POTATO MERISTEM CULTURE TO GENERATE VIRUS-FREE PLANTLETS AND OPTIMIZATION OF CALLUS INDUCTION, TRANSFORMATION AND MICROTUBERIZATION PARAMETERS

M.Sc. Thesis

Year 2024

Submitted to:

Central Department of Biotechnology

Tribhuvan University

Kirtipur, Kathmandu, Nepal

Mr. Kunjan Mishra

Registration Number: 5-2-546-5-2015

POTATO MERISTEM CULTURE TO GENERATE VIRUS-FREE PLANTLETS AND OPTIMIZATION OF CALLUS INDUCTION, TRANSFORMATION AND MICROTUBERIZATION PARAMETERS

M.Sc. Thesis

Year 2024

Submitted to:

Central Department of Biotechnology

Tribhuvan University

Kirtipur, Kathmandu, Nepal

By

Mr. Kunjan Mishra

Registration Number: 5-2-546-5-2015

Supervisor

Dr. Sarbesh Das Dangol

Co-Supervisor

Ms. Sabari Rajbhahak

Date:.....

Recommendation

This is to certify that the research work entitled "Potato meristem culture to generate virus-free plantlets, and optimization of callus induction, transformation and microtuberization parameters" has been carried out by **Mr. Kunjan Mishra** under my supervision.

This thesis work was performed for the partial fulfillment of the Master of Science in Biotechnology under the course code BT 621. The result presented here is his original findings. I/We, hereby, recommend this thesis for final evaluation.

…….

Ms. Sabari Rajbahak

(Co-supervisor)

Central Department of Biotechnology

Tribhuvan University

Kirtipur, Kathmandu, Nepal

…….

Dr. Sarbesh Das Dangol, Ph. D.

(Supervisor)

Central Department of Biotechnology

Tribhuvan University

Kirtipur, Kathmandu, Nepal

Date: 22nd May, 2024

Certificate of Evaluation

This is to certify that this thesis entitled "Potato meristem culture to generate virus-free plantlets, and optimization of callus induction, transformation and microtuberization parameters" presented to evaluation committee by **Mr. Kunjan Mishra** is found satisfactory for the partial fulfillment of Master of Science in Biotechnology.

……

Prof. Dr. Krishna Das Manandhar

(Head of Department)

……

Dr. Hira kaji Manandhar

(External examiner)

……

Asst. prof. Dr. Priti Regmi

(Internal examiner)

……

Dr. Sarbesh Das Dangol, Ph. D.

(Supervisor)

……

Ms. Sabari Rajbahak

(Co-supervisor)

ACKNOWLEDGEMENT

As my research has come to an end, I would like to convey my earnest gratitude to everyone who made this possible.

I sincerely thank my supervisor **Dr. Sarbesh Das Dangol** for his expert advice, encouragement, guidance, motivation, cooperation and full support throughout my project work. I would also like to express my gratitude to my co-supervisor **Ms. Sabari Rajbahak** for her immense guidance, support and cooperation during the project.

I would like to express my appreciation to **Prof. Dr. Krishna Das Manandhar**, Head of Department, Central Department of Biotechnology TU, for his support during the project.

I would also like to express my thanks to **Mr. Roshan Shrestha** of Pragatishil yuva krishak samuha laboratory, Banepa for providing us with resources, guidance and cooperation throughout the study. I would also like to thank Mr. Sashi Adhikari of Khadyanna Tatha Biu Alu, Alaichi, Falful, Nursery Udhyog, Panauti, Kavre for providing us with samples for the study. I would like to extend my gratitude to thank Dr. Surendra Kumar Gautam (Assistant Dean of Institute of Science and Technology, Tribhuvan University), Mr. Deval Bhattarai, Pujan Nepal and Sandhya Parajuli for providing nanoparticle samples. I would like to extend my gratitude to Ms. Anuja Rijal from Nepal Potato Research Program for providing ELISA kits and assisting me through the procedure.

I must also thank my friend Ms. Richa Giri and all the juniors of my laboratory who helped me with my lab work or any other important tasks. Their presence offered me assurance and confidence in my work. I would like to express my sincere thanks to all the faculty members of Central Department of Biotechnology, Tribhuvan University for their encouragements, suggestions and cooperation during the period of this project.

A special thanks to all the teachers, staffs, seniors and juniors of Tribhuvan University's Central Department of Biotechnology, for helping me through my dissertation.

GLOSSARY ACRONYMS

BA: Butyric acid

BAP: 6-benzylamino purine

CIM: Callus Induction Media

CuNP: Copper nanoparticle

DAP: Di-ammonium Phosphate

DAS-ELISA: Double Antibody Sandwich – Enzyme Linked Immune-sorbent Assay

FAO: Food and Agriculture Organization

GA: Gibberellic acid

IBA: Indole Butyric acid

KIN: Kinetin

MS: Murashige and Skoog

NAA: Naphthalene acetic acid

NARC: Nepal Agriculture Research committee

NPRP: Nepal Potato Research Program

PBS: Pre basic seed

PGR: Plant Growth regulator

PLRV: Potato Leaf Roll Virus

PVA: Potato Virus A

PVM: Potato Virus M

PVS: Potato Virus S

PVX: Potato Virus X

PVY: Potato Virus Y

TDZ: Thidiazuron

Table of Contents

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Potato meristem culture to generate virus-free plantlets, and optimization of callus induction, transformation and microtuberization parameters

Potato (*Solanum tuberosum)*, in Nepal, is among the fourth most cultivated food crops in terms of area, first in terms of productivity and techniques developed. Potato apical meristem culture is inevitable for the production of virus-free plants as they do not possess vascular bundle for virus multiplication. Out of eleven different potato cultivars used in this study, the unreleased cultivar 226 showed the highest regeneration with the media containing 0.25 mg/L GA_3 as well as 1.5 mg/L BAP + 1.5 mg/L kinetin. Purple potato showed the highest regeneration in 0.25 mg/L GA_3. Moreover, 0.25 mg/L GA_3 showed the best results for the growth of five cvs. Rosita, Halka Nilo, Purple, Desiree and 226. After DAS-ELISA investigation, six of the cultivars were found to be virus-free. Desiree was selected for microtuberization with copper nanoparticle (CuNP) (CuO). Microtuber formation was observed to be the highest in CuNP 2.0 mg/L with thidiazuron (0.5 mg/L) compared to media with only TDZ or CuNPs. CuNP without hormone didn't yield any microtubers. The biggest microtuber size was also seen in CuNP 2.0 mg/L with thidiazuron (0.5 mg/L) with diameter 4.6 mm weighing 0.31 g. We also optimized potato callus induction in five cvs. MS42.3, Panauti Local (P.L.), Rosita, Purple and Khumal Upahar (K.U.). CIM-5 (2 mg/L kinetin + 1.5 mg/L 2, 4-D) showed the highest callus induction rate for MS42.3 (94.4%) and P.L. (66.67%), and CIM-3 (1.5 mg/L kinetin + 1 mg/L 2, 4-D) for Rosita (55.56%). Callus induction was found to be the highest in CIM-10 (2 mg/L BAP + 1 mg/L NAA) for MS42.3 (94.5%), CIM-11 (2 mg/L BAP + 2 mg/L NAA) for P.L. (94.5%), CIM-12 (2 mg/L BAP + 2 mg/L NAA) for Purple (88.89%) and CIM-9 (2 mg/L BAP + 1 mg/L NAA) for K.U. (83.33%). CIM-12 (2 mg/L BAP + 2 mg/L NAA) showed best callus induction rate (64.44%). Plantlet regeneration rate from callus was found to be the highest in cvs. MS42.3 (59.16%) and P.L. (57.34%). Leaf and internodes of P.L. were infected with *Agrobacterium* strain LBA4404 with pAXY0002 containing *GUSPlus* gene. However, no result was obtained on GUS histochemical analysis. Future work should focus on generation of

improved microtuberization using gene editing technology for pre-basic seed (PBS) generation.

Keywords: meristem culture, DAS-ELISA, callus induction, microtuberization, nanoparticles

CHAPTER-I

INTRODUCTION

1.1 Background:

Potato (Solanum tuberosum L.) holds a significant place in Nepal's agriculture. As the fourth-most cultivated food crop in terms of area and the first in terms of productivity, its role is crucial in the country's food security and economy. (Luitel et al., 2020). It is a great source of carbohydrate and commonly consumed vegetable in local diet or supplementary to other vegetables. In addition, potato farming has become a lucrative prospect for farmers and now viewed as a cash crop with recent developments made in technology to improve productivity, efficiency and reliably produce disease-free plants (Upadhyay & Timilsina, 2020). According to FAO in Nepal in the year 2022, potato is harvested in an area of 198,253 ha with a yield of about 17,204 kg/ha and total production of 3,410,829 tons (FAOSTAT, 2024).

Table 1: List of cultivars released by Nepal Agriculture Research Committee (NARC) (Source: Potato Development Research Programme (PDRP), NARC, Khumaltar, 2022):

SN	Name	Source	Geographical location
1	Kufri Sinduri	India	Terai
2	Kufri Jyoti	India	Mid and high hills
3	Desiree	Netherland	Terai and mid hills
4	Khumal Seto	CIP/Peru	Mid and high hills
5	Khumal Rato	CIP/Peru	Terai
6	Janakdev	CIP/Peru	Terai to high hills

7	Khumal Laxmi	CIP/Peru	Mid hills
8	IPY8	CIP/Peru	Terai
9	Khumal Upahar	CIP/Peru	Terai
10	Khumal Ujwal	CIP/Peru	Terai to high hills
11	Khumal Bikash	NPRP/Nepal	Mid and high hills
12	TPS1	CIP/Peru	Teari to high hills
13	TPS2	CIP/Peru	Terai to high hills
14	Cardinal	Netherland	Terai and mid hills
15	Rosita	Switzerland	High hills
16	MS-42.3	CIP/Peru	Terai and mid hills

Meristem culture is the cultivation of the shoot apical meristem. It is also referred as shoot tip or apical meristem culture. Meristem plays a key function to increase the plant length. It possesses meristematic cells that are continuous, oval, polygonal and rectangle in shape without intercellular spaces (Beyene et al., 2010). Meristem culture involves excision of a lobe of meristematic cells below the shoot or root apex, and are usually 0.2 to 1 mm in length with their length contributing to number of regenerated plants (Marcela & Anca, 2011). Potato seeds are susceptible to seed degradation and can gradually lead to reduced productivity and yield over time due to accumulation of viruses. Viruses that have been commonly associated to potato in almost all the geographical regions include Potato Leaf-Roll Virus (PLRV), Potato Virus S (PVS), Potato Virus X (PVX), Potato Virus Y (PVY), Potato Virus A (PVA) and Potato virus M (PVM) (Campos & Ortiz, 2020). In Nepal, these viruses have been found to infect many

local cultivars (Khatri & Shrestha, 2004). In order to avoid the reduction in yield resulted by these viruses, various procedures such as meristem culture is recommended to produce virus-free pre-basic seeds (Sakha & Rai, 2004). Double Antibody Sandwiched-Enzyme-Linked Immunosorbent Assay (DAS-ELISA) (Clark & Adams, 1977) is serological method commonly used for detection of these viruses and to ensure elimination of the viruses from PBS. RT-PCR (Reverse transcription - polymerase chain reaction) has been found to be sensitive for potato virus detection (Dhital et al., 2010b). DAS-ELISA can be used to detect PLRV, PVA, PVY, PVV, PVM, PVS and PVX from *in vitro* plantlets which are required for production of virus-free potato seeds. This method is widely used for this purpose (Khan et al., 2003).

Nanotechnology is developing area of science that deals with nanoparticles, specifically 1-100 nm (Baker et al., 2015). The International standardization organization (ISO)'s technical committee gave the definition of nanotechnology as "Knowing and handling objects and organisms at nanoscale of 1-100 nm" (Wang et al., 2016). Nanoparticles are known to improve intake and transport of nutrients and thus have great application for increased yield and improved crop production. They can confer greater pathogen resistance, stress tolerance, energy production and lesser waste production and therefore has found wide array of use as pesticides, fertilizers, herbicides, growth promoters, etc. (Pestovsky & Martinez-Antonio, 2017).

Microtuber can be defined as the intermediate stage between *in vitro* plant and minituber production. Microtuber plant decrease acclimatization duration as well as issues regarding area required as seeds are directly produced *in vitro* and it has also been found to improve minituber number per plantlet (Ranalli, 2007). *In vitro* microtuberization which started in Nepal since 2001 requires MS media with 8% sucrose with continuous darkness is required at 20-22 ^{o}C. The explant usually comprises of 2-3 nodes, however higher number of nodes has higher rate of production (Sakha et al., 2004). Thidiazuron (TDZ) hormone is plant growth regulator (PGR) that induces regeneration of recalcitrant plants and induces shoot growth. It also induces somatic embryogenesis at higher concentrations and at low concentration causes organogenesis (Erland et al., 2020). As far as we know, sparse research has been

performed in Nepal using nanoparticles for microtuberization in local Nepali cultivars. So, our study aims to assess the ability of nanoparticles to induce and enhance microtuberization in potato as well as to evaluate its efficiency.

For genetic transformation and genetic engineering, callus induction is an essential step. It involves transformation of explant into a mass of undifferentiated cells that can re-differentiate to produce new plantlets (Ikeuchi et al., 2019). Hence, callus induction is required to regenerate a modified explant via de-differentiation and re-differentiation to produce a new transgene plant (Ikeuchi et al., 2013). Large number of studies have been performed in calli induction of potato. So different procedures, media and hormone composition have been studied for callus induction. Callus induction varies vastly depending on genotypes as well as media composition (Wareh et al., 1989). Studies have also detected genotypes requiring different induction and regeneration media (Gonzalez et al., 2001). Similarly, different explants of same genotype can again have varied callus induction results (Hamdi et al., 2017). Somaclonal variation is performed via callus induction and it is applicable for improving genetic characters and for bacterial and fungal elimination. Hence, it is an excellent method to obtain desirable traits such as increased resistances to environmental stresses (Kaeppler et al., 2000).

Hormones like naphthalene acetic acid (NAA) and kinetin have positive effect on calli induction as well as root formation. The hormone 2,4-Dichlorophenoxyacetic acid (2,4-D) is used in plant cell cultures as a dedifferentiation hormone (callus induction hormone) (Kaviani et al., 2013). Cytokinins promote cell division and play a major role in callus formation as they reduce the lignification of cell walls on the cut surface inducing callus formation (Hoque et al., 2006). Accordingly, this study was performed to evaluate the effect of various hormones on callus formation and plant regeneration of locally important cultivars.

Agrobacterium-mediated plant transformation is the most useful technique for development of transgenic plants via introduction of foreign genes (Gustavo et al., 1998). *Agrobacterium* serves as a carrier for introduction of foreign gene as T-DNA which integrates into the host genome. Upon success, expression of protein encoded by the gene occurs (Chilton et al.,

1977). *Agrobacterium* transformation procedure involves attachment to recognition sites of the plant cell followed by transfer of T-DNA guided by several *vir* genes. *Agrobacterium* T-DNA upon entering plant cells, integrates to plant genome by further actions of *vir* genes (Gelvin, 2000). *Agrobacterium* mediated transformation of *Solanum tuberosum* cv. Desiree was achieved and optimized long ago (Visser, 1991). It has been observed that different genotypes had varied transformation efficiency, even in cases where vectors and targeted genes were always kept constant (Wenzler et al., 1989). Therefore, this research was performed to observe the *Agrobacterium*-mediated transformation in the local potato variety near Kathmandu and to assess its GUS histochemical assay.

1.2 Research Hypothesis:

a) Meristem culture of potato will generate virus-free plants suitable for microtuberization

b) Combination of thidiazuron and nanoparticles will generate microtubers

c) Use of different hormones will help determine the most suitable combination of hormones for callus induction

1.3 Objectives

1.3.1 General Objectives

- To perform meristem culture, generate virus-free plantlets and optimize callus induction and transformation parameters

1.3.2 Specific Objectives

- To develop disease-free local potato cultivars using meristem culture technique
- To optimize meristem culture and calli induction on local potato cultivars
- To generate virus-free microtubers using copper nanoparticles
- To establish the efficient transformation protocol in local potato cultivar using GUS reporter gene

1.11 Rationale of the study

Microtuberization consumes less time and space as compared to the Pre basic seed (PBS) method. However, productivity needs to be optimized in order to determine the viability of this method. With modern development in nanotechnology, need for study of effect of various nanoparticles on many plant species have been rising. Various studies have been performed using different cultivars; however, to our knowledge, no such studies using CuO nanoparticle on local cultivars have been performed. So, the study of nanoparticles to check their ability to induce and enhance microtuberization in potato as compared to those without the use of nanoparticles was performed. Similarly, optimization of callus induction and meristem culture for commonly used agronomic potatoes of Nepal was performed to ease genetic transformation studies in the future.

CHAPTER-II

LITERATURE REVIEW

2.1 Brief History

Potato has been predominant food in Nepali household; however, Nepal is among the lowest average potato yielding countries in the world (Lama et al., 2016). In Nepal, in the last four to five decades, provisions have been made to ensure progress in technology development and research in cultivation of potato due to its prospects in poverty alleviation and opportunities than can be generated in an agriculturally dominated country as means of livelihood (Sharma, 2074).

2.2 Background of potato production in Nepal

Since 1980s, the production of potato seed under the guidance of Swiss government (with agreement of farmers) was being produced and later ousted by Krishak Samuha in 1991. Pre-Basic Seed (PBS) production is now an integral part of potato farming in Nepal. Nepal Potato Research Program (NPRP) under NARC is the main contributor to PBS production in Nepal. Currently around sixteen varieties are available for planting in two seasons. Also, private sectors for PBS productions are also on the rise to its financial prospects (Sharma, 2074).

Typically, potato is cultivated in winter and rainy seasons, nevertheless in Kathmandu valley, cultivation in autumn is being practiced with a great success. In similar way, spring potatoes are also now being produced in the upper hills (Dhital, 2016).

Different aspects of potato agriculture in Nepal:

I. Altitude:

Potato is grown from an altitude of about 100 m mean above sea level (masl) to 4000 m in Nepal. Generally, suitable area for the production of high-quality seed tuber in Nepal is high hill region i.e., 2000 m from mean sea level (Bajracharya & Sapkota, 2017a).

II. Soil type:

Potatoes can be grown in well-drained, loose soil with plenty of organic matters. For seed potato farming, pH 5.5 – 6 is considered good (Bajracharya & Sapkota, 2017a).

III. Irrigation:

Irrigation/ Earthing Up is done if land is dry. Shallow rooted crop, Presowing irrigation, 2nd irrigation at 30 days is performed, then watered weekly (Adhikari, 2018).

IV. Ratio of planting areas to arable land:

In 2018, arable land in Nepal is 2,113,700 ha, which is 14.75% of total land, and potato was planted in 195,268 ha. So, potato is cultivated at 9.23% of arable land in Nepal (World Bank, 2021).

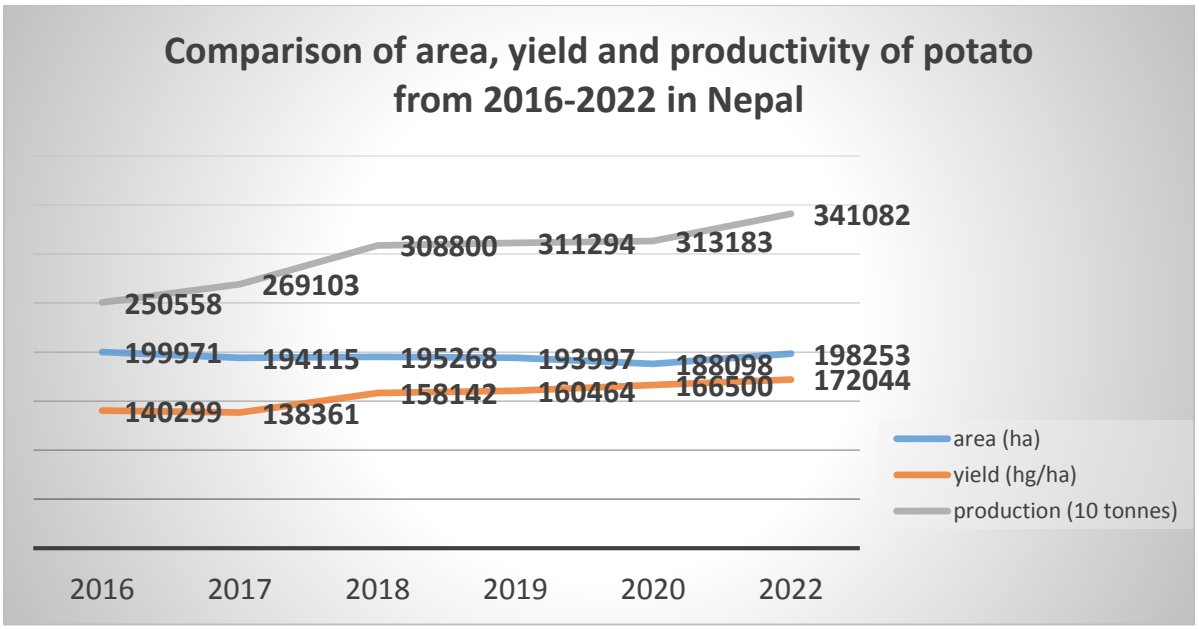


Figure 1: Comparison of potato production, area and yield from 2016-2022. X-axis shows the area, yield and production of potato in Nepal and y-axis denotes progression over time. (Data compiled from FAOSTAT, retrieved from https://www.fao.org/faostat/en/#data/QCL, accessed date: June 23, 2024)

V. Legal provisions for trade:

Food regulation act (1970), seeds regulation act (2013), customs regulation act (2007), are implemented in Nepal. Certificate of origin, permit for import export and import and license for business are required (Government of Nepal, Ministry of Industry, Commerce and Supplies, 2022).

VI. Import and export:

Around 92 percent of required number of potatoes for consumption is self-produced. In 2073/74 B.S., 2,493,603 megatons and NPR 5,311,500,000 worth potatoes were imported from India, China and Bangladesh (from India alone 96.5%). Moreover, just 109.5 megatons worth NPR 1,940,000 potatoes were exported. Exported potatoes are usually grown in the hills in rainy season (Sharma, 2074).

VII. Crops planted before planting potato in Nepal

Usually after paddy, wheat and rice are harvested, potatoes are planted. In Chitwan, good production was observed after potato cultivation following harvesting of potatoes. Crop rotation is also practiced with maize and vegetables in different plots each year (Bajracharya & Sapkota, 2017a).

VIII. Quantity of fertilizer used per hectare

The following quantity of fertilizer is used per hectare.

Compost: Initially during land preparation, 250-300 kg/Ropani (1 ropani=508.7 sq. m =0.05 ha)

Seed tuber plantation: Urea- 2.8 kg/Ropani, DAP- 5.5 kg/ Ropani and Potash: 5 kg/Ropani

Elongation stage: Urea- 1.4 kg/Ropani, ash-10 to 20 g/plant

Seed tuber growth stage: Urea- 1.4 kg/Ropani, ash-10 to 20 g/plant (Bajracharya & Sapkota, 2017b)

IX. Main pests and diseases

The following biotic stresses challenge potato production.

- Insects

Potato tuber moth (*Phthorimaea operculella*), aphid, white fly, termite, white grubs, cutworm

- Viruses

Potato leaf roll virus (PLRV), potato virus A (PVA), potato virus M (PVM), potato virus Y (PVY), Acuba Mosaic Virus

- Bacterial diseases

Bacterial wilt, potato brown rot, potato common scab

- Fungal disease: potato blight, potato wart (Bajracharya & Sapkota, 2017b)

X. Price of Seeds

The prices of seed potatoes in Nepal per ton for the years 2014, 2017 and 2018 were USD 500, USD 115.94 and USD 81.97, respectively (Wamucci, 2024).

Tissue culture method (PBS -pre basic seed production) is the fastest method currently used for disease free potato seed. Small potato seeds free from viral and other diseases which are produced by tissue culture and grown in insect-free screen houses in sand and soil at the ratio 2:1 is termed as pre-basic seeds (Dhital, 2016).

Plantation

Mainly there are two seasons for plantation (winter and rainy season) but autumn season and spring season potato production have been successful in some mid-hill region.

Winter season variety: Cardinal, Desiree, Janakdev, Kufri Sundari, Khuma Rato 2, etc.

Rainy season variety: Janakdev, Kufrii Jyoti, Khumal Seto-1, Cardinal, Desiree, NPI-106, etc.

Generally, in the 1st year, 500 PBS potato covers 45 sq. m area giving 75 kg potato. In 2nd year, 75 kg can be planted in 1 Ropani (508.74 sq. m) to increase potato seed yield to 750 kg. Similarly, it can be increased via plantation in 10 Ropani (5,087 sq. m) to produce 7500 kg in the 3rd year. One hundred Ropani (50,874 square metre) area is used to produce 75,000 kg potato seed in the 4th year (Dhital, 2016).

2.3 Meristem Culture:

Three different commercial variety (Alaska, Spunta and Safrane) of potato tubers were acquired from Ministry of Agriculture in Jordan. Seventy tubers of each variety were selected

to perform meristem culture. To detect virus-free plant growth, DAS-ELISA was performed. Virus infected tubers were then used for meristem culture. For meristem culture, Al-Taleb et al. (2011) used 30 PVY infected sprouts as explants. They were treated with 0.1% hypochlorite and 3 drops tween-20 for 10 min for surface sterilization and then they were washed 3 times with sterile distilled water. Meristem were isolated and cultured in hormone free MS media as a basal media. Ten of the meristems were sub-cultured on MS media without hormones and ten on MS media with 0.5 mg/L NAA and 10 on 0.5 mg/L IBA. In the positive samples, the infection rate for Potato virus Y (PVY) was 21.4% while Spunta had 15.7 % infection rate and Alaska had 12.8%. Meristem culture was performed on all infected cultivars and growth of roots and shoots was observed. It was seen that 0.5 mg/L of indole butyric acid (IBA) was the best for shoots proliferation with average length of 7.71 cm, root induction with average length 9.41 cm and number of shoots with average 2.60 and average number of leaves 15.40 (Al-Taleb et al., 2011).

In vitro production of virus-free potato plants was performed from four common cultivated variety in Bangladesh. Tubers of potato cultivars Diamant, Cardinal, Lalpakri and Multa were taken and meristem culture was performed. Then, plant regeneration from the cultured meristem, shoot and root growth was examined in different phytohormone concentrations and regenerated tubers were tested for presence of viruses by DAS-ELISA. Two Murashige and Skoog (MS) media were used for culture of meristem. Different phytohormones used include Gibberellic acid 3 (GA_3), Kinetin (KIN), Butyric acid (BA) and Indole butyric acid (IBA), both individually and in combination. Among these, 0.5 mg/L GA_3 and 0.04 mg/L kinetin were the best for primary meristem establishment. These primary meristems were then transferred to media with BA or IBA or both. Two mg/L GA_3 was found to be the best among these for shoot induction and was preferred singly rather than in combination with kinetin although, combination of 0.1 mg/L GA_3 and 0.1 mg/L kinetin was more appropriate for root induction and growth. These cultured plants were then acclimatized in green house and growth of virus-free plant was successfully observed by assessing their morphological characteristics. It was observed that shoot tips between 0.2 and 0.5 mm were suitable for virus-free plant regeneration. Explants smaller than 0.2 mm cannot survive and explants

above 0.5 mm may not be virus-free. It was also observed that Diamant and Cardinal showed greater response to phytohormones during development of primary meristem (Nagib et al., 2003).

Study of meristem culture and *in vitro* propagation to produce virus-free plants were performed in Rajshahi, Bangladesh from 2010 to 2012. The extent of shoot elongation and root induction was also studied. Similarly, acclimatization was also performed and characteristics were examined after transfer of *in vitro* plantlets to the field. Four different varieties of *Solanum tuberosum* were studied, namely Diamant, Shilbilati, Diamant and Lalpakri. Meristem wasn't completely extracted, shoot-tip culture was performed and the extracted shoot tip was transferred to liquid MS media. The shoot-tip was placed on the filter paper bridge placed in liquid media. Phytohormones used on media include GA_3, KIN, BA and IBA with different combinations. It was found that 0.1 mg/L KIN + 0.5 mg/L GA_3 was the most suited for primary meristem formation. Then they were transferred to semisolid MS media with 0.5 mg/L BA + 1.0 mg/L IBA, 0.1 mg/L KIN + 0.1 mg/L GA_3; 0.5 mg/L BA + 0.5 mg/L GA_3 and 0.5 mg/L KIN + 0.5 mg/L GA_3 for shoot and root development. Then 0.5 mg/L BA + 0.5 mg/L GA_3 was noted to be the most suitable for growth. Cardinal showed the greatest rate of survival (90%), shorter time to initiate tuber growth, and higher leaf count, tuber size and yield (Azad et al., 2020).

Comparative analysis between the first-generation tubers produced from stem tip cutting and *in vitro* micro propagated potatoes was performed. The tip cutting method was used for numerous multiplication and plants were grown on peat in plastic rolls to produce first generation tuber which were compared with *in vitro* micro tuber. Experiments were performed from 2005 to 2007 with aim to evaluate differences in generation and variety in terms of a plant's total dry mass and tuber's total dry mass obtained from field. Ants and Vigri, two local varieties were selected for the study. Those tubers produced form *in vitro* culture produced comparatively lower dry mass plants and tuber. So, tip cutting was seen to be more efficient for productivity. Similar was the case regarding adaptability to harsher conditions with stem tip cutting seemingly more resistant, however weren't very significant.

The growth rate of *in vitro* was faster and tuber was observed after lower duration than in tip cut variety. Hence, much difference wasn't detected and it was concluded that both methods would be reasonable in production of potato crop (Särekanno et al., 2012).

Three varieties of potato were selected for the study. Axillary buds, tuber sprouts and shoot tips of cvs. Kufri Alankar, Kufri Chandramukhi, Kufri Sindhuri were surface sterilized with 12% Clorox for 12-15 min. The meristem was extracted from tubers buds and shoot tips. They were then cultured on MS media with different concentrations of plant hormones. The phytohormones used in this study are indoleacetic acid (IAA), dichlorophenoxy acetic acid (2,4-D), gibberellic acid (GA) and kinetin. They were used individually as well as in combination. After the study, it was found that regenerating whole plants from callus was problematic whereas meristematic tips are easier, stable and quicker method for vegetative propagation. Similarly, meristems are more tolerant to adverse condition and have added benefit of production of disease-free plants. Thus, the study concluded that the culture of meristems would be the perfect for germplasm cryopreservation (Bajaj, 1980).

Meristem culture is the only known culture method for the production of virus-free plants, as it is assumed that viruses are unable to invade meristematic tissues particularly due to lack of well-developed vascular bundle. Similarly, chemicals methods have presented very little successes for virus-free plant generation. Meristematic cells are actively dividing cells and serve as precursor for plant parts. In the *in vitro* meristem culture, four cultivars from Romania i.e. Amelia, Cristian, Nicoleta and Roclas were selected for meristem culture. The explant involved meristem lobe along with varying number of leaf primordia (one, two- and four-leaf primordia). Nicoleta showed the best shoot development with PM media being the preferable media for plant regeneration. In terms of hormones, auxin and gibberellin showed better growth while cytokinin is more preferrable for calli induction rather than shoot proliferation. The meristem with four lobed leaf primordia exhibited the best growth, this was due to synthesis of auxins. In similar way, hormonal concentration for the best growth was observed on PM medium with 1 mg/L indolyl acetic acid, 1 mg/L indolyl butyric acid, and 0.3 mg/L gibberellic acid for the genotype Nicoleta (Marcela & Anca, 2011).

2.4 Validation of virus-free potato plantlets:

The viruses that have been commonly found in potato throughout the world are Potato leaf roll (PLRV), potato virus A (PVA), potato virus Y (PVY), potato virus V (PVV), potato virus M (PVM), potato virus X (PVX), potato virus S (PVS), potato mop top virus (PMTV) and potato aucuba mosaic virus (PAMV) (Brunt, 2001).

Since these viruses result in decrease in tuber size and reduce the overall productivity, it is essential to devise effective method to detect viruses in *in vitro* plantlets in order to produce disease-free seeds. In this study, six potato varieties namely, Cardinal, Diamant, Dhera, Multa, Cilena and Sieglinde were inoculated with PVA, PVY, PVV, PVM, PVS, PVX and PLRV and subjected to DAS-ELISA. All the cultivars with infected viruses showed positive ELISA reaction except PLRV as inoculation was performed mechanically. DAS-ELISA method was shown to be effective as non-inoculated plantlets didn't give positive reaction (Khan et al., 2003).

In Nepal, National Potato Research Program (NPRP) has been well-established for potato seed production (Sakha & Rai, 2004). Potato tubers undergo degeneration overtime and hence new potato seeds should be produced overtime that are free from viruses via meristem culture. The six common viruses know to infect potato in Nepal are PVX, PVY, PVA, PVM, PVS and PLRV (Akius & Kloos, 1990). In NPRP, meristem culture of 12 different cultivars i.e. Sarkari Seto, Syang Dorje, Kufri Sindhuri, Cardinal, Kathmandu Local, Tharu Local, NPI-106, Kufri Badshah, Jumli Local, CIP 388572.1, CIP 388572.4, Gui Valley were performed and the cultivars were tested for the above six viruses by DAS-ELISA. Prior to meristem culture, the cultivars were found to be infected with single or multiple viruses with PVS infecting eight cultivars and PVM infecting three cultivars. Elimination of viruses was found to be higher in single infected cultivars (Sakha et al., 2007).

In one of the earliest studies of viral detection in potatoes in Nepal, 21 cultivars were studied by PRP, Khumaltar on 1996-1998 CE to discern the various viral diseases present in potato cultivars in Nepal. Nineteen varieties were from hilly region and two were obtained from Terai region. In this study, PVS, PVX and PVY viruses was found in cv. Khodpeli. Two viruses

PVX and PVS were detected in Thakali Red, Lamjunge and Bhotange. PVS was prevalent in almost all the samples and PVY was also highly detected. No PVA presence was observed. As a result of virus detection, tuber size was small; however, Rosita, Khumbule and Lumle Red produced comparatively better tuber sizes (Sakha et al., 2007).

2.5 Nanoparticles:

Nanotechnology can improve agriculture; ensure food security and sustainability, by increasing water efficiency and preventing plant diseases. Nanoparticles can make up for crop nutrient deficiencies, improve crop stress resistance and improve crop quality and yield (El-Tahan et al., 2022). To classify as a nanoparticle, it must have a size range of 1-100 nm. It therefore has higher adsorption efficiency, high surface area to volume ratio, and higher recombination and interaction efficiencies. They can enter plant cells via the plant cell membranes and cell walls. Larger nanoparticles can pass through stomata while smaller nanoparticles can penetrate cell walls. These nanoparticles can be released from the phloem into multiple plant compartments. Nanoparticles can then effectively function in transport of food and water increasing growth, productivity and resistance to stresses (El-Saadony et al., 2022).

In agriculture, various nanoparticles are constantly being used to improve plant productivity and their responses to different stresses. The ones that have shown initial successes include the copper, zinc, molybdenum, manganese, boron, and iron nanoparticles. Nanoparticles show various advantages in comparison to the chemical counterparts to their benefits which aren't as adverse to the environment as well as the consumers. Furthermore, nanoparticles are also quite sustainable and a non-hazardous application for crop improvement. They are found to improve and accelerate seed germination, rate of photosynthesis, seedling elongation and shoot/root growth (Joshi et al., 2022).

It has been found that zinc oxide (ZnO) nanoparticles impart resistance to salinity in crops and improve productivity (Mogazy & Hanafy, 2022). Cerium oxide (CeO2) nanoparticle application also provide tolerance to salinity via antioxidant system stimulation (Chen et al.,

2022). Copper nanoparticles (CuNPs) have been found to absorb antimicrobial properties, so they have great potential in agriculture to induce disease resistance. Likewise, at lower concentration they can enhance germination growth and productivity (Kasana et al., 2016). Nanoparticles in potato culture have also been found to improve microtuberization (Sallam et al., 2022). The minituber production after virus-free *in vitro* plantlet regeneration yields high quality potatoes. However, this requires virus elimination, *in vitro* plantlet production followed by acclimatization and transfer to controlled greenhouse environment (Turkmen et al., 2017).

In potato, copper and silver nanoparticles can improve yield and zinc, silicon and boron can increase salt tolerance (Mahmoud et al., 2020). ZnO and silicon nanoparticles can also induce growth during drought in potato (El-Tahan et al., 2022).

2.6 Microtuber:

Pre basic seeds of potato are now the most common method for potato seed production for agricultural use. The minituber production after virus-free *in vitro* plantlet production produces high quality potatoes. However, this requires virus elimination, *in vitro* plantlet production followed by acclimatization and transfer to controlled greenhouse environment. So, this process requires quite high land resource and quite long time period for acclimatization of plantlets to the soil. Microtuber production *in vitro* allows the production of this seed *in vitro* without transferring to controlled environment as in PBS production. To optimize microtuber production in order to increase efficiency and to provide an alternative to minituber production, a study was performed. Three varieties, three breeding lines and nine different genotypes were subjected to microtuber production. Murashige and Skoog medium with 8% sucrose and 0.1 mg/L TDZ was used to induce microtuber production. For microtuber production, incubation was performed under dark conditions and 22/16 °C (8/16 h) temperature cycle. Moreover, 300135.14, 395017.229, 398180.612, 398190.615, Hermes and MEÇ0908.12 showed greater microtuber per plant. Similarly, Hermes and Marabel displayed greater mean microtuber weight (Turkmen et al., 2017).

Hamza (2019) performed this study to assess the effect of cobalt nanoparticle on potato microtuber formation. Different explants i.e. double nodes, whole plantlets and roots with crown area were cultivated *in vitro* in MS media with different sugar concentration (60 and 80 g/L) and different concentration of cobalt nanoparticles (CoNP) (0.0 and 2.5 mg/L). Hormone used was abscisic acid (ABA) (2.5 µg/L). Incubation of 60 days showed highest microtuber number in MS with 80 g/L sucrose and 2.5 mg/L CoNPs. The same concentration also exhibited the highest size and weight of microtuber in absence of ABA. Plantlet explant gave the highest number of microtuber. So, it was detected in the study that the highest concentration of sucrose used 80 g/L and highest concentration of CoNP used was the most successful in promoting microtuber size, weight and number thereby concluding that sucrose and CoNP promote microtuber production (Hamza, 2019).

The study performed by Ibrahim et al. (2018) was among the first performed in order to identify the properties imparted to microtuberization of potato by Silver Nanoparticles (AgNPs) rather than their ionic form. AgNPs of sizes 2-4 nm were prepared chemically and conformed by Transmission Electron Microscope (TEM). Six different AgNP concentrations i.e. 0.5, 1, 2, 4, 8 and 16 mg/L and ionic AgNO were used to study their effect on microtuber induction and formation. Three to five nodes of potato shoots *S. tuberosum* cv. Desiree were cultured in microtuber induction media with B5 vitamins and BAP and different concentrations of AgNPs. After 6 weeks of culture, the highest average number of microtubers per culture jar was recorded using 1 mg/L of AgNPs i.e. 18.95 microtuber per jar compared to 14.60 microtuber/jar with higher 8 mg/L of AgNPs. Again, 97.4% increase in microtuber production was obtained by using AgNPs in comparison to controls. Similarly, AgNO showed 51.3% more microtuber production. Thus, AgNPs are concluded to be more effective than the ionic AgNO. These findings thus suggest importance of further research in engineering of nanoparticles and protocol optimization to determine suitable nanoparticle for different cultivars and their effect in crop improvement (Ibrahim et al., 2018). Kefi et al. (2000) reported TDZ at concentration 0.1 mg/L to enhance microtuberization.

2.7 Callus Induction:

The study by Khalafalla et al. (2010) was performed to determine optimal hormonal concentration required for calli induction via tuber segment of potato on cv. Almera. Murashige and Skoog's medium with five different concentrations of 2,4-dichlorophenoxy acetic acid (2, 4-D) i.e., 1.0-5.0 mg/L, were used. Within 1-2 weeks, 3 mg/L 2, 4-D showed the fastest calli generation rate however 100% of explants produced calli on MS medium with 2.0-5.0 mg/L of 2, 4-D within 1-2 weeks. The calli were then subcultured after formation of shoot-primordia into a media supplemented with 1.5-5.0 mg/L of TDZ and 2.0-5.0 mg/L of benzyladenine (BA). For shoot regeneration, 5.0 mg/L TDZ displayed the longest shoot as well as higher number of shoots per callus and for root generation 1.0 mg/L IBA was the most successful. Then the plants were acclimatized in pots with soil to sand ratio 3:1 for 15 days and transferred to green house (Khalafalla et al., 2010).

Optimal callus induction and regeneration protocol determination was the objective of the study performed in Iraq. Four different cultivars namely, Arnova, Burren, Provento and Riviera were subjected for callus induction. The internode segments were used for callus induction. The segments were transferred to MS media with three different combinations of hormones BA and NAA (2 mg/L BA +2.5 mg/L NAA, 2 mg/L BA+ 2 mg/L 2,4-D and 2 mg/L 2,4-D). After callus formation, they were transferred to regeneration media with 2.5 mg/L BA+5 mg/L GA_3, 3 mg/L BA+0.5 mg/L GA_3 + 0.03 mg/L NAA, 0.22 mg/L TDZ+ 0.49 mg/L NAA, 5 mg/L TDZ. The varieties with the highest callus induction were found to be Burren and Riviera. They produced calli in shorter time as well. Media with 2 mg/L BA + 2.0 mg/L 2,4-D and 2 mg/L 2,4-D also showed good callus induction. For calli regeneration, 3 mg/L BA+ 0.5 mg/L GA_3 + 0.03 mg/L NAA showed best results for Burren, Provento and Riviera and 0.22 mg/L TDZ+ 0.49 mg/L NAA was better for Arnova variety. However, shoot formation was a failure (Al-Hussaini et al., 2015).

A study examined the impact of plant growth regulators on callus formation, shoot production, and root regeneration in three potato cultivars using nodes and leaf as explants. Cultivars used include Pasinler, Granola and Caspar. The optimal conditions for callus

induction were found to be on MS medium with 3.0 mg L^{-1} benzyl amino purine (BAP) and 2.0 mg L^{-1} naphthalene acetic acid (NAA). Nodes exhibited improved callus induction in comparison to leaf segments. For shoot regeneration, MS media with BAP, Kinetin, TDZ, GA_3 or NAA were used. The best results were obtained on medium with 2.0 mg/L BAP and 0.25 mg/L GA_3. For rooting, media with NAA or IBA & GA_3 were utilized. After rooting, they were acclimatized in pots with sand, soil and manure in ratio 2:1:1 for 3 weeks and then transferred to green house (Kumlay & Ercisli, 2015).

2.8 GUS Assay:

The GUS assay (β-glucuronidase assay) is a reporter gene system that involves the integration of the GUS gene from *Escherichia coli* to detect the expression of the enzyme β-glucuronidase. This enzyme can cleave substrates such as X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide), and produce a visible blue color in tissues where the gene is expressed (Jefferson et al., 1987). Transgenic plant development has been substantial in the analysis of gene and their functions in agriculture industry. Transgenic plants play a significant role in increasing productivity as well as integration of disease resistance as well as other biotic and abiotic stresses. The study by Dangol et al. (2020) was performed to determine optimal protocol for development of transgenic diploid potatoes from tetraploid potatoes as diploid potatoes can be stored as seed unlike the tetraploid potatoes. However, diploid potatoes are problematic to regenerate. So, proper protocol for the transformation of diploid *Solanum chacoense* M6 was determined in the study. Leaf, internodes, and microtubers were used as explants. A binary *Agrobacterium* vector (pBIN19) with *gusA* gene under 35S CaMV promoter was used for infection. Infection was performed for different durations to obtain the optimal inoculation duration i.e. 10, 20 and 30 min. Explants were then subjected to calli induction in MS media with different concentration of hormones BAP, NAA, transzeatin, kinetin and 2, 4-D and then transferred to media with GA_3 and TDZ for shoot generation. After histochemical GUS analysis, the optimal inoculation time for gene transfer was found to be 20 min and optimal hormone concentration was found to be 2 mg L^{-1} BAP and 2 mg L^{-1} NAA for callus induction from 20 min inoculated explants. Further confirmation was performed via RT-qPCR

which showed abundant transcripts of *gusA* gene. GUS assay further confirmed transformation of *GUS* gene (Dangol et al., 2020).

In the study conducted in Bangladesh, two potato cultivars namely, Asterix and Diamant, were selected for *Agrobacterium*-mediated genetic transformation. The internodes were used as explants for inoculation with *Agrobacterium* strain LBA4404/pBI121 containing *GUS* and *nptII* genes. The explants were infected for 20, 30 and 40 min and transferred to co-cultivation media (MS supplemented with 4.0 mg/L BAP, 1.0 mg/L IAA and 100 µmol acetosyringone). Then, they were incubated for 2, 3 and 4 days. Again, they were transferred to media containing 300 mg/L carbenicillin and 200 mg/L kanamycin and calli regeneration was observed. After growth, they were subjected to GUS histochemical assay, 30 min incubation followed by 72 h co-cultivation was the best for transformation of explants. PCR analysis using the genomic DNA isolated from transformed shoots confirmed successful transformation of GUS (Mollika et al., 2020).

CHAPTER-III

MATERIALS AND METHODOLOGY

3.1 Research design:

The research design is given below:

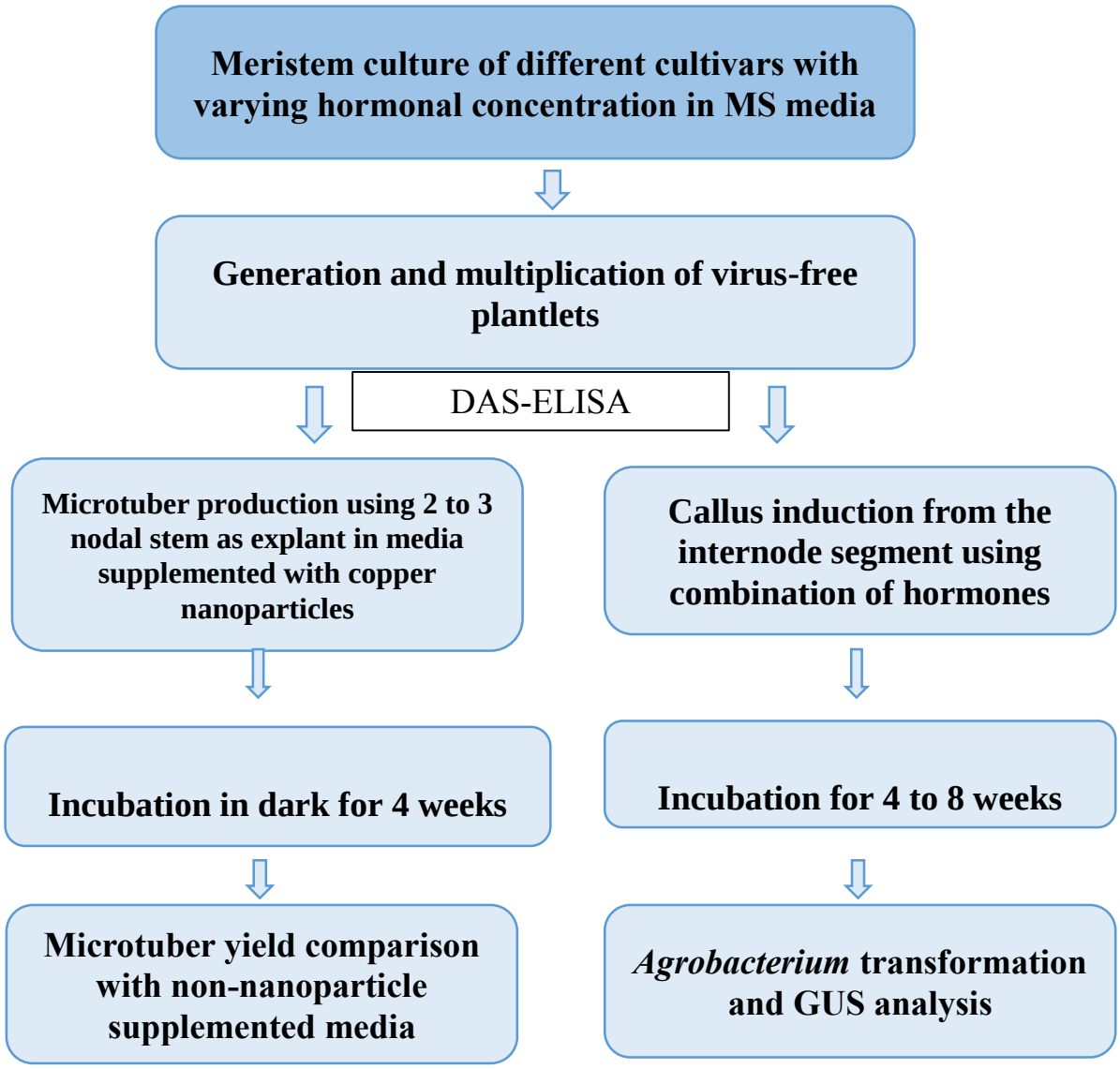


Figure 2: General Outline of Research

3.2 Sample collection:

Potato tubers of eleven different released and unreleased cultivars were collected on 30 August 2022 and 1 December 2022 from Khadyanna Tatha Biu Alu, Alaichi, Falful, Nursery Udhyog, Panauti, Kavre.

The cultivars collected are enumerated below:

I. 226
II. Halka Nilo
III. Purple
IV. MS42.3
V. Khumal Rato
VI. Rosita
VII. Desiree
VIII. Janakdev
IX. Cardinal
X. Khumal Upahar
XI. Khumal Bikas

3.3 Meristem culture:

Meristem culture was performed as described by Marcela and Anca (2011).

3.3.1 Sprouting:

To perform meristem culture from sprouts, tubers were stored in dark room. Then 0.25 mg/L GA_3 were sprayed to induce sprouting.

3.3.2 Surface sterilization:

Sprouts were excised from tuber and sterilized with Tween-20 and washed in running water for 30 min. Then sprouts were then sterilized in beaker with 0.1% $HgCl_2$ for four minutes. Finally, the explants were washed with autoclaved distilled water.

3.3.3 Meristem extraction and culture:

Meristem was then extracted under aseptic condition in laminar air flow hood which was fumigated overnight prior to extraction with formalin (35 mL) and 17.5 g potassium permanganate.

Sprouts were observed under stereoscope and meristematic cell with lobe-like structure was excised and separated from leaf primordia.

The extracted meristem was then transferred to MS media with different hormone concentration for establishment of primary meristem. The MS media were then separated as:

- Meristem culture media 1:
  GA_3: 0.25 mg/L
- Meristem culture media 2 (combination media):
  Kinetin- 1.5, 1.5, 1.5 mg/L
  BAP- 0.5, 1, 1.5 mg/L

The meristems were extracted and cultured at Central Department of Biotechnology laboratory in Tribhuvan University on 21st September 2022 and on 8th December 2022, the meristem cultures were incubated at Pragatishil Yuva Krishak Samuha laboratory in Banepa, Kavre.

Meristem growth was observed in this media after 4 weeks and 8 weeks and transferred to media with 0.25 mg/L GA_3 for further shoot proliferation and root induction.

3.4 Validation of virus-free potato plantlets:

Double Antibody-Sandwiched Enzyme linked Immuno-Sorbent Assay (DAS-ELISA) for six different potato viruses i.e. PLRV, PVA, PVY, PVM, PVX and PVS was performed on 4th May 2023 via kit obtained from International Potato center (CIP), Peru.

3.4.1 Sample processing:

Leaf samples were collected in plastic bags and extraction buffer {four times the volume (mL) of the sample weight (g)} was added. Leaf sap was then collected by completely homogenizing with pressure.

3.4.2 Preparation of ELISA Plates:

Coating solution was prepared with viral IgG and coating buffer as per instruction manual provided by CIP and 100 µL coating solution was added to each well and incubated at 37 ºC for 3-4 h. Then plates were washed with washing buffer and 100 µL of sample was added to the wells. After sealing, plates are incubated overnight at 4 ºC.

After the incubation, plates were washed with washing buffer, and to each well, 90 µL of conjugate buffer was added. It was then again incubated for 3-4 h at 37 ºC. The plates were then washed and 80 µL of substrate solution was added on each well. After allowing reaction to occur for 60 to 90 min, change to yellow coloration was observed for positive reaction and ELISA plate reader was used to determine threshold values as directed by CIP, Peru. If the optical density of sample is less than twice than that of negative control (i.e. threshold value), it is considered as negative and free of viruses.

$$x \geq \bar{x}h \times 2 \text{ ----------Equation (1)}$$

Where, x= threshold value

$\bar{x}h$= average value of healthy controls (negative control)

3.5 Microtuber Production using Nanoparticles

We obtained nanoparticles from Dr. Surendra Kumar Gautam (Assistant Dean of Institute of Science and Technology, Tribhuvan University). CuNp (CuO) of average size 17.24 nm was provided by Mr. Deval Prasad Bhattarai of Amrit Science Campus. For homogenization, the sonicator was used at 100 W and 30 kHz for 45 min as suggested by Hamza (2019).

Different nanoparticles concentration i.e. (1, 2 and 3 mg/L) of CuNP along with plant hormone TDZ (0.5 mg/L) (separately and in combination) were added to liquid MS media in a culture jar with sterile cotton where the media acts as semi-solid media. Explants i.e. shoots with 2-3 nodes of potato cv. Desiree were placed horizontally in the jar (3 per jar). It was then incubated in the dark at 22 ºC for 4-6 weeks and microtuberization was detected (Dangol et al., 2020).

3.6 Callus induction:

Virus-free *in vitro* samples of five different cultivars were subjected to callus induction i.e. MS42.3, Purple, Rosita, P.L. and K.U. were obtained from Khadyanna Tatha Biu Alu, Alaichi, Falful, Nursery Udhyog, Panauti. Internode segments were used as explants for callus induction. Twelve different Callus Induction media (CIM) with varying hormone concentrations were prepared.

CIM 1 to 3 contained constant 1.0 mg/L 2, 4-D with 0.5, 1 and 1.5 mg/L kinetin respectively. CIM 2 to 6 contained constant 2.0 mg/L kinetin with 1.0, 1.5 and 2.0 mg/L 2, 4-D respectively. Meanwhile, CIM 7 to 9 had constant 1.0 mg/L NAA with 1.0, 1.5 and 2.0 mg/L BAP respectively. CIM 10 to 12 had constant 2.0 mg/L BAP with 1.0, 1.5 and 2.0 mg/L NAA respectively. For regeneration media, MS media with 0.25 mg/L GA_3 was used and calli were sub-cultured periodically. Protocol as suggested by Kawochar et al. (2017) was followed.

3.7 *Agrobacterium* Transformation:

The *Agrobacterium* strain was obtained from Plant Genome Editing Laboratory of Central Department of Biotechnology, Tribhuvan University, Kirtipur led by Dr. Sarbesh Das Dangol and Dr. Jarina Joshi funded by University Grants Commission (UGC-CRIG-78/79-S&T-08).

The transformed LBA4404 strain of *Agrobacterium tumefaciens* with pAXY0002 obtained with β-glucuronidase *gusA* gene was grown overnight in LB broth containing 50 mg/L rifampicin and 100 mg/L streptomycin. The leaf and internode explants of *cv.* P. L. were treated with 15 mL of liquid MS medium and 2 mL of transformed *Agrobacterium* was used to infect the internodes (OD value of 0.8 at 600 nm). Next, the explants were dried on blotting paper and transferred to MS media with 100 mg/L of acetosyringone (co-cultivation media) and they were incubated in dark for 3 days. The explants were then washed with 150 mg/L of timentin (Vinterhalter et al., 2008) with shaking. Then they were allowed to dry in blotting paper. Few explants were randomly selected for GUS histochemical assay and immersed in GUS staining solution (1 mM X-gluc, 10 mM EDTA, 100 mM NaH2PO4, 0.1% Triton X100, 50% methanol, pH-8) for 12 h. Remaining explants were dried and transferred to CIM-11 with 150 mg/L timentin for calli induction. After overnight immersion, explants were destained with 70% alcohol and observed under stereomicroscope (Dangol et al., 2020).

CHAPTER-IV

RESULTS

4.1 Meristem culture:

Meristem culture was performed for eleven cultivars in four different calli induction media. Seven samples were first subjected to meristem culture. After 4 weeks, slight green regenerations observed in BAP 1 mg L^{-1} and 1.5 mg L^{-1} kinetin in Khumal Rato.

Table 2: Meristem culture regeneration after 8 weeks

	Khumal rato	khumal upahar	226	Purple	Halka nilo	Rosita	K.B.
Media 1 (0.25 mg/L GA_3)	0	0	1	1	1	1	0
Media 2 (1.5 mg/L kinetin + 0.5 mg/L BAP)	0	0	0	0	0	0	0
Media 3 (1.5 mg/L kinetin + 1.0 mg/L BAP)	1	1	0	0	0	1	0
Media 4 (1.5 mg/L kinetin + 1.5 mg/L BAP)	0	0	1	0	0	0	0

Meristem culture of another six samples was performed on 8th December, 2022. Khumal Bikas showed no growth.

Table 3: Second Meristem culture regeneration after 8 weeks

	Cardinal	Desiree	226	Purple	Janakdev	MS42.3
Media 1 (0.25 mg/L GA_3)	0	1	0	1	0	0
Media 2 (1.5 mg/L kinetin + 0.5 mg/L BAP)	0	0	1	0	0	0
Media 3 (1.5 mg/L kinetin + 1.0 mg/L BAP)	0	0	0	0	1	0
media 4 (1.5 mg/L kinetin + 1.5 mg/L BAP)	1	1	1	0	0	1

Meristem growth was observed in 0.25 mg/L GA_3 in cultivars 226, Rosita, Desiree, Purple and Halka Nilo. Growth was seen in combination media in 0.5 mg/L BAP and 1.5 mg/L kinetin in cv. 226. In 1.0 mg/L BAP and 1.5 mg/L kinetin, growth was seen in Rosita, Khumal upahar (K.U.), Khumal rato and Janakdev. Growth was observed in 1.5 mg/L BAP and 1.5 mg/L kinetin in cvs. 226, MS42.3, Desiree and Cardinal. Further, regenerated cultivars were transferred to regeneration media (0.25 mg L^{-1} GA_3).

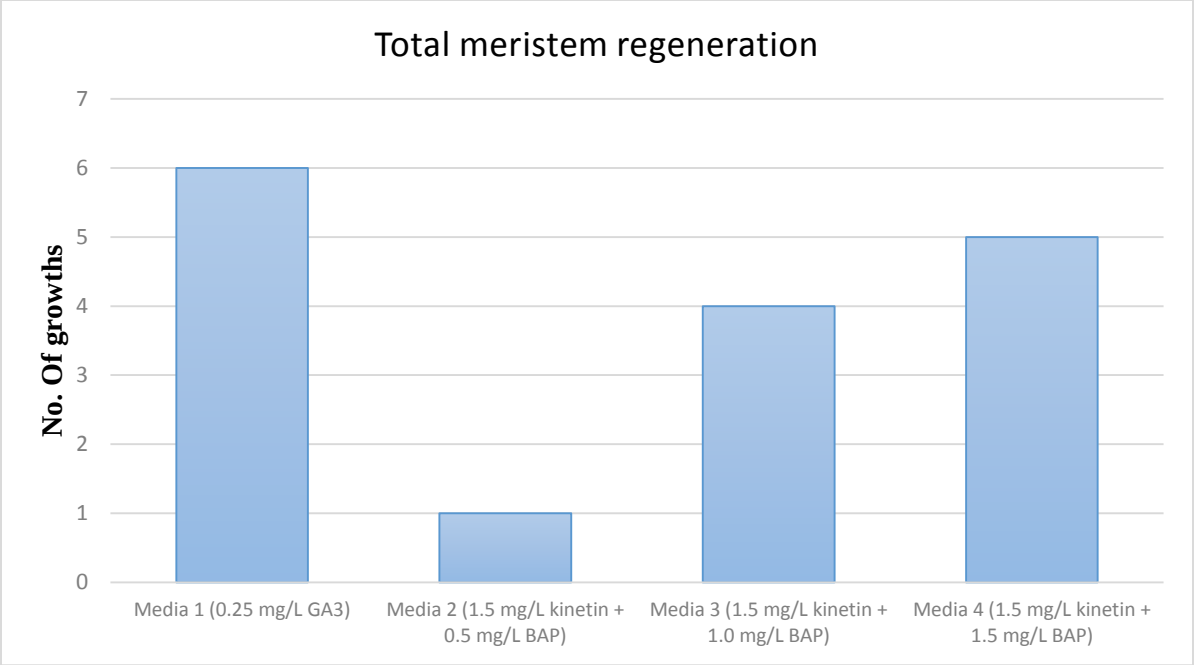


Figure 3: Graph depicting total meristem culture regeneration in different meristem culture media. Greater meristem regeneration rate was seen in 0.25 mg/L GA_3 and with increase in BAP concentration.

Best media for meristem culture observed was on Media 1 with GA_3 (0.25 mg/L) and Media 4 with BAP 1.5 mg/L + kinetin 1.5 mg/L.

4.2 DAS-ELISA:

After meristem regeneration, 14 samples (see Appendix 3) of 10 different cultivars were tested for 6 potato viruses, namely, PVA, PVX, PVM, PVY, PVS and PLRV.

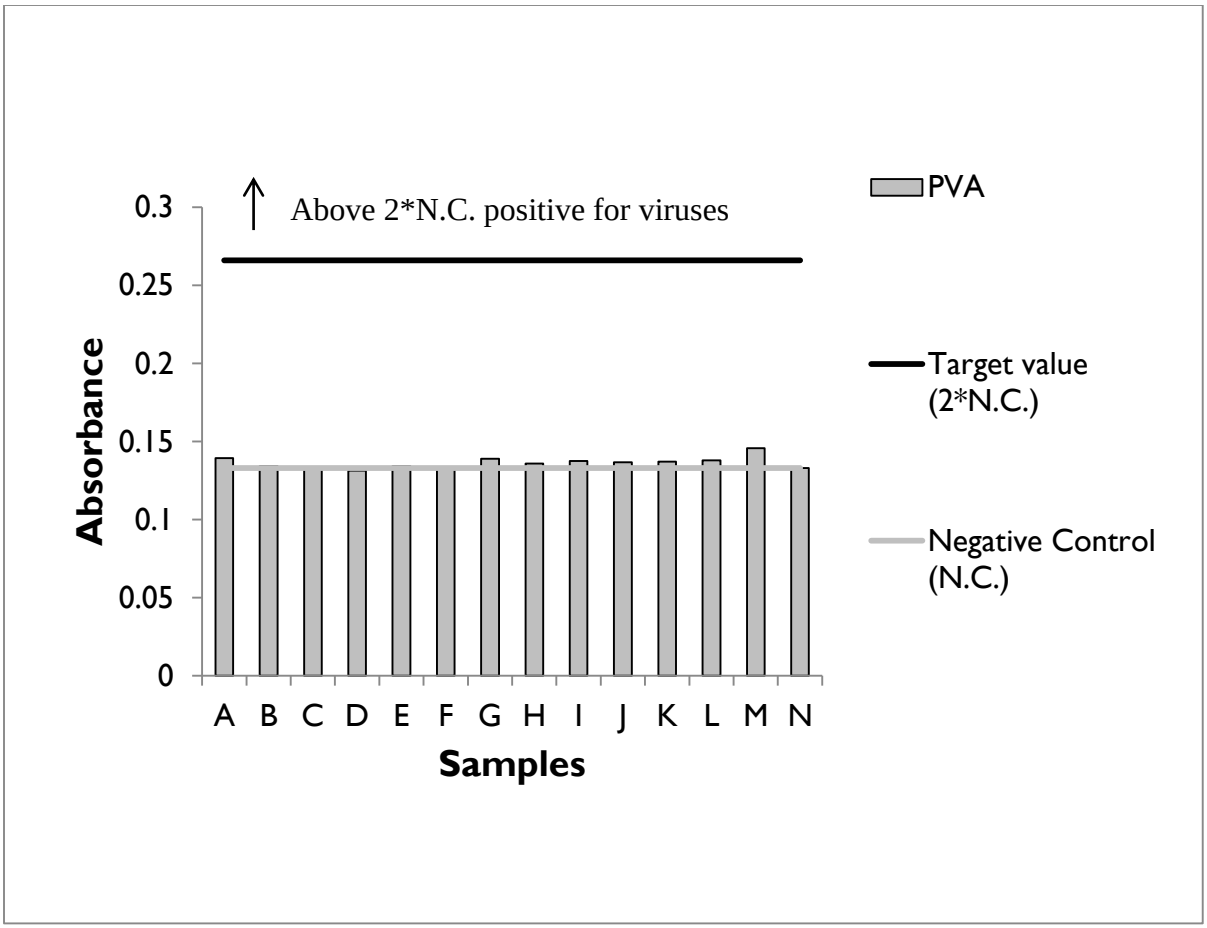


Figure 4: Comparison of absorbance values for all the samples against the threshold values for PVA. Threshold values are twice the absorbance of negative control. Any sample with absorbance approaching threshold value are considered positive for PVA. Here, A= Desiree (0.25 mg/L GA_3), B= J.D. (1.0 mg/ L BAP + 1.5 mg/L kinetin), C= 226 (0.25 mg/L GA_3), D= P.L. (1.0 mg/L BAP + 1.5 mg/L kinetin), E= K.U. (1.5 mg/L BAP + 1.5 mg/L kinetin) F= 226 (1.5 mg/L BAP + 1.5 mg/L kinetin), G= K.R. (1.0 mg/L BAP + 1.5 mg/L kinetin) H= MS42.3 (1.5 mg/L BAP + 1.5 mg/L kinetin), I= Cardinal (1.5 mg/L BAP + 1.5 mg/L kinetin), J= Purple (0.25 mg/L GA_3), K= Desiree (1.5 mg/L BAP + 1.5 mg/L kinetin), L= K.R. (1.5 mg/L BAP + 1.5 mg/L kinetin), M= Rosita (1.5 mg/L BAP + 1.5 mg/L kinetin), N= K.R. (1.5 mg/L BAP + 1.5 mg/L kinetin). All samples are free of PVA.

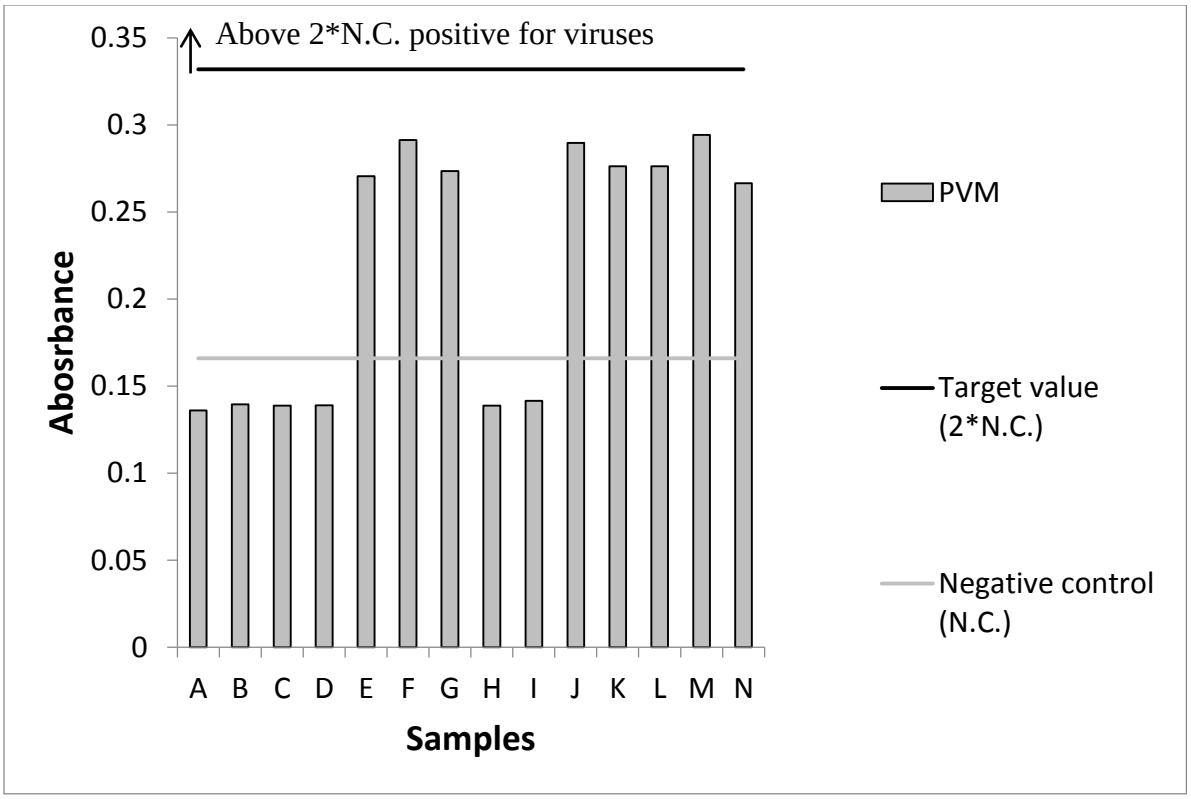


Figure 5: Comparison of absorbance values for all the samples against the threshold values for PVM. Threshold values are twice the absorbance of negative control. Any sample with absorbance approaching threshold value are considered positive for PVM. Here, A= Desiree (0.25 mg/L GA_3), B= J.D. (1.0 mg/ L BAP + 1.5 mg/L kinetin), C= 226 (0.25 mg/L GA_3), D= P.L. (1.0 mg/L BAP + 1.5 mg/L kinetin), E= K.U. (1.5 mg/L BAP + 1.5 mg/L kinetin) F= 226 (1.5 mg/L BAP + 1.5 mg/L kinetin), G= K.R. (1.0 mg/L BAP + 1.5 mg/L kinetin) H= MS42.3 (1.5 mg/L BAP + 1.5 mg/L kinetin), I= Cardinal (1.5 mg/L BAP + 1.5 mg/L kinetin), J= Purple (0.25 mg/L GA_3), K= Desiree (1.5 mg/L BAP + 1.5 mg/L kinetin), L= K.R. (1.5 mg/L BAP + 1.5 mg/L kinetin), M= Rosita (1.5 mg/L BAP + 1.5 mg/L kinetin), N= K.R. (1.5 mg/L BAP + 1.5 mg/L kinetin). Eight samples show low degree positivity.

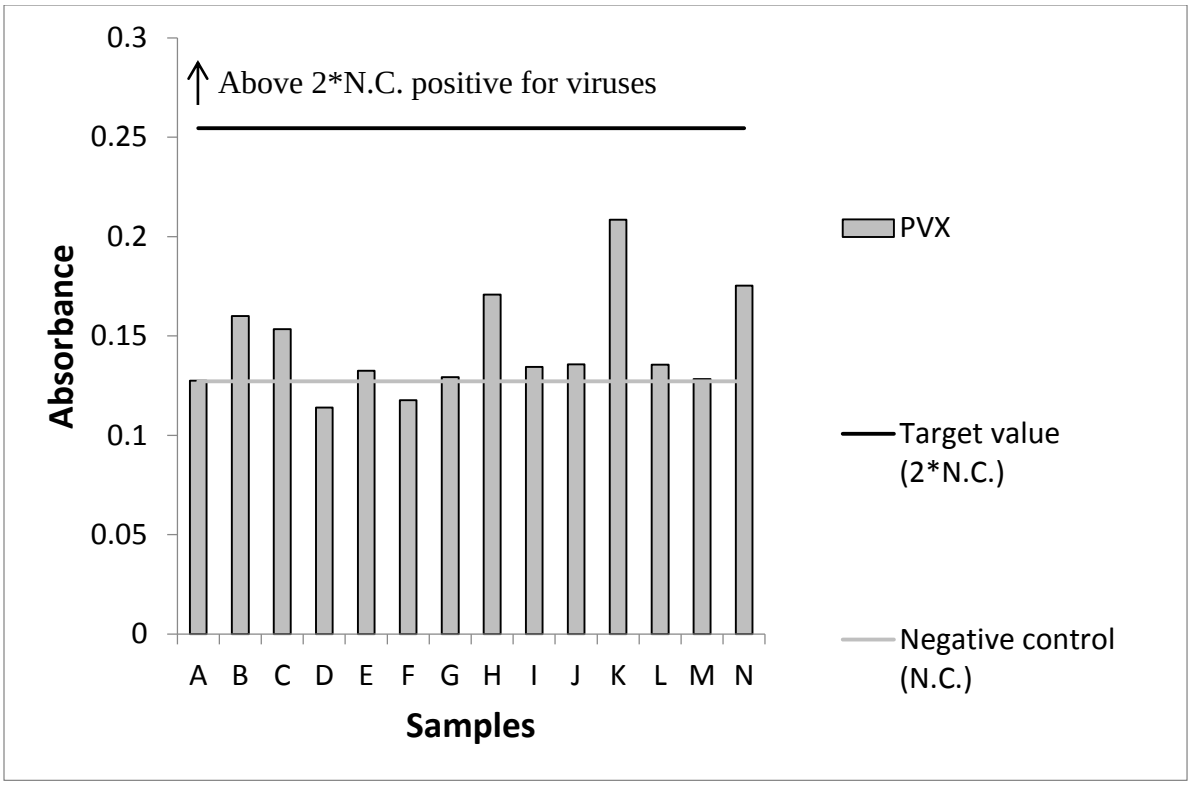


Figure 6: Comparison of absorbance values for all the samples against the threshold values for PVX. Threshold values are twice the absorbance of negative control. Any sample with absorbance approaching threshold value are considered positive for PVX. Here, A= Desiree (0.25 mg/L GA_3), B= J.D. (1.0 mg/ L BAP + 1.5 mg/L kinetin), C= 226 (0.25 mg/L GA_3), D= P.L. (1.0 mg/L BAP + 1.5 mg/L kinetin), E= K.U. (1.5 mg/L BAP + 1.5 mg/L kinetin) F= 226 (1.5 mg/L BAP + 1.5 mg/L kinetin), G= K.R. (1.0 mg/L BAP + 1.5 mg/L kinetin) H= MS42.3 (1.5 mg/L BAP + 1.5 mg/L kinetin), I= Cardinal (1.5 mg/L BAP + 1.5 mg/L kinetin), J= Purple (0.25 mg/L GA_3), K= Desiree (1.5 mg/L BAP + 1.5 mg/L kinetin), L= K.R. (1.5 mg/L BAP + 1.5 mg/L kinetin), M= Rosita (1.5 mg/L BAP + 1.5 mg/L kinetin), N= K.R. (1.5 mg/L BAP + 1.5 mg/L kinetin). Three samples show low degree positivity.

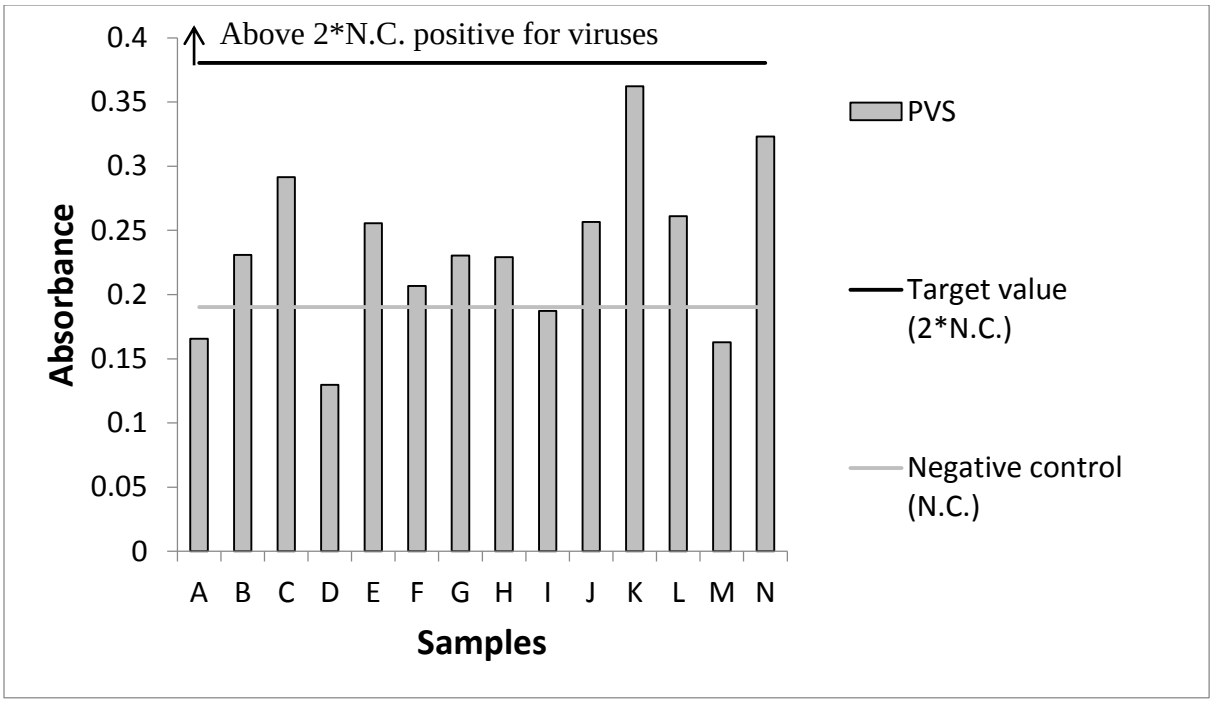


Figure 7: Comparison of absorbance values for all the samples against the threshold values for PVS. Threshold values are twice the absorbance of negative control. Any sample with absorbance approaching threshold value are considered positive for PVS. Here, A= Desiree (0.25 mg/L GA_3), B= J.D. (1.0 mg/ L BAP + 1.5 mg/L kinetin), C= 226 (0.25 mg/L GA_3), D= P.L. (1.0 mg/L BAP + 1.5 mg/L kinetin), E= K.U. (1.5 mg/L BAP + 1.5 mg/L kinetin) F= 226 (1.5 mg/L BAP + 1.5 mg/L kinetin), G= K.R. (1.0 mg/L BAP + 1.5 mg/L kinetin) H= MS42.3 (1.5 mg/L BAP + 1.5 mg/L kinetin), I= Cardinal (1.5 mg/L BAP + 1.5 mg/L kinetin), J= Purple (0.25 mg/L GA_3), K= Desiree (1.5 mg/L BAP + 1.5 mg/L kinetin), L= K.R. (1.5 mg/L BAP + 1.5 mg/L kinetin), M= Rosita (1.5 mg/L BAP + 1.5 mg/L kinetin), N= K.R. (1.5 mg/L BAP + 1.5 mg/L kinetin). Seven samples show low degree positivity.

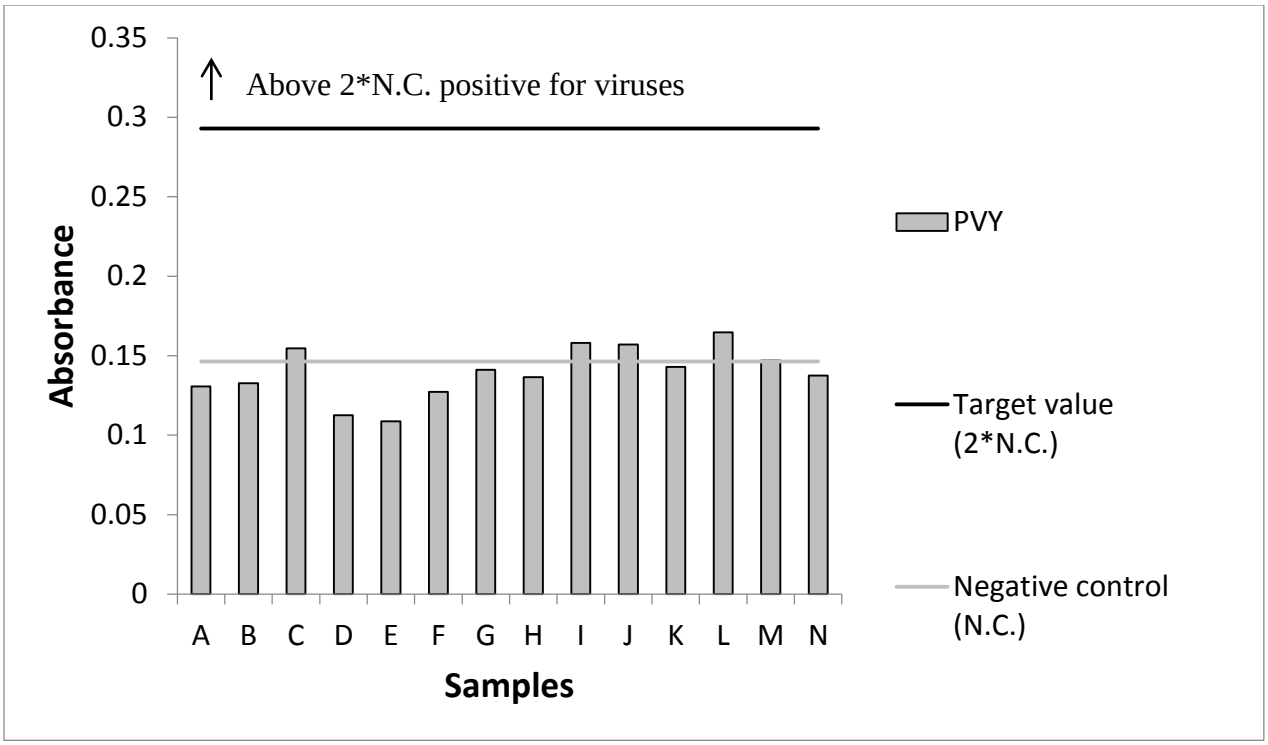


Figure 8: Comparison of absorbance values for all the samples against the threshold values for PVY. Threshold values are twice the absorbance of negative control. Here, A= Desiree (0.25 mg/L GA_3), B= J.D. (1.0 mg/ L BAP + 1.5 mg/L kinetin), C= 226 (0.25 mg/L GA_3), D= P.L. (1.0 mg/L BAP + 1.5 mg/L kinetin), E= K.U. (1.5 mg/L BAP + 1.5 mg/L kinetin) F= 226 (1.5 mg/L BAP + 1.5 mg/L kinetin), G= K.R. (1.0 mg/L BAP + 1.5 mg/L kinetin) H= MS42.3 (1.5 mg/L BAP + 1.5 mg/L kinetin), I= Cardinal (1.5 mg/L BAP + 1.5 mg/L kinetin), J= Purple (0.25 mg/L GA_3), K= Desiree (1.5 mg/L BAP + 1.5 mg/L kinetin), L= K.R. (1.5 mg/L BAP + 1.5 mg/L kinetin), M= Rosita (1.5 mg/L BAP + 1.5 mg/L kinetin), N= K.R. (1.5 mg/L BAP + 1.5 mg/L kinetin). All samples show negative result.

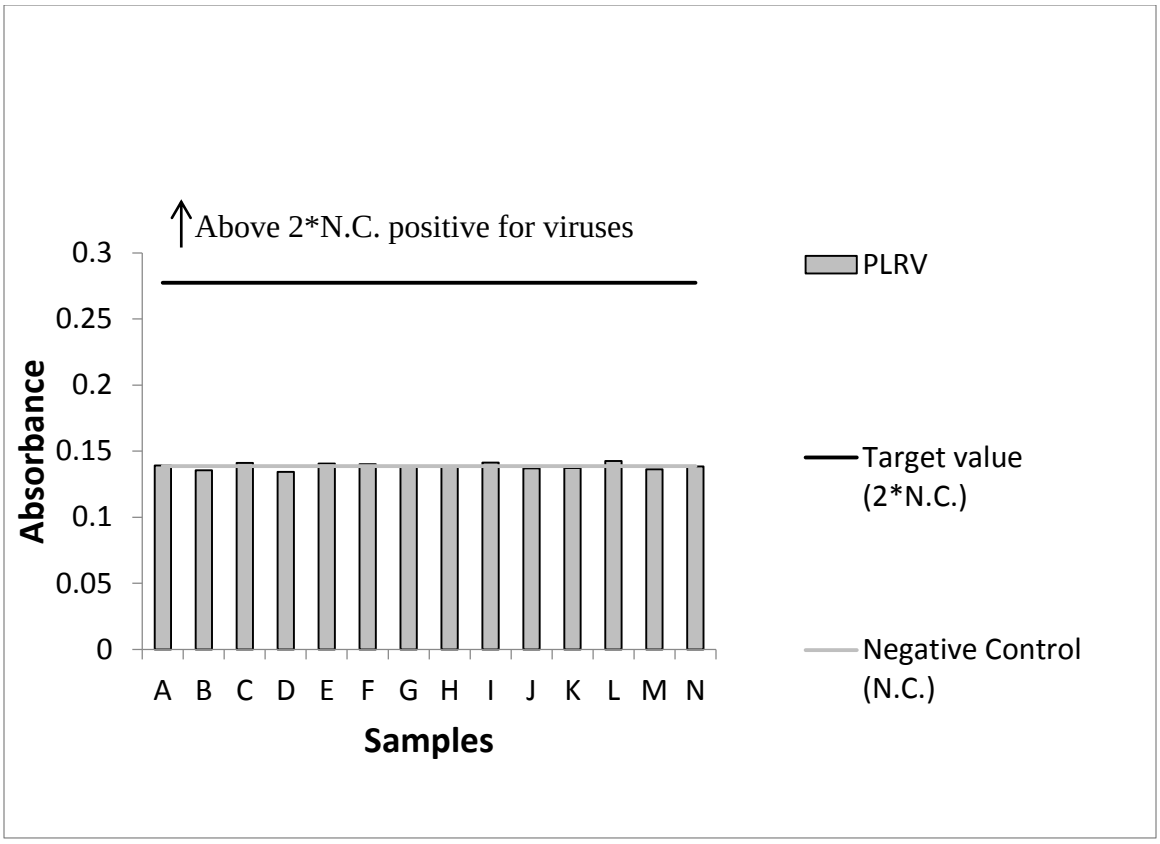


Figure 9: Comparison of absorbance values for all the samples against the threshold values for PLRV. Threshold values are twice the absorbance of negative control. Any sample with absorbance approaching threshold value are considered positive for PLRV. Here, A= Desiree (0.25 mg/L GA_3), B= J.D. (1.0 mg/ L BAP + 1.5 mg/L kinetin), C= 226 (0.25 mg/L GA_3), D= P.L. (1.0 mg/L BAP + 1.5 mg/L kinetin), E= K.U. (1.5 mg/L BAP + 1.5 mg/L kinetin) F= 226 (1.5 mg/L BAP + 1.5 mg/L kinetin), G= K.R. (1.0 mg/L BAP + 1.5 mg/L kinetin) H= MS42.3 (1.5 mg/L BAP + 1.5 mg/L kinetin), I= Cardinal (1.5 mg/L BAP + 1.5 mg/L kinetin), J= Purple (0.25 mg/L GA_3), K= Desiree (1.5 mg/L BAP + 1.5 mg/L kinetin), L= K.R. (1.5 mg/L BAP + 1.5 mg/L kinetin), M= Rosita (1.5 mg/L BAP + 1.5 mg/L kinetin), N= K.R. (1.5 mg/L BAP + 1.5 mg/L kinetin). PLRV is absent in all the samples.

Table 4: DAS-ELISA results of different cultivars

	PVA	PVM	PVS	PVY	PLRV	PVX
Desiree	Negative	Negative	Negative	Negative	Negative	Negative
Janakdev	Negative	Negative	Negative	Negative	Negative	Negative
226	Negative	Negative	Negative	Negative	Negative	Negative
Panauti local	Negative	Negative	Negative	Negative	Negative	Negative
Khumal Upahar	Negative	low degree positive	Negative	Negative	Negative	Negative
226	Negative	low degree positive	Negative	Negative	Negative	Negative
Khumal Rato	Negative	low degree positive	Negative	Negative	Negative	Negative
MS-42	Negative	Negative	Negative	Negative	Negative	Negative
Cardinal	Negative	Negative	Negative	Negative	Negative	Negative
Purple	Negative	low degree positive	Negative	Negative	Negative	Negative
Desiree	Negative	low degree positive	low degree positive	Negative	Negative	low degree positive
Khumal Rato	Negative	low degree positive	Negative	Negative	Negative	Negative
Rosita	Negative	low degree positive	Negative	Negative	Negative	Negative
Khumal Rato	Negative	low degree positive	low degree positive	Negative	Negative	Negative

If the optical density of sample i.e. threshold value is less than twice than that of negative control, it is considered as negative and free of viruses. Wavelength for O.D. was 405 nm and readings were taken by ELISA reader.

By performing DAS-ELISA, the cvs. P.L., Desiree, Janakdev, 226, MS-42.3 and Cardinal were confirmed to be free of viruses. P.L. was then selected for *Agrobacterium* transformation and cv. Desiree for microtuber production.

4.3 Microtuberization with Nanoparticles:

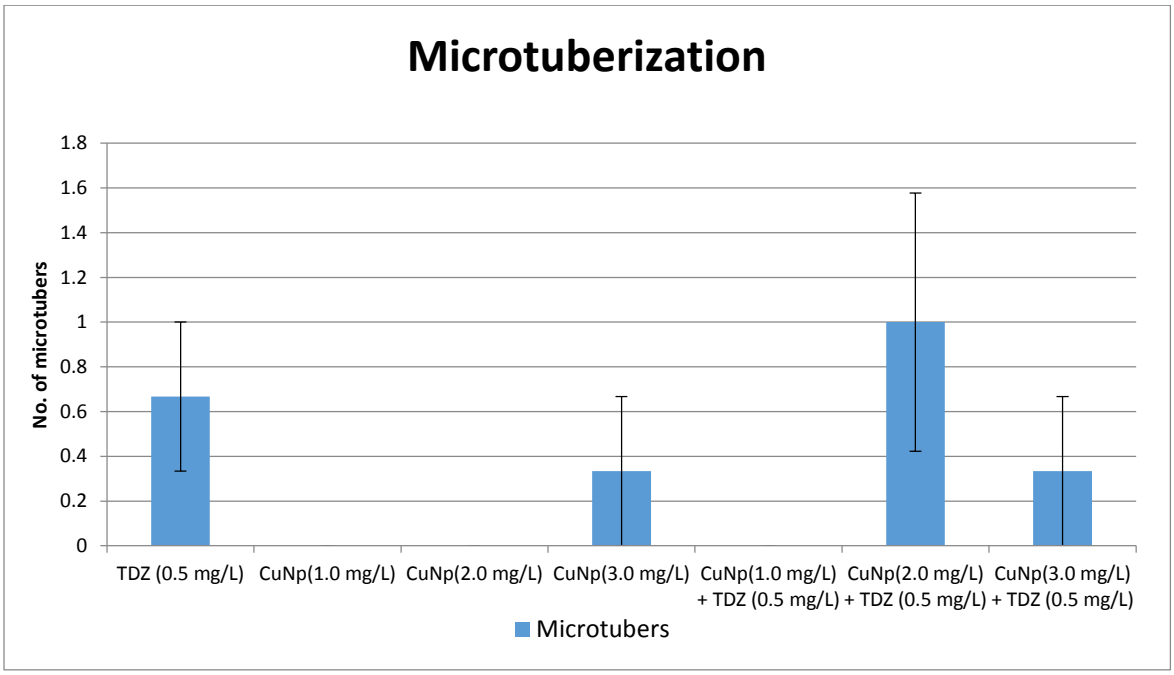


Figure 10: Graph depicting microtuberization in TDZ hormone, CuNP and in combination media with both TDZ and CuNP. Microtuberization was sparse with slight improvement in combination media.

Microtuber formation was quite low as shown in Figure 10. CuNP 2.0 mg/L with TDZ (0.5 mg/L) produced highest number of microtubers. It showed slight enhancement compared to microtuberization with only either hormone or CuNPs. CuNPs without hormone didn't produce any microtuber. The highest microtuber size was in CuNP 2.0 mg/L with TDZ (0.5 mg/L) yielding a size of diameter 4.6 mm with 0.31 g. Rest of the microtubers were less than 2 mm in diameter. However, data obtained wasn't much reliable due to high standard error.

4.4 Callus Induction:

Calli induction for five different cultivars MS42.3, P.L., Rosita, Purple and K.U. was performed. Callus was observed after 4 weeks and regeneration after 4 weeks.

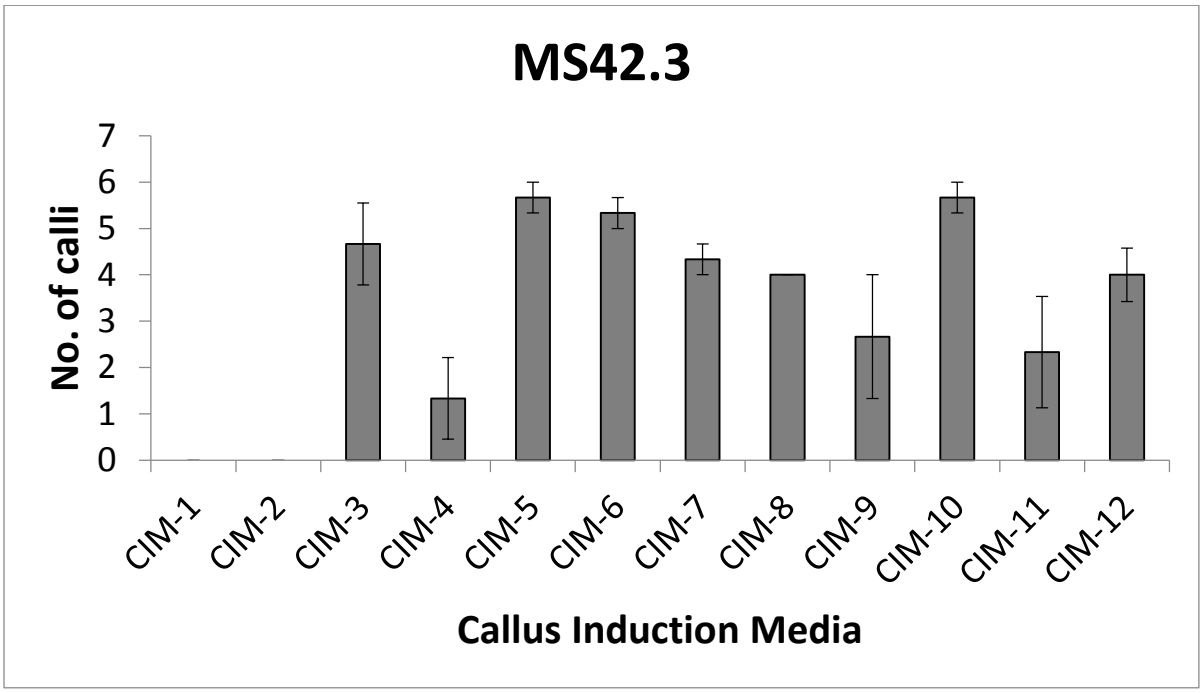


Figure 11: Average number of calli generated in different callus induction media (CIM) for cv. MS42.3. (See Appendix 1 for different CIM compositions). Calli formation was high in CIM-5 with 2.0 mg/L kinetin and 1.5 mg/L 2,4-D (94.4%) and in CIM-10 with 2.0 mg/L BAP and 1.0 mg/L NAA (94.4%).

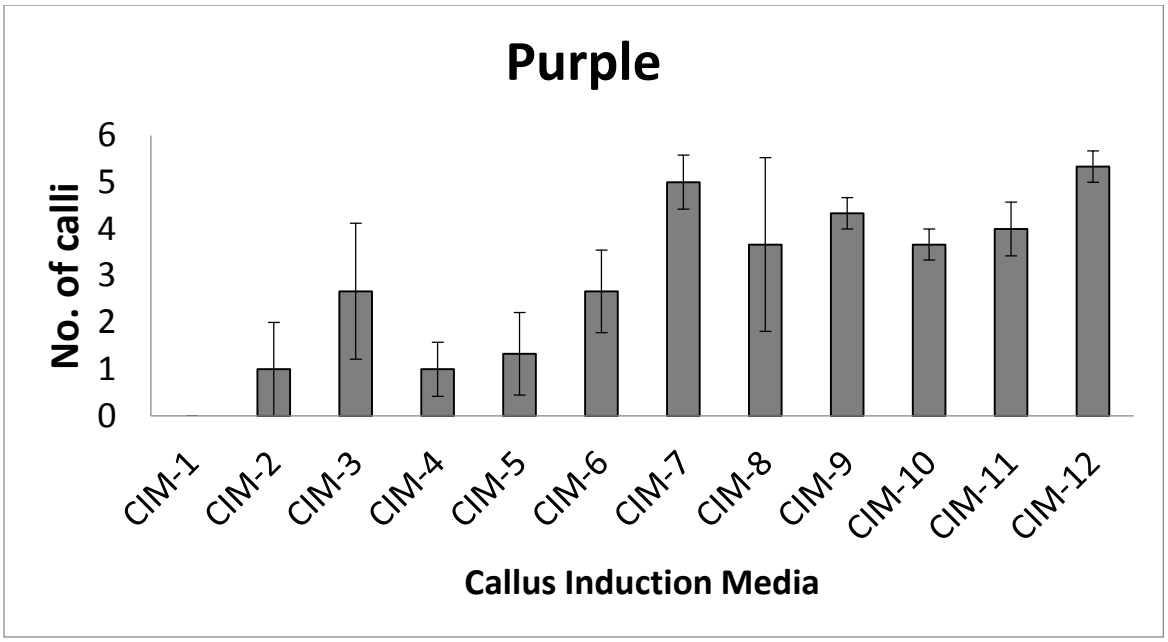


Figure 12: Average number of calli generated in different Callus Induction media (CIM) for cv. Purple. (see Appendix 1 for different CIM composition). Calli formation was high in CIM-12 with 2.0 mg/L BAP and 2.0 mg/L NAA (88.89%).

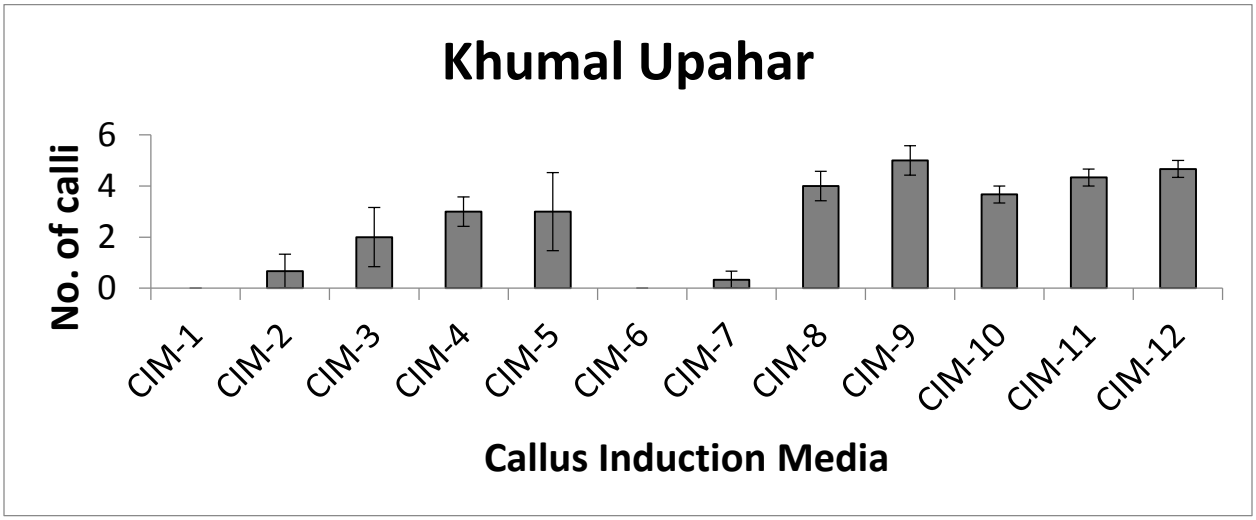


Figure 13: Average number of calli generated in different Callus Induction media (CIM) for cv. K.U. (see Appendix 1 for different CIM composition). Calli formation was low in CIM 1-6 with kinetin and 2,4-D. Callus induction was highest in CIM-9 (83.33%) with 2.0 mg/L BAP and 1.0 mg/L NAA.

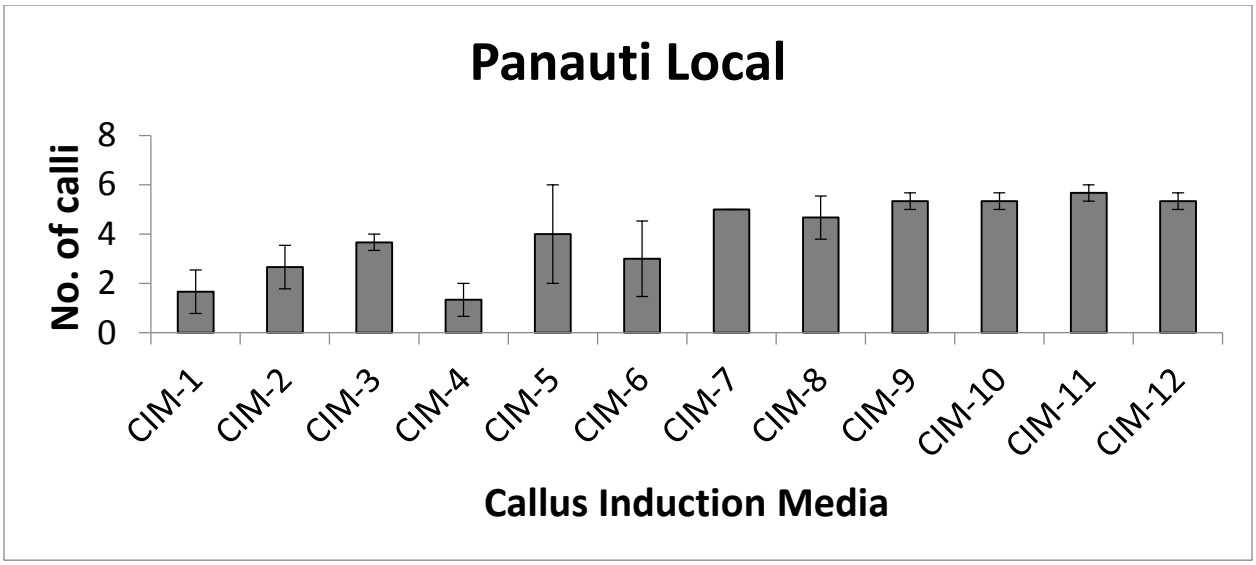


Figure 14: Average number of calli generated in different callus induction media (CIM) for cv. P.L. (see Appendix 1 for different CIM composition). Callus induction was the highest in CIM-11 (94.4%) with 2.0 mg/L BAP and 1.5 mg/L NAA.

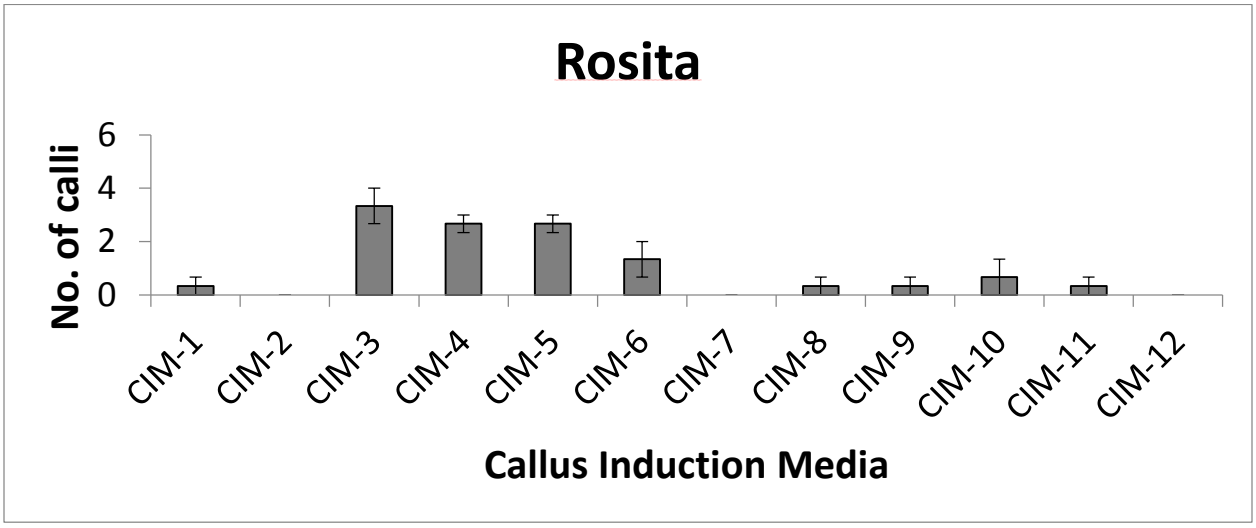


Figure 15: Average number of calli generated in different Callus Induction media (CIM) for cv. Rosita. (Appendix 1 for different CIM composition). Calli formation was observed to be highest in CIM 3 (55.56%) with 1.5 mg/L kinetin and 1.0 mg/L 2,4-D. Callus induction was very low in CIM 7-12 with BAP and NAA.

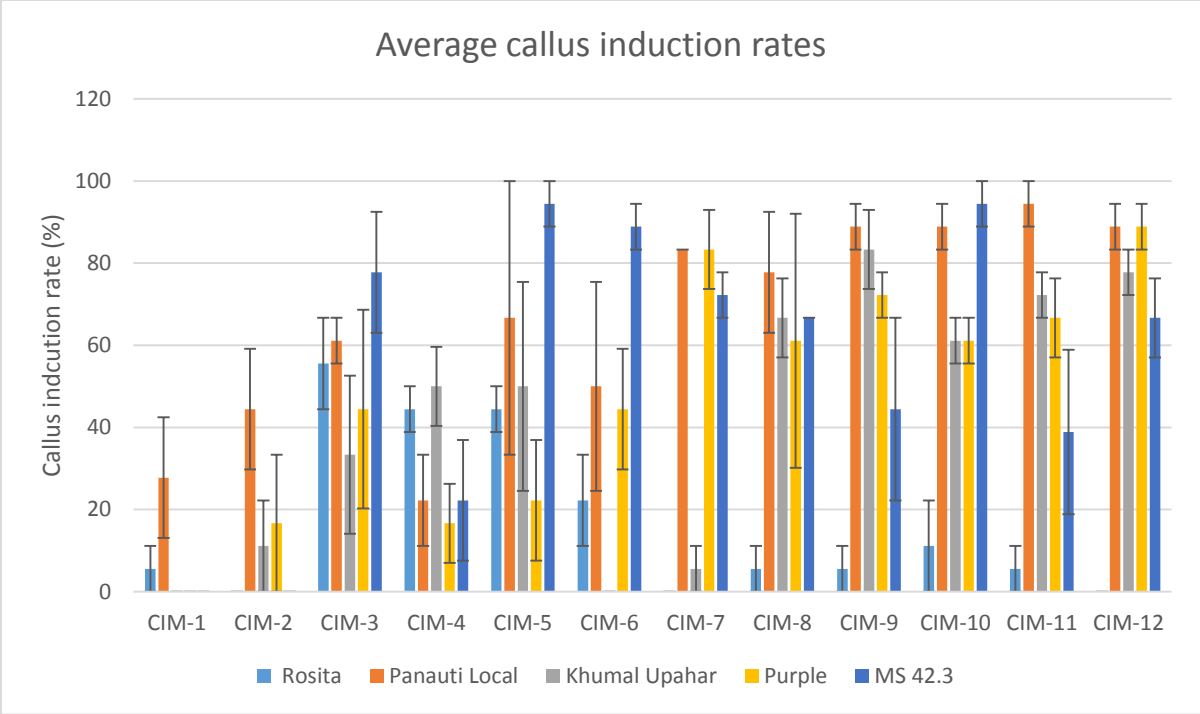


Figure 16: Callus induction rate in different Callus Induction Media (CIM) for different potato cultivars. Y-axis denotes total callus induction rate and X-axis indicates different callus induction media. CIM-12 with 2mg/L BAP+ 2mg/L NAA shows best average callus induction rate (64.44%).

Plantlet regeneration

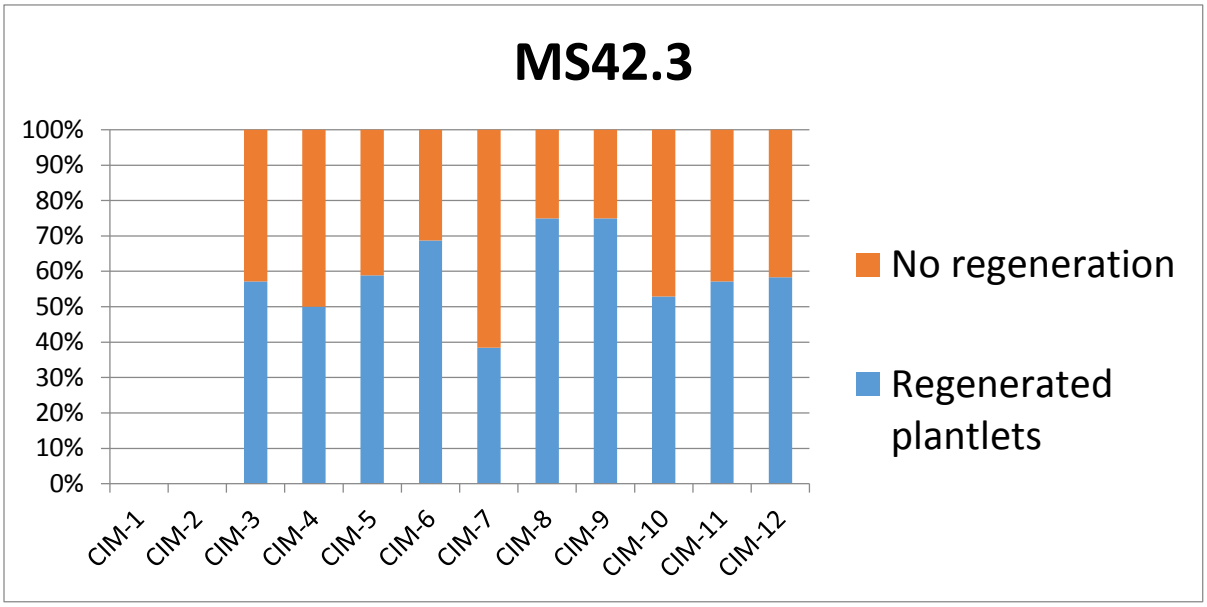


Figure 17: Percentage of plantlet regeneration of cv. MS42.3. Y-axis shows the percentage of plantlets regenerated from total callus induced and X-axis indicated the media callus was induced from.

During callus regeneration after transfer into regeneration media, MS42.3 had average regeneration rate of 59.167% with CIM-9 calluses showing high regeneration rate.

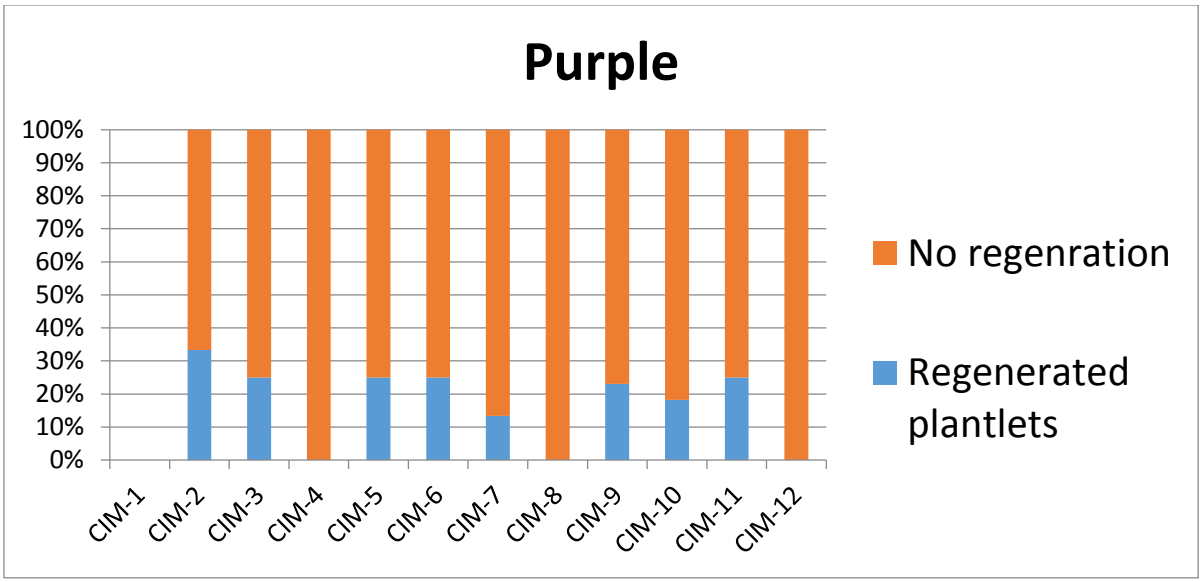


Figure 18: Percentage of plantlet regeneration of cv. Purple. Y-axis shows the percentage of plantlets regenerated from total callus induced and X-axis indicated the media callus was induced from.

Potato cv. Purple showed very low regeneration rate of 15.38%.

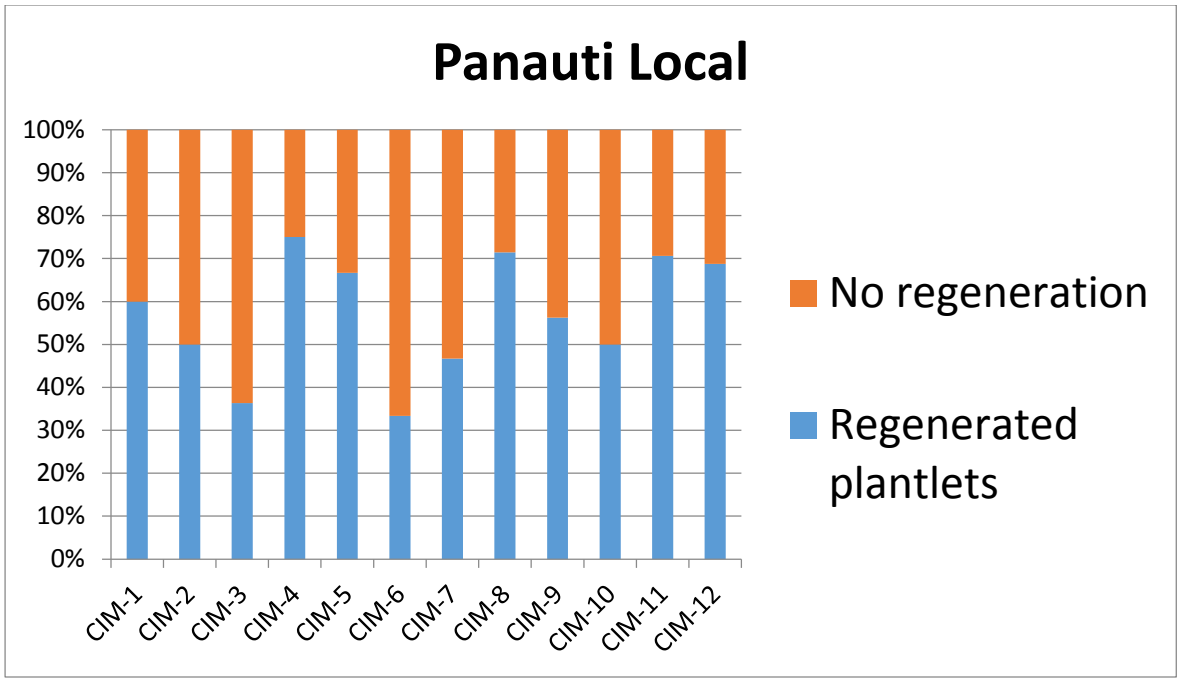


Figure 19: Percentage of plantlet regeneration of cv. Panauti Local. Y-axis shows the percentage of plantlets regenerated from total callus induced and X-axis indicated the media callus was induced from.

P.L. displayed regeneration rate of 57.34% with CIM-5 induced callus showing high regeneration. Cultivars K.U. and Rosita failed to regenerate.

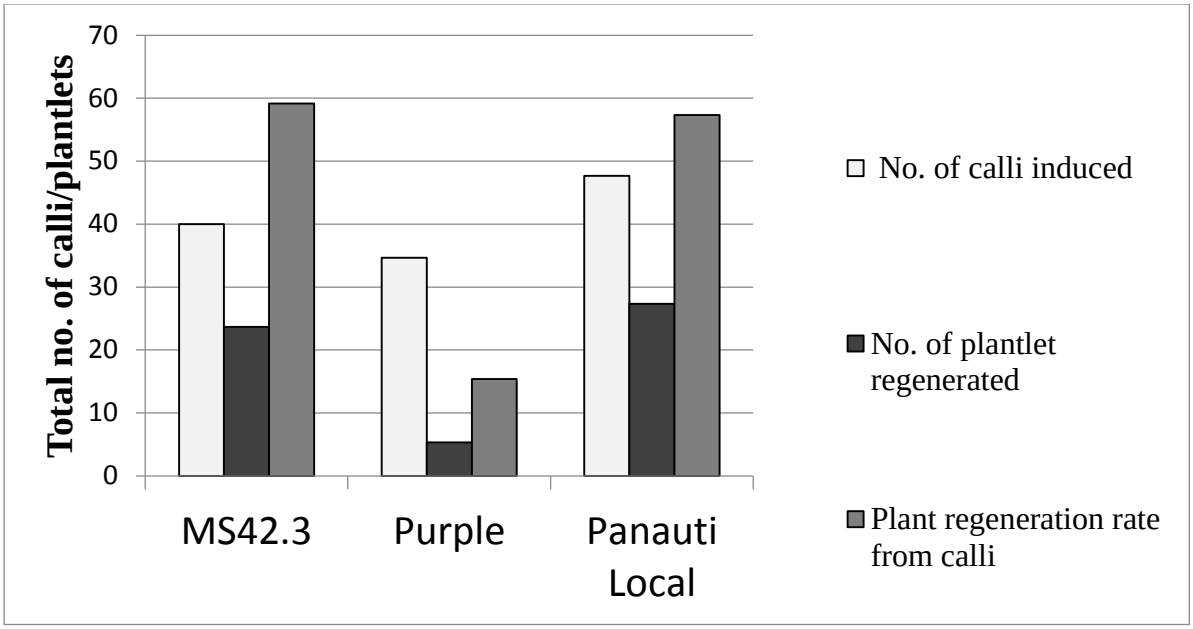


Figure 20: Graph comparing total callus obtained, total plantlet regenerated and the plantlet regeneration from calli in different cultivars. P.L. showed higher calli induction however MS42.3 had higher regeneration rate from callus.

From Figure 20, comparing the total plant regeneration from calli induced on CIM media, greater callus induction rate was observed for P.L. Both MS42.3 and P.L. showed high regeneration rates. Therefore, best cultivars for calli induction and regeneration observed were MS42.3 and P.L.

4.5 *Agrobacterium* transformation and GUS analysis:

Panauti local explants i.e. leaf and internodes were infected with *Agrobacterium* strain LBA4404 containing *GUSPlus* gene. After immersing in GUS solution overnight, explants were observed for infection in stereomicroscope. However, no evidence of transformation was observed indicating failure in *Agrobacterium* transformation.

CHAPTER-V

DISCUSSION

5.1 Meristem culture:

In this study of eleven different cultivars, meristem growth was observed in 0.25 mg/L GA_3 in cvs. 226, Rosita, Desiree, Purple and Halka Nilo; 0.5 mg/L BAP and 1.5 mg/L kinetin in cultivar 226; 1.0 mg/L BAP and 1.5 mg/L kinetin in Rosita, K.U., Khumal Rato and Janakdev; 1.5 mg/L BAP and 1.5 mg/L kinetin in 226, MS42.3, Desiree and Cardinal. Among those, the unreleased cv. 226 showed great regeneration with both 0.25 mg/L GA_3 and 1.5 mg/L BAP+1.5 mg/L kinetin. Similarly, cv. Purple showed greater regeneration in 0.25 mg/L GA_3. Among the hormones, 0.25 mg/L GA_3 showed the best results with growth of five cvs. Rosita, Halka Nilo, Purple, Desiree and 226. In this study, the concentration of kinetin was kept constant (1.5 mg/L) with three different BAP concentrations i.e., 0.5 mg/L, 1.0 mg/L and 1.5 mg/L. Among these combinations, one with 0.5 mg/L supported very low growth of meristem. While 1.5 mg/L BAP concentration of BAP displayed best result with 5 cultivars displaying growth. BAP is cytokinin and has property of inducing cell division. At lower concentration hormone concentration maybe insufficient to induce enough cell division. At high concentration, it may hinder growth by inhibiting apical dominance (Badoni & Chauhan, 2009). Cytokinin like kinetin and BAP are plant hormones that are derivatives of the purine adenine and thus the lower growth rate in media with kinetin maybe attributed to high kinetin concentration resulting to inhibition of apical dominance (Kieber, 2002).

GA is essential component in plant regeneration due to its great activity resulting in shoot growth. So, they are used for regeneration of meristem from callus as well. They contribute to increase in length of regenerated plants. Hence, greater number of cultivars could have been regenerated using GA_3 as phytohormone (Marcela & Anca, 2011). The evaluation of GA_3 hormone showed similar positive growth effects on micro-propagation of potato (Pereira & Fortes, 2003). Badoni and Chauhan (2009) study showed (0.25 mg/L) was the best one for the

development of complete plants from meristem culture which showed similar findings as in our study.

The lack of growth of certain cultivars could be attributed due to their recalcitrant nature which makes certain genotype difficult to be cultured in vitro and complicates the process of producing virus-free healthy seeds (Heeres et al., 2002). Similarly, various factors that may contribute to such low levels of plant regeneration include lack of quality seeds of good varieties, non-cultivability of potato seeds *in vitro* and response to different hormones. Potatoes are usually cultivated by vegetative propagation and hence are quite vulnerable to viral diseases. Shoots of an apical meristem and the first set of primordial leaves aren't connected to vascular bundle and therefore aren't vulnerable to viruses as viruses pass through the vascular bundle. So, if meristem of a plant is excised aseptically and cultured *in vitro,* it is possible to generate completely virus-free plants. (Azad et al., 2020)

However, the excision process needs to be quite precise. If the meristem is too small, no regeneration is obtained. As such could be the case for lower rate of regeneration. Similarly, larger meristem size may result in failure to solely isolate the virus-free meristem. Hence, explant could be contaminated with virus, impeding the regeneration or causing development of virus infected plants. Studies have shown that the survival rate of potato plants after meristem culture doesn't depend necessarily on varieties and are more influenced by other factors such as size of meristem, precaution and handling during extraction and culture and also incubation conditions (Ali et al., 2013).

5.2 Validation of virus-free potato plantlets:

DAS-ELISA was performed in order to ensure that the meristem culture procedure proceeded successfully. Via DAS-ELISA, the cvs. P.L., Desiree, Janakdev, 226, MS42.3 and Cardinal were confirmed to be free of viruses. ELISA involves hydrolysis of substrate nitrophenyl phosphate by alkaline phosphate producing characteristic yellow color. Its sensitivity is 1 to 10 ng/mL (Regenmortel, 1982).

All the cultivars were found to be free of PVA. However, low degree negativity or low prevalence of PVM was found in eight samples. Further analysis is necessary to ensure virus free plantlets in such cases. Hence, such samples weren't selected for further studies. PVX was detected in three of the samples. Low degree of prevalence of PVS was detected in seven cultivars. No prevalence of PVY and PLRV was observed. Potato viruses could have been detected as a result of large meristem excision resulting in failure to isolate only the virus-free meristems.

Virus infection of potato, for a long time, was a problem affecting the yield and productivity for farmers. Hence, proper method to eliminate viruses is necessary. ELISA is an effective immunogenic assay to detect virus infecting the potato in order to devise and optimize methods required to eliminate these viral diseases (Salazar, 1994). In our study, PVS, PVX and PVY viruses were found in our samples which were similar to study conducted previously by Potato Research Program, Khumaltar. They found high prevalence of PVY and PVS and absence of PVA in local cultivars (Sakha et al., 2007) similar to observation in our study. Virus-free cv. Desiree was then selected for microtuberization and cv. P.L. for *Agrobacterium* transformation.

5.3 Microtuberization with Nanoparticle:

Microtuberization is an alternate method of seed production. Nanoparticles have now been known to improve crop yield by enhancing transport as well as making plant sturdier to stresses. Microtuberization depends on various factors such as hormones, genotypes etc. Previous studies showed that quality and yield varied highly based on genotypes (Kefi et al., 2000). Microtuber formation was the highest in CuNP 2.0 mg/L with TDZ (0.5 mg/L). It showed slight enhancement compared to microtuberization with only either hormone or CuNPs. CuNP without hormone didn't yield any microtubers. The highest microtuber size was in CuNP 2.0 mg/L with Thidiazuron (0.5 mg/L) with diameter 4.6 mm with 0.31 g. Rest of the microtubers were less than 2 mm in diameter.

The microtuber sizes and weight was quite low compared to studies performed by Ibrahim et al. (2018) in cv. Desiree using AgNPs. The highest average weight of microtuber was 312.18 mg and the average diameter of microtuber was 10.15 mm in Desiree using 1 mg/L AgNPs (Ibrahim et al., 2018).

Microtuber production, from our study, seems to be positively affected by TDZ (Turkmen et al., 2017) as well by common effect of TDZ and CuNP with 2.0 mg/L CuNP and 0.5 mg/L TDZ producing the best results. Microtuberization without hormone, in presence of only the nanoparticle has diminished effect. This effect could be due to the ability of CuNP to improve nutrient transport and absorption by plant (Mahmoud et al., 2020), therefore enhancing the effect of TDZ hormone on microtuber production. Lower size of potato microtuber could be due to temperature as well as early harvesting of seeds (Randhawa & Chandra, 1990).

5.4 Callus induction:

We performed callus induction in five different cvs. MS42.3, P.L., Rosita, Purple, K.U. Optimization of callus induction in these five cultivars in different concentration of two different combinations of plant hormones was performed. In this study, combination of hormones used were kinetin+2,4-D (CIM-1 to CIM-6) and BAP+NAA (CIM-7 to CIM-12). Hormone concentration of one was kept constant while varying another (three different concentrations) to study the individual effects as well.

In MS42.3, calli formation was the highest in CIM-5 with 2.0 mg/L kinetin and 1.5 mg/L 2,4-D and in second combination callus induction was the highest in CIM-10 with 2.0 mg/L BAP and 1.0 mg/L NAA. BAP and NAA combination displayed better induction compared to kinetin and 2,4-D. There was no callus induction in lower concentration of kinetin (0.5 and 1 mg/L). In Purple, calli formation was high in CIM-6 with 2.0 mg/L kinetin and 2.0 mg/L 2,4-D and for second combination, it was the highest in CIM-12 with 2.0 mg/L BAP and 2.0 mg/L NAA. Again, BAP and NAA combination displayed better induction compared to kinetin and 2,4-D and no callus induction was observed in low concentration of kinetin (0.5 mg/L).

In cv. K.U., calli formation was low in CIM-1 to CIM-6 with the best results in CIM-4 and CIM-5 with kinetin and 2,4-D. Callus induction was the highest in CIM-9 with 2.0 mg/L BAP and 1.0 mg/L NAA. BAP and NAA combination was better for calli induction compared to kinetin and 2,4-D. There was callus induction only in higher kinetin and 2,4-D concentrations. In P.L., calli formation was high in CIM-5 with 2.0 mg/L kinetin and 1.5 mg/L 2,4-D. Callus induction was the highest in CIM-11 with 2.0 mg/L BAP and 1.5 mg/L NAA. In cv. Rosita, calli formation was the highest in CIM-3 with 1.5 mg/L kinetin and 1.0 mg/L 2,4-D. Callus induction was very low in CIM-7 to CIM-12 with BAP and NAA. Rosita is the only variety tested that showed better callus induction results in kinetin and 2,4-D.

Callus induction rate depends on diameter and the size of callus. In addition, the potato cultivars, type of explants and plant hormones used extensively affect callus induction (Kumlay & Ercisli, 2015). Cytokinins promote cell division and play a major role in callus formation as they reduce the lignification of cell walls on the cut surface inducing callus formation (Hoque et al., 2006). Cytokinins also contribute to adventitious shoot formation (Makunga et al., 2005).

Yasmin et al. (2003) reported high (95%) callus induction with media containing 2.0 mg/L BAP and NAA. In this study, we also observed high callus induction with 2.0 mg/L BAP and NAA (CIM-12). Laboney et al. (2013) reported high callus formation (95%) in 2 mg/L 2,4-D. In similar concentrations of 2,4-D (1.5 and 2 mg/L) in CIM-5 and CIM-6, we observed better growth in almost all the cultivars.

5.5 Regeneration of plantlets:

It has been well known that GA_3 promotes cell division, cell elongation and it also promotes shoot development (Dhital et al., 2010a). Dhital et.al. (2016) observed the highest root regeneration using 0.1 mg/L GA_3 with NAA and zeatin. For shooting, Laboney et al. (2013) observed that the shoot regeneration was the fastest using 0.5 mg/L GA_3 and 1.0 mg/L BAP took about 15 days. The hormone GA_3 was used for plant regeneration and we observed regeneration in most calluses after 3 to 4 weeks.

MS42.3 had average regeneration rate of 59.167% with CIM-9 calluses showing high regeneration rate. Potato cv. Purple showed very low regeneration rate of 15.38%. P.L. displayed regeneration rate of 57.34% with CIM-5 induced callus showing high regeneration. Cultivars K.U. and Rosita failed to regenerate. MS42.3 and P.L. showed high regeneration rates. Hence, we identified optimal hormone concentration for calli induction and regeneration in local cultivars.

5.5 *Agrobacterium* Transformation and GUS analysis:

Transformation in potato is dependent on cultivars (Veale et al., 2012). Donmez et al. (2019) used LBA4404 with pBIN19 to optimize *Agrobacterium* transformation in different cultivars and found it to be quite effective either by leaf or internode as explant. In similar way, Bakhsh et al. (2014) found *Agrobacterium tumefaciens* strain LB4404 to be more infective and efficient than other strains for transformation in dicots and internode was found more susceptible to infection by LBA4404 containing pBIN19 expression vector with β-glucuronidase *gusA* gene under the control of 35S CaMV promoter.

Leaf and internodes *Solanum tuberosum* cv. P.L. were infected with *Agrobacterium* strain LBA4404 with pAXY0002 containing *GUSPlus* gene. After immersing in GUS solution overnight, explants were observed for infection in stereomicroscope. However, no evidence of transformation was observed indicating failure in *Agrobacterium* transformation. This study was performed to study infection rate on local cultivar however no result was obtained. This may be due to various factors such as low infectivity or compatibility. The failure of transformation could also be attributed to the in-effectivity of certain *Agrobacterium* strain for a particular cultivar.

CHAPTER-VI

CONCLUSION

We successfully obtained virus free regeneration of local potato cultivars using meristem culture. We also observed some potato viruses that may be present on local samples via DAS-ELISA. We made an effort to optimize meristem culture protocol as well as callus induction and microtuberization protocol on locally important cultivars. We determined optimal hormone and Copper Nanoparticle concentration for microtuberization. This research was among the first in Nepal in terms of CuO Nanoparticle for microtuberization of local cultivar. We performed *Agrobacterium* transformation for local cultivar; however, we did not observe success due to limited trials. The utilization of virus-free potatoes for microtuberization to generate pre-basic seeds using combination of nanoparticle and TDZ as well as transformation optimization parameters performed in this study will help gene manipulation and editing studies in potato for better tuberization. This study highlights the importance of implementing the proper protocols for integration of modern nanotechnology into conventional plant technology and describes the improvement it brings to crop production and yield. Accurate comprehension and further studies to understand dynamics of such technologies within the rapidly advancing agriculture sector is absolutely crucial for leading Nepali agriculture to modern era.

CHAPTER-VII

RECOMMENDATIONS

I. It is recommended to expand similar technology to other local potato cultivars.

II. It is suggested that more diverse samples be obtained in terms of geographical characteristics.

III. Our findings highlight the importance of modern nanotechnology in agriculture and new plans should be established for its application on a wider scale.

REFERENCES:

Adhikari, S. (2018). *Potato Cultivation Practices in Nepal*, B.Sc. Ag, Environment, Horticulture. Retrieved from https://agrisujan.wordpress.com/2018/06/13/potato-cultivation-practices-in-nepal/

Akius, M., & Kloos, J. P. (1990). Viral diseases spread and detection in Nepal. *11th European Association for potato Research (EAPR)*, 66-67. https://doi.org/10.3126/ijasbt.v6i2.19702

Al-Hussaini, Z. A., Yousif, S. A., & Al-Ajeely, S.A. (2015). Effect of Different Medium on Callus Induction and Regeneration in Potato Cultivars. *International Journal Current Microbiology and Applied Science, 4*(5), 856-865.

Ali, M. A., Nasiruddin, K. M., Haque, M. S., & Faisal, S. M. (2013). Virus Elimination in Potato through Meristem Culture Followed by Thermotherapy. *SAARC Journal of agriculture, 11*(1): 71-80. https://doi.org/10.3329/sja.v11i1.18376

Al-taleb, M. M., Hassawi, D. S., & Abu-romman, S. M. (2011). Production of Virus-Free Potato Plants Using Meristem Culture from Cultivars Grown under Jordanian Environment Biotechnology Department. *American-Eurasian J. Agric. & Environ. Sci., 11*(4), 467–472.

Azad, M. A., Khatun, Z., Eaton, T. E. J., Hossen, M. I., Haque, M. K., & Soren, E. B. (2020). Generation of Virus-Free Potato Plantlets through Meristem Culture and Their Field Evaluation. *American Journal of Plant Sciences, 11*(11), 1827–1846. https://doi.org/10.4236/ajps.2020.1111131

Badoni, A., & Chauhan, J. S. (2009). Effect of Growth Regulators on Meristem-tip Development and in vitro Multiplication of Potato Cultivar "Kufri Himalini". *Nature and Science, 7*(9)*,* 3*1*-34.

Bajaj, Y. (1980). Regeneration Of Plants from Potato Meristems Freeze Preserved For 24 Months. *Euphytica, 30*, 141-145.

Bajracharya, M., & Sapkota, M. (2017a). Profitability and productivity of potato (*Solanum tuberosum*) in Baglung district, Nepal. *Agriculture &Amp; Food Security, 6*(1). https://doi.org/10.1186/s40066-017-0125-5

Bajracharya, M., & Sapkota, M. (2017b). Resource use efficiency analysis for Potato production in Nepal. Ministry of Agriculture. *Journal of Nepal Agricultural Research Council, 4*(1), 54. https://doi.org/10.3126/jnarc.v4i1.19690

Baker, S., Kumar, K., Santosh, P., & Satish, S. (2015). Extracellular synthesis of silver nanoparticles by novel Pseudomonas veronii AS41G inhabiting *Annona squamosa* L. and their bactericidal activity. *Spectrochimica Acta, Part A: Molecul. Biomolecul. Spect., 136,* 1434-1440. https://doi.org/10.1016/j.saa.2014.10.033

Bakhsh, A., Anayol, E., & Ozcan, S. F. (2014). Comparison of transformation efficiency of five *Agrobacterium tumefaciens* strains in *Nicotina tobacum* L. *Emir J Food Agric*, *26*(3), 259-264. https://doi.org/10.9755/ejfa.v26i3.16437

Beyene, D., Feyissa, T., & Bedada, G. (2010). Micropropagation of Selected Cassava Varieties (Manihot esculenta Crantz) from Meristem Culture. Retrieved from https://www.researchgate.net/publication/261552350

Brunt, A. A. (2001). The Main Viruses Infecting Potato Crops. *Virus and Virus-like Diseases of Potatoes and Production of Seed-Potatoes*, 65-67.

Campos, H. (Ed.), & Ortiz, O. (Ed.). (2020). The Potato Crop, its Agricultural, Nutritional and Social Contribution to Humankind. *Springer Nature Switzerland AG*: 389-430. https://doi.org/10.1007/978-3-030-28683-5

Chen, L., Peng, Y., Zhu, L., Huang, Y., Bie, Z., & Wu, H. (2022). CeO2 nanoparticles improved cucumber salt tolerance is associated with its induced early stimulation on antioxidant system. *Chemosphere*, *299*, 134474. https://doi.org/10.1016/j.chemosphere.2022.134474

Chilton, M. D., Drummond, M., Merlo, D., Gordon, M., Sciaky, D., Montoya, A., & Nester, E. (1977). Stable incorporation of plasmid DNA into higher plant cells; the molecular basis of crown gall tumorigenesis. *Cell, 11*(2), 263–271. https://doi.org/10.1016/0092-8674(77)90043-5

Clark, M. F., & Adams, A. (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *J. Gen. Virol*, *34,* 475-783. https://doi.org/10.1099/0022-1317-34-3-475

Dangol, S. D., Yel, I., & Caliskan, M. (2020). Manipulating genome of diploid potato inbred line *Solanum chacoense* M6 using selectable marker gene. *Turkish Journal of Agriculture and Forestry, 44*(4), 399-407. https://doi.org/10.3906/tar-1910-13

Dhital, S. P. (2016). *Potato seed PDP manual: Potato seed management and production technology.* Nepal Agricultural Research Centre (NARC).

Dhital, S. P., Lim, H. T., & Manandhar, H. K. (2010a). Direct and Efficient Plant Regeneration from Different Explants Sources of Potato Cultivars as Influenced by Plant Growth Regulators. *Nepal Journal of Science and Technology*, *12*, 1-6. https://doi.org/10.3126/njst.v12i0.6471

Dhital, S. P., Sakha, B. M., & Nepal, R. B. (2010b). Effects of Season on Rapid Multiplication of Virus-free Plantlets under In Vitro and on Production of Pre-basic Seeds of Potato (*Solanum tuberosum* L.) under In-Vivo Conditions. *Nepal Agric. Res. J. Vol. 10*, 75-82.

Donmez, B. A., Dangol, S. D., & Bakhsh, A. (2019). Transformation efficiency of five Agrobacterium strains in diploid and tetraploid potatoes. *Sarhad Journal of Agriculture, 35*(4), 1344-1350. http://dx.doi.org/10.17582/journal.sja/2019/35.4.1344.1350

El-Saadony, M. T., Saad, A. M., Soliman, S. M., Salem, H. M., Desoky, E. S. M., & Babalghith, A. O. (2022). Role of Nanoparticles in Enhancing Crop Tolerance to Abiotic Stress: A Comprehensive Review. *Frontiers in Plant Scienc*e, *13*, 146.

El-tahan, I., Azab, E. S., Abdelrazik, E., & Elhamahmy, M. (2022). Physiological Parameters of Potato Explants as Affected by Silicon Nanoparticles under Drought Stress. *Journal of Plant Production Sciences, 11*(1), 11-20.

Erland, L. A., Giebelhaus, R. T., Victor, J. M., Murch, S. J., & Saxena, P. K. (2020). The morphoregulatory role of thidiazuron: Metabolomics-guided hypothesis generation for mechanisms of activity. *Biomolecules, 10*(9), 1253. https://doi.org/10.3390/biom10091253

FAOSTAT (2024). Crops and livestock products [online]. Retrieved June 23, 2024, from https://www.fao.org/faostat/en/#data/QCL

Gelvin, S. B. (2000). Agrobacterium and plant genes involved in T-DNA transfer and integration. *Plant physiology and Plant molecular biology*, *51,* 223-256.

Gonzalez, J. M., Friero, E., & Jouve, N. (2001). Influence of genotype and culture medium on callus formation and plant regeneration from immature embryos of *Triticum turgidum* Desf. Cultivars. *Plant Breeding*, *120*(6), 513-517. https://doi.org/10.1046/j.1439-0523.2001.00661.x

Government of Nepal. Ministry of industry, commerce and supplies. Retrieved February 2, 2022, from https://moics.gov.np/en/list/resource/1282?parent=11742

Gustavo, A., De la Riva, J. G., Cabrera, R. V., & Padron, C. A. (1998). *Agrobacterium tumefaciens* a natural tool for plant transformation. *Electronic J. Biotechnol*, *1,* 118-133.

Hamdi, B., Khawla, A., & Messaoud, B. (2017). The effect of growth regulators and explants on callus induction in four cultivars of potato (*Solanum tuberosum* L.). *Journal of Bioresources Valorization*, *2*(1), 34-41.

Hamza, E. M. (2019). Improvement of Potato Micropropagation and Microtubers Formation as Affected by Nanoparticles. *Middle East Journal of Agriculture Research, 8*(2), 525-532.

Heeres, P., Schippers-Rozenboom, M., Jacobsen, E., & Visser, R. (2002). Transformation of a large number of potato varieties: genotype-dependent variation in efficiency and somaclonal variability. *Euphytica, 124*, 13–22. https://doi.org/10.1023/A:1015689112703

Hoque, A., Nahar, A., & Razvy, M. A. (2006). Micropropagation of water chestnut (Trapa sp.) through local varieties of Rajshahi Division. *Asian Journal of Plant Science*, *5*(3), 409–413. https://doi.org/10.3923/ajps.2006.409.413

Ibrahim, A. D., Fahmy, A. H., & Ahmed, S. S. (2018). Silver Nanoparticles Promote Microtuberization in Potato (*Solanum tuberosum* L.) cv. Desirée. *Journal of Horticultural Science & Ornamental Plants, 10*(3), 129-139.

Ikeuchi, M., Favero, D. S., Sakamoto, Y., Iwase, A., Coleman, D., Rymen, B., & Sugimoto, K. (2019). Molecular mechanisms of plant regeneration. *Annu. Rev. Plant Biol.*, *70*, 377–406.

Ikeuchi, M., Iwase, A., & Sugimoto, K. (2013). Plant Callus: Mechanisms of Induction and Repression. *The Plant Cell, 25*(9), 3159–3173. https://doi.org/10.1105/tpc.113.116053

Jefferson, R. A., Kavanagh, T. A., & Bevan, M. W. (1987). GUS fusions: B-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *The EMBO Journal, 6*(13), 3901 - 3907.

Joshi, N., Pathak, A., Upadhyay, D. C., Krishna, S. B. N., & Upadhyay, C. P. (2022). Synthesis of biocompatible Fe3O4 and MnO2 nanoparticles for enhanced tuberization in potato (*Solanum tuberosum* L.). *Biocatalysis and Agricultural Biotechnology*, *39*, 3455. https://doi.org/10.1016/j.bcab.2021.102258

Kaeppler, S. M., Kaeppler, H. F., & Rhee, Y. (2000). Epigenetic aspects of somaclonal variation in plants. *Plant Molecular Biology*, *43,* 179-188. https://doi.org/10.1023/A:1006423110134

Kartha, K. K., Gamborg, O. L., Constabel, F., & Shyluk, J. P. (1974). Regeneration of cassava plants from apical meristems. *Plant Science Letters, 2*(1), 107-113. https://doi.org/10.1016/0304-4211(74)90066-2

Kasana, R. C., Panwar, N. R., Kaul, R. K., & Kumar, P. (2016). Copper Nanoparticles in Agriculture: Biological Synthesis and Antimicrobial Activity. *Nanoscience in Food and Agriculture 3. Sustainable Agriculture Reviews*, 23. https://doi.org/10.1007/978-3-319-48009-1

Kaviani, B., Hesar, A. A., Tarang, A., Zanjani, S. B., Hashemabadi, D., & Ansari, M. H. (2013). Effect of kinetin (Kn) and naphthalene acetic acid (NAA) on the micro-propagation of *Matthiola incana* using shoot tips, and callus induction and root formation on the leaf explants. *African Journal of Agricultural Research, 8*(30), 4134-4139. https://doi.org/10.5897/AJAR11.921

Kawochar, A., Ahmed, N. U., Hossain, I., & Ferdous, J. (2017). Role of Explants and NAA on Callus Induction of Potato (*Solanum tuberosum*). *American Journal of Life Sciences, 5*(5), 140-144. https://doi.org/10.11648/j.ajls.20170505.14

Kefi, S., Pavlista, A. D., Read, P. E., & Kachman, S. D. (2000). Comparison of Thidiazuron and Two Nitroguanidines to Kinetin on Potato Microtuberization in vitro under Short and Long Days. *Journal of plant growth regulation*, *19*(4), 429-436. https://doi.org/10.1007/s003440000019

Khalafalla, M. M., Khadiga, G., Elaleem, A., & Modawi, R. S. (2010). Callus formation and organogenesis of potato (*Solanum Tuberosum* l.) Cultivar almera. *Journal of Phytology, 2*(5), 40–46.

Khan, M. S., Hoque, M. I., Sarker, R. H., & Muehlbach, H. P. (2003). Detection of Important Plant Viruses in In-vitro Regenerated Potato Plants by Double Antibody Sandwich Method of ELISA. *Plant Tissue Culture, 13*(1), 21-29.

Khatri, B., & Shrestha, S. (2004). Some Local Potato Cultivars and their Reaction to Viruses, N*epal Agric. Res. J., 5*(1), 19.

Kieber, J. J. (2002). Cytokinins. *The Arabidopsis book, 1*: e0063. https://doi.org/10.1199/tab.0063

Kumlay, A. M., & Ercisli, S. (2015). Callus induction, shoot proliferation and root regeneration of potato (*Solanum tuberosum* L.) stem node and leaf explants under long-day conditions. *Biotechnology & Biotechnological Equipment, 29*(6), 1075-1084. https://doi.org/10.1080/13102818.2015.1077685

Laboney, U. Z., Biswas G. C., & Abdullah-Al-Shoeb, M. (2013). Callus induction and regeneration of potato shoot tip culture. *International Journal of Agricultural Sciences, 3,* 40–45.

Lama, T. L., Khatri, B. B., & Dhakal, S. P. (2016). Status and prospects of potato research and development in Nepal. *Horticulture in last 6 decades, Nepal Horticulture Society*, 145-156.

Luitel, B. P., Bhandari, B. B., & Thapa, B. (2020). Evaluation of Potato Genotypes for Plant and Yield Characters in Field at Dailekh. *Nepal Journal of Science and Technology*, *19*(2), 16–24. https://doi.org/10.3126/njst.v20i1.39380

Mahmoud, A. W., Abdeldaym, E. A., Abdelaziz, S. M., El-Sawy, M. B., & Mottaleb, S. A. (2020). Synergetic effects of zinc, boron, silicon, and zeolite nanoparticles on confer tolerance in potato plants subjected to salinity. *Agronomy*, *10*(1), 19.

Makunga, N. P., Jager, A. K., & Staden, J. V. (2005). An improved system for the in vitro regeneration of *Thapsia garganica* via direct organogenesis influence of auxins and cytokinins. *Plant Cell Tissue and Organ Culture, 82*, 271-280.

Marcela, D. O., & Anca, B. (2011). Potato (*Solanum tuberosum* L.) regeneration using the technique of meristem tip culture. *Journal of Horticulture, Forestry and Biotechnology, 15*(4), 175–178.

Mogazy, A. M., & Hanafy, R. S. (2022). Foliar Spray of Biosynthesized Zinc Oxide Nanoparticles Alleviate Salinity Stress Effect on *Vicia faba* Plants. *Journal of Soil Science and Plant Nutrition, 22*, 2647-2662.

Mollika, S. R., Sarker, R., & Hoque, M. (2020). In vitro Regeneration and Agrobacterium-mediated Genetic Transformation of a Cultivated Potato Variety Using Marker Genes. *Plant Tissue Culture and Biotechnology, 30*(1), 149–160. https://doi.org/10.3329/ptcb.v30i1.47800

Nagib, A., Hossain, S. A., & Alam, M. F. (2011). Virus-Free Potato Seed Tuber Production through Meristem Culture in Tropical Asia. *Asian Journal of Plant Sciences, 2*(8), 616–622.

Nepal Arable Land 1961-2022. Macrotrends. Retrieved September 14, 2022, from https://www.macrotrends.net/countries/NPL/nepal/arable-land

Pereira, J. E., & Fortes, G. R. (2003). Protocol for potato propagative material production in liquid medium. *Pesquisa Agropecuária Brasileira, 38*(9), 1035-1043. https://doi.org/10.1590/S0100-204X2003000900003

Pestovsky, Y. S., & Martínez-Antonio, A. (2017). The Use of Nanoparticles and Nanoformulations in Agriculture, *Journal of Nanoscience and Nanotechnology*, *17*(12), 8699-8730. https://doi.org/10.1166/jnn.2017.15041

Ranalli, P. (2007). The Canon of Potato Science. *Potato Res, 50*(24), 301-304.

Randhawa, G. J., & Chandra, R. (1990). In vitro tuberization in six Indian potato cultivars. *Journal of Indian Potato Association, 17*(4), 199-201.

Regenmortel, M. H. V. (1982). *Serology and Immunochemistry of Plant Viruses*, 1-302.

Sakha, B. M., & Rai, G. P. (2004). Performance of in vitro plantlets and stem cuttings for pre-basic seed potato production under glasshouse condition in Nepal. *4th National Horticulture Research Workshop,* 186-189.

Sakha, B. M., Bhatia, A. K., Batra, V. K., Chaudhary, V. K., Batra, P., & Khurana, S. C. (2004). In vitro micro-tuberization in potato (*Solanum tuberosum* L.). *Indian Journal of Experimental Biology, 12*(12), 1245-1247.

Sakha, B. M., Rai, G. P., Dhital, S. P., & Nepal, R. B. (2007). Disease-free Pre-Basic Seed Potato Production through Tissue Culture in Nepal. *Nepal Agriculture Research Journal*, *8,* 7-13. https://doi.org/10.3126/narj.v8i0.11564

Salazar, L. F. (1994). Virus detection and management in developing countries. *Advances in potato pest biology and management, 1-8.*

Sallam, A. R., Mahdi, A. A., & Farroh, K. Y. (2022). Improving drought stress tolerance in potato (*solanum tuberosum* L.) using magnetite and zinc oxide nanoparticles. *Plant cell biotechnology and molecular biology*, 1-16.

Särekanno, M., Kadaja, J., Kotkas, K., Rosenberg, V., & Eremeev, V. (2011). Development of field-grown potato plants derived from meristem plants multiplied with different methods. *Acta Agriculturae Scandinavica, Section B. Soil & Plant Science*, *62*(2), 114–124. https://doi.org/10.1080/09064710.2011.581680

Sharma, K. P. (2074). *Alubali bibaran pustika*. National Potato Development program. Vegetable development directorate. Ministry of agriculture, Government of Nepal.

Turkmen, A. K., Yavuz, C., Dangol, S. D., Tarim, C., Demirel, U., & Caliskanomer, M. E. (2017). Evaluation of Micro Tuberization Performances of Different Genotypes. *Turkish Journal of Agriculture -Food Science and Technology*, *5*(4), 353-357. https://doi.org/10.24925/turjaf.v5i4.353-357.1203

Upadhyay, K. P., & Timilsina, A. P. (2020). Bung: A traditional practice for potato cultivation in eastern hills of Nepal. *Journal of Agriculture and Natural Resources, 3*(1), 38-50. https://doi.org/10.3126/janr.v3i1.27004

Veale, M. A., Slabbert M. M., & Van Emmenes, L. (2012). Agrobacterium-mediated transformation of potato cv. Mnandi for resistance to the potato tuber moth (*Phthorimaea operculella*). *South African Journal of Botany, 80*, 67-74. https://doi.org/10.1016/j.sajb.2012.02.007

Vinterhalter, D., Korac, S., & Mitic, N. (2008). Protocols of Agrobacterium Mediated Transformation of potato. *Fruit, Vegetables and Cereal biotechnology*, 1-15.

Visser, R. G. (1991). Regeneration and transformation of potato by *Agrobacterium tumefaciens*. *Plant tissue culture manual, Springer,* 301-309. https://doi.org/10.1007/978-94-009-0103-2_16

Wamucci, S. (2024). Nepal Seed Potato Prices in 2024. Retrieved April 21, 2024, from https://www.selinawamucii.com/insights/prices/nepal/seed-potatoes/

Wang, P., Lombi, E., Zhao, F., & Kopittke, P. (2016). Nanotechnology: a new opportunity in plant sciences Trend. *P. Sci., 21*(8), 22-27. https://doi.org/10.1016/j.tplants.2016.04.005

Wareh, H. A., Trolinder, N. L., & Gooding, J. R. (1989). Callus initiation, shoot regeneration, and micro-propagation of three potato cultivars. *Hort. sci*., *24*(4), 680-682. https://doi.org/10.21273/HORTSCI.24.4.680

Wenzler, H., Mignery, G., May, G., & Park, W. (1989). A rapid and efficient transformation method for the production of large numbers of transgenic potato plants. *Plant Sciences, 63,* 79-85. https://doi.org/10.1016/0168-9452(89)90103-9

World Bank Group. Retrieved September 18, 2022, from https://data.worldbank.org/indicator/AG.LND.ARBL.ZS?locations=NP

Yasmin, S., Nasiruddin, K. M., & Begum, R. (2003). Regeneration and establishment of potato plantlet through callus formation with BAP and NAA. *Asian J Plant Sci*., *2*(12), 936–940. https://doi.org/10.3923/ajps.2003.936.940

Oral Presentation at the Conference derived from this Master's Thesis

Kunjan Mishra, Sabari Rajbahak, Sarbesh Das Dangol. Potato meristem culture to generate virus-free plantlets, and optimization of callus induction, transformation and microtuberization parameters. Biotechnology Society of Nepal. April 25, 2024. (Oral Presentation)

Master's Thesis Submitted for Publication

Kunjan Mishra, Sabari Rajbahak, Sarbesh Das Dangol. (2024). Virus-Free Meristem Culture, Calli Induction and Regeneration Optimization of Cultivated and Unreleased Potatoes of Nepal. Submitted to Turkish Journal of Botany.

CURRICULUM VITAE

Kunjan Mishra was born on November 17, 1996 in Kathmandu, Nepal. He completed his higher secondary education (Major: Physics, Chemistry, Biology, Mathematics) in Prasadi College, Kathmandu from 2012-2014. He joined Xavier International College, Maitighar, Kathmandu for Bachelors in Microbiology from 2015-2019. He enrolled for Master of Bio-technology in Tribhuvan University (Nepal) from 2019. He knows Nepal Bhasa, Nepali and English languages. He is currently working in research project on meristem culture, plant transformation optimization and generation of microtubers using nanoparticles. He was involved in seminar on capacity building in flowcytometry (2021) and has worked as tissue culture lab technician at Krishi Samuha tissue culture lab in Banepa.

APPENDICES

Appendix 1: Media Preparation protocols

Callus Induction Media composition:

	Kinetin (mg/L)	**2,4-D (mg/L)**
CIM-1	0.5	1.0
CIM-2	1.0	1.0
CIM-3	1.5	1.0
CIM-4	2.0	1.0
CIM-5	2.0	1.5
CIM-6	2.0	2.0
	BAP (mg/L)	**NAA (mg/L)**
CIM-7	1.0	1.0
CIM-8	1.5	1.0
CIM-9	2.0	1.0
CIM-10	2.0	1.0
CIM-11	2.0	1.5
CIM-12	2.0	2.0

MS media preparation:

Stock I: Prepare 500 mL of 20X stock solution I:

Note: For 20x dissolve original concentration in 500 mL

Chemicals	10X(mg L^{-1})	20x
KNO_3	19000	19
NH_4NO_3	16500	16.5
$MgSO_4.7H_2O$	3700	3.7
$CaCl_2.2H_2O$	4400	4.4
KH_2PO_4	1700	1.7
Final volume	1000 mL	500 mL

Stock II: Prepare 100 mL of 1000X stock solution II:

Note: For 1000X dissolve original concentration in 100 mL

Chemicals	100X(mg L^{-1})	1000x
KI	83	0.083
H_3BO_3	620	0.62
$MnSO_4.4H_2O$	2230	2.23
$ZnSO_4.7H_2O$	860	0.860
$Na_2MoO4.2H_2O$	25	0.025
$CuSO_4.5H_2O$	2.5	0.0025
$CuCl_2.6H_2O$	2.5	0.0025
Final volume	1000 mL	100 mL

Stock III: Prepare 100 mL of 100X stock solution III: For 100X dissolve original concentration in 100 mL

Chemicals	10X(mg L^{-1})	100x
Na_2EDTA	373	0.373
$FeSO_4.7H_2O$	278	0.278
Final volume	1000 mL	100 mL

Stock IV: Prepare 100 mL of 1000X stock solution IV: For 1000x dissolve original concentration in 100 mL

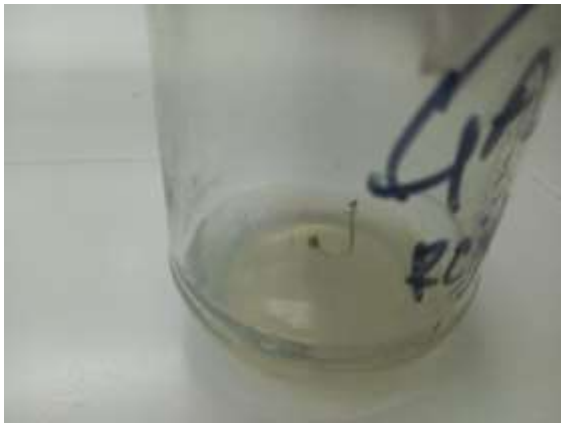
Chemicals	10X(mg L^{-1})	100x
Glycine	200	0.2
Nicotinic acid	50	0.05
Pyridoxin HCl	50	0.05
Thiamine HCl	10	0.01
Final volume	1000 mL	100 mL

Reagents	Amount
Stock I	50 mL
Stock II	1 mL
Stock III	10 mL
Stock IV	1 mL
Myo-inositol	100 mg
Sucrose	30 g
Make final volume	1000 mL
Adjust P^H	5.6-5.8

Appendix 2: Photographs

Meristem extraction

Meristem transfer to various culture media



Plantlet regeneration from meristem

Various Plantlets regenerated

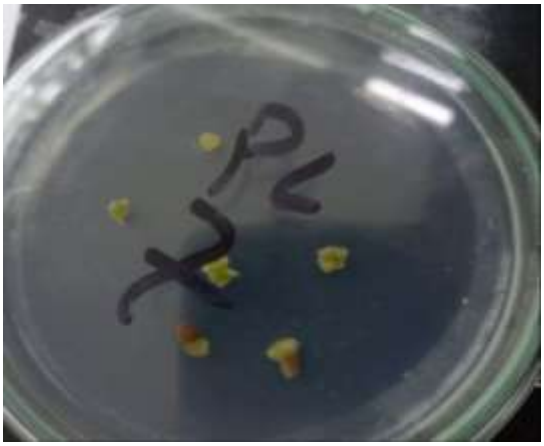
Plant regeneration via meristem culture

ELISA reaction for PVM, PVX, PVA, PVS, PVY and PLRV

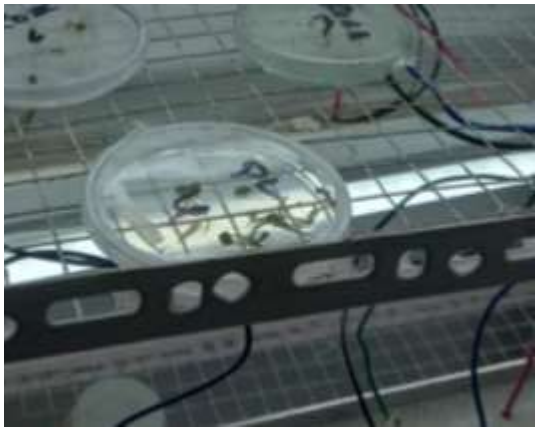
Elisa reading via reader for PVA, PVM, PVY, PVX, PVS and PLRV

Microtuberization in potato cv. Desiree

Callus induction of P.L. in CIM-9



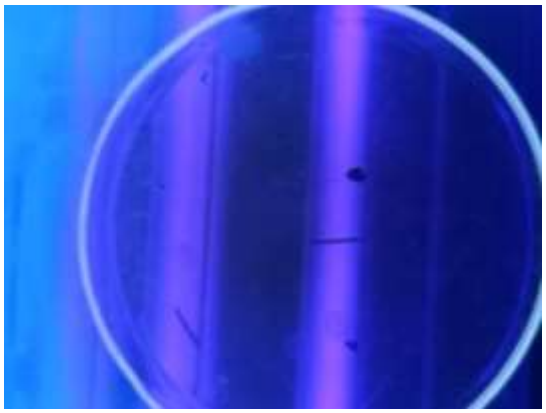
Callus induction of P.L. in CIM-7



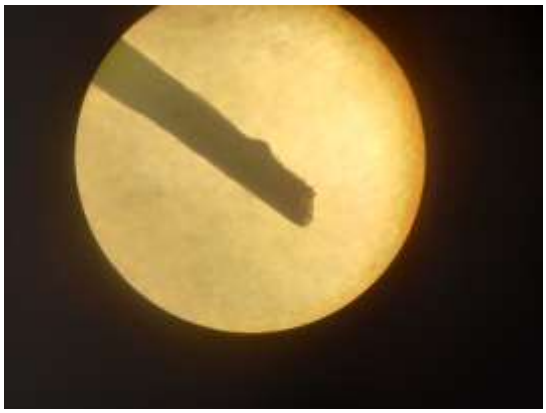
Callus regeneration

Plantlet regeneration from callus

Figure: Plant regeneration from callus in P.L.



GUS analysis negative via UV illumination



GUS analysis negative via microscopy

Performing ELISA

Performing Callus induction

Appendix 3: Miscellaneous

I. Meristem culture media

S.N.	Hormones	Concentration (mgL^{-1})
1	Gibberellic acid-3 (GA_3)	0.25
2	6-benzyl amino purine (BAP) kinetin	0.5 1.5
3	BAP kinetin	1.0 1.5
4	BAP kinetin	1.5 1.5

II. Callus induction media:

	Kinetin (mgL^{-1})	2,4-D ($mg\ L^{-1}$)
CIM-1	0.5	1.0
CIM-2	1.0	1.0
CIM-3	1.5	1.0
CIM-4	2.0	1.0
CIM-5	2.0	1.5
CIM-6	2.0	2.0
	BAP ($mg\ L^{-1}$)	NAA ($mg\ L^{-1}$)
CIM-7	1.0	1.0

CIM-8	**1.5**	**1.0**
CIM-9	**2.0**	**1.0**
CIM-10	**2.0**	**1.0**
CIM-11	**2.0**	**1.5**
CIM-12	**2.0**	**2.0**

III. Callus induction results

Cultivar	**Media**	**NO. of calli in triplicates**		
		1st replicate	**2nd replicate**	**3rd replicate**
MS42.3	**CIM-1**	**X**	**X**	**X**
	CIM-2	**0**	**0**	**X**
	CIM-3	**6**	**5**	**3**
	CIM-4	**3**	**x**	**1**
	CIM-5	**6**	**6**	**5**
	CIM-6	**6**	**5**	**5**
Cultivar	**Media**	**NO. of calli in triplicates**		
		1st replicate	**2nd replicate**	**3rd replicate**
	CIM-1	**0**	**0**	**0**
	CIM-2	**3**	**0**	**0**

Purple	**CIM-3**	**0**	**5**	**3**
	CIM-4	**1**	**0**	**2**
	CIM-5	**3**	**0**	**1**
	CIM-6	**4**	**3**	**1**
Cultivar	**Media**	**NO. of calli in triplicates**		
		1st replicate	**2nd replicate**	**3rd replicate**
Rosita	**CIM-1**	**0**	**0**	**1**
	CIM-2	**0**	**0**	**0**
	CIM-3	**4**	**4**	**2**
	CIM-4	**3**	**2**	**3**
	CIM-5	**2**	**3**	**3**
	CIM-6	**0**	**2**	**2**
Cultivar	**Media**	**NO. of calli in triplicates**		
		1st replicate	**2nd replicate**	**3rd replicate**
	CIM-1	**3**	**2**	**0**
	CIM-2	**4**	**1**	**3**
P.L.	**CIM-3**	**4**	**4**	**3**

	CIM-4	**2**	**0**	**2**
	CIM-5	**0**	**6**	**6**
	CIM-6	**0**	**4**	**5**
Cultivar	**Media**	**No. of calli in triplicates**		
		1st replicate	**2nd replicate**	**3rd replicate**
K.U.	**CIM-1**	**0**	**0**	**0**
	CIM-2	**0**	**0**	**2**
	CIM-3	**2**	**4**	**0**
	CIM-4	**2**	**3**	**4**
	CIM-5	**0**	**4**	**5**
	CIM-6	**X**	**X**	**X**
Cultivar	**Media**	**NO. of calli in triplicates**		
		1st replicate	**2nd replicate**	**3rd replicate**
MS42.3	**CIM-7**	**5**	**4**	**4**
	CIM-8	**4**	**4**	**4**
	CIM-9	**4**	**X**	**4**
	CIM-10	**6**	**6**	**5**

	CIM-11	**X**	**3**	**4**
	CIM-12	**5**	**4**	**3**
Cultivar	**Media**	**NO. of calli in triplicates**		
		1st replicate	**2nd replicate**	**3rd replicate**
Purple	**CIM-7**	**5**	**6**	**4**
	CIM-8	**5**	**X**	**6**
	CIM-9	**4**	**4**	**5**
	CIM-10	**4**	**3**	**4**
	CIM-11	**4**	**3**	**5**
	CIM-12	**6**	**5**	**5**
Cultivar	**Media**	**NO. of calli in triplicates**		
		1st replicate	**2nd replicate**	**3rd replicate**
Rosita	**CIM-7**	**0**	**0**	**0**
	CIM-8	**0**	**1**	**0**
	CIM-9	**0**	**0**	**1**
	CIM-10	**0**	**2**	**0**
	CIM-11	**0**	**0**	**1**

	CIM-12	**0**	**0**	**0**
Cultivar	**Media**	**NO. of calli in triplicates**		
		1st replicate	**2nd replicate**	**3rd replicate**
P.L.	**CIM-7**	**5**	**5**	**5**
	CIM-8	**6**	**3**	**5**
	CIM-9	**5**	**5**	**6**
	CIM-10	**6**	**5**	**5**
	CIM-11	**6**	**5**	**6**
	CIM-12	**5**	**5**	**6**
Cultivar	**Media**	**NO. of calli in triplicates**		
		1st replicate	**2nd replicate**	**3rd replicate**
Khumal Upahar	**CIM-7**	**X**	**X**	**1**
	CIM-8	**3**	**4**	**5**
	CIM-9	**6**	**5**	**4**
	CIM-10	**4**	**4**	**3**
	CIM-11	**4**	**5**	**4**
	CIM-12	**5**	**5**	**4**

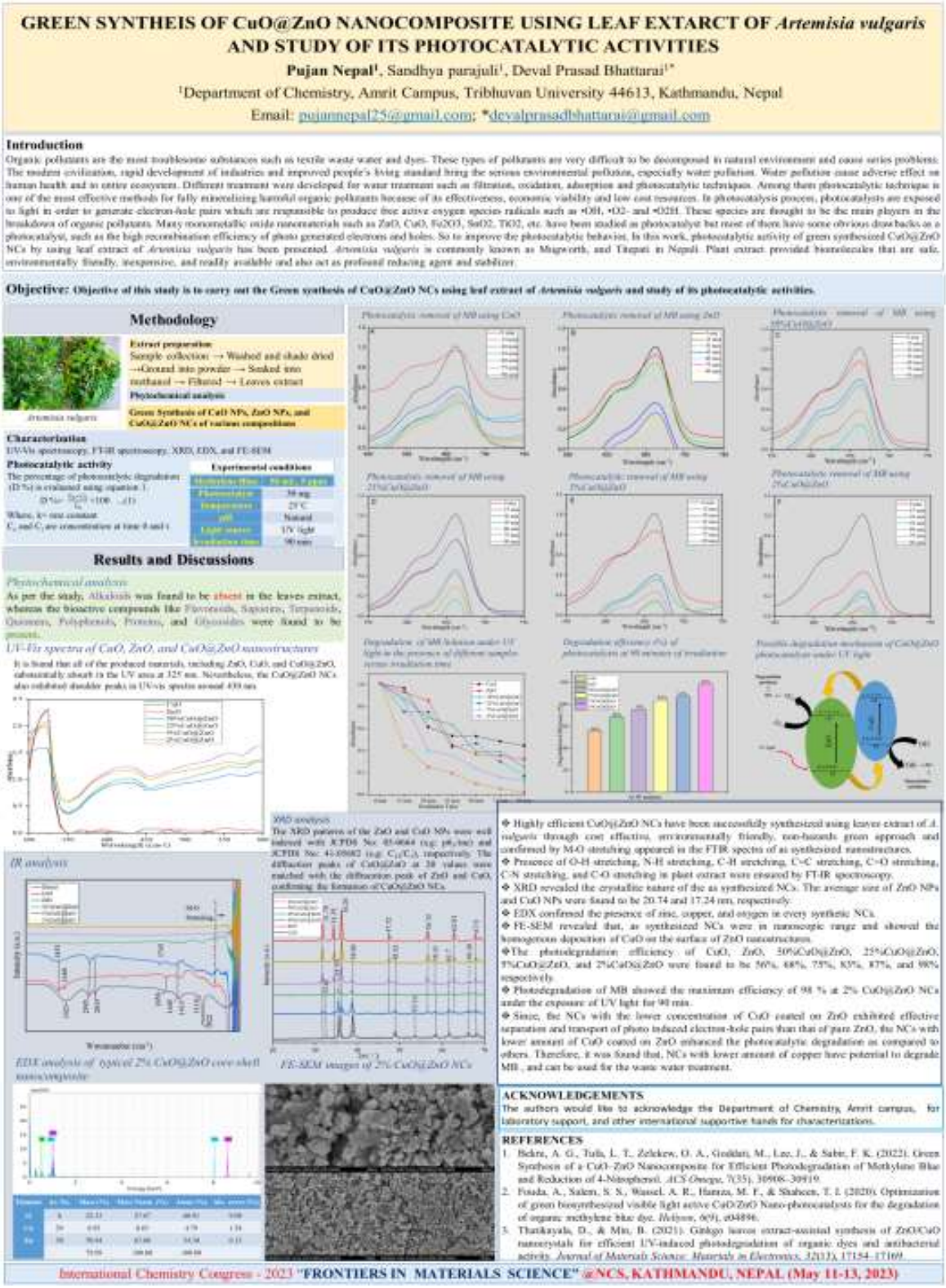
Elisa Samples

S. no.	Cultivars	hormones (mg L^{-1})	Well no.
1.	Desiree	GA_3 0.25 mg L^{-1}	D1-4
2.	Janakdev	BAP- 1 mg L^{-1} Kin- 1.5 mg L^{-1}	D5-8
3.	226	GA_3 0.25 mg L^{-1}	D9-12
4.	P.L.	BAP- 1 mg L^{-1} Kin- 1.5 mg L^{-1}	E1-4
5.	K.U.	BAP- 1.5 mg L^{-1} Kin- 1.5 mg L^{-1}	E5-8
6.	226	BAP- 1.5 mg L^{-1} Kin- 1.5 mg L^{-1}	E9-12
7.	K.R.	BAP- 1 mg L^{-1} Kin- 1.5 mg L^{-1}	F1-4
8.	MS42.3	BAP- 1.5 mg L^{-1} Kin- 1.5 mg L^{-1}	F5-8
9.	Cardinal	BAP- 1.5 mg L^{-1}	F9-12

		Kin- 1.5 mg L^{-1}	
10.	**Purple**	**GA_3 0.25 mg L^{-1}**	**G1-4**
11.	**Desiree**	**BAP- 1.5 mg L^{-1}** **Kin- 1.5 mg L^{-1}**	**G5-8**
12.	**Khumal Rato**	**BAP- 1.5 mg L^{-1}** **Kin- 1.5 mg L^{-1}**	**G9-12**
13.	**Rosita**	**BAP- 1.5 mg L^{-1}** **Kin- 1.5 mg L^{-1}**	**H1-4**
14.	**K.R.**	**BAP- 1.5 mg L^{-1}** **Kin- 1.5 mg L^{-1}**	**H5-8**

IV Source of CuO Nanoparticles:

Provided by: Deval Prasad Bhattarai, Pujan Nepal and Sandhya Parajuli



GREEN SYNTHEIS OF CuO@ZnO NANOCOMPOSITE USING LEAF EXTARCT OF *Artemisia vulgaris* AND STUDY OF ITS PHOTOCATALYTIC ACTIVITIES

Pujan Nepal[1], Sandhya parajuli[1], Deval Prasad Bhattarai[1*]

[1]Department of Chemistry, Amrit Campus, Tribhuvan University 44613, Kathmandu, Nepal

Email: pujannepal25@gmail.com; *devalprasadbhattarai@gmail.com

Introduction

Organic pollutants are the most troublesome substances such as textile waste water and dyes. These types of pollutants are very difficult to be decomposed in natural environment and cause series problems. The modern civilization, rapid development of industries and improved people's living standard bring the serious environmental pollution, especially water pollution. Water pollution cause adverse effect on human health and to entire ecosystem. Different treatment were developed for water treatment such as filtration, oxidation, adsorption and photocatalytic techniques. Among them photocatalytic technique is one of the most effective methods for fully mineralizing harmful organic pollutants because of its effectiveness, economic viability and low cost resources. In photocatalysis process, photocatalysts are exposed to light in order to generate electron-hole pairs which are responsible to produce free active oxygen species radicals such as •OH, •O2- and •O2H. These species are thought to be the main players in the breakdown of organic pollutants. Many monometallic oxide nanomaterials such as ZnO, CuO, Fe2O3, SnO2, TiO2, etc. have been studied as photocatalyst but most of them have some obvious drawbacks as a photocatalyst, such as the high recombination efficiency of photo generated electrons and holes. So to improve the photocatalytic behavior, In this work, photocatalytic activity of green synthesized CuO@ZnO NCs by using leaf extract of *Artemisia vulgaris* has been presented. *Artemisia vulgaris* is commonly known as Mugwerth, and Titepati in Nepali. Plant extract provided biomolecules that are safe, environmentally friendly, inexpensive, and readily available and also act as profound reducing agent and stabilizer.

Objective: **Objective of this study is to carry out the Green synthesis of CuO@ZnO NCs using leaf extract of *Artemisia vulgaris* and study of its photocatalytic activities.**

Methodology

Artemisia vulgaris

Extract preparation

Sample collection → Washed and shade dried →Ground into powder → Soaked into methanol → Filtered → Leaves extract

Phytochemical analysis

Green Synthesis of CuO NPs, ZnO NPs, and CuO@ZnO NCs of various compositions

Characterization

UV-Vis spectroscopy, FT-IR spectroscopy, XRD, EDX, and FE-SEM

Photocatalytic activity

The percentage of photocatalytic degradation (D %) is evaluated using equation 1

$$D\% = \frac{C_0 - C}{C_0} \times 100 \quad \ldots(1)$$

Where, k= rate constant

C_0 and C_t are concentration at time 0 and t

Experimental conditions	
Quantity (mg)	50 mg/ 100 ml
Photocatalyst	50 mg
Temperature	25°C
pH	Natural
Light source	UV light
Irradiation time	90 min

Results and Discussions

Photochemical analysis

As per the study, Alkaloids was found to be absent in the leaves extract, whereas the bioactive compounds like Flavonoids, Saponins, Terpenoids, Quinones, Polyphenols, Proteins, and Glycosides were found to be present.

UV-Vis spectra of CuO, ZnO, and CuO@ZnO nanostructures

It is found that all of the produced materials, including ZnO, CuO, and CuO@ZnO, substantially absorb in the UV area at 325 nm. Nevertheless, the CuO@ZnO NCs also exhibited shoulder peaks in UV-vis spectra around 410 nm.

XRD analysis

The XRD patterns of the ZnO and CuO NPs were well indexed with JCPDS No: 05-0664 (e.g. pb,mc) and JCPDS No: 41-0902 (e.g. C_2/C_1), respectively. The diffraction peaks of CuO@ZnO at 2θ values were matched with the diffraction peak of ZnO and CuO, confirming the formation of CuO@ZnO NCs.

IR analysis

EDX analysis of 0.5% of 2% CuO@ZnO core shell nanocomposite

FE-SEM images of 2% CuO@ZnO NCs

- Highly efficient CuO@ZnO NCs have been successfully synthesized using leaves extract of *A. vulgaris* through cost effective, environmentally friendly, non-hazards green approach and confirmed by M-O stretching appeared in the FTIR spectra of as synthesized nanostructures.
- Presence of O-H stretching, N-H stretching, C-H stretching, C=C stretching, C=O stretching, C-N stretching, and C-O stretching in plant extract were ensured by FT-IR spectroscopy.
- XRD revealed the crystallite nature of the as synthesized NCs. The average size of ZnO NPs and CuO NPs were found to be 20.74 and 17.24 nm, respectively.
- EDX confirmed the presence of zinc, copper, and oxygen in every synthetic NCs.
- FE-SEM revealed that, as synthesized NCs were in nanoscopic range and showed the homogenous deposition of CuO on the surface of ZnO nanostructures.
- The photodegradation efficiency of CuO, ZnO, 50%CuO@ZnO, 25%CuO@ZnO, 5%CuO@ZnO, and 2%CuO@ZnO were found to be 56%, 68%, 75%, 83%, 87%, and 98% respectively.
- Photodegradation of MB showed the maximum efficiency of 98 % at 2% CuO@ZnO NCs under the exposure of UV light for 90 min.
- Since, the NCs with the lower concentration of CuO coated on ZnO exhibited effective separation and transport of photo induced electron-hole pairs than that of pure ZnO, the NCs with lower amount of CuO coated on ZnO enhanced the photocatalytic degradation as compared to others. Therefore, it was found that, NCs with lower amount of copper have potential to degrade MB , and can be used for the waste water treatment.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the Department of Chemistry, Amrit campus, for laboratory support, and other international supportive hands for characterizations.

REFERENCES

1. Bekre, A. G., Tufa, L. T., Zelekew, O. A., Goddati, M., Lee, J., & Sabir, F. K. (2022). Green Synthesis of a CuO–ZnO Nanocomposite for Efficient Photodegradation of Methylene Blue and Reduction of 4-Nitrophenol. *ACS Omega*, 7(35), 30908–30919.
2. Fouda, A., Salem, S. S., Wassel, A. R., Hamza, M. F., & Shaheen, T. I. (2020). Optimization of green biosynthesized visible light active CuO/ZnO Nano-photocatalysts for the degradation of organic methylene blue dye. *Heliyon*, 6(9), e04896.
3. Thatikayala, D., & Min, B. (2021). Ginkgo leaves extract-assisted synthesis of ZnO/CuO nanocrystals for efficient UV-induced photodegradation of organic dyes and antibacterial activity. *Journal of Materials Science: Materials in Electronics*, 32(13), 17154–17169.

International Chemistry Congress - 2023 "FRONTIERS IN MATERIALS SCIENCE" @NCS, KATHMANDU, NEPAL (May 11-13, 2023)

kunjan MSc Thesis 6-4.docx

ORIGINALITY REPORT

17%

SIMILARITY INDEX

PRIMARY SOURCES

1	worldwidescience.org Internet	237 words — 1%
2	rulrepository.ru.ac.bd Internet	191 words — 1%
3	"Plants for Immunity and Conservation Strategies", Springer Science and Business Media LLC, 2023 Crossref	172 words — 1%
4	prr.hec.gov.pk Internet	167 words — 1%
5	studentsrepo.um.edu.my Internet	159 words — 1%
6	pdfcookie.com Internet	140 words — 1%
7	www.science.gov Internet	101 words — 1%
8	www.researchgate.net Internet	92 words — 1%
9	Onditi, John. "Epidemiology and Control of Potato Virus Y in Kenya.", Wageningen University and Research, 2021 ProQuest	87 words — 1%
10	"Plant Tissue Culture: Propagation, Conservation and Crop Improvement", Springer Science and Business Media LLC, 2016 Crossref	79 words — < 1%
11	Tsokyi Choera, Brittney Jung-Hynes, Derrick J. Chen. " Comparative study of GenePDC GBS LB Assay and GeneXpert GBS LB Assay for the detection of Group B in prenatal screening samples ", Cold Spring Harbor Laboratory, 2019 Crossref Posted Content	79 words — < 1%
12	Nandy, Debmalya. "Covariate Information: A Novel Approach to Sufficient Dimension Reduction & Feature Screening for Ultrahigh-Dimensional Covariates in Supervised Problems.", The Pennsylvania State University, 2020 ProQuest	68 words — < 1%
13	eprints.hec.gov.pk Internet	58 words — < 1%