The holes were covered with wire mesh to prevent the sand from falling. Then the sterilized sand was transferred in the tray about half level about 2 cm and then the clean water is sprayed in the sand to wet it.

Transfer the plantlets in the sand

Finally the plantlets were transferred into the sand tray. The plantlets were kept 2 cm apart. Then the tray was kept in the skin house to maintain the humidity.

Results

Germination of seeds

The first germination of M1 and M2 samples in concentration of 50µl GA3 was seen after 10 days and 13 of culture respectively.

The first germination of trifoliate was seen after 23 days. The other cultured seeds were also germinated in a time interval of 12-15 days.

Where in *ex-vivo* condition the seed germination was seen after 35 days.

ELISA Plate Reading of DAS-ELISA

All the *in vitro* and *ex-vivo* culture seeds of citrus species namely *Poncirus trifoliata, citrus reticulata* and germinated plantlets from these respective species were tested for CTV by DAS-ELISA method and O.D reading was taken using ELISA plate reader.

The samples ranging less than 0.2 were considered negative, 0.2-0.3 were mild, 0.3-0.4 were moderate and greater than 0.4 were considered severely infected.

Out of 19 samples 3 were negative (M1 E, Tri (B) c and Tri (B) A) which are from infected mother plants and were grown in *in-vitro* condition.

And 11 were mild positive (Tp6, M1(A), M1(C), M1(D), M1(F), M1(G), M2(S), Tri(D)b, Tri€, Tri(B), Tri(D)d, among these Tp6 sample was grown in skin -house and other was culture *in-vitro* condition.

Also 3 were moderate positive, M1,M2 and which were CTV positive mother plants.

Then also, 2 were severe positive (L1 and M3) which were also already CTV positive mother plant.

S.NO.	Name of Sample	O.D (Avg ± S.D)
1	Mother (M1)	0.351 ± 0.074
2	Mother (M2)	0.315 ± 0.0464
3	Mother (T1)	0.370 ± 0.1127
4	Mother (M3)	0.433 ± 0.04
5	Mother (L1)	0.536 ± 0.0272
6	Poly House (Tp6)	0.230 ± 0.0036
7	M1(A)	0.234 ± 0.0464
8	M1©	0.240 ± 0.0273
9	M1(D)	0.287 ± 0.0624
10	M1(F)	0.249 ± 0.0716
11	M1€	0.088 ± 0.0223
12	M1(G)	0.239 ± 0.0133
13	M2(S)	0.247 ± 0.0516
14	Tri(D)b	0.244 ± 0.0191
15	Tri(B)c	0.075 ± 0.0139
16	Tri€	0.291 ± 0.0498
17	Tri(B)	0.286 ± 0.0415
18	Tri(B)A	0.061 ± 0.0075
19	Tri(D)d	0.299 ± 0.0257

Table 2: Optical density or Absorbance at 405 nm of different samples of citrus for CTV.

Index

- 0.2-0.29 (Mild) Negative
- 0.3-0.39 (Moderate)
- 0.4< (Severe)

Figure 1: O.D of Mandarin sample at 405 nm.

Figure 2: O.D of Trifoliate and lime at 405 nm.

Media optimization for shoot multiplication

MS optimization media with NAA+BAP

In the solid media consisting of different concentrations of BAP and NAA, the best average shoot multiplication and shoot length after 9 weeks of culture was observed in 1mg/lt BAP +0.1 mg/lt NAA. As we have discussed earlier we have three different concentrations of media I,e 0.5, 1 and 2 mg/lt. The highest average shoot length is 4 cm, 2.25 cm and 2.2 cm after 9 weeks in 1 mg/lt, 0.5 mg/lt and 2 mg/lt media respectively. The highest average shoot multiplication is 6, 3.5 and 3 after 9 weeks in 1 mg/lt, 0.5 mg/lt and 2 mg/lt media respectively.

Figure 3: Average length of shoot grown in each hormone concentration.

Figure 4: Average number of shoot multiplication in each hormone concentration.

Sample name	Effect of BAP+ NAA on negative Sample				
	Parameter	BAP + NAA Mg/ It	Average ± S.D		
			3weeks	6weeks	9weeks
M1€	No. of shoot Multiplication	0.5	1 ± 0	2 ± 0	3.5 ± 0.5
	Height(cm)		0.65 ± 0.15	1.4 ± 0.2	2.25 ± 0.05
	No. of shoot Multiplication	1	2 ± 0	3 ± 0.5	3.5 ± 0.5
	Height (cm)		1 ± 0.05	1.65 ± 0.07	3.15 ± 0.25
	No. of shoot Multiplication	2	1 ± 0	1.5 ± 0.05	2.5 ± 0.5
	Height (cm)		0.45 ± 0.05	0.95 ± 0.05	1.2 ± 0.1
Tri(B)c	No. of shoot Multiplication	0.5	1 ± 0	1.5 ± 0.5	2.5 ± 0.5
	Height (cm)		1.5 ± 0.1	1.85 ± 0.05	2.15 ± 0.05
	No. of shoot Multiplication	1	2.5 ± 0.5	3.5 ± 0.5	5.5 ± 0.5
	Height(cm)		2.4 ± 0.1	3.3 ± 0.5	3.85 ± 0.05
	No. of shoot Multiplication	2	1.5 ± 0.5	2 ± 0	3 ± 0
	Height(cm)		1.55 ± 0.05	1.95 ± 0.05	2.2 ± 0.1
Tri(B)A	No. of shoot Multiplication	0.5	1.5 ± 0.5	2 ± 0	3.5 ± 0.5
	Height(cm)		1.25 ± 0.15	1.7 ± 0.2	2 ± 0.1
	No. of shoot Multiplication	1	3 ± 0	4.5 ± 0.5	6 ± 0.1
	Height(cm)		2.35 ± 0.15	3.25 ± 0.05	4 ± 0.1
	No. of shoot Multiplication	2	1 ± 0	2 ± 0	2.5 ± 0.5
	Height(cm)		1.2 ± 0.1	1.75 ± 0.15	1.95 ± 0.05

Table 3: Effects of plant growth regulators BAP and NAA on number of shoot multiplication and height (in cm).

Media optimization for rooting

MS optimization media with NAA

In media optimization for rooting, we have prepared different concentrations of rooting hormone(NAA) I,e (0.25, 0.5 and 1) mg/lt. The best response was observed in the media concentration of 1 mg/lt of NAA. The highest average length of root after 9 weeks was 6.55 cm, 3.1 cm, 3.05 cm in concentration of 1,0.5 and 0.25 mg/lt media respectively. Similarly the highest average number of root after 9 weeks was found to be 11,6,3.5 in concentration of 1,0.5 and 0.25 mg/lt media respectively.

Table 4: Effects of plant growth regulators NAA on number of roots and root length (in cm).

Sample Name	Parameter	NAA mg/lt	Average±S.D 3weeks	6weeks	9weeks
M1€	No.Of roots	0.25	1±0	1.5±0.5	2.5±0.5
	Root length(in cm)		1.35±0.25	1.85±0.05	2.05±0.05
	No.Of roots	0.5	2.5±0.5	3.5±0.5	4.5±0.5
	Root length(in cm)		1.95±0.05	2.45±0.05	3.05±0.15
	No.Of roots	1	4.5±0.5	6.5±0.5	9±1
	Root length(in cm)		3±0.1	3.55±0.35	4.65±0.45
Tri(B)c	No.Of roots	0.25	2.5±0.1	3±1	3.5±0.5
	Root length(in cm)		1.35±0.05	1.6±0.1	2.1±0.1
	No.Of roots	0.5	2.5±0.5	4±1	6±1
	Root length(in cm)		2.25±0.15	2.7±0.2	3.1±0.1
	No.Of roots	1	3.5±0.5	5.5±0.5	8.5±0.5
	Root length(in cm)		1.75±0.25	3.6±0.6	5.7±0.7
Tri(B)A	No.Of roots	0.25	1±0	1.5±0.5	2±0
	Root length(in cm)		1.75±0.2	2.1±0.1	3.05±0.05
	No.Of roots	0.5	1.5±0.5	2.5±0.5	4.5±0.05
	Root length(in cm)		1.75±0.15	2.3±0.2	3±0.1
	No.Of roots	1	4.5±0.5	6.5±0.5	11±1
	Root length(in cm)		2.25±0.25	4.55±0.35	6.55±0.0.25

Figure 5: Average number of roots in different hormone concentration.

Figure 6: Average root length at different hormone concentration.

Discussion

Seed germination

In-vitro seed Germination

As we have discussed earlier, the first work we study is *in-vitro* seed culture. For this we have cultured total of 20 seeds of Trifoliate (T1), 30 seeds of Mandarin (M1) and 10 seeds of Mandarin (M2). Among these 15 out of 20 seeds of Trifoliate were germinated (i,e. about 20%.). Similarly out of 30 seeds all the seeds of mandarin (M1) were germinated and only 1 out of 10 seeds of mandarin (M2) was germinated (i,e. 10%).

The first germination of mandarin seeds were after 10 days of culture and trifoliate was seen after 23 Days. But all the seeds of mandarin (M2) were not grown until 4 months.

From the experiment we can also say that less number of seed germination on Mandarin (M2) can be due to several reasons like immature seeds of mandarin, chemical compositions of seeds, water content of seeds, defects on endosperm that cannot absorb enough nutrients.

Similarly, according to Ferreira (1969) germination of trifoliate seeds is delayed because they are dried for a longer time. And other reasons for late germination of seeds can be moisture content in the media, appropriate photoperiod, appropriate P.H of media and humidity can be considered.

Ex-vivo seed germination

For *ex-vivo*, 10 seeds of mandarin (M1), 10 seeds of Mandarin (M2) and 20 seeds of trifoliate were cultured. Among these all the seeds of trifoliate were germinated after 35 but the germination did not appear on M1 and M2 samples.

Reason behind not germination of the Mandarin sample could be excess water content in the tray which leads to seed dead, immature seeds of mandarin, temperature and moisture can be considered.

DAS- ELISA

Once all the *in-vitro* plants and *ex-vivo* plants had reached their certain length, the healthiest plants among them were taken for DAS-ELISA procedure.

While we were doing DAS-ELISA, out of 19 test samples we have five already CTV infected mother plants (M1,M2,M3,T1 and L1) and 13 are *in-vitro* grown plants from respective virus infected plants seeds M2(S) from (M2 seed), (M1(A), M1(C), M1(D), M1€, M1(F), M1(G) (from M1 seed) and Tri(D)b, Tri(B)c, Tri(B), Tri€, Tri(B) A, Tri(D)d from (T1 seed) and one is *ex-vivo* sample (Tp6). These all samples are performed DAS-ELISA at 405 nm. Among these overall 19 samples Two sample name (L1 and M3) were found severely infected, three sample name(M1,M2 and T1 were found to be moderately infected, 11 samples name(M1(A), M1(C), M1(D), M1(F), M1(G), Tri(D)b, Tri(B), Tri€, Tri(D)d, M2(s) and Tp6 were found to be mildly infected and only three samples (M1€, Tri(B)A and Tri(B)c were found to be negative.

In our present study, samples at O.D(405nm), 0.20-0.29 is considered mildly infected, 0.30-0.40 was considered moderately infected, >0.4 was considered severely infected and < 0.2 was

considered as negative. The sample names M3 and L1 are positive controls.

As Wallece, 1978 described the CTV is not a seed-borne disease. And here in our study, among 14 samples which were germinated from infected mother plants, only 3 were test negative and others were seen mildly and moderately infected. The reason behind mildly infected *in-vitro* plants could be contamination during the experiment, older antibody, and unsterilized motor pestle as we are using the same mortar and pestle for both positive and negative samples and also can be experimental errors like washing, adding samples etc. Also samples from the skin house were also seen mildly infected and this could be because of unmanaged screen house that aphids can easily enter into the skin house.

Media optimization

Media optimization for shooting

Maximum shoot length and shoot multiplication in different concentrations of BAP+NAA was measured in different negative samples. In the M1€ sample average shoot length was 2.25, 3.15 cm and 1.2 cm in concentration of 0.5mg/l, 1 mg/l and 2 mg/l respectively. Here maximum shoot development was observed in 1 mg/l media and then in 0.5 mg/l and 2 mg/l media. Whereas least shoot development was seen in 2 mg/l concentration. Similarly in the case of sample Tri (B)c and Tri (B)A maximum shoot length was seen in 1 mg/l concentration and it was followed by 0.5mg/l. Least development of shoot was seen in 2 mg/l.

In the case of the M1€ sample, the average highest number of shoot multiplication was 3.5 which was observed in 0.5 mg/l and 1mg/l. Whereas the highest number of shoot multiplication in Tri(B)c and Tri(B)A was 5.5 and 6 respectively which was found in 1 mg/l concentration of BAP+NAA.

Chamandosti, 2020 described that MS media supplement with 1 mg/l BAP and 0.01 mg/l of NAA is the best media for shoot elongation and multiplication.

Here in our experiment both shoot length and shoot multiplication were seen well on 1 mg/l of BAP compared with other two concentrations. The plants on 1 mg/l of BAP+NAA was well developed but in media 2 mg/l there is induction of callus on the plants which could be due to high hormone concentration and if we look on the plants at 0.5 mg/l of BAP+NAA these are developed well but not better than the 1 mg/l of BAP+NAA.

So from this we can say that the maximum number of shoot multiplication and better shoot length can be seen on media supplied with 1 mg/l of BAP+NAA.

Media optimization for rooting

The apical shoot tips of the negative sample were excised aseptically and inoculated in MS-media containing different concentrations of auxin (NAA) i.e. 0.25, 0.5,1 mg/ml. The first generation of root was on media 1 mg/l after 25 days of culture in rooting media. The maximum average number of roots in sample M1€ was 2.5, 4.5 and 9 in medium concentration of (0.25, 0.5, 1) mg/l respectively. Here maximum no of roots were grown on media concentration of 1 mg/l than other two concentrations. And in sample Tri (B) A and Tri (B) c maximum number of roots was seen on media concentration of 1 mg/l I,e. 11 and 8.5 respectively.

The maximum average root length observed in the sample M1€ was 4.65 cm, 3.05 cm and 2.05 cm in media concentration of 0.25, 0.5 and 1 mg/ml NAA respectively. And in sample Tri (B) A and Tri (B) c maximum root length was seen on media concentration of 1 mg/l I,e. 6.5 cm and 8.5cm respectively.

From experiment and above data it is clear that the better root length and number of roots can be seen on media concentration of 1 mg/l than other two concentration 0.25 and 0.5. Also from this data we can also say that higher the concentration of auxin, the more will be growth in root.

Conclusion

From our study following conclusion can be made;

- Mandarin orange seeds take less germination time as compared to trifoliate oranges.
- CTV virus is not transmitted from infected mother plants to its seedlings.
- 1 mg/l BAP was effective for shoot multiplication and shoot length growth.
- 1 mg/l NAA was effective for root multiplication and root growth.

Thus, from this we can conclude that CTV virus free citrus plants can be produced.

Acknowledgement

We would like to express the deepest gratitude to our supervisor Prof. Dr. Mukunda Ranjit who is also the academic Director of Kantipur Valley College for his support, suggestions, understanding, guidance and encouragement in each and every step of our research work.

Our appreciation also extends for our respected mam Mrs. Keshari Maiya Rajkarnikar who has helped us in every step of our research. We also would like to thank our senior Sabita Tamang Bomjan for continuous support in the laboratory.

We would like to express our sincere gratitude to Kantipur Valley College for funding us in our research and express our appreciation to our principal Mr. Pushpa Man Amatya and very thankful to our coordinator Mr. Sagar Thapa for providing the necessary laboratory facilities and for his suggestions and support during the research work.

We also like to thank our friend Sagar Koirala, seniors Lasta Maharjhan, Purnima Tamrakar, Menuka Gurung, Bijay Rana Magar and Ashok Limbu for guiding us during our work.

And for providing us with samples infected with CTV for our research, we want to thank Prof. Dr. Mukunda Ranjit once again.

We would also like to thank our teachers, seniors and friends; who helped us directly or indirectly during research.

Finally, we would like to express our sincere gratitude to our parents and family who always support and encourage us in every step of our life.

Bibliography

1. A Gautam., *et al*. "Economics of production and marketing of mandarin in gulmi, NEPAL". *Food and Agribusiness Management (FABM)* 1 (2020): 1-4.
2. S Dahal., *et al*. Food and Agribusiness Management (FABM). 1.1 (2020): 47-53.
3. S Atta., *et al*. "Distribution and Research Advances of *Citrus tristeza* virus". *Journal of Integrative Agriculture* 11.3 (2012): 346-358.
4. M L Edwards and J I Cooper. "Plant virus detection using a new form of indirect ELISA". *Journal of Virological Methods* 11.4 (1985): 309-319.
5. F Chamandoosti. "The utilities of Citrus tissue culture". *International Journal of Agriculture and Environmental Research* 3.9 (2017): 36-46.
6. "RS5687_Master Plan for Horticulture Development Fruit Production in Nepal-scr".
7. P Acharya. "Study on *Citrus Tristeza* Virus (Ctv) Vectors in a Mandarin Farm of Kavrepalanchowk District , Nepal". (2017).
8. N S Poudel and K Khanal. "Viral Diseases of Crops in Nepal". *International Journal of Applied Sciences and Biotechnology* 6.2 (2018): 75-80.
9. P Moreno., *et al*. "*Citrus tristeza* virus: A pathogen that changed the course of the citrus industry". *Molecular Plant Pathology* 9.2 (2008): 251-268.
10. M Alhajj and A Farhana. "Enzyme Linked Immunosorbent Assay". Treasure Island (FL), (2022).
11. S Korkmaz., *et al*. "Detection of *Citrus tristeza* virus (CTV) from Satsuma Owari mandarins (*Citris unshiu)* by direct tissue blot immunoassay (DTBIA), DAS-ELISA, and biological indexing". *New Zealand Journal of Crop and Horticultural Science* 36.4 (2008): 239-246.
12. MA Rocha-Peña and R F Lee. "Serological techniques for detection of *citrus tristeza* virus". *Journal of Virological Methods* 34.3 (1991): 311-331.
13. TA Thorpe. "History of plant tissue culture.". *Molecular Biotechnology* 37.2 (2007): 169-180.
14. T Murashige. "Plant Propagation Through Tissue Cultures". *Annual Review of Plant Biology* 25.1 (1974): 135-166.
15. MZ Abdin., *et al*. "Plant biotechnology: Principles and applications". March. (2017).
16. A Hussain., *et al*. "Plant Tissue Culture: Current Status and Opportunities". A. Leva and L. M. R. Rinaldi, Eds. Rijeka: IntechOpen, (2012): 1.
17. Resham Babu Amgai., *et al*. "Hormonal Effect on Mandarin Orange (*Citrus reticulata* Blanco)". *Nepal Journal of Biotechnology* 4.1 (2016): 33-36.
18. TM Campus. "Journal of Tikapur Multiple Campus". 5 (2022): 152-167.
19. AA Sofian., *et al*. "Effect of IBA and BAP on Shoot Growth of Tawangmangu Tangerine (*Citrus reticulate*) by *In-Vitro"*. *Biosaintifika Journal of Biology and Biology Education* 10.2 (2018): 379-387.