IN VITRO CLONAL PROPAGATION OF SPINE GOURD (*Momordica dioica* Roxb.)

THESIS

Submitted to
Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola
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Enrolment Number – OO-1835

2023

DECLARATION OF STUDENT

I hereby declare that the experimental work and its interpretation in the thesis entitled **"IN VITRO CLONAL PROPAGATION OF SPINE GOURD (*Momordica dioica* Roxb.)"** or part there of has neither been submitted for any other degree or diploma of any university, nor the data have been derived from any thesis / publication of any university or scientific organization. The source of material used and all assistance received during the course of investigation have been duly acknowledged.

Place: Akola **(Gajbhiye Amar Jayprakash)**

Date: / / 2024 **Enrolment No. OO-1835**

CERTIFICATE

This is to certify that thesis entitled **"IN VITRO CLONAL PROPAGATION OF SPINE GOURD (*Momordica dioica* Roxb.)"** submitted in partial fulfillment of the requirement for the degree of **"Master of Science in Agriculture (Agricultural Biotechnology)"** of the Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola is a record of bonafide research work carried out by **Gajbhiye Amar Jayprakash** under my guidance and supervision.

The subject of the thesis has been approved by the Students Advisory Committee.

Place: Akola
Date: / /2024

(Dr. D. R. Rathod)
Chairman
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Place : Akola **(Gajbhiye Amar Jayprakash)**

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(D) LIST OF ABBREVIATIONS

/	:	Per
%	:	Per cent
±	:	Plus or minus
^0C	:	Degree Celsius
pH	:	Hydrogen ion concentration
LAF	:	Laminar air flow
½ MS	:	Half strength Murashige and Skoog medium
2, 4-D	:	2, 4-Dichloro phenoxy acetic acid
$2H_2O$	:	Dihydrogen monoxide
LGA	:	L-Glutamic Acid
ABA	:	Abscisic Acid
BA	:	Benzyl adenine
$AdSO_4$	:	Adenine sulphate
Av.	:	Average
FYM	:	Farm Yard Manure
BAP	:	Benzyl Adenine Purine
$CaCl_2$	:	Calcium chloride
$CaCl_2.2H_2O$	:	Calcium Chloride Dihydrate
CH	:	Casein hydrolysate
µM	:	Micromolar
N	:	Normality
cm	:	Centimetre
CRD	:	Completely randomized design
DDW	:	Double distilled water
et al.	:	Et. Alibis (and associates)
Fig.	:	Figure
viz.	:	Videlicet (namely)
@	:	At the rate

v/v	:	Volume by volume basis
w/v	:	Weight by volume basis
ml	:	Mililiter
g	:	gram
mg	:	Miligram
mg/L	:	Milligram per litter
g/L	:	Gram per liter
GA_3	:	Gibberellic acid
HCl	:	Hydrochloric acid
NaOH	:	Sodium hydroxide
HEPA	:	High Efficiency Particulate Air
$HgCl_2$	:	Mercuric chloride
hr.	:	Hour
i.e.	:	That is
IAA	:	Indole 3-acetic acid
IBA	:	Indole 3- butyric acid
NAA	:	Naphthalene acetic acid
Kn	:	Kinetin
S.E.	:	Standard Error
CD	:	Critical Difference

E) THESIS ABSTRACT

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b)	Full name of student	:	**Gajbhiye Amar Jayprakash**
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ABSTRACT

The present study entitled "*In-vitro* clonal propagation of Spine gourd (*Momordica dioica* Roxb.)" was conducted during years 2021-22 and 2022-23 at plant tissue culture laboratory of Dr. PDKV, Akola. Objectives were to identify suitable explant for multiple shoot proliferation and to optimize nutrient media for explant establishment and multiple shoot formation using CRD design with three replications and 11 treatments of nutrient media combinations.

Results were obtained with pooled means. Sterilization of spine gourd local genotype using shoot tip and nodal segment excised from female plants with Bavistin 0.1% for 25-30 min., Streptomycin and $HgCl_2$ both at 0.1% for 5 min, respectively. Nodal segment showed significantly higher rate of survival, initial culture establishment, high rate of multiple shoots and shoot-let proliferation as compare to the shoot tips of female explants. Therefore, only nodal segment was used for further *in-vitro* propagation studies. The effect of MS medium with BAP and L-Glutamic acid using nodal explant female plants of Spine gourd, MS + BAP (2.0mg/L) + LGA (2.0mg/L) showed highest survival percentage of 75.47%, minimum day for initiation (7.08 days), maximum number of shoot per explant (7.61 shoot multiples), number of leaves per culture was 7.59, and same treatment results into 5.46cm average shoot length after 4^{th} week of inoculation. During subsequent sub-culturing studies observations made about the commencement, proliferation, and growth of multiple shoots revealed that multiplication index of shoot was always seen more after doing subculture. It has also noticed that among all the treatment, T_9 with MS + BAP (2.0mg/L) + LGA (2.0mg/L) performed significantly better as compared to other treatments for all traits under study. After 2^{nd} and 3^{rd} subculture, the maximum multiplication index of shoot was obtained in MS + BAP (2.0 mg/L) + LGA (2.0 mg/L) with 8.89 and 8.97, maximum number of leaves 10.62 and 11.93 and maximum length of shoot 6.10 cm and 6.89 cm were observed in MS + BAP (1.0 mg/L), respectively. The proposed protocol can be effectively used for nodal segment used as explant source only for achieving initial culture establishment, good survival and getting high shoot multiplication rate in spine gourd.

CHAPTER I

INTRODUCTION

1.1 Background Information

Spine gourd (*Momordica dioica* Roxb.) (2n=28) having vernacular names such as Teasle gourd in English, Spine gourd or Kakrol in Hindi, Kartule, Kartoli in Marathi, Meetha Karela, Bhaat Karela, etc. it is an important but underexploited rare vegetable crop. It is a dioecious, perennial, herbaceous climber having tuberous roots belonging to cucurbitaceae family, under the natural order cucurbitales.

Spine gourd is believed to be originated in the tropics of old world. Spine gourd is probably native of India, plants are distributed from Himalayas to Sri Lanka, up to an altitude of 1500m. Plants of Spine gourd are found naturally growing in hilly tract of Rajmahal, Hazaribagh and Rajgir of Jharkhand and Wet hills of Maharashtra, Assam and West Bengal. West Bengal and Karnataka are two major Indian States that grow Spine gourd commercially. Spine gourd is largely cultivated in Malda and Nadia districts of West Bengal.

Spine gourd is grown for its immature nutritious tender fruits. This high economic value and export oriented crop has been originated in tropical Asia*.,* Indian sub-continent and distributed through-out the country up to an altitude of 1500 m (Shekhawat *et al*., 2011). Spine gourd has glabrous stem, broad ovate leaves, entire deeply 3-5 lobed; yellow solitary flowers, ovoid or ellipsoid fruits, 2.5-6.3 cm long, shortly beaked, densely echinate with soft spines; seeds slightly compressed, 67 mm long, irregularly corrugated. Spine gourd fruits have very good shelf life along with fruits, young leaves, flowers and seeds also edible (Ghive *et al*., 2006a). Spine gourd grows in warm and humid weather and tuberous roots are planted in pits. Plantation is done at the onset of summer with the first shower, in the month of May while flowering starts from June-July and fruiting ends in September to December. The plants undergoes dormant during winter season (Rasul *et al*., 2007). It is a high demandable seasonal vegetable and medicinal plant, fruit yield varies at 75-100 q/ha at optimum management (Bharathi, 2007). It is free from

cholesterol and has adequate amount of high energy (45.74 Kcal), water (84%), minerals and vitamins (Gopaln *et al*., 1994). Fruits are rich source of protein, calcium, phosphorus, iron and highest amount of carotene (162mg/100g edible portion) among cucurbits (Ram *et al*., 2002). Fruits are also a rich source of lectins, triterpinse of ursolic acid, momordic ursenol, vitamins, iodine, flavonoids and glycosides (Ali and Srivastava 1998; Singh *et al*. 2009) and is described as “Good supplement for nutrient” (Aberoumand, 2010).

1.1.1 Nutritional Value

Spine gourd possess high nutritional value. Nutritional value of Spine gourd per 100 g edible fruits consists of Moisture (84.14 g), Carbohydrates (4.2 g), Protein (3.1 g), Fat (1.0 g), Minerals (1.1 g), Fiber (3 g), Calcium (33 mg), Phosphorus (42 mg), Iron (4.6 mg), Carotene (4.6 mg), Thiamine (1.62 mg), Riboflavin (0.18 mg) and Niacin (0.6 mg) (Ram *et. al*., 2002).

1.1.2 Medicinal Background

The medicinal properties of spine gourd are sex-specific and only female plants have medicinal values (Shastri *et al.,* 1962). Fruits have its own value in preparation of medicines against ulcer, piles, sores and obstruction of liver and spleen as appetizer and astringent (Ghive *et al.,* 2006c). Fruits are also used to cure the asthma, leprosy, tumor, excessive salivation and heart diseases. Ayurvedic practitioner prescribed spine gourd fruit to cure diabetes and fruit powder is used to induce sneezing leading to nasal clearance. Leaf decoction of female spine gourd is used as aphrodisiac, to eliminate parasites in intestine, fever and respiratory disorder (Kumar and Prajapati, 2003). Seeds are quite helpful for relieving chest problem and urinary discharge. The roots are used in bleeding piles, bowl infection, urinary complaint and also in headache, kidney stone, jaundice and root paste is applied on body as sedative (Jain and Singh, 2010). Root paste is applied on body as sedative (Chakravorty, 1959). In traditional days it was used in treatment of Snake bite, Scorpion sting and also used as abortifacient. Along with these medicinal properties, they also possess anti-bacterial, anti-oxidant, anti-inflammatory, anti-lipid, anti-cancerous, anti-fertility, anti-allergic,

peroxidative and nematicidal. It has prominent position among cucurbitaceous vegetables owing to its good nutritional and medicinal value, rich taste, high keeping quality, ability to withstand long distant transportation, high economic return and good export potential (Rasul *et al.,* 2007).

Considering to multifold uses of Spine gourd, the systemic improvement in cultivation would be a boon both for horticulture as well as pharmaceutical industries especially in Ayurveda.

1.2 Importance and need of the study.

Spine gourd is well acclimatized to Indian climate, but still it is underutilized. Its popularization will add one more vegetable to Indian dishes, it will help improving per capita consumption of vegetables of Indians. Present per capita consumption of country is around 140 g, which is very low than requirement. The major hurdle in its popularization is unavailability of sufficient and quality planting material.

The process of *in vitro* propagation includes the selection and isolation of a cell, a tissue or organ which are termed as explants, the surface sterilization and incubation of isolated explants in a growth-promoting medium having essential nutrients, phytoharmones with controlled pH, temperature, humidity in its environment. The fact behind the *in vitro* cloning of isolated single cells demonstrated the fact that somatic cells, under an appropriate condition can differentiate to a whole plant. The basic principle of tissue culture is the potential of cells or tissue to regenerate a plant is termed totipotency. This principle of totipotency was discovered by Gottlieb Haberlandts in 1902.

Spine gourd has high demand in market but still remain underutilized and underexploited mainly due to its vegetative mode of propagation and dioecious nature. Conventional commercial propagation of Spine gourd largely depends on tuberous roots (Nabi *et al.* 2002), followed by stem cuttings, and seeds. Commercial multiplication using the tuberous roots and stem cuttings are critically limited due to inadequate availability of tuberous roots and late availability of stem cuttings in fruiting season. Tuberous roots have low multiplication rate and occupies the valuable

cultivable land until next planting season. Male plants dominate natural populations and sex determination is possible only when the plants start flowering. Since fruits are the main edible portion of this species, which are harvested on female plants, it is desirable to have commercial fields with a large proportion of female plants.

This vegetable has immense potential for contribution to a particular pocket of food production because they are well adapted to existing as well as adverse climatic condition and are generally resistant to pests and pathogens. This popular vegetable has high demand in market but still remained as underutilized, underexploited and minor cucurbitaceous vegetable due to its dioecious nature and conventional method of vegetative propagation (Bharathi *et al.,* 2007). The commercial multiplication using tuberous roots is critically limited due to low rate of multiplication and dormancy, because of which it occupies a valuable cultivable land until next planting season. The problem arises with the stem cutting are late availability in fruiting season, along with stem cuttings containing 2-3 nodes from dark green vines of 2-3 months old plants are planted, but only 36 % of plants will sprout and survive. The difficulties in seed propagation are hard seed coat, prolonged seed dormancy (4-5 month), limited seed germination (10%) and unpredictable sex ratio in seedling progenies before flowering (Mondal *et al.,* 2006). Due to its dioecious nature, poor natural pollination of female flowers results in lower yield. To compensate this problem plantation needs 5-10 % male plants as pollinizer and it is imperative for good fruit set (Rasul, 2007). It is highly cross pollinated in nature and exhibit genetic variation for morphology and growth parameters.

As the conventional methods of spine gourd propagation impose several limitations for large scale production of sex specific plants, an efficient clonal propagation is must. Improvement of plant species via biotechnological approach depends on plant tissue culture. Micropropagation helps to overcome above said problems in great extent and systematic improvement is boon for horticulture, pharmaceutical industry and Ayurveda. High multiplication ratio achieved rapid multiplication of disease and pest free elite plant within short span of time and space (Ghive, 2006b). An *in vitro*

propagation system offers unlimited availability of planting material early in the planting season. It can be employed as an alternative means for genetic upgradation and its application largely depends on a reliable plant regeneration system. The application of micropropagation is well-established for rapid large-scale propagation of many crops including cucurbitaceous vegetables. Micropropagation has been reported in shoot tip and nodal explants of Cucurbitaceae such as *Citrullus lanatus* (Barnes *et al.,* 1978) and *Cucumis sativus* (Vasudevan *et al.,* 2001; Ahmed and Anis, 2005).

1.3 Objectives of study

Present study is carried out with the following objectives. Objective of the study are-

i. To identify suitable explant for multiple shoot proliferation

ii. To optimize nutrient media for explant establishment and multiple shoot formation

1.4 Hypothesis

Spine gourd has number of problems, including poor natural pollination of female flowers and low yield. Germination is very difficult or impossible because of its hard seed coat. Moreover it is impossible to predict sex of the seed producing plants before flowering. Propagation by tuberous roots is limited due to low multiplication rate (Mondal *et al.,* 2006) and occupies the valuable cultivation land until next planting season.

1.5 Scope and limitations of the study

Modern biotechnology offers viable tools that are beneficial to any crop improvement programmes. That includes minimizing duration and dependence on seasonal factor for any crop breeding programme through tissue culture. *In-vitro* propagation in tissue culture is the foundation on which all biotechnological research tests, as almost all uses of plants biotechnology ultimately require the successful culture of plant cells, tissue or organ. *In-vitro* propagation in a plant tissue culture technique is used for producing many clone plantlets and implies the culture of the aseptic controlled environmental condition.

The present study reveals some limitations of the Spine gourd vines in *in vitro* propagation.

1) Availability of explant: As the Spine gourd is seasonal cucurbitaceaous vine, mostly available in monsoon season. It is the important limiting factor in *in-vitro* propagation of Spine gourd. This problem is solved by conserving Spine gourd vines in protected structures for further inoculation.

2) Handling of plant material: Proper handling during *in vitro* propagation is necessary to reduce contamination losses from *in vitro* condition to acclimatization stage. Careful handling is necessary as the explants that is vines are delicate.

3) Cost of Plants: Initial cost of per plant is high due to high cost of machineries and skilled labours are required in laboratory.

CHAPTER II

REVIEW OF LITERATURE

Spine gourd (*Momordica dioica* Roxb.) is one of the underexploited vegetable crop. Very little efforts have been made to improve this crop. The present investigation entitled *in-vitro* shoot proliferation studies in Spine gourd (*Momordica dioica* Roxb.) was conducted with objectives to optimize the parameters for initial establishment of explants and to optimize the media composition for shoot multiplication.

Earlier worker's contribution in respect of Spine gourd on *in vitro* conditions, and relevant cucurbitaceous vegetables are summarized as review of their contribution as under.

Murashige and Skoog (1962) published a well-defined medium for tobacco culture, which has probably been cited more than any other media for culture of wide variety of plant species including both dicots and monocots.

Vasil and Hildebrandt (1965) obtained differentiation of completely organized Tobacco plant from single cell.

Lineberger R. D. (1980) gave a general protocol for *In-vitro* propagation. The growing tissues were first surface sterilized then placed on nutrient media and observed rapid shoot proliferation with elongated shoot tip and adventitious shoots in 8 to 12 weeks. Growing shoots were transferred on fresh media at regular intervals to continue proliferation. The small shoots were removed and rooted in a separate sterile medium.

Owe*n et al.* (1991) reported that growing plants can utilize nutrients only if the growth medium has optimum pH. Hence, pH becomes the most important and crucial factor in plant tissue culture. The pH of the basal medium can be adjusted with 1N HCl or NaOH.

2.1. *In vitro* culture establishment

2.1.1 Parameters for Initial establishment of explants

2.1.1.1 Selection of Explants

Explants are small pieces of plant parts or tissues that are aseptically cut and used to initiate a culture in a nutrient medium. Different

parts of the plants are used as explants for micro propagation. The correct choice of explant material can have an important effect on the success of tissue culture. The choice of explant depends the kind of culture to be initiated, purpose of the proposed culture and the plant species to be use. Depending upon the goal or objective the choice of the explants differs. For the clonal propagation, the lateral or terminal bud or shoot will serve as better explant. However, for the callus induction, pieces of cotyledons, hypocotyl, stem, leaf or embryo may be used as explant depending upon the crop and genotype. The studies have been carried out in case of spine gourd for the selection of explants are summarized briefly hereunder suitable subheadings.

The culture establishment mainly depends on type, size and age of explant, the methods of sterilization followed and the nutrient medium used for culture establishment.

Senevirate and Flegman (1996) reported that, for better performance in *In-vitro* conditions axillary buds which are neither too closer nor too far from apex is to be selected in *Hevea brasiliencis.*

Geeta (1999) found that the axillary buds of 20mm size gave maximum establishment of 85.5% than apex bud (40%) and 20 mm size of explant is better than 10 mm size of explant for establishment in Spine gourd.

Kulkarni (1999) conducted micropropagation studies in Kartoli by using nodal segment as an explant and developed a proper method of *in-vitro* regeneration and multiplication. MSHP + 80 ppm $AdSO_4$ + 10 ppm BAP + 5.0 ppm IBA +100 mg/L myo-inositol + 0.8% Agar agar + 3% sucrose gave the best results (75%). The same medium gave the maximum multiple shoots per culture (81±1.28) at the end of 4^{th} subculture. It was found that the nodal segment cultures of Kartoli initiated maximum rooting response (86.66%) to the medium, MS basal+ 3.0 ppm NAA + 0.8% Agar agar + 3% sucrose + 0.2% activated charcoal. Among the different potting mixture compositions tried for hardening of the in-vitro developed plantlets vermiculite alone gave maximum (77.33%) survival and the lowest survival was observed by using FYM (20%) alone.

Deokar *et al.* (2003) reported that response of explants viz. shoot tips, axillary buds, hypocotyl and cotyledon of Spine gourd for *in vitro* establishment and they observed that 0.5 to 1.0cm long axillary buds of Spine gourd were proved to be the best for Micro-propagation.

Abdul-Awal *et al.* (2005) reported that in *Trichosanthes cucumerina* the rate of shoot multiplication was maximum 12.00±0.70 after 4th sub culture at concentration of 1.0 mg/L BAP in combination with lower amount of 0.1 mg/L NAA. Out of different chemical combinations used, 100% multiple shoot formation was noticed in 1.0 mg/L BAP + 0.2 mg/L NAA.

Sultana (2005) studied the effect of different levels of pH and agar infused with different concentrations of sucrose on shoot induction in *Momordica Charantia*. Maximum shoot induction was recorded in medium containing MS + (2.0 mg/L) BAP + (0.2 mg/L) NAA, with 30 g/L sucrose, 7 g/L agar and 5.5-6.0 level pH.

Ghive *et al.* (2006) tried different explants for standardizing the protocol for micropropagation of Spine gourd (*Momordica dioica* Roxb) and opined that axillary buds were the best explant for in-vitro propagation. Further, they concluded that combination of MS + (1.5 mg/L) BAP + (1.0 mg/L) NAA was found to be the best for establishment and initiation of explant. The treatment combination of MS + $AdSO_4$ (70 mg/L or 80 mg/L) + BAP (1.0 mg/L) + NAA (1.0 mg/L) was adjudged to be superior for multiple shoot development. In addition to this, they opined that MS + $AdSO_4$ (80 mg/L) + IBA (1.0 mg/L) was found to be the best treatment for induction and development roots. The maximum survival of plantlets in primary hardening (54.44) was observed on soilrite:cocopeat (3:1) while as regards the local genotypes under study, during secondary hardening, they found that 95 per cent plantlets were survived in case of AKSG-35 followed by AKSG-5 (90%) and AKSGM-1 (75%).

Rai *et al*. (2012) reported an efficient protocol for rapid *in vitro* clonal propagation of Spine gourd (*Momordica dioica* Roxb.) in genotype RSR/DR15 (female) and DR/NKB-28 (male) was developed through enhanced axillary shoot proliferation from nodal segments. Maximum shoot proliferation of 6.2 shoots per explant with 100% shoot regeneration

frequency was obtained from the female genotype on Murashige and Skoog's (1962) medium supplemented with 0.9µM N6-benzyladenine (BA) and 200 mg/L casein hydrolysate (CH). While from the male genotype the optimum shoot regeneration frequency (86.6%) and 6.4 shoots per explant was obtained on MS medium supplemented with 2.2µM BA. CH induced vigorous shoots, promoted callus formation, and proved inhibitory for shoot differentiation and shoot length, especially in explants from male genotype.

Rajashekharan *et al.* (2012) conducted investigations on the *in vitro* propagation and conservation of *Momordica sahyadrica* species. The various explants from *in vitro* grown seedling were cultured on modified MS medium (BAP, BAP + NAA/IBA). They observed shoot differentiation on MS medium supplemented with BAP. Further, shoot as well as root differentiation was obtained on medium containing BAP + IBA/NAA. Multiple shoots with roots were formed on MS medium without hormones. Rooting was induced on shoots in medium containing IBA and 40% of the plants survived successfully when transferred to the field. *In vitro* grown shoots were conserved for six months without subculture.

Thiruvengadam *et al.* (2012) developed efficient protocol for *in vitro* regeneration by using encapsulated shoot tip as an explant in Spine gourd. They obtained 100% conversion into plantlets from encapsulated shoot tip explants when placed on 0.5µM BAP supplemented full strength MS containing the 0.7% agar. Hardened and acclimatized plant in field reported the 90% survival rate and grew well without considerable variation in Spine gourd.

Kausar *et al.* (2013) noted that in *Beninca hispida* shoot tip showed the highest rate of multiple shoots at (1.5 mg/L) BAP + (0.2 mg/L) GA_3, where normal number of shoots per culture recorded was 5.55. The lower concentration of GA_3 induced multiple shoots effectively.

2.1.1.2 Disinfection of explants

Explants should be free from surface contaminants to achieve growth and development under aseptic conditions and this is accomplished by

surface sterilization. The sterilization treatment should be so selected that it kills the microbes without disturbing the plant tissue adversely.

De Fossard (1985) suggested a combination of physical methods (aimed at reducing the size of microbial populations) and chemical methods (killing remaining microbes) leading to microbe free culture.

Bhat (1992) reported that 90% aseptic cultures from excised lateral buds of Rose when surface sterilized with $HgCl_2$ (0.1%) solution for 8 minutes followed by rinsing with sterile distilled water.

Geeta (1999) obtained significantly superior aseptic cultures of Spine gourd by surface sterilization with $HgCl_2$ (0.1%) for 10 minutes over all the treatments of saturated chlorine water and sterile double distilled water.

Salvi *et al*. (2000) noted that when explants washed thoroughly with detergent and tap water. They were further rinsed with 70% alcohol for 30sec and sterilized with 0.1% $HgCl_2$ for five min followed by six washes with autoclaved distilled water showed less contamination.

Deokar *et al.* (2003) reported that the treatment of 0.1% $Hgcl_2$ for 5 minutes for sterilization was effective for initiation of aseptic cultures of Spine gourd.

Robinson *et al.* (2009) washed the nodal segments of Insulin plant under running tap water followed by a detergent, Tween-20 for a min. After continuous washes in double distilled water, surface sterilization was done with 0.1% $HgCl_2$ for 4-8 min. The results showed only 15% of contamination.

Mustafa *et al*. (2012) reported that good amount of compact and green callus from nodal cultures of *Momordica dioica*, could be achieved on MS medium fortified with 2.0 mg/L 2,4-D + 1.0 mg/L BAP. They also reported that high frequency of regeneration of plantlets on the BAP + 1.0 mg/L IAA supplemented medium after the sub culture.

Jadhav *et al.* (2015) studied the effect of two different sterilizing agents *viz*., ethanol and $HgCl_2$ at different concentration and duration on young leaves and nodal part of Insulin plant. The result confirmed that the $HgCl_2$ 0.3% for 5 min proved best as it resulted in least contamination.

Jamatia (2016) developed an efficient plant regeneration protocol in spine guard by using two explants viz. *in vivo* nodal and leaf as well as *in vitro* nodal and leaf explants respectively. Among the different treatments of TDZ and BAP studied, she reported maximum shoot induction (71.6%) in a media containing 1.5 mg/L TDZ while the highest shoot induction response (83.3%) was noticed in 1.5 mg/L BAP supplemented media. The 100% shoot regeneration was obtained from *in vitro* nodal segment in TDZ (0.5-2.5 mg/L) alone, BAP (1.0-2.0 mg/L) alone and NAA fortified media. Further she opined that for shoot multiplication, 0.5 mg/L BAP alone and in combination with 1.0 mg/L kinetin were most suitable.

2.1.2 Parameters for media composition for shoot multiplication

2.1.2.1 Culture medium

For obtaining desired responses in tissue culture, the role of culture medium, growth regulators and their concentration play a major role in their growth. The most important development in the tissue culture of the plants were made with the discovery of growth regulators such as auxins, gibberellins, cytokinins and abscissic acid and other organic compounds.

Murashige and Skoog (1962) introduced the basal media for the *in-vitro* propagation of herbaceous perennial plant species by supplementing it with distinct combination of growth regulators.

Geeta (1999) reported size and type of explants of Spine gourd, the axillary buds of 20mm size gave maximum establishment of 85.5% than basal, buds of vine (22.5%) in culture media MSHP + (80 ppm) $AdSo_4$ + (10 ppm) BA + (5 ppm) IBA + (0.8%) Agar + (3%) Sucrose.

Deokar *et al.* (2003) found that axillary buds gave maximum culture establishment (80%) in medium MSHP + (10 mg/L) Kin + (5.0 mg/L) IBA + (0.9%) agar agar. It was observed that higher concentration of cytokinin than auxin in combination gave better results with agar agar than gelrite.

2.1.2.2. Composition for Shoot multiplication

Hoque *et al*. (1982) found that a combination results of 1.5 mg/L Glutamic acid were more suitable for adventitious multiple shoot induction,

where as in our investigation (2.0 mg/L) BAP and (2.0 mg/L) LGA was to be the proved best for the production of multiple shoots.

Hoque *et al.* (2000) noted shoot differentiation from excised cotyledons of developing green fruits (18 days after anthesis) of tetraploid *Momordica dioica* to standardize a method for the rapid multiplication of *Momordica dioica* through *in-vitro* regeneration. High frequency callus formation occurred on Murashige & Skoog's (MS) medium supplemented with (0.2-1 mg/L) 2,4-D alone, or with (0.2-1mg/L) NAA alone or in combination with (1-2 mg/L) benzyladenine or (1-2 mg/L) kinetin. Growth, morphological nature and organogenic potentiality of the calluses varied with the growth regulator supplements. All combinations of benzyladenine with NAA increased the organogenic potential of the primary callus. The medium containing both (2 mg/L) benzyladenine and (0.5 mg/L) NAA elicited the highest shoot regeneration rate (58.1% from the apical and 71.3% from the basal half of cotyledon explants). However, the maximum number of shoots per cotyledon explant regenerated on the medium containing (2 mg/L) benzyladenine and (0.2 mg/L) NAA. Callus culture was maintained for a long time through subculturing on MS medium + benzyladenine or kinetin only, or in combination with NAA or IAA for shoot regeneration. The *in vitro* regenerated shoots were rooted on MS medium supplemented with (1 mg/L) IAA and successfully transferred to soil.

Ahamad and Anis (2005) reported addition of casein hydrolysate 200mg/L to the shoot induction medium (MS + BAP) significantly enhanced the number of multiple shoots in *Cucumis sativus* L. but Casein hydrolysate 200mg/L + 0.9µM BAP helped in enhancing the axillary shoot proliferation in case of nodal explants of Spine gourd. Highest number of shoots i.e., 6.2 shoots per explants was recorded with the 100% shoot regeneration frequency. Especially in case of male genotype CH helped in inducing the callus formation healthy shoots and proved inhibitory action for the shoot length and shoot differentiation.

Sultana *et al*. (2005) studied the effects of different concentrations of sucrose, agar and different levels of pH on *in vitro* axillary shoot multiplication of *Momordica charantea* L. The nodal segments from the

field grown plant were used as testing plant material. For maximum number of shoot induction and multiplication in MS medium containing 2.0 mg/L BAP +0.2 mg/L NAA with 30 g/L of sucrose, 7 g/L of agar and 5.5-6.0 levels of pH proved more effective.

Ghive *et al.* (2006) studied, “Micro propagation in Spine gourd (*Momordica dioica* Roxb)” with different explants, in which axillary buds proved to be the best explant for *in vitro* propagation. Different concentrations at cytokinins and auxins were used for establishment of axillary buds of Spine gourd, where, the treatment combination, MS + BAP 1.5 mg/L + NAA 10 mg/L was found to be the best for establishment and initiation of explant. The treatment combination of MS + $AdSO_4$ (70 mg/L / 80 mg/L) + BAP (1.0 mg/L) + NAA (1.0 mg/L) was found to be superior for multiple shoot development.

Haque *et al.* (2008) found that the best hormone for shoot multiplication of pumpkin was BAP (2 mg/L), in case of Ash-Gourd BAP was 1.5 mg/L. For callus induction BAP+ 2,4-D was best combination for pumpkin and it was 2.0+0.1 mg/L when in ash gourd BAP+NAA was the best combination.

Mahazabin *et al.* (2008) achived Micropropagation in Pumpkin (*Cucurbita maxima* Duch.) by using shoot tip of *in vitro* grown seed derived plants of two cultivars namely, Bikrompuri and Baromasi of Bangladesh. The excised shoot tips were cultured on MS medium containing KIN, BA, NAA at various levels of concentration and combination for shoot induction and proliferation, and best response was found at 3.0 mg/L of BA. Shoots were rooted most effectively in ½ MS medium supplemented with 1.0mg/L IBA. Bikrompuri was found more responsive than Baromasi for rapid clonal propagation.

Devendra *et al.* (2009) reported, plant regeneration via. organogenesis *in Momordica dioica* Roxb. using leaf derived callus. The maximum morphogenic callus induction rate (80%) was observed from leaf explant by culturing in MS medium supplemented with (1.0mg/L) 2,4-D + (2.0 mg/L) BAP. Callus size and fresh weight increased substantially through sub culturing. The highest percentage of shoot regeneration (70%) and highest mean number of shoots (12.33) per culture were obtained with (1.5 mg/L)

BAP + (1.5 mg/L) Kn. Leaf explants were more responsive than node explants in terms of callus induction and subsequent regeneration.

Karim and Ahmed (2010) studied *in vitro* somatic embryogenesis and subsequent plant regeneration in callus cultures derived from internode, node, shoot tip, petiole and leaf explant of Teasle gourd plant on semi-solid Murashige and Skoog (MS) basal salts and growth regulators supplemented with 1.0 mg/L BAP, 0.1 mg/L NAA and 30 g/L, (w/v) sucrose. Somatic embryos proliferated rapidly by somatic embryogenesis after 4 weeks. The embyogenic callus germinated on MS salts and growth regulators supplemented with 1.0 mg/L BAP and 0.1 mg/L NAA.

Shekhawat *et al.* (2011) studied, an *in vitro* propagation method for female plants of *Momordica dioica* Roxb. The nodal segments were harvested and the cut ends of the explants were sealed with wax and then surface sterilized and cultured. Bud breaking occurred on Murashige and Skoog's (MS) agar-gelled medium + 2.0 mg/L 6-Benzylaminopurine (BAP) + 0.1 mg/L Indole-3 acetic acid (IAA). The cultures were amplified by passages on MS medium supplemented with 1.0 mg/L BAP + 0.1 mg/L IAA. Further, shoot amplification (29.2 shoots per vessel) was achieved by sub culturing of *in vitro* regenerated shoot clump on MS medium + 0.5 mg/L BAP + 0.1 mg/L IAA.

Debnath *et al.* (2013) noted high frequency *in vitro* regeneration and multiplication of shoots of female *Momordica dioica* Roxb. has been established from callus derived from nodal explants culture. Callus was established on Murashige and Skoog's (MS) agar gelled medium supplemented with 2.0 mg/L 2,4-D and 0.5 mg/L BAP Coconut milk (15% v/v). Multiple shoot buds were developed from the callus when they were cultured on MS medium containing 4.0 mg/L 6-Benzylaminopurine (BAP) and CM (15% v/v). Further shoot multiplication (86 ± 3.44) was achieved by sub culturing of regenerated shoot clump on the same fresh medium. The shoots multiplication could be repeated for more than five cycles with normal morphogenesis without returning to the original explants source.

Kausar *et al.* (2013) studied the effect of external application of phytohormone on explants viz., shoot tips and nodal segments of Ash gourd

(*Benincasa hispida* L.). Shoot tips and nodal segments were cultured on MS medium supplemented with different concentrations and combinations of cytokinins (BAP, Kinetin), auxin (IBA, NAA) and gibberellic acid (GA_3) for multiple shoot formation and root induction. The highest number (up to 90%) of multiple shoot formation was obtained from the shoot tips in MS medium fortified with 1.5 mg/L BAP + 0.2 mg/L GA_3, where average number of shoots per culture was 5.55. In case of nodal segment, better response (up to 78%) for shoot multiplication was found in MS with 2.0 mg/L BAP + 0.2 mg/L GA_3.

Mustafa *et al.* (2013) reported that the, MS medium supplemented with 2.0 mg/L 6-Benzylaminopurine BAP + 2.0 mg/L L-Glutamic acid, the explants produced little amount of callus and shoot buds in Spine gourd. The shoot buds on successive subcultures for twice on the same medium produced multiple shoots. Shoot proliferation was further continued even after six months.

Parvin *et al.* (2013) studied rapid and efficient protocol for *in vitro* propagation of Muskmelon (*Cucumis melo* L.). Shoot tips, nodal segments and cotyledonary nodes from *in vitro* grown seedlings were used as explants. The explants were inoculated on MS medium fortified with different combinations and concentrations of growth regulators viz., BAP, NAA, GA_3 and IBA for multiple shoot regeneration. Effective result was found on MS medium supplemented with 2.0 mg/L BAP, in which 90% and 70% cultures induced multiple shoots from nodal segments and shoot tip explants, respectively. Whereas, 70% cultures of cotyledonary nodes were found to induced shoots on MS medium with 1.5 mg/L BAP + 0.1 mg/L GA_3.

Margareate (2014) reported that high frequency of multiple shoot regeneration was achieved on MS medium containing 1.0 mg/L BAP + 0.2 mg/L NAA + 20 mg/L L-glutamine while elongation of shoots were achieved by adding 0.5 mg/L GA_3 in *Cucumis anguria*.

Verma *et al.* (2014) reported MS with 0.5 mg/L BAP in monoecious Bitter melon more number of shoots (3.4) after 3rd sub culture with shoot length (2.7cm).

Jadhav (2015) carried out research on four different genotypes of Spine Gourd (*Momordica dioica* Roxb.) to standardize a reliable procedure for shoot and root initiation. The cultures were initiated using nodal explants of genotypes R1P17, R2P5, R9P7 and R11P5. He found that shoot initiation and shoot proliferation were greatly affected by the genotypes, medium combinations and their interactions. Further he reported MS + 1.0 mg/L BAP + 0.2 mg/L NAA medium for genotype R1P17 for initiation of early shoot while genotype R11 P5 produced a greater number of shoots in the same medium. Among all the genotypes under study, highest shoot length was produced by the R11P5 genotypes. The results revealed that the MS medium containing 0.5 mg/L NAA was the most effective medium for the root initiation in regenerated shoots of all the genotypes. The highest root length was reported by R1P17 genotype on MS + 1.0 mg/L IBA. The medium MS + 1.5 mg/L NAA recorded significantly minimum number of day to initiation of root and MS + 0.5 mg/L IBA recorded significantly maximum root length. The rooted shoots were successfully established in polythene bags containing sand, soil and FYM in 1:2:1 ratio. The established plants were finally transplanted in the field conditions.

Patel (2015) developed an efficient protocol for *in-vitro* shoot multiplication and regenerations of spine gourd as well as to check its antidiabetic activity by using nodal segments as explants. MS agar-gelled medium with optimum concentration of 1.5 mg/L BAP + 0.1 mg/L NAA and 0.5 mg/L NAA + 0.5 mg/L NB6 had an effect on callus production. Shoot multiplication was found best in 0.5 mg/L NB6 + 0.5 mg/L BAP. After 15 days, shoot length of 5.2±0.37cm and shoot numbers 10±1.4 were observed. In the investigations, he developed a novel method by which multiple shoot can be induced on MS medium supplemented with cytokinins (BAP, NAA and NB6). This is the first report in Momordica dioica, that he used the NB6 growth hormones for induction of callus and multiplication of shoot.

Jasmin and Mian (2016) noted a reliable and reproducible protocol to get healthy and wellformed callus from juvenile explants of Cucumber. The sterilized seeds of Cucumber cultivar were cultured on MS basal medium (Murashige and Skoog, 1962). The seeds germinated after 7

days of culture with 24 hours dark photoperiod. Explants from germinated seedlings were cultured on MS medium supplemented with individual treatments of different auxins [2,4-dichloro-phenoxyacetic acid (2,4D), α naphthalene acetic acid (NAA) or cytokinins (benzyl aminopurine (BAP)]. Plant parts such as leaves, stems and cotyledons were used as source of explants. Callus were initiated from leaves, stems and cotyledons after 4 weeks of culture. The optimum medium for callus induction from leave, stem and cotyledon explants was MS medium supplemented with 0.5 mg/L BAP added with 1.0 mg/L NAA. The highest percentage of callus was obtained from stem explants (89.0 ± 0.75 %) followed by leave (79.05 ± 3.28%) and cotyledon (74.43 ± 1.30 %) explants. Maximum callus induction in stems (73.05 ± 2.1%) was obtained in 1.0 mg/L concentration of BAP.

Choudhary *et al.* (2017) reported an improved and efficient micropropagation method for wild female *Momordica dioica* using nodal explants. Shoot amplification was achieved using sub-culturing of *in vitro* raised shoots on MS medium supplemented with various concentrations of BAP alone or in combination with IAA. The maximum number of shoots (45.30±3.83) with an average length 6.52±0.89 cm were differentiated on MS medium containing 0.5 mg/L BAP, 0.1 mg/L IAA and additives (50 mg/L 1 ascorbic acid, 25 mg/L each of adenine sulphate, citric acid and L-arginine). The cloned shoots were rooted ex-vitro. Each shoot treated with 250 mg/L IBA for 5 min, produced 12.3±1.33 roots with a mean length 5.4±0.73 cm. More than 85% (46 plants) of *ex-vitro* rooted plantlets were successfully hardened in a greenhouse with normal growth characteristics. In order to evaluate the genetic stability of micropropagated plants, the two PCR-based techniques, Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) were used. The amplification patterns of the micropropagated and mother plant were monomorphic thus depicting genetic stability of the micropropagation system.

Kapadia (2018) optimized the culture medium for higher multiplication and efficient micropropagation of spine gourd by using nodal explants. He reported that that supplementation of 1.0 mg/L NAA along with 1.0 mg/L BAP induced vigorous and healthy shoots with highest (4.75±0.25)

mean number of shoots with average shoot length of around 5.10±0.04 cm. Further, the regenerated shoots of 4cm in length were used for rooting purpose. The highest rooting percent (86.67%), number of roots per culture (9.6±0.50) and root length (4.59±0.09cm) with lowest percent callusing at cut ends (33.33%) was reported in the treatment M2 (½MS + IBA 2.0 mg/L). The well-developed shoots with roots were deliberately transferred to the polythene small size glass containing equal mixture of soil and vermicompost. The established plants were finely transplanted in the field conditions.

CHAPTER III

MATERIALS AND METHODS

The present investigation entitled "*In vitro* clonal propagation of Spine gourd (*Momordica dioica* Roxb.) was conducted at Centre of Excellence in Plant Biotechnology, Biotechnology Centre, Department of Agricultural Botany, Post Graduate Institute (PGI) Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Dist. Akola during the academic year 2021-22 and 22-2023. The detail of methods and methodology are given as follow.

3.1 Materials

3.1.1 Source of explants

The Spine gourd is a perennial climber and only vines are available in monsoon season, mostly found in wild areas, and farm borders of farmer's field. The material that is female vines used for present study are collected from Instructional Farm, Department of Vegetable Science, Dr. Panjabarao Deshmukh Krishi Vidyapeeth, Akola.

3.1.2 Collection of explants from plant material

The healthy tops of 4-5 months old spine gourd (female) genotype wild were collected immediately 1-2hr. before using the plant materials for research and kept in cool place.

3.1.3 Selection and Preparation of explant

3.1.3.1 Nodal segment

A stem part of 3.0 cm length containing an axillary bud was separated from the vein of matured field grown spine gourd vine. After sterilizing it, the final length was reduced to 2.6 cm by cutting 0.2 cm at both the cut ends without injuring the buds for further inoculation on culture media.

3.1.3.2 Shoot tip

The young top most shoot tips were collected along with the apical buds. The small leaves and tendrils are carefully removed from shoot tip without damaging the apical bud. Shoot tip of 3.0cm were taken and after sterilization only 0.2 cm of base portion of the shoot tip were removed and

further used for inoculation on culture media containing various combination of growth regulators.

3.2 Chemicals

The major, minor elements and other ingredients required for the preparation of media, sterilization of media were obtained from Himedia Laboratories Limited, Mumbai, India. The amino acids, vitamins and plant growth regulators used were of plant tissue culture grade from Himedia.

The details of the various laboratory chemicals used for media preparation and surface sterilization of the explants are given below:

a) Chemicals for media preparation

1. Salts of macro and micro elements of analytical grade.
2. Vitamins and amino acids.
3. Sucrose as a carbon source.
4. Myo-inositol.
5. Agar as a solidifying agent.
6. 1N HCl and 1N NaOH for pH adjustment.

b) Chemicals for surface sterilization

1. Bavistin
2. Tween 20
3. Streptomycin sulphate
4. Labolene
5. Mercuric chloride
6. Carbendazim
7. Ethanol/ Sprit

c) Plant growth regulators

1. Cytokinins: Benzyl amino purine (BAP).
2. L- Glutamic Acid

3.2.1 Glasswares and Laboratory Equipment's

1. Conical flasks having capacity of 250, 500 1000, 2000 and 3000 ml.
2. Beakers having capacity of 100, 250, 500 and 1000 ml.
3. Measuring cylinders of capacity 10, 100, 250, 500 and 1000 ml.
4. Pipettes of capacity 5 and 10 ml.
5. Micropipettes of capacity 20-100µl and 200-1000µl.
6. Autoclavable reagent bottles having capacity of (50, 100, 250, 500, 1000 and 2000 ml).
7. Culture bottles with polyurethane polyvinyl cap.

Table 3.1. Use of Laboratory equipments

Sr. No.	Instrument	Use
1	Refrigerator	To store stock solutions and plant growth regulators.
2	Autoclave	To sterilized glassware, DDW and nutrient media.
3	Hot air oven	To sterilize glass wares using dry heat.
4	Electronic digital balance	For weighing of chemicals.
5	Double distillation unit	To get double distilled water (DDW).
6	pH meter	To test / adjust pH of solutions/Media.
7	Shaker	Used for shaking explants
8	Laminar air flow unit (LAFU)	To carry out inoculation, sterilization work under aseptic condition.
9	Incubator	To incubate the cultures at required temperature and light.

3.2.2 Other materials

Materials which are of day use like trays, caps, autoclavable bags, zip lock bags for sample collection, parafilm, aluminum foil, nonabsorbent cotton, spirit lamp, forceps, scalpels, surgical blade, wash bottle, cotton, glass bead etc. were used for maintaining the aseptic culture.

3.2.3 Culture medium

In the present study, Murashige and Skoog (MS) (Murashige and Skoog, 1962) media was used in combination with different plant hormones for *in vitro* micro-propagation. The different stocks used to prepare MS media along with its compositions and concentrations of the respective elements are given in Table 3.2.

Table 3.2. Composition of Murashige and Skoog (MS) as basal media

Stock solutions	Constituent elements	Quantity used (g) (1 lit)	Stock solutions used (ml/L)
A	Ammonium Nitrate (NH_4NO_3)	33.0	50
	Potassium Nitrate (KNO_3) *	38.0	
	Calcium Chloride ($CaCl_2$. $2H_20$)	8.8	
	Manganese Sulphate ($MgSO_4.4H_20$)	7.4	
	Potassium dihydrogen Phosphate (KH_2PO_4)	3.4	
B	Potassium Iodide (KI)	0.83	5
	Boric acid (H_3BO_3)	6.22	
	Manganese sulphate ($MnSO_4.4H_2O$)	22.40	
	Zinc sulphate ($ZnSO_4.7H_20$)	8.60	
	Sodium molybdate (Na_2MoO_4 . $2H_2O$)	0.25	
	Copper sulphate ($CuSO_4$.$5H_2O$)	25	
	Cobaltous chloride ($CaCl_2$.$6H_2O$)	25	
C	Ferrous sulphate (FeSO4.6H20)	5.56	5
	Disodium salt of EDTA (Na2 EDTA)	7.5	
D	Thiamine HCl	10mg	5
	Nicotinic acid	50mg	
	Pyridoxine HCl	10mg	
	Glycine	200mg	

*Dissolved in hot water # Store in amber colour bottle

3.3 Methodology

3.2.1 Normal plant tissue culture procedure was followed for the execution of the present experiment

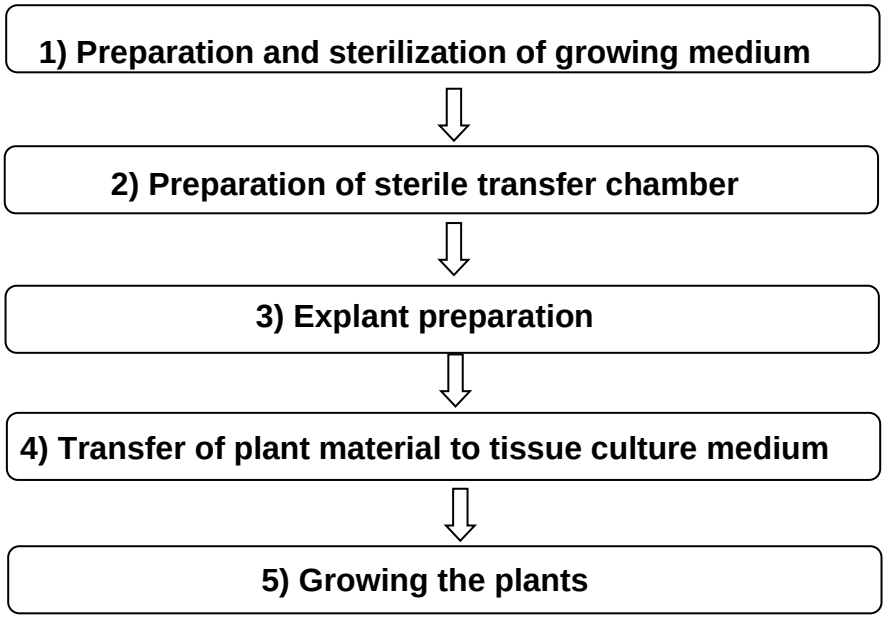


Fig. 3.1.Flow chart of procedure of plant tissue culture

Table 3.3 Experimental Details

(1) Crop	Spine gourd (*Momordica dioica* Roxb.)
(2) Variety	Local
(3) Experimental design	CRD
(4) Number of Treatment	11
(5) Number of replications	03
(6) No. of explants/ treatment	30
(7) Year	2022-2023
(8) Location	Center of Excellence in Plant Biotechnology, Biotechnology Centre, Department of Agricultural Botany, Dr. P. D. K. V. Akola

Table 3.4 Treatment Details

Treatment	Treatment Details
(1)	(2)
T_1	MS control media
T_2	MS+BAP(1mg)
T_3	MS+BAP (2 mg)
T_4	MS + BAP (1mg) + LGA (1.5mg)
T_5	MS+ BAP (1mg) + LGA (2mg)
T_6	MS+ BAP (1mg) + LGA (2.5 mg)
T_7	MS+ BAP (1mg) + LGA (3mg)
T_8	MS+ BAP (2mg) + LGA (1.5 mg)
T_9	MS+ BAP (2mg) + LGA (2mg)
T_{10}	MS + BAP (2mg) + LGA (2.5mg)
T_{11}	MS + BAP (2mg) + LGA (3 mg)

3.3.1 Washing of glasswares

All the culture vessels and glass wares used in preparation of the media and for other purpose were cleaned in Chromic acid (Potassium dichromate in sulphuric acid) by soaking the glass wares overnight. On the following day, they were thoroughly washed in tap water to remove all the traces of the chromic acid solution. Then they were washed with detergent solution (labolene) and again washed thoroughly with tap water and rinsed with double distilled water. They were allowed to dry on draining racks and stored in dust free cabinet till further they are sterilized.

3.3.2 Sterilization of Glasswares and Equipments

The washed glassware were sterilized in oven at 121°C.The culture bottles are closed with caps and autoclave at 121^{o}C. The petri plates and other glass wares were wrapped in plastic bags and then autoclaved at 15lbs/ psi pressure for 60 minutes and then stored in dust proof room for further use. Aseptic conditions were maintained by performing all operations in laminar air flow chamber. For the maintenance of working balance of laminar air, HEPA filters were cleaned after every 6 months. Before starting

any operation, the floor/walls of the laminar airflow were sterilized with 70% alcohol and UV exposure for 30-60 min.

Before transfer of the explants, all the surgical instruments were first dipped in rectified spirit or steripot then flamed on spirit lamp and cooled.

3.3.3 Preparation of 1 N NaOH and 1 N HCl solution

For preparation of 1N NaOH, 4 g of NaOH pellets were dissolved in reduced amount distilled water and volume made up to 100 ml. And 8.33 ml of 37% HCl solution mixed in 91.67ml distilled water gives 1N HCl solution. This solution is further used for adjusting pH of media.

3.3.4. Media Preparation

The modified MS basal media was utilized for *in-vitro* culture of Spine gourd explants. Media was prepared in double distilled water by using different chemical constituents.

3.3.5 Preparation of stock solutions

The stock solution A, B, C and D for the preparation of MS basal medium were prepared by dissolving the required amount of chemicals in reduced volume of distilled water as mentioned in Table 3.2. The procedure for preparation respective of stock solution is given below.

Stock A

The constituent salts given in Table 3.2 for the preparation of stock A were weighed and dissolved in distilled water separately. Then all solutions were mixed and the volume was made up to 1000 ml. The completely dissolved stock was autoclaved for 20 min at 15 psi pressure and stored at 4°C.

Stock B

Required amount of Potassium iodide, Boric acid, Manganese sulphate and Zinc sulphate were taken in required amount and dissolved in reduced volume of water, Sodium molybdate, Copper sulphate and Cobaltous chloride in required quantity were dissolved in 100 ml of double distilled water and 10 ml of each was taken to prepare 1lit of stock B along with rest of the micro elements.

Stock C

EDTA-disodium salt and ferrous sulphate were dissolved in reduced volume of boiling water then volume was made up to 1000 ml. Stock was autoclaved and stored in amber color reagent bottle at 4°C.

Stock D

All the vitamins were dissolved separately in distilled water and then mixed together to make up the volume to 1000 ml. As the vitamins are heat labile, the stock is prepared in autoclaved double distilled water and stored at 4°C.

Carbon Source

Sucrose was used as main source of carbon for plant regeneration. Sucrose was added just prior of adjusting the pH of the medium.

Table 3.5. Working composition of tissue culture nutrient media

Sr. No.	Compositions	Half MS (1000 ml)	Full MS (1000 ml)
(1)	(2)	(3)	(4)
1	Stock A (Macronutrients 20X)	25ml	50ml
2	Stock B (Micronutrients 50X)	2.5ml	5ml
3	Stock C (Salts and chelating agent 50X)	2.5ml	5ml
4	Stock D (Vitamins 50X)	2.5ml	5ml
5	Myo-inositol	50mg	100mg
7	Sucrose	30g	30g
8	Agar-Agar	8g	8g
9	pH	5.8	5.8

3.3.6. Preparation of growth regulator solutions

The stock solutions of plant growth regulators such as auxins and cytokinins, gibberellins etc. of concentration 1.0 mg/ml were prepared by dissolving required amount of respective solvent for particular plant growth

regulator (Table 3.4). The final volume of each completely dissolved growth regulator was made to required volume by gently adding distilled water to avoid precipitation. The stock solutions thus prepared were stored in reagent bottles and kept in refrigerator at 4°C temperature and used whenever required.

Table 3.6. Solvents for plant growth regulators

Name and Abbreviation of Plant growth regulators	Solvent
Benzyl aminopurine (BAP)	1N NaOH
L-Glutamic acid (L-GA)	Water

3.3.7. Preparation of culture media

- For the preparation of one liter culture medium, 50 ml of stock A was transferred to a clean sterile conical flask.
- To the same flask 5 ml stock B, 5 ml stock C and 5 ml stock D were transferred and mixed gently.
- Myo-inositol (100 mg/l) is dissolved in double distilled water.
- The basal MS medium was manipulated by adding required quantity of growth regulators needed for the experiment.
- Sucrose at 3% was added as carbon source prior of adjusting pH.
- The final volume was made up to one liter using distilled water and the pH of the medium was adjusted to 5.8 with systronic pH meter using 1N NaOH or 1N HCl.
- Media were kept for heating and 8 g agar was added gently. The medium was stirred regularly to avoid formation of agar clumps while boiling till it gets dissolved.
- The medium was poured hot at the rate of 20-25 ml per sterilized culture bottle.
- The culture bottles were capped with sterilized cap.

3.3.8. Sterilization of media

Closed bottles with media were loaded in the autoclave. The medium was autoclaved at 15psi (1.06 kg/cm^2) pressure and 121°C for 2 minutes. After sterilization the medium was cooled to room temperature and stored in sterile, cool and dry place till further use.

3.3.9. Transfer area and aseptic manipulations

All the aseptic manipulations, such as surface sterilization of explants, preparation and inoculation of explants and subsequent sub culturing were carried out under aseptic conditions in the hood of clean laminar air flow (LAF) cabinet.

The working platform of the laminar air flow chamber was first wiped and cleaned with cotton dipped in 70% rectified spirit and then by switched on the ultra violet (UV) lights for 20-25 minutes. Sterilized petri dishes as well as instruments like surgical scalpel handle along with disposable blades, scissors, forceps etc. were also cleaned with cotton dipped in rectified spirit and sterilized by flaming over spirit lamp for repeated use. Hands were also wiped with a hand disinfectant before inoculation.

3.4. Preparation of Spine gourd vines for culture inoculation

The female Spine gourd vines were collected from nearby villages and from the Instructional Farm of Department of Vegetable Science, Dr. PDKV, Akola. Spine gourd vine were cleaned thoroughly under running tap water for 20 min then again washed with few drops of Tween 20 for 10 min, then treated with a solution of Bavistin @ 0.5% for 25 min. The explants were washed four times with sterile distilled water and again surface sterilized using antibiotics Streptomycin @ 0.5% for 20 min, followed by four times wash with sterile distilled water to remove traces of antibiotics.

The cleaned explants were finally treated with 0.1% (w/v) $HgCl_2$ for 5 min under aseptic conditions and washed four times with sterile distilled water to remove traces of $HgCl_2$. After surface sterilization, explants were transferred individually on autoclaved blotting sheets to prepare them for inoculation on MS basal media and different combination of growth regulators.

3.4.1. Micro-propagation of explants of Spine gourd to obtain shoot initiation

The nodal explants were inoculated on MS medium supplemented with different concentrations and combinations of BAP @ 1.0 mg/L and 2.0 mg/L and L-Glutamic acid @ 1.5, 2, 2.5 and 3 mg/L (Table 3.5) to obtain shoot initiation. MS media without any growth regulators/ hormones was treated as control. Percent survival of explants was observed after a week to fourth week.

3.4.2. Transfer of Cultured Bottles

The inoculated culture bottles were incubated in a culture room where temperature is 25±2ºC, humidity 90% and photoperiod of 16hrs light and 8hrs dark conditions were maintained.

3.4.3. Sub-culture of micro-propagated shoots

The successfully established cultures were excised by trimming the discolored tissue and they were longitudinally split into two equal halves and cultured in the same media after 4 weeks.

3.5. Observations

3.5.1 Average number of days required for shoot initiation

From the day of inoculation to the day of explants growth initiation is considered as the days required to shoot initiation. Average number of days required for shoot initiation were taken from the mean of treatments.

3.5.2 Percent survival

The number of plants survived out of total inoculated was converted into percent.

3.5.3 Average number of shoots per explants

Total number of shoots per explant were taken after 30 days from the day of inoculation.

$$\text{Average no. of Shoot} = \frac{\text{Total number of Shoot}}{\text{Number of explants}}$$

3.5.4 Average number of leaves per explants

Total number of leaves per explant were taken after 30 days from the day of inoculation.

$$\text{Average no. of leaves} = \frac{\text{Total number of leaves}}{\text{Number of explants}}$$

3.5.5 Average length of shoot (cm)

Length of shoot is calculated after 30 days of inoculation of explant and the average was calculated by considering the mean of all explants in single treatment.

3.5.6 Multiplication index

The multiplication index was defined as the number of newly formed shoot (>0.5 cm) per initial shoot tip recorded after the subculture interval.

During the research experiment, observations mentioned in following tables of different treatment combinations will be recorded upto 3rd subculture. Statistical analysis will be carried out using CRD with 3 replications.

3.5.7. Statistically Analysis

The experiment was conducted to develop a protocol of Spine gourd, hence various treatment combinations were formulated and the treatment set was replicated into three. A completely randomized design (CRD) of statistics was used to analyze data like mean, SE (m) and CD% to derive a valid conclusion from the result obtained that during an experiment. As per these simple and first degree statistics, discrete data were analyzed to draw a valid conclusion which is detailed in next chapter under results and discussion.

CHAPTER IV

RESULT AND DISCUSSION

The present investigation entitled "*In vitro* clonal propagation of Spine gourd" was undertaken to standardize standard operating protocoal of micropropagation of female spine Gourd (*Momordica dioica* Roxb.) at Center of Excellence in Plant Biotechnology, Biotechnology Centre, Department of Agricultural Botany, Post Graduate Institute (PGI), Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Dist. Akola during the academic years 2021-22 and 2022-23. The shoot tip and nodal part were used as explant source excised from healthy mother plant vines. The observations were recorded for different parameters and the experimental results are presented in this chapter.

Spine gourd (*Momordica dioica* Roxb.) is one of the underutilized vegetable plant having Ayurvedic importance. Spine gourd is mostly raised through tubers; but because of dormancy and low multiplication rate, it is limited. It is highly cross pollinated because of dioecious nature and exhibit variation. High multiplication ratio can be achieved by micropropagation technique which will enable to produce genetically uniform disease free elite plants within short time.

Therefore, for exploiting technique for commercial purpose, it is absolutely necessary to develop *in-vitro* methods for propagation of spine gourd plants which suit the local conditions because *in-vitro* performance of plant tissue is dependent on a number of factors, which ultimately are related with the physiological state of the donor plant and the explants (Geeta, 1999).

The experimental work consisting of *in vitro* propagation of explants *Momordica dioica* Roxb. by using shoot tip and nodal segment as a source of to generate true to type plantlets using MS media with different combinations of growth regulators. In view of the above facts, the observations were recorded on various aspects related to *in-vitro* propagation of Spine gourd are presented and explained under the appropriate heads having the following objectives.

i To identify suitable explant for multiple shoot proliferation

ii To optimize nutrient media for explant establishment and multiple shoot formation

The present experiment was initiated with the selection of local variety of Spine gourd from the Instructional Farm of Department of Vegetable Science, Dr. PDKV, Akola.

4.1 Effect of surface sterilization agents on initial *in vitro* establishment of Spine gourd explants

Surface sterilization or disinfection is one of the important and basic operation for successful micropropagation in any crop plants. Removing contaminants from the surface of the explants is of prime concern. The shoot tip and nodal part of the female Spine gourd vine is used as explant for experiment. To overcome contamination problem sterilization of explants were done with $HgCl_2$, fungicide Bavistin, bactericide Streptomycin with double distilled water.

In this experiment, 30 explants for each treatment were sterilized, whole vine is selected and then it is washed under running tap water for 4-5 times to remove dust and other traces from vine. It was then followed by washing through Tween-20 for 15 min. Followed by washing with distilled water. Then, whole vines bearing explants source were sterilized with 0.1% Bavistin and Streptomycin for 20 min further washing with distilled water.

Washing was given again with DDSW to remove traces of antibiotic streptomycin and $HgCl_2$, systemic fungicide (Bavistin) and Bactericide (streptomycin). These combinations were kept at 0.1% for Bavistin, Streptomycin for 25-30 min. respectively, and concentration of $HgCl_2$ at 0.1% for 5 min. of duration. Geeta (1999) obtained significantly superior aseptic cultures of Spine gourd by surface sterilization with $HgCl_2$ (0.1%) for 10 min. Deokar *et al.* (2003) got similar results by using 0.1% $HgCl_2$ for 5 min. the sterilization was effective for initiation of aseptic cultures of Spine gourd.

Similar results were earlier found by Robinson *et al*. (2009) in Insulin plant, after washing under running tap water followed by detergent, Tween-20 for a minute nodal segments of insulin plant were washed with

double distilled water and surface sterilization was done with 0.1% $HgCl_2$ for 4-8 min. The results showed only 15% of contamination.

4.2 Response of different explants types of spine gourd for *in vitro* cultural initiation and their optimization

In an attempt to standardize explants for tissue culture of spine gourd, two types of explants were tried for *in-vitro* shoot regeneration and multiplication. The response observed from each explant is presented in Table 4.1.

Table 4.1 Response of different of explants types of Spine gourd (*Momordica dioica Roxb.*) on *in vitro* condition

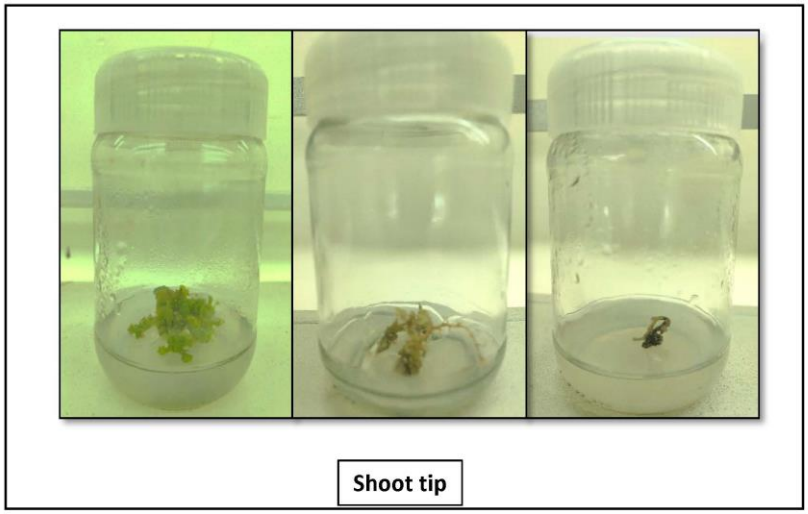
Explant	Callus	Shoot proliferation	Shoot multiplication
(1)	(2)	(3)	(4)
Shoot tip	+	_	_
Nodal segment	+	+	+

\+ Positive response

_ Negative response

Two types of explants shoot tip and node were cultured on media and it was observed that explants exhibited differential response to the *in vitro* conditions. Both explants produced whitish brown friable callus on MS medium but only nodal explant followed by the formation of shoot proliferation, shoot multiplication. As a result, nodal explant survival, shoot proliferation, and multiplication responses were preferred over shoot tip explant responses.

The major focus of the researchers working in tissue culture of crops was concentrated on the percentage of shoot induction. However, the number of days required for shoot initiation also plays an important role in rapid multiplication from the various explants. The nodal shoot segments proved to be most suitable explants material for regeneration of shoots in *Momordica* species in short period of time as reported by Agarwal and Kamal (2004) and Sultana *et al*. (2005).



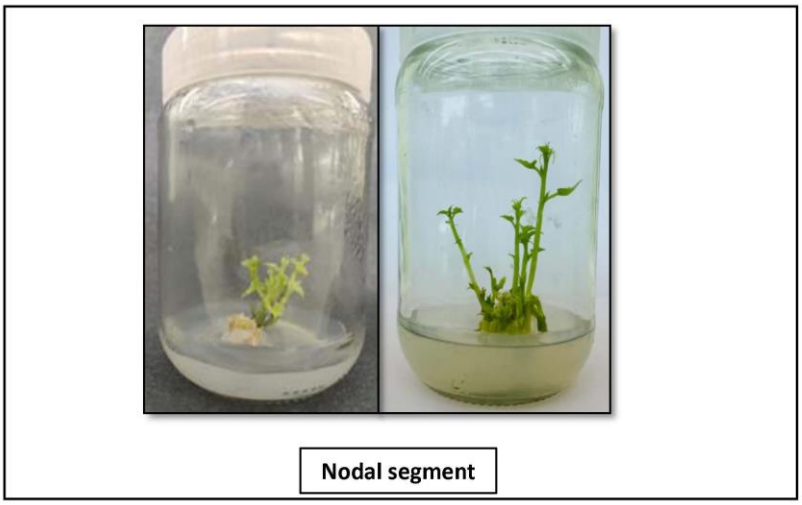


Plate 4.1. Response of shoot tip and nodal segment explants of spine gourd on *in-vitro* condition

4.3 Effect of MS medium supplemented with BAP and L-Glutamic acid on survival percentage during *in-vitro* shoots initiated of Spine gourd

MS media supplemented with growth regulators with different combinations and concentrations was used. The effect on the survival percentage after 1st, 2nd, 3rd and 4th week of inoculation in established cultures. The results are shown in Table no 4.2. Significant differences at the level of 5% were noticed for survival of nodal explant after inoculation.

After 1st week of inoculation, the treatment MS + BAP (2.0 mg/L) + LGA (2.0 mg/L) T_9 showed highest survival percentage of 93.33% followed by MS + BAP (2.0 mg/L) + LGA (2.5 mg/L) T_{10} whereas treatment MS + BAP (2.0 mg/L) + LGA (2.5 mg/L) T_{11} showed lowest percentage of shoot survival of 70.00%. Similar trend of decreasing of shoot survival percent was noticed after 2nd week from the date of inoculation. The treatment MS + BAP (2.0 mg/L) + LGA (2.0 mg/L) T_9 showed highest shoot survival percentage of 89.63% followed by MS control T_1 which showed 80.10% survival, while MS + BAP (2.0 mg/L) + LGA (2.5 mg/L) T_{11} showed lowest shoot survival percentage of 66.67%.

After 3rd week of inoculation, the treatment MS + BAP (2.0 mg/L) + LGA (2.0 mg/L) T_9 showed highest survival percentage of 81.76% followed by MS + BAP (2.0 mg/L) + LGA (2.5 mg/L) T_{10} which showed 72.78% whereas MS + BAP (2.0 mg/L) + LGA (2.5 mg/L) T_{11} showed lowest percentage of shoot survival of 61.43%.

Similar trend of decreasing of shoot survival per cent was noticed after 4th week from the date of inoculation. The treatment MS + BAP (2.0 mg/L) + LGA (2.0 mg/L) T_9 showed highest shoot survival percentage of 75.47% followed by MS + BAP (2.0 mg/L) + LGA (2.5 mg/L) T_{10} which showed 71.03% survival, while MS + BAP (2.0 mg/L) + LGA (2.5 mg/L) T_{11} showed lowest shoot survival percentage of 54.23%.

Similar results were found by Geeta (1999), axillary bud explants of Spine gourd of size 20mm gave maximum establishment of 85.50% than basal bud vine explants (22.5%) in culture medium MS supplemented with 80 ppm $AdSO_4$, 10 ppm BA and 5 ppm IBA.

Table 4.2 Effect of MS medium supplemented with BAP and L-Glutamic acid on survival percentage during *in vitro* shoots initiated of Spine gourd

Treatment	MS media supplemented with (BAP, L-Glutamic acid). mg/L	No. of explants inoculated	Survival % after			
			1st wk	2nd wk	3rd wk	4th wk
1	2	3	4	5	6	7
T1	MS control	30	83.33	80.10	69.36	66.67
T2	MS+BAP(1mg)	30	83.33	73.33	68.25	66.03
T3	MS+BAP(2mg)	30	73.33	70.47	67.97	62.10
T4	MS+BAP(1mg)+LGA(1.5mg)	30	76.67	71.48	69.61	61.27
T5	MS+BAP(1mg)+LGA(2mg)	30	76.67	70.83	65.00	60.31
T6	MS+BAP(1mg)+LGA(2.5mg)	30	80.00	75.92	71.85	56.02
T7	MS+BAP(1mg)+LGA(3mg)	30	76.67	70.83	65.56	58.33
T8	MS+BAP(2mg)+LGA(1.5mg)	30	83.33	70.83	67.50	63.33
T9	MS+BAP(2mg)+LGA(2mg)	30	93.33	89.63	81.76	75.47
T10	MS+BAP(2mg)+LGA(2.5mg)	30	86.67	73.06	72.78	71.03
T11	MS+BAP(2mg)+LGA(3mg)	30	70.00	67.14	61.43	54.23
	SE (m)±		**3.80**	**3.43**	**1.95**	**1.75**
	CD 5%		**11.15**	**10.05**	**5.73**	**5.15**

Similarly, Deokar *et al.* (2003) found that axillary buds gave maximum culture establishment (80%). After 4th week of inoculation, the survived explants were transferred to fresh MS medium supplemented with different growth regulators combinations like BAP (1.0 mg/L and 2.0 mg/L) and L-Glutamic acid (1.5, 2.0, 2.5, 3.0 mg/L) for the multiple shoot formation. The cultures were maintained by the regular subculture on fresh medium.

4.4 Effect of MS medium supplemented with BAP and L-Glutamic acid on *in vitro* shoots multiplication of Spine gourd after 4th weeks of inoculation

Micro-propagation of plants mainly depends on various factors and culture conditions. The optimization of culture media pH is necessary but critical step to achieve rapid and efficient growth of plantlets by using *in vitro* practices. As a matter of fact, plant growth regulators are one of the main factors affecting growth and development of explants in tissue culture. Observation shown in Plate 4.2 and Fig.4.1.

4.4.1 Number of days for initiation

The number of days required for shoot initiation from the nodal explant of spine gourd placed on MS media fortified with L-Glutamic acid and their combinations with different concentrations on shoot initiation in the Spine gourd var. Local have been studied and the results obtained are presented in Table 4.3 Significant differences concerning the no. of day for initiation at the level of 5% among the applied different treatments. The minimum number of days i.e. 7.08 days required for shoot initiation was observed in the nodal explant placed in treatment (T_9) MS + BAP (2.0 mg/L) + LGA (2.0 mg/L) followed by treatment (T_{10}) MS + BAP (2.0 mg/L) + LGA (2.5 mg/L) with 7.10, however the maximum 8.59 days were reported in treatment (T_2) MS + BAP (1.0 mg/L) for shoot initiation.

4.4.2 Number of shoots per explant

The effect of growth regulator along with L-Glutamic acid and their combinations with different concentrations on shoot initiation in the Spine gourd var. Local have been studied and the results obtained are presented in Table 4.3 Significant differences concerning the number of Shoot/explants

Table 4.3 Effect of MS medium supplemented with BAP and L-Glutamic acid on *in vitro* shoots multiplication of Spine gourd after 4th weeks of nodal segment explants inoculation

Treatment	MS media supplemented with (BAP, L-Glutamic acid). mg/L	No. of explants inoculated	Number of days for shoot initiation	No. of shoots /explant	No. of leaves /shoot	Average shoot length/ Explant (cm)
(1)	(2)	(3)	(4)	(5)	(6)	(7)
T1	MS control	30	8.04	1.07	0.87	2.38
T2	MS+BAP(1mg)	30	8.59	2.54	1.67	3.49
T3	MS+BAP(2mg)	30	8.43	2.93	1.83	2.74
T4	MS+BAP(1mg) +LGA(1.5mg)	30	8.16	3.34	2.68	3.41
T5	MS+BAP(1mg) +LGA(2mg)	30	8.12	3.51	3.35	4.25
T6	MS+BAP(1mg) +LGA(2.5mg)	30	7.82	5.03	3.73	3.33
T7	MS+BAP(1mg) +LGA(3mg)	30	7.97	5.90	5.76	3.11
T8	MS+BAP(2mg) +LGA(1.5mg)	30	7.84	6.35	6.66	4.69
T9	MS+BAP(2mg) +LGA(2mg)	30	7.08	7.61	7.59	4.92
T10	MS+BAP(2mg) +LGA(2.5mg)	30	7.10	7.13	7.25	4.18
T11	MS+BAP(2mg) +LGA(3mg)	30	7.93	5.37	5.59	3.33
	SE (m)		**0.31**	**0.38**	**0.29**	**0.51**
	CD 5%		**0.91**	**1.12**	**0.86**	**1.51**

at the level of 5% among the applied different treatments. Maximum number of shoots per explants were found in the treatment (T_9) MS+ BAP (2.0 mg/L) + LGA (2.0 mg/L) with 7.61 followed by treatment (T_{10}) MS+ BAP (2.0 mg/L) + LGA (2.5 mg/L) with 7.13 while treatment (T_1) MS Medium Control showed minimum result with 1.07.

4.4.3 Number leaves per explant

Data tabulated in Table 4.3 clearly showed significant differences concerning the number of leaves/shoot at the level of 5% among the applied different treatments. The maximum number of leaves in the treatment (T_9) MS+ BAP (2.0 mg/L) + LGA (2.0 mg/L) were recorded with 7.59 followed by treatment (T_{10}) MS + BAP (2.0 mg/L) + LGA (2.5 mg/L) in which number of leaves is 7.25. However, the minimum number of leaves/shoot appeared in treatment (T_1) MS media control which is 0.70.

4.4.4 Average length of shoot (cm)

The average length of shoots per explant indicated significant differences at the level of 5% among the various media supplementations with BAP and LGA used for the nodal explants of Spine gourd (Table 4.3). The minimum length 2.38 cm was recorded in the nodal explants placed on treatment (T_1) MS control medium. The maximum length of shoot 4.92cm was registered in the nodal explant in treatment (T_9) MS + BAP (2.0 mg/L) + LGA (2.0 mg/L) followed by the treatment (T_8) MS + BAP (2.0 mg/L) + LGA (1.5 mg/L) with 4.69cm.

As per the results found the synergistic effect of L-Glutamic acid in combination with BAP in the formation of multiple shoots has been reported for other members of Cucurbitaceae family such as *Citrullus lanateus* and *Momordica charanitia* reported by Sultana *et al.* (2005).

The positive effect of MS medium augmented with BAP on increasing of shoots number was also found by Kausar *et al.* (2013) who reported that, concentration of BAP (1.5 mg/L) along with lower concentration of GA_3 was found to be the optimum for highest rate of multiple shoots in *Beninca hispida*. The higher levels of cytokinin and auxin might have induced the callus formation which in turn reduced the length of shoots of plants. The

T_1

MS control

T_9

MS + BAP (2.0mg/L) + LGA (2.0mg/L)

T_{10}

MS + BAP (2.0mg/L) + LGA (2.5mg/L)

Plate 4.2. Effect of MS medium with BAP and LGA on *in-vitro* shoots proliferation of Spine gourd, after 4 weeks of inoculation

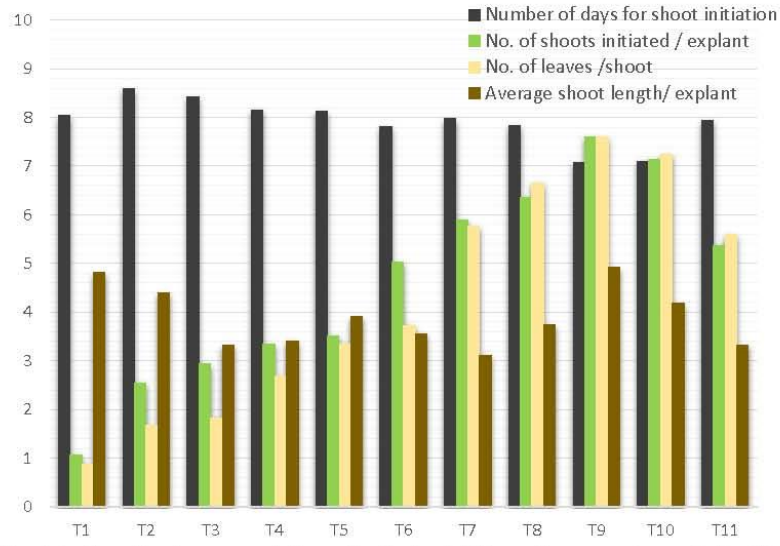


Fig. 4.1. Graphical representation of effect of MS medium supplemented with BAP and LGA on *in-vitro* shoots multiplication of Spine gourd after 4th weeks of inoculation

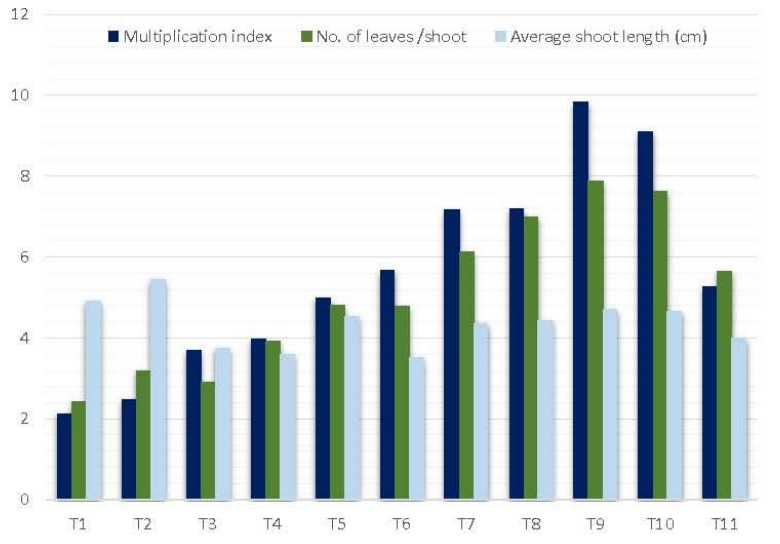


Fig. 4.2 Graphical representation of effect of MS medium supplemented with BAP and LGA on *in-vitro* shoots multiplication of Spine gourd after 1st subculture

present findings are in conformity with those reported by *Rai et al.* (2012), Patel *et al.* (2015) and Jamatia *et al.* (2016) in Spine gourd.

4.5 Effect of MS medium supplemented with BAP and L-Glutamic acid for *in vitro* shoots multiplication of Spine gourd after sub-cultures

In the present study the observations recorded on initiation, proliferation and development of multiple shoots indicated that the shoot proliferation from the established culture occurred after 30 days of their culturing on test media. Preliminary visual observations showed that growth of main shoot was rapid than that of lateral shoots in explants.

Different treatments of BAP and L-Glutamic acid showed different results, while shoot number was always seen more after doing subculture. Combinations of BAP and LGA at different concentrations has been subjected to continued sub culturing and the results are presented in Table 4.4, 4.5 and 4.6.

4.6 Effect of MS medium supplemented with BAP and L-Glutamic acid on *in vitro* shoots multiplication of Spine gourd after 1st subculture

After 4th week of inoculation, the survived explants were transferred to fresh MS medium supplemented with different growth regulators combinations like BAP (1.0, 2.0 mg/L) and L-Glutamic acid (1.5, 2.0, 2.5, 3.0 mg/L) for the multiple shoot formation presented in Plate 4.3(a). The cultures were maintained by the regular subculture on fresh medium and observation shown in Fig.4.2.

4.6.1 Multiplication index

The effect of growth regulator along with L-Glutamic acid and their combinations with different concentrations on multiplication index of shoot in the Spine gourd var. Local have been studied and the results obtained are presented in Table 4.4 Significant differences concerning the number of shoot/explants at the level of 5% among the applied different treatments.

Table 4.4 Effect of MS medium supplemented with BAP and L-Glutamic acid on *in vitro* shoots multiplication of Spine gourd after 1st subculture

Treatment	MS media supplemented with (BAP, L-Glutamic acid). mg/L	Multiplication index	No. of leaves /shoot	Average shoot length (cm)
(1)	(2)	(3)	(4)	(5)
T1	MS control	2.13	2.42	4.91
T2	MS+BAP(1mg)	2.48	3.20	5.46
T3	MS+BAP(2mg)	3.69	2.90	3.74
T4	MS+BAP(1mg) +LGA(1.5mg)	3.98	3.92	3.60
T5	MS+BAP(1mg) +LGA(2mg)	5.00	4.80	4.52
T6	MS+BAP(1mg) +LGA(2.5mg)	5.67	4.78	3.51
T7	MS+BAP(1mg) +LGA(3mg)	7.17	6.12	4.36
T8	MS+BAP(2mg) +LGA(1.5mg)	7.19	7.00	4.43
T9	MS+BAP(2mg) +LGA(2mg)	9.83	7.88	4.70
T10	MS+BAP(2mg) +LGA(2.5mg)	9.10	7.63	4.66
T11	MS+BAP(2mg) +LGA(3mg)	5.27	5.65	3.99
	SE (m)	**0.71**	**0.46**	**0.37**
	CD 5%	**2.07**	**1.36**	**1.10**

4.6.2 Number leaves per explant

Data tabulated in Table 4.4 Clearly showed significant differences concerning the number of leaves/shoot at the level of 5% among the applied different treatments. The maximum number of leaves in the treatment (T_9) MS + BAP (2.0 mg/L) + LGA (2.0 mg/L) were recorded with 7.88 followed by treatment (T_{10}) MS + BAP (2.0 mg/L) + LGA (2.5 mg/L) in which number of leaves is 7.63. However, the minimum number of leaves/shoot appeared in treatment (T_1) MS media control which is 2.42.

4.6.3 Average length of shoot (cm)

The average length of shoots indicated significant differences at the 5% among the various media supplementations with BAP and LGA (Table 4.4). The minimum shoot length 3.51cm was recorded in treatment (T_6) MS + BAP (1.0 mg/L) + LGA (2.5 mg/L). The maximum length of shoot 5.46cm was registered in treatment (T_2) MS + BAP (1.0 mg/L) followed by the treatment (T_1) MS media control with 4.91cm.

The present results are similar to Hoque *et al*. (1982) when they found that a combination results of (1.5 mg/L) Glutamic acid were more suitable for adventitious multiple shoot induction, where as in our investigation BAP (2.0 mg/L) and LGA (2.0 mg/L) was to be the proved best for the production of multiple shoots.

4.7 Effect of MS medium supplemented with BAP and L-Glutamic acid on *in vitro* shoots multiplication of Spine gourd after 2nd subculture

In-vitro multiplication of shoots per culture is considered as one of the most crucial stages deciding the viability, feasibility and effectiveness of micropropagation techniques. The stage is characterized by induction and development of adventitious buds from the well-established shoot tip or axillary bud culture.

After 4th week of subculture 1st, the survived explants were transferred to fresh MS medium supplemented with different growth regulators combinations like BAP (1.0 mg/L and 2.0 mg/L) and L- Glutamic

Table 4.5 Effect of MS medium supplemented with BAP and L-Glutamic acid on *in vitro* shoots multiplication of spine gourd after 2nd subculture

Treatment	MS media supplemented with (BAP, L-Glutamic acid). mg/L	Multiplication index	No. of leaves/shoot	Average shoot length (cm)
(1)	(2)	(3)	(4)	(5)
T1	MS control	3.13	2.42	4.44
T2	MS+BAP(1mg)	3.95	3.38	6.10
T3	MS+BAP(2mg)	4.54	4.46	3.86
T4	MS+BAP(1mg) +LGA(1.5mg)	5.93	4.76	4.02
T5	MS+BAP(1mg) +LGA(2mg)	6.44	5.23	4.43
T6	MS+BAP(1mg) +LGA(2.5mg)	6.59	6.69	3.42
T7	MS+BAP(1mg) +LGA(3mg)	6.83	8.93	4.33
T8	MS+BAP(2mg) +LGA(1.5mg)	7.14	9.63	4.45
T9	MS+BAP(2mg) +LGA(2mg)	8.89	10.62	4.79
T10	MS+BAP(2mg) +LGA(2.5mg)	7.36	9.98	4.68
T11	MS+BAP(2mg) +LGA(3mg)	6.75	6.41	4.20
	SE (m)	**0.59**	**0.58**	**0.44**
	CD 5%	**1.73**	**1.71**	**1.29**

acid (1.5, 2.0, 2.5, 3.0 mg/L) for the multiple shoot formation. The cultures were maintained by the regular subculture on fresh medium and result were shown in Fig. 4.3 and Plate 4.3(b).

4.7.1 Multiplication index

The effect of growth regulator along with L-Glutamic acid and their combinations with different concentrations on multiplication of shoot in the Spine gourd var. Local have been studied and the results obtained are presented in Table 4.5 Significant differences concerning the number of shoot/explants at the level of 5% among the applied different treatments. Maximum multiplication of shoot were found in the treatment (T_9) of MS+ BAP (2.0 mg/L) + LGA (2.0 mg/L) with 8.89 followed by treatment (T_{10}) MS+ BAP (2.0 mg/L) + LGA (2.5 mg/L) with 7.36 while treatment (T_1) of MS Medium Control showed minimum result with 3.13.

4.7.2 Number leaves per explant

Data tabulated in Table 4.5 clearly showed significant differences concerning the number of leaves/shoot at the level of 5% among the applied different treatments. The maximum number of leaves in the treatment (T_9) of MS+ BAP (2.0 mg/L) + LGA (2.0 mg/L) were recorded with 10.62 followed by treatment T_{10} of MS+ BAP (2.0 mg/L) + LGA (2.5 mg/L) in which number of leaves is 9.98. However, the minimum number of leaves/shoot appeared in treatment T_1 of MS media control which is 2.42.

4.7.3 Average length of shoot (cm)

The average length of shoots per explant indicated significant differences at the level of 5% among the various media supplementations with BAP and LGA (Table 4.5). The minimum length 3.42cm was recorded in the treatment (T_6) MS media supplemented with BAP (1.0 mg/L) + LGA (2.5 mg/L). The maximum length of shoot 6.10 cm was registered in treatment (T_2) of MS media supplemented with (1.0 mg/L) BAP followed by the treatment (T_9) of MS + BAP (2.0 mg/L) + LGA (2.0 mg/L) with 4.79 cm.

Similar results were found by Haque *et al.* (2008), where best hormone for shoot multiplication of pumpkin was BAP (2.0 mg/L), in case of Ash-Gourd BAP was 1.5 mg/L. For callus induction BAP + 2,4-D was best

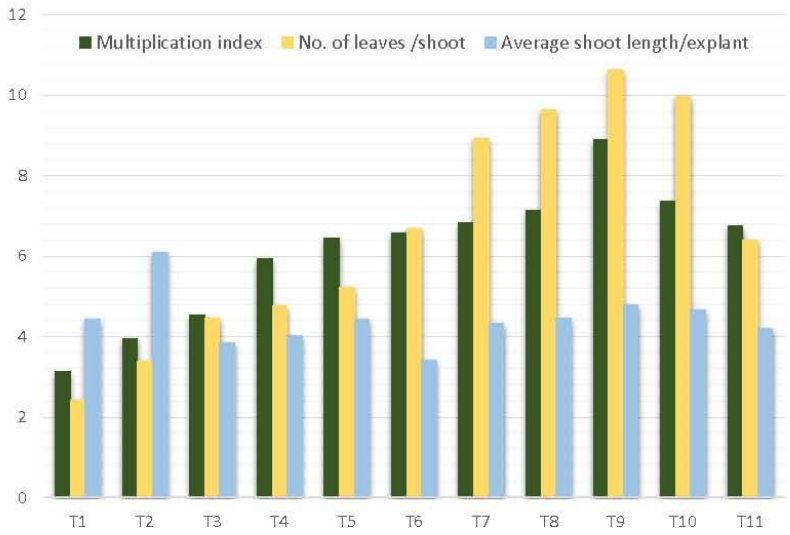


Fig. 4.3. Graphical representation of effect of MS medium supplemented with BAP and LGA on *in-vitro* shoots multiplication of spine gourd after 2nd subculture

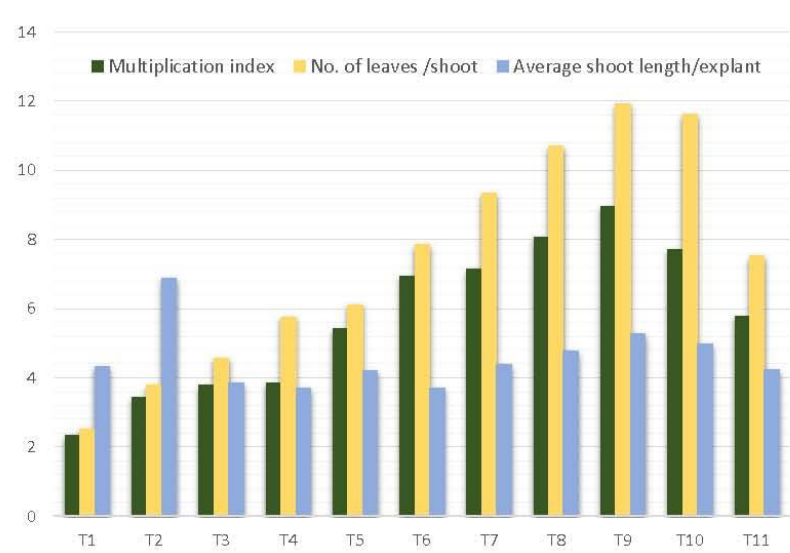


Fig. 4.4. Graphical representation of effect of MS medium supplemented with BAP and LGA on *in-vitro* shoots multiplication of spine gourd after 3rd subculture

combination for pumpkin and it was 2.0 + 0.1 mg/L when in ash gourd BAP + NAA was the best combination.

4.8 Effect of MS medium supplemented with BAP and L-Glutamic acid on *in vitro* shoots multiplication of Spine gourd after 3rd subculture

After 4th week of subculture 3rd, the survived explants were transferred to fresh MS medium supplemented with different growth regulators combinations like BAP (1.0 mg/L and 2.0 mg/L) and L-Glutamic acid (1.5, 2.0, 2.5, 3.0 mg/L) for the multiple shoot formation. The cultures were maintained by the regular subculture on fresh medium and result shown in Plate 4.3.(c) and Fig.4.3.

4.8.1 Multiplication index

The effect of growth regulator along with L-Glutamic acid and their combinations with different concentrations on multiplication index of shoot in the Spine gourd var. Local have been studied and the results obtained are presented in Table 4.6 Significant differences concerning the number of shoot/explants at the level of 5% among the applied different treatments. Maximum multiplication index of shoot were found in the treatment (T_9) MS + BAP (2.0 mg/L) + LGA (2.0 mg/L) with 8.97 followed by treatment (T_{10}) MS + BAP (2.0 mg/L) + LGA (2.5 mg/L) with 7.72 while MS Medium Control (T_1) showed minimum result with 2.34.

4.8.2 Number leaves per explant

Data tabulated in Table 4.6 Clearly showed significant differences concerning the number of leaves/shoot at the level of 5% among the applied different treatments. The maximum number of leaves in the treatment (T_9) of MS + BAP (2.0 mg/L) + LGA (2.0 mg/L) were recorded with 11.93 followed by treatment (T_{10}) of MS + BAP (2.0 mg/L) + LGA (2.5 mg/L) in which number of leaves is 11.62. However, the minimum number of leaves/shoot appeared in MS control (T_1) which is 2.53.

Table 4.6 Effect of MS medium supplemented with BAP and L-Glutamic acid on *in vitro* shoots multiplication of Spine gourd after 3rd subculture

Treatment	MS media supplemented with (BAP, L-Glutamic acid). mg/L	Multiplication index	No. of leaves /shoot	Average shoot length (cm)
(1)	(2)	(3)	(4)	(5)
T1	MS control	2.34	2.53	4.33
T2	MS+BAP(1mg)	3.44	3.79	6.89
T3	MS+BAP(2mg)	3.78	4.57	3.85
T4	MS+BAP(1mg) +LGA(1.5mg)	3.86	5.76	3.70
T5	MS+BAP(1mg) +LGA(2mg)	5.43	6.12	4.20
T6	MS+BAP(1mg) +LGA(2.5mg)	6.94	7.85	3.69
T7	MS+BAP(1mg) +LGA(3mg)	7.14	9.33	4.38
T8	MS+BAP(2mg) +LGA(1.5mg)	8.07	10.70	4.78
T9	MS+BAP(2mg) +LGA(2mg)	8.97	11.93	5.27
T10	MS+BAP(2mg) +LGA(2.5mg)	7.72	11.62	4.98
T11	MS+BAP(2mg) +LGA(3mg)	5.79	7.52	4.24
	SE (m)	**0.70**	**0.40**	**0.52**
	CD 5%	**2.06**	**1.18**	**1.51**

4.8.3 Average length of shoot (cm)

The average length of shoots per explant indicated significant differences at the level of 5% among the various media supplementations with BAP and LGA (Table 4.6). The minimum shoot length 3.69 cm was recorded in the treatment (T_6) of MS media supplemented with BAP (1.0 mg/L) + LGA (2.5 mg/L). The maximum length of shoot 6.89 cm was registered in treatment (T_2) MS + BAP (1.0 mg/L) followed by the treatment (T_9) of MS + BAP (2.0 mg/L) + LGA (2.0 mg/L) with 5.27 cm.

Similar results were found by Mustafa *et al.* (2013), MS medium supplemented with BAP (2.0 mg/L) and LGA (2.0 mg/L), the explants produced little amount of callus and shoot buds in Spine gourd. The shoot buds on successive subcultures for twice on the same medium produced multiple shoots. Shoot proliferation was further continued even after six months.

Similarly, Debnath *et al.* (2013) noted maximum numbers of shoots from nodal explants of Spine gourd in MS media supplemented with 2,4-D (2.0 mg/L) and BAP (0.5 mg/L).

Similar result was also reported by Varma *et al.* (2014), in which MS media supplemented with BAP (0.5 mg/L) in monoecious Bitter melon has exhibits more number of shoots after 3rd subculture. Hence, during the present investigations, it has noticed that the nodal segment shows significantly higher rate of survival, initial culture establishment, high rate of multiple shoots and shoot-let proliferation as compare to the shoot tips. Overall, during two years study, it was noticed that among all the treatment, T_9 treatment MS supplemented with BAP (2.0 mg/L) + LGA (2.0 mg/L) gives better results as compare to the other treatments in spine gourd.

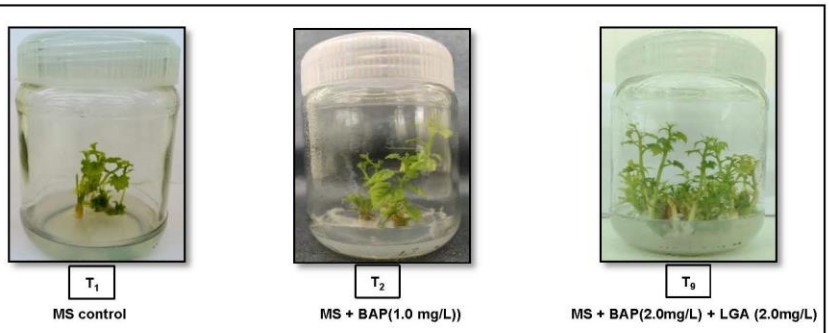


Plate 4.3(a). Effect of MS medium with BAP and LGA on *in-vitro* shoots multiplication of Spine gourd, after 1st Subculture

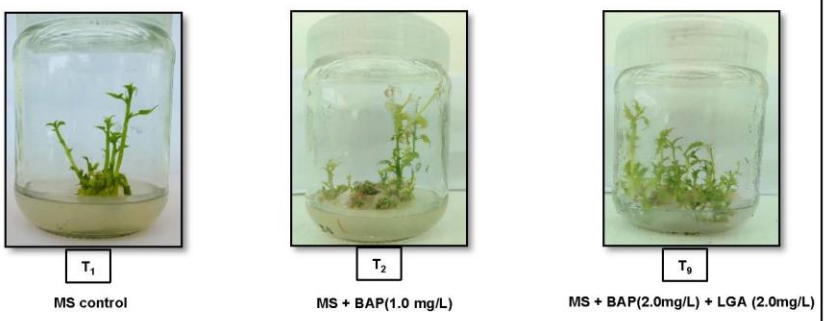


Plate 4.3(b). Effect of MS medium with BAP and LGA on *in-vitro* shoots multiplication of Spine gourd, after 2nd Subculture

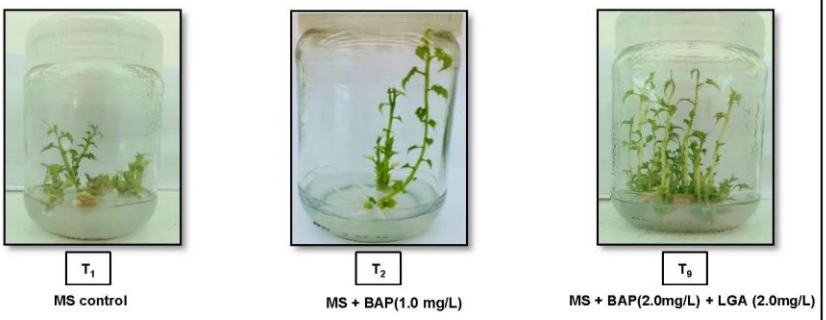


Plate 4.3(c). Effect of MS medium with BAP and LGA on *in-vitro* shoots multiplication of Spine gourd, after 3rd Subculture

CHAPTER V

SUMMARY AND CONCLUSION

The present investigation entitled "*In-vitro* clonal propagation of Spine gourd (*Momordica dioica* Roxb.)" was conducted during year 2021-22 and 2022-23 at plant tissue culture laboratory of Dr. PDKV, Akola with objectives to identify suitable explant for multiple shoot proliferation and to optimize nutrient media for explant establishment and multiple shoot formation. The experiment was laid out in completely randomized design (CRD). The pooled mean results obtained during the period of investigation are summarized in this chapter.

For the sterilization of shoot tip and nodal segment, systemic fungicide (Bavistin) and bactericide (Streptomycin) and $HgCl_2$ was used. These combinations were kept at 0.1% for Bavistin, Streptomycin for 25-30 min. respectively, and concentration of $HgCl_2$ at 0.1% for 5 min.

Two types of explants shoot tip and node were cultured on media and it was observed that explants exhibited differential response to the *in vitro* conditions. As a result, nodal explant survival, shoot proliferation, and multiplication responses were preferred over shoot tip explant responses.

Depending on the inoculation of nodal segment were used for *in-vitro* propagation studies. The effect of MS medium with BAP and L-Glutamic acid on the survival percentage of nodal explant in tissue culture of Spine gourd after 1^{st}, 2^{nd}, 3^{rd} and 4^{th} week of inoculation. The highest survival percentages after each week were found in treatments MS + BAP (2.0 mg/L) + LGA (2.0 mg/L), after 4^{th} week, treatment showed highest survival percentage of 75.47%. The minimum number of days i.e. 7.08 days required for shoot initiation was observed on MS + BAP (2.0 mg/L) + LGA (2.0 mg/L). Maximum number of shoots per explants were found in MS+ BAP (2.0 mg/L) + LGA (2.0 mg/L) with 7.61. The maximum number of leaves in MS+ BAP (2.0 mg/L) + LGA (2.0 mg/L) were recorded with 7.59. The maximum length of shoot 4.98 cm was registered in the nodal explant on MS + BAP (2.0 mg/L) + LGA (2.0 mg/L).

Effect of MS medium with BAP and L-Glutamic acid in *in vitro* shoots multiplication after sub-culturing studies. The observations made about the commencement, proliferation, and growth of multiple shoots revealed that, following 30 days of culturing on test media, the established culture's shoots began to proliferate. Different treatments of BAP and L-Glutamic acid showed different results, while shoot growth was always seen more after doing subculture.

Subculture 1st after 4th week of inoculation, the maximum multiplication index of shoots were found in MS+ BAP (2.0 mg/L) + LGA (2.0 mg/L) with 9.83. The maximum number of leaves in MS+ BAP (2.0 mg/L) + LGA (2.0 mg/L) were recorded with 7.88. The maximum length of shoot 5.46cm was registered in MS + BAP (1.0 mg/L).

After subculture 2nd, the maximum multiplication index of shoot were found in MS+ BAP (2.0 mg/L) + LGA (2.0 mg/L) with 8.89. The maximum number of leaves in MS+ BAP (2.0 mg/L) + LGA (2.0 mg/L) were recorded with 10.62. The maximum length of shoot 6.10cm was registered in MS + BAP (1.0mg/L).

After subculture 3rd, the maximum multiplication index of shoots were found in MS + BAP (2.0 mg/L) + LGA (2.0 mg/L) with 8.97. The maximum number of leaves in MS + BAP (2.0 mg/L) + LGA (2.0 mg/L) were recorded with 11.93. The maximum length of shoot 6.89 cm was registered in the nodal explant in MS + BAP (1.0 mg/L).

Conclusion:

- During the present investigations, it has noticed that the nodal segment shows significantly higher rate of survival, initial culture establishment, high rate of multiple shoots and shoot-let proliferation as compared to the shoot tips.
- Overall, across two years study, it was noticed that among all the treatment, T_9 treatment MS + BAP (2.0 mg/L) + LGA (2.0 mg/L) gives better results as compare to the other treatments in spine gourd.

CHAPTER VI

IMPLICATION

The important implication of present work entitled "*In-vitro* clonal propagation of Spine gourd (*Momordica dioica* Roxb.)" is to contribute for establishment of efficient *in- vitro* protocol, that was composed of MS media and hormonal combination with basal MS + BAP (2.0 mg/L) + LGA (2.0 mg/L). The proposed protocol will be effectively used for nodal segment used as explants source. Further the proposed treatment combination will be helpful for obtaining initial culture establishment, good survival and shoot multiplication rate in spine gourd.

CHAPTER VII

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