IN VITRO PROPAGATION STUDIES IN GARLIC (*ALLIUM SATIVUM)*

Thesis

by

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CERTIFICATE-I

This is to certify that the thesis titled **"*In vitro* propagation studies in garlic (*Allium sativum*)"** submitted in partial fulfillment of the requirements for the award of the degree of **Master of Science Molecular Biology and Biotechnology** in the discipline of **Biotechnology** to Dr. Yashwant Singh Parmar University of Horticulture and Forestry, (Nauni) Solan (HP) – 173 230 is a bonafide research work carried out by **Ms Riddhi Rathore (H-2021-37-M)** daughter of Mr Dayal Singh Rathore under my supervision and that no part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of this investigation have been fully acknowledged.

Place: Nauni, Solan (HP)
Dated:

Dr Anupama Singh
Major advisor

CERTIFICATE-II

This is to certify that the thesis titled, "***In vitro* propagation studies in garlic (*Allium sativum*)**" submitted by **Ms Riddhi Rathore (H-2021-37-M)** son of Mr Dayal Singh Rathore to the Dr. Yashwant Singh Parmar University of Horticulture & Forestry, (Nauni) Solan (HP) – 173 230 India in partial fulfilment of the requirements for the degree of **Master of Science Molecular Biology and Biotechnology** in the discipline of **Biotechnology** has been approved by the Advisory Committee after an oral examination of the student in collaboration with an External Examiner.

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CONTENTS

ABBREVIATIONS USED

$AlCl_3$	:	Aluminium chloride
ANOVA	:	Analysis of variance
BAP	:	6-Benzylaminopurine
BDS	:	Basal Dunstan Short
CD	:	Critical Difference
CH_3COOK	:	Potassium acetate
cm	:	Centimetre
DMRT	:	Duncan's Multiple Range Test
DPPH	:	2,2-diphenylpicrylhydrazyl
CRD	:	Completely Randomized Design
cv	:	Cultivar
2, 4 D	:	2,4-dichlorophenoxyacetic acid
FC reagent	:	Folin Ciocalteu reagent
gL^{-1}	:	Gram per litre
GA3	:	Gibberellic acid
GAE	:	Gallic acid equivalent
HCl	:	Hydrochloric acid
h/day	:	Hours per day
$HgCl_2$	:	Mercuric chloride
IAA	:	Indole
IBA	:	Indole 3-acetic acid
2 iP	:	Indole 3-butyric acid
JA	:	Jasmonic acid
kin	:	Kinetin
$lbs/inch^2$	:	Pounds per square inch
M	:	Molar
mgL^{-1}	:	Milligram per Litre
min	:	Minute(s)
mL	:	Millilitre
mM	:	Milli molar
MS	:	Murashige and Skoog

N	:	Normal
NAA	:	Naphthalene acetic acid
Na_2CO_3	:	Sodium carbonate
NaOH	:	Sodium hydroxide
OD	:	Optical density
%	:	Percent
QE	:	Quercetin equivalent
SE	:	Standard error
sec	:	Seconds
SS	:	Sum of squares
t/ha	:	Tonnes per hectare
TSS	:	Total soluble solids
UV	:	Ultra violet
µL	:	Micro litre
µM	:	Micro molar
v/v	:	Volume by volume
var	:	Variety
viz	:	Licet /as follow
w/v	:	Weight by volume

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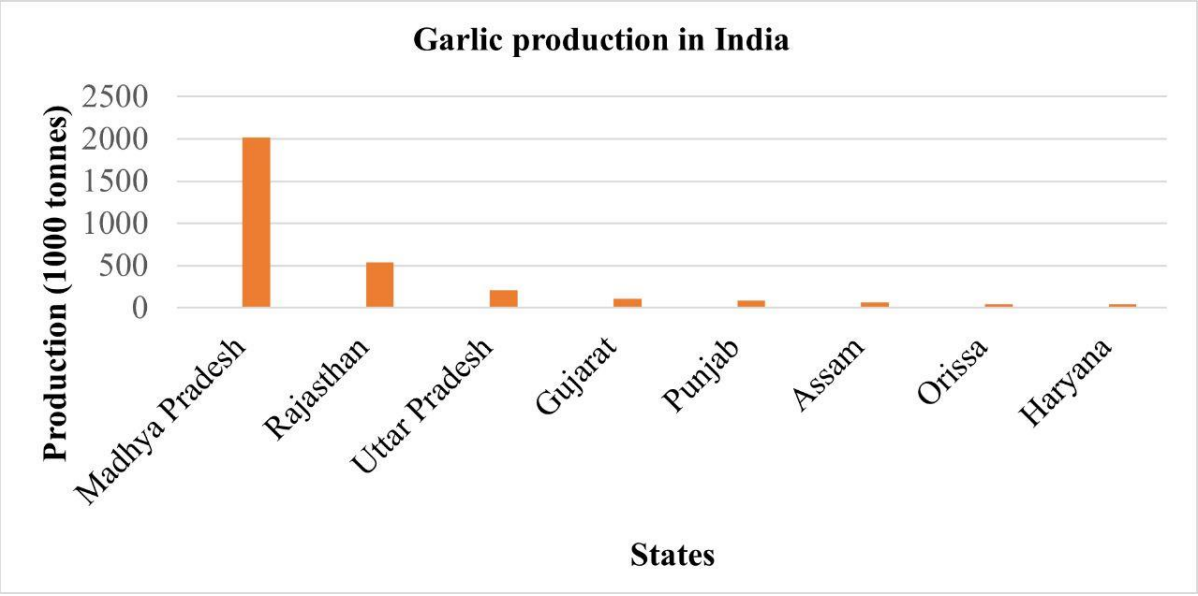
Chapter-1

INTRODUCTION

Garlic (*Allium sativum*) is an important monocotyledonous aromatic bulb crop belonging to the family Alliaceae. It is native to Central Asia and has long been cultivated across the world for culinary and medicinal purposes.

In past, garlic extract has been used in the treatment of wide range of disorders. Garlic contains a number of chemical compounds like phytosterols, carotenoids, phenolic compounds, phytoestrogens, organo-sulphur compounds such as allicin (diallyl disulphide oxide), glucosinolates, their degradation products and dietary fibre (Quintin et al. 2019) which impart several health-enhancing properties such as antioxidant, anticarcinogenic, antimicrobial, cardioprotective, lowering blood pressure and cholesterol, fighting against infectious diseases, anti-diabetic properties along with antibiotic, antitumor and antithrombic effects (Sata et al. 2000). Garlic is also known worldwide for its aroma and characteristic flavour (Kumar and Prasad 2015).



Source: National Horticulture Board (NHB)*2021-22 (1st Advance Estimate)

Figure 1: **Production of garlic in different states of India**

Based on the data from Food and Agriculture Organization (2022), China is the largest producer of garlic followed by India and Bangladesh. Predominant garlic producing

states in India are Madhya Pradesh, Rajasthan, Uttar Pradesh, Gujarat, Punjab and Himachal Pradesh. Madhya Pradesh is the largest producer of garlic followed by Rajasthan (NHB 2022) (Fig 1). Total production in Himachal Pradesh was 5,140 tonnes (NHB 2022). Garlic is included in important spice crops of sub-tropical areas of Himachal Pradesh. Garlic is grown on a large scale across the Sirmaur district of Himachal Pradesh. Garlic is a high value crop and generates high income. Garlic cultivation is suitable for farmers with small land holdings and thus has a great role in hill agriculture (Sharma et al. 2018).

Garlic is a perennial flowering plant with an erect stem that grows in height up to 1 m. Leaves typically arise from short hard stem above the bulb or arise from shorter pseudo stem made up of overlapping leaf sheaths. The bulbs enclose up to 20 edible bulblets called cloves covered with membranous skin. Bulb is the main economic part of the plant.

Garlic has been classified into hard neck and soft neck type based on the tendency of each variety to develop flower stalks, hardness and pattern of clove formation (Kshirsagar et al. 2018). Soft neck garlic can be stored well as compared to hard neck garlic. Garlic varieties can be of long day and short day on the basis of critical day length required for bulbing. Short-day garlic requires 10-12 h photoperiod while long-day garlic requires 13-14 h photoperiod (https://dogr.icar.gov.in). Some of the short-day varieties are 'Yamuna Safed (G1)', 'Yamuna Safed-2 (G-50)', 'Yamuna Safed-3 (G-282), 'Yamuna Safed-3 (G-323)', 'Yamuna Safed-4 (G-189)' and 'Agrifound White (G-41)' whereas long-day varieties are 'VL Garlic 1', 'VL Lahsun 2', 'Agrifound Parvati' and 'CITH'. 'VL Garlic 1' is recommended for mid hills of North-Western and North Eastern India. It was developed by clonal selection from exotic germplasm 'EC 158250'. 'Yamuna Safed' is recommended for growing in Maharashtra, Gujarat and Madhya Pradesh. It was developed by NHRDF by mass selection from local collection (https://dogr.icar.gov.in).

Garlic crop can adapt well in wide range of climatic conditions. It requires an optimum temperature range of 25-30ºC to induce bulbs. It grows well in loamy soil with high humus and potash content (Nainwal et al. 2015).

Garlic is propagated vegetatively by cloves (Novak 2018). Most garlic cultivars have lost their ability to flower due to repeated vegetative propagation and are sexually sterile (Kim et al. 2003). This makes conventional breeding impossible. New cultivars are developed mainly by clonal selection of landraces and spontaneous mutants. Traditional

vegetative propagation show low rate of multiplication and have high chances of disease transmission such as viruses (Khan et al. 2017; Nagasawa and Finer 1988). Therefore, tissue culture techniques are necessary to increase multiplication rate and to produce disease free planting material.

Plant tissue culture is a technique in which small tissue or organ pieces are removed from whole plants and cultured on a nutrient medium under aseptic environmental conditions. Plant tissue culture is important not only for micropropagation but also for application of other biotechnological tools like genetic transformation, *in vitro* mutagenesis, *in vitro* germplasm conservation and *in vitro* selection of somaclonal variants. Tissue culture protocols depend greatly on the genotype used and hence, it becomes necessary to standardize the media and culture conditions for different genotypes (Barandiaran et al. 1999).

The application of biotechnology along with conventional breeding methods can be useful in any garlic improvement research program (Haider et al. 2015). Use of techniques like meristem tip culture, somaclonal variations and genetic transformation can help in developing new resistant garlic cultivars. Above all, tissue culture techniques can favour long term conservation of garlic.

Keeping in view, the importance of tissue culture in garlic, the research was taken up with following objectives:

- ***In vitro* establishment of shoot culture of garlic**
- ***In vitro* root induction and acclimatisation of regenerants**

Chapter-2

REVIEW OF LITERATURE

Literature related to **"*In vitro* propagation studies in garlic (*Allium sativum*)"** is reviewed as follows:

2.1 Morphological and Biochemical characterization

2.2 *In vitro* regeneration

2.2.1 *In vitro* direct regeneration

2.2.2 *In vitro* indirect regeneration

2.1 Morphological and Biochemical characterization

Morphological and biochemical characterization of different parts of garlic have been studied in various garlic genotypes. It has been discussed below:

Gvozdanovic et al. (2000) collected ecotypes of winter and spring garlic from main production region in Yugoslavia and studied the variability of their characteristics. The analysis on ecotypes was performed for variability of characteristics like height of the above ground plant part, number of cloves, dry matter content and yield. The results showed that the winter and spring garlic were significantly variable with respect to the number of outer leaves of bulb, bulb mass, clove mass, number of cloves, dry matter content and yield.

Bocchini et al. (2001) estimated allicin content in garlic by HPLC-UV detection. Allicin content in non-purified garlic extract was 4.6mg/g, 3.8mg/g and 3.6 mg/g using HPLC-UV, HPLC hv-UV and HPLC-h-ED respectively whereas in purified garlic extract, allicin content was 1.84 mg/g, 1.7 mg/g and 2.1 mg/g using HPLC-UV, HPLC-h-UV and HPLC-h-ED respectively.

KC et al. (2004) collected a total of 179 garlic accessions from 51 districts and carried out experiment to study physio-morphological variation, crop duration and yield potentiality in Nepalese indigenous garlic germplasm. The collected bulbs were characterized and were planted for the evaluation. Phenological and physio-morphological characters were recorded and multivariate analysis was performed on only 158 bulb producing germplasms. On the basis of cluster analysis, 8 different natural groups were identified with similar characteristics.

Panthee et al. (2006) collected a total of 179 garlic (*Allium* sativum) accessions from various parts of Nepal. All accessions were characterized for leaf erectness, leaf colour, leaf wax, leaf cross section, bulb regularity, bulb skin colour, bulb outer scales number, days to emergence, bulbing period, number of green leaves at 135 days after planting, days to maturity, plant height, bulb weight, bulb diameter, number of cloves per bulb, clove diameter and bulb yield. Data was analysed by using principal component and cluster analysis procedures to reveal three major clusters. Four principal components were identified explaining more than 86% of total variation.

Stavelikova (2008) evaluated a collection of 613 genotypes according to the Descriptor list for *Allium spp*. (International Plant Genetic Resources Institute 2001) from Siberia and Central Asia. The genotypes were divided into the three main morphological groups according to the ability to produce the scape: the first group with the scape (bolting) – 300 genotypes, the second group without the scape (non-bolting garlic) 205 genotypes and the third group presented semi-bolting garlic – 108 genotypes with bulbils in the different parts of the pseudostem.

Batth et al. (2013) carried out a study to characterize germplasm collection of 25 garlic genotypes from different garlic growing regions of India. GGE biplot analyses were used for multi-trait selection to identify accessions on the basis of collective desirable trait combinations in individual genotypes. Seven genotypes (AC-50, AC-200, JG-03-263, PG-17, PG-1, PG-18 and RAUG-5) having combinations of desirable morphological variation were identified. Three traits i.e., plant height, bulb length and number of cloves per bulb were indicated to be the main determinants of bulb weight. A maximum positive correlation was exhibited in bulb length and clove length.

Panse et al. (2013) studied 56 garlic lines for various traits of cloves and bulbs like polar diameter of bulb, equatorial diameter of bulb, average weight of 10 cloves and plant height. The study showed that high significant variation exists for all the traits except leaf width.

Khalid et al. (2014) studied total soluble solids (TSS) in *Allium tuberosum* and *Allium sativum*. A total soluble solid content of 17.95% was reported in *Allium tuberosum* while *Allium sativum* had a total solid content of 16.45%.

Mohammadi et al. (2014) evaluated 16 local accessions collected from growers in 16 localities and geographical origins in 5 Provinces of Iran and evaluated them for agro-

morphological characters to analyse patterns of variation among different landraces of garlic and their relation with geographical locations. High level of diversity was detected. It was reported to be predominantly due to genotype. A relationship was derived between genetic divergence and geographical origins based on cluster analysis. Genotypes 'G1-14' and 'GI-15' from Isfahan and Markazi Provinces respectively were reported to have the ability to flower and were bolting types.

Kumar M (2015) characterized 15 garlic cultivars and variation was observed in plant height, number of leaves per plant, leaf length, leaf width, bulb diameter, bulb weight, single clove weight and cloves per bulb.

Tarique et al. (2016) studied characters like number of cloves per bulb, polar diameter of bulb, equatorial diameter of bulb, bulb colour, colour of cloves and found considerable variation among them.

Nandini et al. (2018) studied morphologically and biochemically nine genotypes of garlic (*Allium sativum* L.). The analysis revealed highest TSS content of 40.38% in 'Agrifound White' while maximum pyruvate content of 27.19 µM/g fresh weight was observed in 'Yamuna Safed'. Maximum equatorial diameter of 3.96 cm, polar diameter of 4.42 cm, average bulb weight of 19.01 g and highest yield of 8.65 t/ha was observed in genotype 'Bhima Purple'.

Petropoulos et al. (2018) assessed garlic genotypes from the main production regions of Greece for their chemical composition, quality and bulb morphology. The results showed significant diversity in quality features and bulb morphology not only from different growing regions but from same growing regions too.

Ayed et al. (2019) studied phenotypic diversity of thirty-six local garlic landraces on the basis of fourteen quantitative characteristics from garlic producing areas of Tunisia. High diversity was detected among Tunisian garlic landraces. Most significant variation of the yield was observed in bulb weight and bulb diameter. The cluster analysis sorted the 36 genotypes into three main groups as cluster 1 (11 accessions), cluster 2 (20 accessions), and cluster 3 (5 accessions).

Salahuddin et al. (2019) characterized a total of twenty-five garlic accessions for various morphological characters like number of leaves, height of plant, leaf length, fresh

weight of leaf, dry weight of leaf, number of cloves per bulb, length and diameter of bulb, dry weight of bulb, fresh weight of bulb, yield per plot and yield per hectare. Garlic accession 'G49' from Vietnam was found to be best performing.

Selen (2019) studied variation in morphology and biochemical in different garlic cultivars. Studied traits were bulb and clove weight, clove height, number of cloves per bulb, colour, pH, titrable acidity (TA), total soluble solids (TSS), dry matter (DM), total phenolic content (TPC) and antioxidant activity (AA). The results showed that Turkish variety had the highest TA (1.21%), TSS (36.80%) and AA (62.58%) while the highest DM was obtained from French variety. In contrast, the highest level of TPC (32.17 mg GAE g^{-1} fresh $weight^{-1}$) was found in Chinese variety.

Umaretiya et al. (2019) studied in total 21 garlic genotypes for TSS, crude protein content, dry matter and crude fat. Genotype 'RGP- 1' revealed maximum total sugar content of 6.66% and maximum reducing sugar content of 4.46%. Study reported highest non reducing sugar content of 3.79% in genotype 'RGP-114' and maximum crude protein content of 10.43% in genotype 'RGP-585'. Genotype 'RGP-513' had highest dry matter content of 44.14% and genotype 'RGP-114' had maximum crude fat content of 0.592%.

Thapa et al. (2021) carried out phenotypic characterization and genetic diversity assessment of thirty-seven local garlic landraces. The phenotypic diversity was analysed based on fifteen qualitative and nine quantitative characters by single non replicated rod row design following the Descriptors developed by International Plant Genetic Resources Institute (2001). Four clusters were identified and 'CO 10307', 'CO 10482' and 'CO 10615' of fourth cluster were superior in terms of quantitative characters.

Akan (2022) measured morphological characters including biometric parameters based on 18 morphological traits in genotypes of garlic obtained from different cultivation location in Turkey. The study proved significant variation in the Turkish genotypes for the Morphological Descriptors and the results showed besides cultivation area, environmental conditions effect the differentiation in garlic genotypes.

Kirac et al. (2022) determined morphological and biochemical characteristics of 39 garlic genotypes which were widely cultivated in Turkey in terms of features like fresh weight, dry weight and bulb diameter. The study concluded that despite its clonal

propagation, some level of morphological and molecular variation is present in garlic plant.

Shree et al. (2022) performed morphological characterization of 25 genotypes collected locally as well as from different research centres of India. A significant variation was observed in heights of plants along with length and breadth of leaves. Highest bulb yield was obtained in garlic line '650' which was reported to be significantly superior to all other genotypes.

Shreshtha et al. (2022) collected and characterized 37 garlic landraces from Nepal genebank by using phenotypic trait maps or agro-morphological markers. A significant intra landrace diversity was revealed for both quantitative and qualitative traits when Shannon and Weaver diversity index (H') analysis was done. Thirty-seven accessions were grouped into five distinct group. The Accession 'CO4816' was found superior in terms of yield attributing characters such as number of cloves per bulb, weight of cloves and yield but shorter in plant height. High significant correlation was revealed among 8 quantitative traits i.e., leaf length and leaf width, leaf length and shaft length, leaf length and yield, weight of cloves and yield when Pearson correlation analysis was performed.

2.2 *In vitro* regeneration

2.2.1 *In vitro* direct regeneration

Kehr et al. (1976) reported differentiation when garlic explants excised from growing points of air-dried bulbs were cultured in MS or Nitsch medium with or without growth regulators. Vegetative cell differentiation was observed when explants were cultured on MS media supplemented with 1.0 mgL^{-1} IAA; 0.5 mgL^{-1} 2,4 D; 1.0 mgL^{-1} kinetin and coconut milk. However, differentiated plants were observed on MS medium with 1.0 mgL^{-1} IAA and 0.2 mgL^{-1} kinetin or on Nitsch medium without hormones.

Bhojwani (1980) obtained both axillary and adventitious shoot proliferation from shoot buds about 5-8 mm long on B5 as a basal medium supplemented with 0.5 mgL^{-1} 2 iP and 0.1 mgL^{-1} NAA. Eight times increase in shoot number was observed every six weeks and shoots were readily rooted in B5 + 0.01 mgL^{-1} NAA. When transferred to pots, about 70% formed established plants which retained the diploid condition of the parents. MS media proved to be an effective medium, supplemented with 0.3 mgL^{-1} IAA, 2.0 mgL^{-1} 2 iP and 30.0 gm sucrose L^{-1}, for shoot multiplication from shoot tip explants.

El-Gizawy and Ford-Lloyd (1984) kept cultures in varying temperature and media to maintain its viability. A range of *Allium sativum* varieties were cultured on B5 media supplemented with NAA (1.0 mgL^{-1}) at 4, 8, 12 and 26 ºC which proved possible to maintain all the varieties for 12 months.

Bovo and Mroginski (1985) cultured 0.4-0.6 mm meristems on MS medium supplemented with 0.01-0.1 mgL^{-1} NAA and 0.3-3.0 mgL^{-1} GA_3. They reported highest plant regeneration of 60-74% in MS medium containing 0.1 mgL^{-1} NAA.

Choi et al. (1985) observed that when only apical meristems were cultured, it resulted in 16% survival after four weeks in culture and slow subsequent shoot formation, whereas the retention of one or two leaf primordia resulted in survival up to 40% survival and plantlets were produced even in the unsupplemented medium.

Lassocinski et al. (1985) used scales with shoot apex of garlic to initiate successful cultures using Gamborg's B5 medium containing 10.0 mgL^{-1} kinetin and 0.1 mgL^{-1} NAA. Large number of regenerated plants were produced.

Mosella and Fernandez (1985) cultured shoot tips on a medium containing 0.05 mgL^{-1} BAP and 126.0 mgL^{-1} phloroglucinol for 20 days and then transferred it to a rooting medium with 0.5-2.0 mgL^{-1} NAA. Percentage rooting observed was highest (92% after 30 days) with 1.0 mgL^{-1} NAA along with microbulbs that developed in 80% of the rooted shoots while still in culture.

Edney et al. (1988) reported that 70% more roots were initiated in MS medium with a modified potassium concentration (7.577 gL^{-1} as potassium dibasic phosphate) and 21% more shoots than MS medium with a regular potassium content (4.839 gL^{-1} as potassium monobasic phosphate).

Rauber and Grunewaldt (1988) attempted shoot regeneration by using leaf disc as an explant from *Allium sativum* L., *A. porrum* L and *Allium schoenoprasum* L. The results showed that regeneration rate from leaf disc was low and shoot regeneration ability was strongly controlled by the genotype.

Moriconi et al. (1990) obtained numerous adventitious shoots from shoot tip explants of two different clonal types of garlic. Two different media were used to culture

the explants and further subcultured on 5 concentrations of growth regulator. The cultures were kept under 16 h/day photoperiod and observations were made every 50-60 days. The results showed that initiation and growth were best on MS medium enriched with 0.4 mgL^{-1} thiamine, 100.0 mgL^{-1} myo-inositol, 0.5 mgL^{-1} nicotinic acid, 0.5 mgL^{-1} pyridoxine, 2.0 mgL^{-1} glycine, 80.0 mgL^{-1} adenine sulphate, 3% sucrose, 0.1 mgL^{-1} IAA and 0.1 mgL^{-1} kinetin in 0.65% agar.

Ravnikar et al. (1993) observed that JA significantly contributed to enhancement of shoot and bulb development when present at 1.0-10.0 µM. An initiation medium containing 10.0 µM JA + 5.0 µM 2 iP resulted in the highest rate of shoot formation with an average of 30 shoots per explant as observed after 6 weeks of culture.

Haque et al. (1997) cultured root tips on medium supplemented with 1.0 µM NAA and 10.0 µM BAP to develop an effective and innovative method of direct *in vitro* regeneration. The cultured root tips produced *in vitro* shoots in 75% of the explants.

Ayabe and Sumi (1998) used 'Stem disc culture' for micropropagation of garlic. Stem disc was sliced into four pieces, which were then cultured in Petri dishes over Linsmaier and Skoog medium. Cultures were then incubated at 25°C for 16 hours with fluorescent light at 3000 lux. Numerous shoot buds were observed after two weeks from each explant. Another 2 weeks of culturing resulted in shoot growth of buds into 1 cm tall shoots.

Robert et al. (1998) made use of thermotherapy for virus elimination. Multiple shoots were produced when treated meristems were cultured initially on initiation medium supplemented with 1.0 µM NAA and 1.0 µM BAP and then on multiplication medium with 5.0 µM JA and 5.0 µM 2 iP. On an average, 6-7 shoots were formed from one meristem with no callus formation.

Sata et al. (2000) established an efficient novel method for direct somatic embryogenesis from basal plate of garlic cloves. The best plant growth regulator combination observed was 2,4 D and kinetin at 1.0 mgL^{-1} and 5.0 mgL^{-1} respectively.

Martin-Urdiroz et al. (2004) carried out research to determine the protocol for *in vitro* micropropagation methods in Tunceli garlic. MS medium supplemented with different combinations of 2,4 D and NAA for root culture and NAA, IAA with BAP for shoot culture were used. The results showed that the method in which root tip was used was not proper for

shoot proliferation. On the other hand, shoot culture method was found efficient for shoot proliferation. Each explant produced on an average 1 or 2 shoots. Lower doses of IAA (0.1 mgL^{-1}) and BAP (0.1 mgL^{-1}) resulted in better shoot proliferation.

Kenel et al. (2010) recovered transgenic plants directly from immature leaves by selective culture following *Agrobacterium*-mediated transformation. The method utilized binary vector containing the *mgfp-ER* reported gene and *hpt* selectable marker. The methodology followed was similar to the protocol previously developed for the transformation of immature onion embryos. The resulting improvement in frequency and use of clonal commercial 'Printanor' germplasm facilitated the integration of useful agronomic and quality traits into this crop.

Keller and Senula (2013) standardised *in vitro* regeneration in garlic by using bulbs or bulbils as explants on MS media containing NAA, 6- 9 dimethylallyl aminopurine and 2 iP. Shoot regeneration was observed along with rooting.

Taskin et al. (2013) regenerated virus-free plants of *A. tuncelianum* and *A. sativum* on two different media. Good results were shown for both on MS medium supplemented with 2.0 mgL^{-1} BAP and 0.5 mgL^{-1} IBA. The regenerants were analysed for elimination of virus through real-time PCR assay for micropropagation by comparing shoot tip culture with meristem culture.

Mubarrat et al. (2018) reported maximum shoot regeneration rate, when only 3.0 mgL^{-1} kinetin was used or a combination of 2.5 mgL^{-1} kinetin along with 1.0 mgL^{-1} 2, 4-D. The study emphasized the effect of kinetin and 2,4-D on *in vitro* propagation of garlic (*Allium sativum*). After 3 weeks, the longest shoot length recorded was 34.50 cm.

Wen et al. (2020) performed experiments to develop an efficient and widely applicable shoot regeneration system in garlic by using garlic inflorescence as explant. A mean shoot regeneration rate of 97% and mean shoot number of 23.4 per explant were achieved in 14 commercial garlic cultivars with various characteristics and origin by optimising the type of basal medium, pH value and explant size. Histological studies showed that regenerated shoots were obtained through direct organogenesis.

Konchigeri et al. (2022) carried out study to optimize the media for direct shoot organogenesis in garlic genotype of Kodaikanal hills. The study aimed to produce virus free

planting material. *In vitro* responses in shoot organogenesis of clove basal meristematic regions were studied. According to study conducted, MS media supplemented with 2.0 mgL^{-1} BAP and 0.1 mgL^{-1} NAA produced an average of 3.1 shoots per clove. The study ensured that findings would be useful for scaling up the protocol for shoot organogenesis and subsequent bulbet induction in garlic.

2.2.2 *In vitro* indirect regeneration

Havranek and Novak (1973) used young leaf tissues of common garlic (*Allium* sativum) to derive callus on MS medium supplemented with IAA; 2,4 D and kinetin. The study compared the effect of these growth regulators from which 2,4 D appeared to be a strong dedifferential factor. Bud formation from callus was observed when concentration of 2,4 D was altered.

EI-Nil (1977) studied organogenesis and embryogenesis in callus cultures of garlic (*Allium sativum* L.). Callus cultures were subcultured into AZ (Abo-El Nil and Zettler) medium containing 10.0 µM Kinetin and 10.0 µM IAA. Organogenesis occurred as shoot was formed on modified AZ medium containing 18.0 µM ammonium nitrogen and 40.0 µM nitrate nitrogen supplemented with 10.0 µM kinetin and 10.0 µM IAA. Organogenesis was also observed in callus cells on modified AZ medium supplemented only with 20.0 µM kinetin and 10.0 µM IAA.

Lu et al. (1982) reported that green shoots resulted from differentiation of callus that was induced from young leaves in 11% of explants on MS medium supplemented with 0.5 mgL^{-1} NAA and 3.0 mgL^{-1} BAP. Rooting was achieved in 84% of cases on MS medium alone. Highest percentage of shoot and root formation was observed in meristems from cloves incubated for 24h in 50 ppm GA, 25 ppm adenine and cultured on a basal MS medium with 50 ppm IBA.

Tapia (1987) cultured meristematic root tumours on MS medium containing 0.125 mgL^{-1} 2,4 D and stems on MS medium supplemented with 0.5 mgL^{-1} 2,4 D in light to produce callus. Strong well-formed roots and green spots were observed within 60 days on MS medium with kinetin and IAA. It took 150 days to generate a whole plant.

Lee et al. (1988) observed that when MS medium supplemented with 2.0 mgL^{-1} kinetin and 0.5 mgL^{-1} NAA, multiple shoots were obtained from apical meristem comprising single leaf primordium. Most roots were formed on media supplemented with 0.5 mgL^{-1}

kinetin and 0.5 mgL^{-1} NAA. Greatest callus proliferation and shoot production from callus culture were observed on the media containing IAA at concentration 2.0 mgL^{-1}. However, IBA and BAP had no to little effect.

Richart et al. (1988) cultured divided parts of young garlic leaves on MS medium supplemented with varying concentrations of the auxins (2,4 D; 2,4,5-T; Dicamba or Pilcoram). Highest embryogenic callus was obtained in ten to twelve weeks in explants cultured on MS medium fortified with 2.5 µM 2,4,5-T. The results showed that plant regeneration via somatic embryogenesis and adventitious bud formation could be achieved on BDS- medium supplemented with 10.0 µM IAA and 0.5 µM NAA.

Xue et al. (1991) reported formation of embryogenic callus from basal plate of garlic on AZ (Abo-El Nil and Zettler) medium supplemented with 1.0-10.0 µM 2, 4-D; 10.0 µM p-CPA and 1.0 µM kinetin. Similar results were obtained when basal plates were cultured on BDS medium containing 1.0-10.0 µM NAA. The study revealed that 2,4 D was more effective for callus induction than p-CPA and BAP. Further, plants were regenerated from embryogenic callus by culturing callus in 1.0 µM BAP and were successfully transferred to soil and grown to maturity.

Barandiaran et al. (1999) investigated if different callus induction media influenced the regeneration process in garlic. They found that combinations of growth regulators commonly used for dicot tissue culture resulted in high levels of callus induction and regeneration that could be efficiently used in a genetic transformation program.

Zhen (2001) initiated callus and globular bodies from young leaves on MS medium supplemented with 500.0 mgL^{-1} casein hydrolysate, 1000.0 mgL^{-1} yeast extract and 2.0 mgL^{-1} 2,4 D. When globular bodies were cultured on MS medium enriched with 2.0 mgL^{-1} kinetin and 0.5 mgL^{-1} IAA, shoots and roots were obtained.

Fereol et al. (2002) standardised a protocol for somatic embryogenesis and plant regeneration in garlic variety ‘Rouge de la Reunion’. Young leaf section or roots obtained from *in vitro* plants were used as explants. Higher embryogenic potential was expressed in young leaves while callus was optimal in root sections derived explants.

Mariani et al. (2003) studied the effect of 2,4 D on indirect somatic embryogenesis and surface structural changes in garlic (*Allium sativum*). Root tips were used as explants and

cultured on embryogenic callus induction medium. This was followed by transfer of somatic embryos to embryo maturation medium, desiccation and transfer to the somatic embryogenesis medium repeatedly. They evaluated the effect of 2,4 D on induction of somatic embryogenesis and observed the developmental stages of somatic embryo along with surface structural changes. Observations were made using dissecting microscope which revealed that embryogenic callus and somatic embryo were formed on embryogenic callus induction medium containing 0.1 µM 2,4 D and developed into mature somatic embryos on medium containing 0.01 µM 2,4 D.

Khan et al. (2004) utilized root tips as explant to obtain indirect shoot proliferation through callus formation. Higher frequency of callus formation was reported in medium enriched with 5.0 mgL^{-1} kinetin and 1.5 mgL^{-1} 2,4 D.

Luciani et al. (2006) performed evaluation with an objective to study the effect of different explants and growth regulators on callus induction and plant regeneration in garlic (*Allium sativum*). Furthermore, they intended to evaluate different types of calluses on morphological basis by light microscopy to relate them with their abilities to generate plants in red garlic cultivar '069'.

Chowdhary et al. (2008) reported use of somatic embryogenesis to achieve plant regeneration and bulblet development. Leaf explants were collected and cultured on agar solidified MS media containing various concentrations of 2,4-D alone or in conjunction with IAA or NAA for callus induction and embryo production. The addition of IAA or NAA to 2,4-D containing medium was found to be necessary for embryogenic callus development. Medium enriched with 1.5 mgL^{-1} 2,4-D and 1.0 mgL^{-1} IAA presented 70% embryogenic callus formation.

Ahn et al. (2009) cultured garlic stem disc on MS medium containing 1.0 mgL^{-1} 2,4 D and 0.2 mgL^{-1} IAA for callus induction and proliferation. Callus formation was activated by MS media in combination with 1.0 mgL^{-1} 2,4-D and 0.2 mgL^{-1} IAA. Callus regeneration rate reported for 'Euseong' was 76.1% and that of 'Danyang' was 81.2%. Shoot regeneration rate was 51.5% and 56.6% for 'Danyang' and 'Euseong' respectively.

Cheng et al. (2012) observed callus proliferation on MS medium supplemented with 3.0 mgL^{-1} BAP and 0.1 mgL^{-1} IAA. The results showed that higher ratio of cytokinin to auxin

was required for shoot induction. Medium supplemented with 3.0 mgL^{-1} BAP and 0.1 mgL^{-1} of IAA showed highest shoot induction.

Kapoor et al. (2012) performed experiment to investigate the regeneration potential of a garlic variety 'Yamuna Safed (G1)', as well as to develop an efficient protocol for regeneration of garlic through callus induction. Higher percentage of callus was observed in medium with combination of BAP and 2,4 D at 2.0 mgL^{-1} and 0.25 mgL^{-1} respectively. Callus from roots were induced in individual shoots after shifting to medium without any growth regulator. The plantlets were established in the soil after acclimatization.

Mehta et al. (2013) regenerated somatic embryos via callus formation. MS medium containing 0.5 mgL^{-1} kinetin and 0.25 mgL^{-1} 2,4 D resulted in largest embryos. Multiple shoots were most easily induced from embryos on MS medium supplemented with 1.0 mgL^{-1} kinetin. Different concentrations of IBA were supplemented for rooting. Maximum percent rooting was observed on MS medium supplemented with 1.0 mgL^{-1} IBA.

Scotton et al. (2013) used root segments as explants to analyze the *in vitro* regeneration of eight cultivars of garlic. Bulblet-derived explants of each genotype were isolated and introduced into MS medium supplemented with 2,4 D and 2 iP. Further, callus obtained were shifted to MS medium supplemented with 8.8 mM BAP and 0.1 mM NAA, or with 4.6 mM kinetin alone. Regeneration frequencies of callus were then evaluated after maintaining *in vitro* culture for 60 days.

Metwally et al. (2014) studied 3 garlic cultivars for *in vitro* regeneration. Shoot and root apices were cultured in 8 callus induction treatments. A combination of 1.0 mgL^{-1} 2,4-D; 5.0 mgL^{-1} BAP and 5.0 mgL^{-1} NAA gave 100% of callus induction from root apices for all garlic cultivars subjected to callus induction treatments.

Khan et al. (2017) standardised an efficient protocol for callus induction in garlic by using roots as explants which were derived by clove germination on MS medium. Optimum callus was produced on MS medium containing 0.5 mgL^{-1} 2,4 D and 0.5 mgL^{-1} kinetin. For shoot regeneration from callus, the callus was cultured after 4-6 weeks in MS medium fortified with 0.5 mgL^{-1} BAP alone or 1.0 mgL^{-1} BAP in combination with 0.5 mgL^{-1} kinetin. MS medium containing 2.0 mgL^{-1} IAA and 0.5 mgL^{-1} NAA resulted in optimum rooting.

Mostafa et al. (2020) observed significant differences in callus formation in garlic varieties. The results showed that tip explants were best source to produce callus and 91.05% of the explants from 4 varieties on an average formed callus after 45 days of first culturing. Lower values were obtained from upper leaf explants. The callus was produced in shortest time from tip explant of variety 'T141' and had the greatest callus fresh weight at day 45.

Hailu et al. (2021) reported *in vitro* callus formation along with shoot proliferation on BDS or MS medium fortified with 2,4 D and kinetin or with 2,4 D and BAP.

Chapter-3

MATERIALS AND METHODS

The present research on **"*In vitro* propagation studies in garlic (*Allium sativum*)"** was carried out in the Department of Biotechnology, Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan. Outline of research work carried out has been mentioned in following sub headings.

3.1 Collection of plant material

3.2 Morphological analysis

3.3 Medium preparation

3.3.1 Cleaning of glassware

3.3.2 Preparation of stock solution

3.3.3 Preparation of culture medium

3.4 Sterilization of explants

3.5 *In vitro* regeneration in 'Yamuna Safed'

3.5.1 Surface sterilization

3.5.2 *In vitro* establishment

3.5.3 *In vitro* induction of shoots

3.6 *In vitro* regeneration in 'VL Garlic 1'

3.6.1 Surface sterilization

3.6.2 *In vitro* establishment

3.6.3 *In vitro* callus induction

3.6.4 *In vitro* induction of shoots from callus

3.7 Hardening of the *in vitro* raised micro plants

3.8 Biochemical analysis

3.8.1 Quantitative estimation of Total Soluble Solid content

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3.8.3 Quantitative estimation of Total Phenol

3.8.4 Quantitative estimation of Flavonoids

3.8.5 Quantitative estimation of Antioxidants

3.9 Statistical analysis

3.1 Collection of plant material

Bulbs of 'VL Garlic 1' were procured from 'Vivekananda Parvatiya Krishi Anusandhan Sansthan', Almora, Uttarakhand while that of 'Yamuna Safed' were procured from 'KNK College of Horticulture', Mandsaur, M.P.

3.2 Morphological analysis

Garlic bulbs of 'VL Garlic 1' and 'Yamuna Safed' were analysed for various morphological characteristics. Bulb and clove characteristics were visually studied using Descriptor of Distinctiveness, Uniformity and Stability (DUS) guideline for garlic from Plant Protection Variety and Farmer's Rights Authority (Anonymous 2009). The characteristics studied were as follows: Bulb shape, Shape of base, Compactness, Colour of external scales, Position of cloves, Arrangement of cloves, Average bulb weight, Average clove weight, Average bulb diameter, Average clove diameter and Number of Cloves of each cultivar. The above characteristics were measured in 3 samples and average was taken. Bulb weight and clove weight were measured using digital weighing machine while bulb diameter and clove diameter were measured longitudinally and equatorially with the help of vernier calliper and average was taken.

3.3 Medium preparation

3.3.1 Cleaning of glassware

All the laboratory glassware to be used in research work were washed in solution of teepol with the help of cleaning brush under running tap water. After that, they were kept in hot air oven at 160ºC for 2 hours for the purpose of sterilization.

3.3.2 Preparation of stock solution

Concentrated MS stock solutions of salts, vitamins and plant growth regulators were prepared separately and stored at 4ºC in refrigerator. Four stock solutions viz a viz Stock I, Stock II, Stock III and Stock IV were prepared. Stock solutions of cytokinin (BAP, 2 iP and TDZ) and auxin (2,4 D; NAA and IAA) were prepared in the ratio 1:1 and stored in refrigerator at 4ºC. Cytokinin (BAP and 2 iP) were dissolved in few drops of 1N NaOH, auxin (2,4 D; NAA and IAA) was dissolved in few drops of alcohol and TDZ was dissolved

in dimethyl sulfoxide (DMSO). Chemicals used in preparation of stock solutions were of tissue culture grade and procured from firms like HIMEDIA, SRL, Sigma Aldrich.

3.3.3 Preparation of culture medium

Culture medium was prepared by adding standard quantities of all four stock solutions i.e., Stock I (25 mL), Stock II (1 mL), Stock III (5 mL) and Stock IV (5 mL) in distilled water in a beaker. Sucrose (30.0 gL^{-1}), meso-inositol (100.0 mgL^{-1}) and required growth regulators (cytokinin, auxin and gibberellin) were added and final volume was made up to 1 L with distilled water. The pH of medium was taken each time after preparation of media with pH meter and pH was adjusted to 5.8 with the help of 1N NaOH and 1 N HCl. Agar was added (8.0 gL^{-1}) as a solidifying agent and medium was heated in oven to dissolve and homogenize the same.

The prepared medium in hot liquid state was poured in 100 mL flasks in equal quantities followed by plugging them with cotton plugs. The flasks containing medium were autoclaved under 15 $lbs/inch^2$, at 121 ºC for 15-20 minutes for steam sterilization.

3.4 Sterilization of explants

Explants (basal plate, roots, plumule tips) were washed with 2-3 drops of teepol and kept under running tap water for 15-20 minutes. Explants were thereafter sterilized with carbendazim and mercuric chloride in LAF chamber. Treatment of varying concentrations of carbendazim and mercuric chloride was given for different time durations to explants (Table 1). Sterilized explants were then washed in jar with autoclaved distilled water. The treatments of carbendazim and mercuric chloride were given to remove traces of fungus and bacteria respectively present on explants.

Table 1: Different sterilant treatments given to explants of 'VL Garlic 1' and 'Yamuna Safed'

S. No.	Treatments	Carbendazim (%)	Duration	$HgCl_2$ (%)	Duration
1.	Control	-	-	-	-
2.	T1	0.1	2 min	0.1	1 min
3.	T2	0.1	3 min	0.1	1 min
4.	T3	0.2	3 min 30 sec	0.1	1 min 30 sec
5.	T4	0.2	4 min	0.1	2 min
6.	T5	0.2	5 min	0.1	2 min
7.	T6	0.2	5 min	0.1	3 min
8.	T7	0.2	5 min	0.2	3 min 30 sec
9.	T8	0.2	5 min	0.2	4 min

Where, T = Treatment

3.5 *In vitro* regeneration in 'Yamuna Safed'

Various combinations of plant growth regulators were tried to observe established root and shoot cultures of garlic. Out of all the tried combinations, root establishment was observed on MS media supplemented with 2,4 D alone as well as 2,4 D along with BAP. Thereafter, concentration of BAP and NAA was varied and GA_3 was supplied to obtain shoots in established cultures.

3.5.1 Surface sterilization

Floor of laminar air flow (LAF) chamber was wiped with 70% alcohol for carrying out aseptic manipulations. Autoclaved medium containing flasks and autoclaved scalpel handle, forceps were placed inside LAF chamber, after which UV light was switched 'ON' for 15-20 minutes. UV light kills harmful micro-organisms. After switching 'OFF' UV light, fluorescent light and airflow were turned 'ON' and further aseptic manipulations were carried out. Autoclaved scalpel handle and forceps were flame sterilized using spirit lamp. Hands were disinfected with rectified spirit inside LAF chamber and alcohol was allowed to dry before carrying out inoculation process.

3.5.2 *In vitro* establishment

For *in vitro* establishment of 'Yamuna Safed', basal plate and roots of *in vitro* generated plants were used as explants. Different concentrations of auxin (2,4 D and IAA) and cytokinin (BAP) were used (Table 2). *In vitro* establishment of 'Yamuna Safed' was achieved on MS medium supplemented with 2,4 D alone and along with BAP too. The excised basal plates and roots were cultured with an objective to obtain callus.

3.5.3 *In vitro* induction of shoots

The *in vitro* established cultures with roots were cultured on shoot induction media. MS media was supplemented with different combinations and varying concentrations of auxin (2,4 D and NAA), cytokinin (BAP and 2 iP), gibberellin (GA_3) and JA (Table 3).

Table 2: Medium codes with different concentrations of plant growth regulators for *in vitro* establishment of 'Yamuna Safed' from basal plates

S. No.	Media code	MS + Growth regulators		
		2,4D (mgL^{-1})	BAP (mgL^{-1})	NAA (mgL^{-1})
1.	Control	0	-	-
2.	M1	0.5	-	-
3.	M2	1.0	-	-
4.	M3	1.5	-	-
5.	M4	2.0	-	-
6.	M5	0	0.5	-
7.	M6	0.5	1.0	-
8.	M7	1.0	1.5	-
9.	M8	1.5	2.0	-
10.	M9	2.0	2.5	-
11.	M10	-	0.5	0.1
12.	M11	-	1.0	0.2
13.	M12	-	1.5	0.3
14.	M13	-	2	0.4
15.	M14	-	2.5	0.5

Where, M = Media

Table 3: Medium codes with different concentrations of plant growth regulators for *in vitro* establishment of shoot culture in 'Yamuna Safed'

S. No.	Media code	MS + Growth regulator					
		NAA (mgL^{-1})	2,4D (mgL^{-1})	BAP (mgL^{-1})	2 iP (mgL^{-1})	JA (µL)	GA_3 (mgL^{-1})
1.	Control	-	-	-	-	-	-
2.	SE1	0.2	-	0.5	-	-	-
3.	SE2	0.3	-	1.0	-	-	-
4.	SE3	0.4	-	1.5	-	-	-
5.	SE4	0.5	-	2.0	-	-	-
6.	SE5	-	0.5	1.0	-	-	0.5
7.	SE6	-	1.0	2.0	-	-	1.0
8.	SE7	-	1.5	3.0	-	-	1.5
9.	SE8	-	0.5	1.0	-	-	-
10.	SE9	-	1.0	2.0	-	-	-
11.	SE10	-	1.5	3.0	-	-	-
12.	SE11	-	-	-	4.0	0.5	-
13.	SE12	-	-	-	6.0	1.0	-
14.	SE13	-	-	-	8.0	1.5	-
15.	SE14	-	-	-	10.0	2.0	-

Where, SE= Shoot Establishment

3.6 *In vitro* regeneration in 'VL Garlic 1'

3.6.1 Surface sterilization

Floor of laminar air flow chamber was wiped with 70% alcohol for carrying out aseptic manipulations. Autoclaved medium containing flasks and autoclaved scalpel handle, forceps were placed inside LAF chamber, after which UV light was switched 'ON' for 15-20 minutes. UV light kills harmful micro-organisms. After switching 'OFF' UV light, fluorescent light and airflow were turned 'ON' and further aseptic manipulations were carried out. Autoclaved scalpel handle and forceps were flame sterilized using spirit lamp. Hands were disinfected with rectified spirit inside LAF chamber and alcohol was allowed to dry before carrying out inoculation process.

3.6.2 *In vitro* establishment

The shoot tips with basal plates were cultured on MS medium with different concentrations of auxin (NAA), cytokinin (BAP), gibberellin (GA_3) and activated charcoal to achieve *in vitro* regeneration (Table 4).

Table 4: Medium codes with different concentrations of plant growth regulators for *in vitro* establishment of 'VL Garlic 1' from shoot tips along with basal plates

Sr.No.	Medium code	MS + Growth regulators			
		BAP (mgL^{-1})	GA_3 (mgL^{-1})	NAA (mgL^{-1})	Activated charcoal (gL^{-1})
1.	Control	-	-	-	-
2	E1	0.5	0.1	0.1	-
3.	E2	0.5	0.1	0.2	-
4.	E3	1.0	0.2	0.3	-
5.	E4	1.0	0.2	0.4	-
6.	E5	2.0	0.3	0.5	-
7.	E6	2.0	0.3	0.6	-
8.	E7	0.5	0.1	0.1	0.1
9.	E8	0.5	0.1	0.2	2.0
10.	E9	1.0	0.2	0.3	3.0
11.	E10	1.0	0.2	0.4	4.0
12.	E11	2.0	0.3	0.5	5.0
13	E12	2.0	0.3	0.6	6.0

Where, E = Establishment

3.6.3 *In vitro* callus induction

The roots obtained from *in vitro* regenerated plants were cultured on MS medium containing different combinations of auxin (2,4 D and IAA), KNO_3 and TDZ (Table 5) with varying concentrations. The objective was to obtain callus for indirect regeneration in 'VL Garlic 1'.

Table 5: Medium codes with different concentrations of plant growth regulators for callus induction from *in vitro* roots in 'VL Garlic 1'

S. No.	Media code	Medium composition with MS		Medium composition with B5		KNO_3 (g)
		2,4 D (mgL^{-1})	IAA (mgL^{-1})	TDZ (mgL^{-1})	Picloram	
1.	Control	-	-	-	-	-
2.	CFR1	0.1	-	-	-	-
3.	CFR2	0.2	0.1	-	-	-
4.	CFR3	0.3	0.2	-	-	-
5.	CFR4	0.4	0.3	-	-	-
6.	CFR5	0.5	0.4	-	-	-
7.	CFR6	0.6	0.5	-	-	-
8.	CFR7	0.2	0.1	-	-	0.5
9.	CFR8	0.3	0.2	-	-	1.0
10.	CFR9	0.4	0.3	-	-	1.5
11.	CFR10	0.5	0.4	-	-	2.0
12.	CFR11	0.6	0.5	-	-	2.5
13.	CFR12	-	-	0.5	1	-
14.	CFR13	-	-	1.0	2	-
15.	CFR14	-	-	1.5	3	-
16.	CFR15	-	-	2.0	4	-

Where, CFR = Callus from Roots

3.6.4 *In vitro* induction of shoots from callus

The callus obtained was further culture on MS medium in combination with varying concentrations of auxin (2,4 D and IAA), cytokinin (2 iP and BAP) and gibberellin (GA_3) with an objective to obtain shoots (Table 6).

Table 6: Medium codes with different concentrations of growth regulators for shoot induction from callus in 'VL Garlic 1'

S. No.	Media code	MS + Growth regulator				
		2,4 D (mgL^{-1})	BAP (mgL^{-1})	GA_3 (mgL^{-1})	IAA (mgL^{-1})	2 iP (mgL^{-1})
1.	Control	-	-	-	-	-
2.	SFC1	0.5	1.0	0.5	-	-
3.	SFC2	1.0	2.0	1.0	-	-
4.	SFC3	1.5	3.0	1.5	-	-
5.	SFC4	2.0	4.0	2.0	-	-
6.	SFC5	0.2	-	-	0.1	0.2
7.	SFC6	0.3	-	-	0.2	0.3
8.	SFC7	0.4	-	-	0.3	0.4
9.	SFC8	0.5	-	-	0.4	0.5
10.	SFC9	0.6	-	-	0.5	0.6

Where, SFC = Shoot From Callus

3.7 Hardening of the *in vitro* raised micro plants

Hardening of the obtained *in vitro* plants of 'Yamuna Safed' was done in potting mixture having coco-peat, vermiculite and perlite in ratio 2:1:1.

3.8 Biochemical analysis

Biochemical analysis was done of cloves of both cultivars. Estimation of TSS, Ascorbic acid content, Total Phenol content, Flavonoids and Antioxidants was done.

3.8.1 Quantitative estimation of Total Soluble Solid content

Quantitative estimation of Total Soluble Solid was performed as described by Ranganna (2009). Digital refractometer was used to measure TSS and the results were expressed in degree Brix (^oB).

3.8.2 Quantitative estimation of Ascorbic Acid content

Quantitative estimation of Ascorbic acid content was performed by using 2,6-dichlorophenol indophenol visual titration method as described by Rao and Deshpande (2006).

3.8.2.1 Chemicals and reagents

L-Ascorbic acid; 2,6-dichlorophenolindophenol dye; Metaphosphoric acid solution (3%); Sodium bicarbonate.

3.8.2.2 Preparation of dye

Sodium bicarbonate (42 mg) was dissolved in 150 mL boiled distilled water. Dye was prepared by adding 50 mg of dye to sodium bicarbonate solution and final volume was made up to 200 mL with distilled water.

3.8.2.3 Standardization of dye factor

Dye factor was standardized by dissolving 100 mg Ascorbic acid in 3% metaphosphoric acid solution and final volume was prepared up to 100 mL. From this 100 mL prepared solution, 10 mL of working solution is taken and titrated against dye to get titre value.

$$\text{Dye factor} = 0.5/ \text{ Titre value}$$

3.8.2.4 Estimation

Cloves of both garlic varieties were crushed in pestle and mortar separately. Further, garlic extract obtained (25 g) was pressed with the help of muslin cloth and strained into two different flasks. Ten millilitre (mL) garlic juice of both the varieties was obtained from garlic extract. Metaphosphoric acid solution was made up to 100 mL by adding distilled water. From the above prepared solution, 10 mL of solution was taken and titrated against 2,6-dichlorophenolindophenol dye. Titration was done until pink colour appeared.

The results were expressed as mg/100 g of sample and calculated as per formula:

$$\text{Ascorbic acid (mg/100 g)} = \frac{\text{Titre value}\times*\text{Dye factor}\times\text{Dilution}\times 100}{\text{Weight of sample}\times\text{Volume of sample}}$$

3.8.3 Quantitative estimation of Total Phenol

Quantitative estimation of Total Phenol content was performed by method as described by Bray and Thorpe (1954).

3.8.3.1 Chemicals required

Chemicals required were 80% ethanol, 20% Na_2CO_3 and 1N FCR. FCR (1N) was prepared by mixing in distilled water in 1:1 ratio.

3.8.3.2 Preparation of standard curve

Stock of standard Gallic acid was prepared by dissolving 0.01 g in 10 mL of 80% ethanol. From this stock solution, 1 mL aliquot was taken and final volume was made up to 10 mL with 80% ethanol. Working standard solution of Gallic acid viz 0.2 mL, 0.4 mL, 0.6 mL, 0.8 mL and 1 mL were taken in different test tubes and final volume was made up to 3 mL with distilled water. FC reagent (0.5 mL) was added in each test tube. After waiting for 5 minutes, 2 mL Na_2CO_3 was added in each test tube. All the test tubes were kept in hot boiling water bath for 1 minute. This was followed by taking OD in spectrophotometer at 765 nm and standard curve was plotted.

Table 7: Standard table for Total Phenol estimation

S. No.	Volume of working standard solution (mL)	Volume of distilled water (mL)	Folin Ciocalteu Reagent (mL)	Na_2CO_3 (mL)	OD at 765 nm
1.	-	3.0	0.5	2 mL	0
2.	0.2	2.8	0.5	2 mL	0.4734
3.	0.4	2.6	0.5	2 mL	0.7507
4.	0.6	2.4	0.5	2 mL	1.0799
5.	0.8	2.2	0.5	2 mL	1.5297
6.	1.0	2.0	0.5	2 mL	1.8031

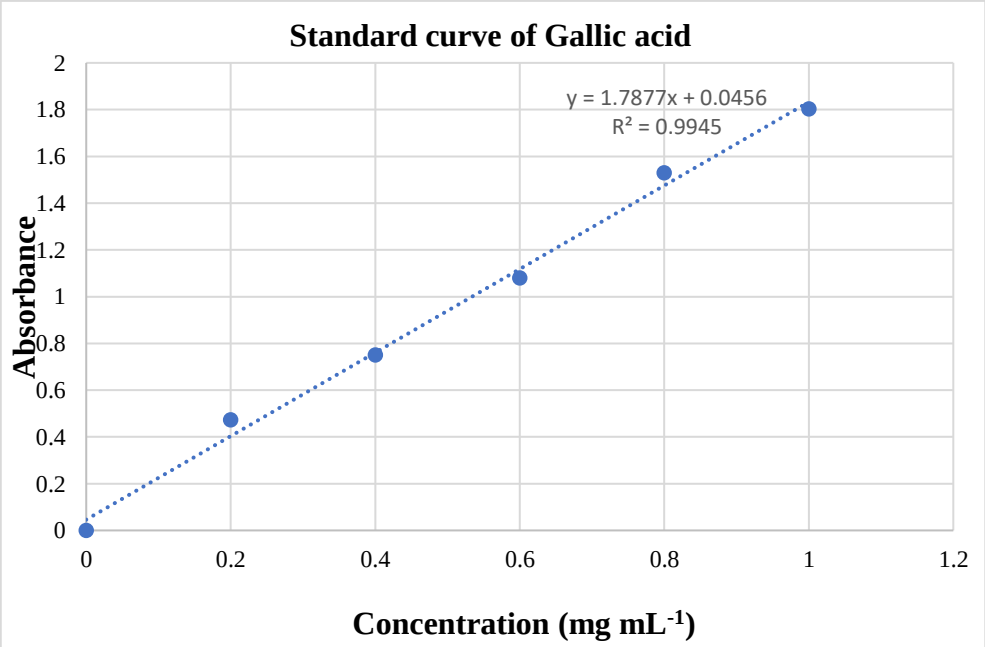


Figure 2: Standard curve of Gallic acid

3.8.3.3 Sample preparation

Garlic cloves were crushed with the help of mortar and pestle. The extract was strained to obtain 1 mL garlic juice and 9 mL of 80% ethanol was added to it.

3.8.3.4 Estimation

Prepared sample (0.5 mL) was taken in a test tube and 3 mL distilled water was added. It was followed by addition of 0.5 mL FCR and 2 mL Na_2CO_3. Bluish colour appeared. The prepared solution was kept in hot water bath for 2 minutes. It was allowed

to cool down and OD was taken at 765 nm. Standard curve was prepared using Gallic acid as standard.

3.8.4 Quantitative estimation of Flavonoids

Quantitative estimation of flavonoids was performed following method as explained by Chang et al. (2002).

3.8.4.1 Chemicals required

Pure methanol, 80% ethanol, 10% $AlCl_3$ and 10% CH_3COOK

3.8.4.2 Preparation of standard curve

Stock of standard Quercetin was prepared by dissolving 100 mg in 100 mL pure methanol. Working standard solution of Quercetin viz 0.1 mL, 0.2 mL, 0.3 mL and 0.4 mL were taken in different test tubes and final volume was made up to 10 mL with methanol. From each working standard solution, 0.1 ml aliquot was taken from each of the test tube in 5 different test tubes after which 1 mL of 80% ethanol, 0.1 mL of 10% $AlCl_3$ and 0.1 mL of 10% CH_3COOK was added in each of these test tubes. It was followed by addition of 4.3 mL of distilled water in each test tube. Finally, the solutions were kept at room temperature for 40 minutes and OD was taken in spectrophotometer at 415 nm.

Table 8: Standard table for Total Flavonoid estimation

S. No.	Volume of working standard solution (mL)	80% Ethanol (mL)	10% $AlCl_3$ (mL)	10% CH_3COOK (mL)	Distilled water (mL)	OD at 415 nm
1.	-	1	0.1	0.1	4.3	0
2	0.1	1	0.1	0.1	4.3	1.008
3	0.2	1	0.1	0.1	4.3	2.155
4	0.3	1	0.1	0.1	4.3	3.089
5	0.4	1	0.1	0.1	4.3	4.0

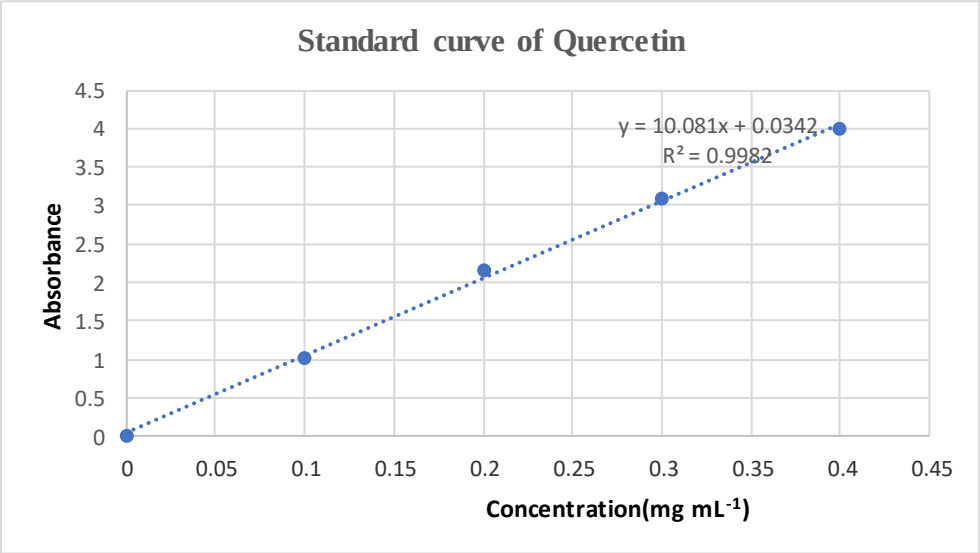


Figure 3: Standard curve of Quercetin

3.8.4.3 Sample preparation

Garlic cloves were crushed with the help of mortar and pestle. The extract was strained with the help of muslin cloth and garlic juice was obtained.

3.8.4.4 Estimation

From the obtained sample, 0.1 mL garlic juice was taken and was added in 1 mL of 80% ethanol followed by addition of 0.1 mL of 10% $AlCl_3$ and CH_3COOK. Distilled water (4.3 mL) was added and OD was taken at 415 nm as against the reagent blank.

3.8.5 Quantitative estimation of Antioxidants

Quantitative estimation of antioxidants was performed following the method as described by Ranganna (2009).

3.8.5.1 Reagents

Reagents required were pure methanol and 0.1 M DPPH. DPPH (0.1 M) was prepared by adding 23 mg in 1 L pure methanol.

3.8.5.2 Sample preparation

Garlic cloves were crushed with the help of mortar and pestle. The garlic juice was obtained by straining extract using muslin cloth and 1 mL garlic juice was taken. It was mixed with 3 mL methanol using a stirrer. The sample was filtered using cotton.

3.8.5.3 Estimation

From the filtrate, 0.1 mL was added in 3.9 mL DPPH. It was kept in dark for 30 minutes. OD was taken at 515 nm as against the pure methanol used as blank.
Antioxidant activity was calculated using following formula:

$$\% \text{ Antioxidant activity} = \frac{OD\ of\ DPPH - OD\ of\ sample}{OD\ of\ DPPH} \times 100$$

3.9 Statistical analysis

Morphological and Biochemical data was analysed using t test through OPSTAT software. Data was recorded in mean of three replicates. All data collected was subjected to analysis of variance at 5% level of significance. Data was analysed according to Completely Randomized Design (CRD) analysis using standard statistical OPSTAT software (Gomez and Gomez 1984), followed by Duncan's Multiple Range Test (DMRT) for comparison between sample means.

Chapter-4

RESULTS AND DISCUSSION

The results achieved in course of research on the topic **"*In vitro* propagation studies in garlic (*Allium sativum*)"** has been discussed under following headings:

4.1 Morphological analysis

4.2 Explant for *in vitro* regeneration in garlic

4.3 Sterilization of explants for *in vitro* regeneration in garlic

4.4 *In vitro* regeneration in 'Yamuna Safed'

4.4.1 *In vitro* establishment

4.4.2 *In vitro* induction of shoots

4.5 *In vitro* regeneration in 'VL Garlic 1'

4.5.1 *In vitro* establishment

4.5.2 *In vitro* callus induction

4.5.3 *In vitro* induction of shoots from callus

4.6 Hardening of the *in vitro* raised micro plants

4.7 Biochemical analysis

4.7.1 Quantitative estimation of Total Soluble Solid content

4.7.2 Quantitative estimation of Ascorbic Acid content

4.7.3 Quantitative estimation of Total Phenol

4.7.4 Quantitative estimation of Flavonoids

4.7.5 Quantitative estimation of Antioxidants

4.1 Morphological analysis

Morphological analysis of two garlic varieties, 'VL Garlic 1' and 'Yamuna Safed' was carried out using Distinctiveness, Uniformity and Stability (DUS) Descriptors of garlic from Protection of Plant Variety and Farmer's Rights Authority (PPV & FRA) (Anonymous 2009). Bulb and clove characteristics of both 'VL Garlic 1' and 'Yamuna Safed' were studied

(Table 9). 'VL Garlic 1' was of white colour whereas 'Yamuna Safed' was silvery white in colour. Anthocyanin strips were absent on both of them. 'VL Garlic 1' had elliptical bulb shape whereas bulb of 'Yamuna Safed' was of ovate shape (Plate 1). However, shape observed was circular and ovate for 'VL Garlic 1' and 'Yamuna Safed' respectively when bulb was cut in cross section. Mean of 3 samples of each variety was taken to measure average bulb diameter and average clove weight.

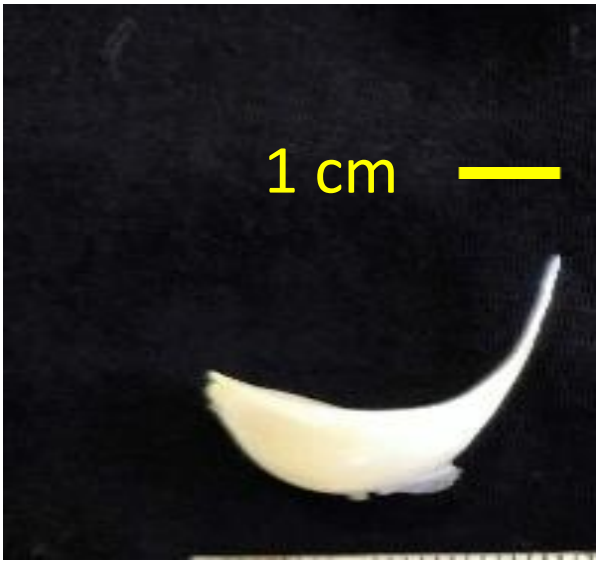
Table 9: Morphological characteristics of bulb and clove of garlic cultivars

BULB CHARACTERISTICS			
S. No.	**Qualitative characters**	**VL Garlic 1**	**Yamuna Safed**
1.	Bulb shape (longitudinal section)	Elliptic	Ovate
2.	Bulb shape (cross section)	Circular	Oval
3.	Anthocyanin strips	Present	Absent
4.	Shape of base	Recessed	Recessed
5.	Compactness	Compact	Compact
6.	Bulb colour	White	Silvery white
S. No.	**Quantitative characters**		
1.	Average bulb weight (g)	30.62±0.98	28.60±0.25
2.	Average bulb diameter (mm)	54.02±1.18	53.10±1.01
CLOVE CHARACTERISTICS			
S. No.	**Qualitative characters**	**VL Garlic 1**	**Yamuna Safed**
1.	Colour of scale	Pale yellow	White
2.	Colour of flesh	Yellow	Creamy white
3.	Clove position at tip of base	Exerted	Inserted
4.	External cloves	Absent	Absent
5.	Distribution of cloves	Radial	Radial
S. No.	**Quantitative characters**		
1.	Average clove weight (g)	3.83±0.62	3.61±1.30
2.	Average clove diameter (mm)	22.20±0.54	23.13±0.60
3.	Number of cloves	8-10	8-10

4.2 Explant for *in vitro* regeneration in garlic

Different kinds of explants have been used in various researches carried out till date. This includes excised roots (Bhojwani 1980; Haque et al. 1997). Ayabe and Sumi (1998) used stem disc which is found just under the basement of immature foliage leaves. Shoot meristems and root tips were used as explants by Haque et al. (2003). Sata et al. (2000) found cloves to be highly suitable for multiple shoot production. Also, young leaf sections or root sections from *in vitro* plants were used as explants by Nagasawa (1988) and Fereol (2002) for

A)



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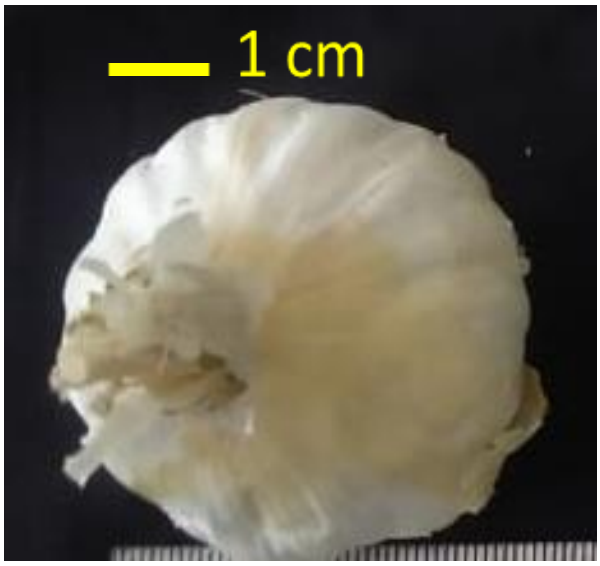
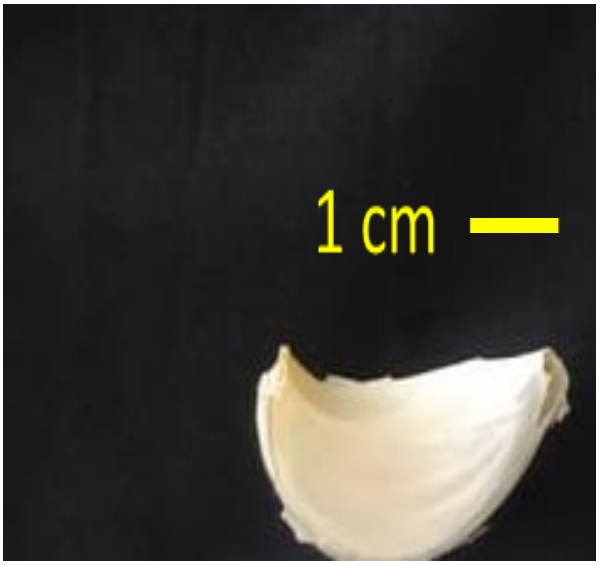


C)



D)

A) Bulb of 'VL Garlic 1'

B) Clove of 'VL Garlic 1'

C) Bulb of 'Yamuna Safed'

D) Clove of 'Yamuna Safed'

Plate 1: Bulbs and cloves of both the cultivars

callus induction. In present study, basal plate and excised roots were used as explants. The basal plate used was cut from the base of peeled clove while the roots used were excised from *in vitro* regenerated plants. The basal plates responded well in case of 'Yamuna Safed' while did not respond at all in case of 'VL Garlic 1'. *In vitro* roots of 'Yamuna Safed' did not respond in any media. However, *in vitro* roots of 'VL Garlic 1' produced callus. *In vitro* direct regeneration was observed in 'Yamuna Safed' and regeneration via callus formation was observed in 'VL Garlic 1'.

4.3 Sterilization of explants for *in vitro* regeneration in garlic

Sterilants were used at a particular concentration for different time intervals and their effect on mean percent uncontaminated explants was recorded. The explants (*in vitro* roots and basal plate) were first washed with teepol under running tap water for 15-20 minutes. Further, the explants were given treatment with different concentrations and combinations of sterilants for different durations. Finally, the treated explants were washed with autoclaved distilled water.

Effective sterilization was observed on 0.1% $HgCl_2$ and 0.2% carbendazim when treated for 3 minutes and 5 minutes respectively. Same treatment was found effective for the sterilization of explants of 'Agrifound Parvati' and 'CITH' (Tandon 2022). $HgCl_2$ has previously been demonstrated as an effective sterilant for *in vitro* regeneration in garlic (Pandey et al. 1992; Kamstaityte and Stanys 2004) and other *Allium* species like onion. Khar et al. (2005) reported that treatment with carbendazim increased the mean percent of non-contaminated explants. The results showed that on treatment T6 i.e., 0.2% carbendazim and 0.1% $HgCl_2$ for 5 and 3 minutes respectively resulted in maximum percent uncontamination of 90.34% for explants of 'VL Garlic 1'. Maximum percent survival of 91.50% was achieved for cultivar 'VL Garlic 1' (Table 10). In 'Yamuna Safed', maximum percent uncontamination of 90.34% and maximum percent survival of 90.89% was achieved using T6 treatment (Table 11). It has been proved in previous experiments that $HgCl_2$ treatment not only reduces contamination but also mean percent survival of explants. This means on increasing the concentration of $HgCl_2$ up to 0.2%, mean percent survival gets reduced. It has been reported by Patel et al. (2015) that $HgCl_2$ (0.1%) and carbendazim (0.2%) are effective in reducing microbial contamination in garlic. Various scientists have used different concentrations and duration of sterilant like $HgCl_2$, carbendazim and alcohol. Sata et al. (2000) reported that contamination is eliminated by surface sterilization with 0.1% $HgCl_2$ for 15 minutes and 70%

alcohol for 30 seconds. Haque et al. (2003) reported an efficient sterilization when the explants were rinsed with 70% alcohol only.

Table 10: Effect of different sterilant treatments for different time duration on *in vitro* survival of explants of 'VL Garlic 1'

S. No.	Treatments	Carbendazim (%)	Duration	$HgCl_2$ (%)	Duration	Mean percent uncontaminated explants	Mean percent survival
1.	Control	-	-	-	-	0.00^{h}	0.00^{i}
2.	T1	0.1	2 min	0.1	1 min	$21.22(27.42)^{f}$	$18.41(25.40)^{f}$
3.	T2	0.1	3 min	0.1	1 min	$56.62(48.78)^{e}$	$58.44(49.84)^{e}$
4.	T3	0.2	3 min 30 sec	0.1	1 min 30 sec	$65.41(53.96)^{d}$	$68.23(55.67)^{d}$
5.	T4	0.2	4 min	0.1	2 min	$80.00(63.41)^{c}$	$81.72(64.67)^{c}$
6.	T5	0.2	5 min	0.1	2 min	$85.33(67.52)^{b}$	$86.04(68.06)^{b}$
7.	**T6**	**0.2**	**5 min**	**0.1**	**3 min**	**$90.34(71.87)^{a}$**	**$91.50(73.17)^{a}$**
8.	T7	0.2	5 min	0.2	3 min 30 sec	$80.10(63.49)^{c}$	$10.56(18.95)^{g}$
9.	T8	0.2	5 min	0.2	4 min	$80.27(63.60)^{c}$	$2.82(9.66)^{h}$
$CD_{0.05}$						2.37(1.77)	2.57(2.20)
SE±						1.10(0.58)	0.85(0.73)

Where, T = Treatment

Here, CD = Critical Difference

SE = Standard Error

Table 11: Effect of different sterilant treatments for different time duration on *in vitro* survival of explants of 'Yamuna Safed'

S. No.	Treatments	Carbendazim (%)	Duration	$HgCl_2$ (%)	Duration	Mean percent uncontaminated explants	Mean percent survival
1.	Control	-	-	-	-	0^{h}	0^{i}
2.	T1	0.1	2 min 30 sec	0.1	30 sec	$20.05(26.59)^{g}$	$18.04(25.12)^{f}$
3.	T2	0.1	3 min	0.1	1 min	$52.65(46.50)^{f}$	$58.39(49.81)^{e}$
4.	T3	0.2	3 min 30 sec	0.1	1 min 30 sec	$60.31(50.93)^{e}$	$66.13(54.39)^{d}$
5.	T4	0.2	4 min	0.1	2 min	$82.02(64.89)^{c}$	$80.27(63.64)^{c}$
6.	T5	0.2	4 min 30 sec	0.1	2 min 30 sec	$87.11(68.94)^{b}$	$86.30(68.34)^{b}$
7.	**T6**	**0.2**	**5 min**	0.1	**3 min**	**$90.50(72.16)^{a}$**	**$90.89(72.58)^{a}$**
8.	T7	0.2	5 min 30 sec	0.2	3 mins 30 secs	$75.31(30.19)^{d}$	$10.62(19.01)^{g}$
9.	T8	0.2	6 min	0.2	4 min	$80.22(26.71)^{c}$	$3.71((11.10)^{h}$
$CD_{0.05}$						2.75(2.24)	3.40(2.93)
SE±						0.91(0.74)	1.12(0.97)

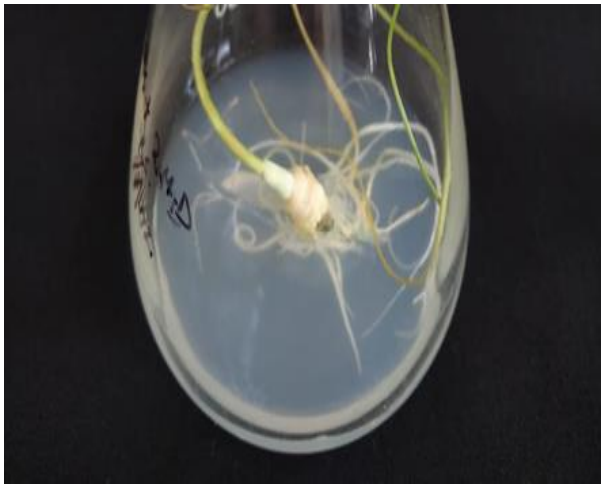
Where, T = Treatment

Here, CD = Critical Difference

SE = Standard Error

A) Day 3

B) Day 10



C) Day 20

D) Day 40

D) Day 60

Plate 2 : *In vitro* establishment of 'Yamuna Safed' using basal plate as explant on M4 medium (MS + 2mgL^{-1} 2,4 D)

4.4 *In vitro* regeneration in 'Yamuna Safed'

4.4.1 *In vitro* establishment

For *in vitro* establishment of cultures of 'Yamuna Safed', basal plates used as explants were placed in MS medium containing different concentrations of growth regulators (2,4 D; BAP; NAA). Root induction was observed first in basal plates in medium containing 2 mgL^{-1} 2,4 D after 10 days without callus formation. Percent rooting, average number of roots and average length of roots observed were most in M4 medium (MS + 2 mgL^{-1} 2,4 D) (Plate 2). Highest percent rooting, average number of roots and average length of roots observed was 73.33(58.89) %, 8.00±0.01 and 6.50±0.09 cm respectively (Table 12)**.**

Table 12: Effect of different concentrations of growth regulators on *in vitro* establishment of 'Yamuna Safed' from excised basal plate

S. No.	Media code	MS + Growth regulators			Percent establishment (%)	Average number of roots	Average root length (cm)	Number of days taken for establishment	Average number of shoots
		2,4D (mgL^{-1})	BAP (mgL^{-1})	NAA (mgL^{-1})					
1.	Control	-	-	-	0^{k}	0^{h}	0^{d}	-	0^{b}
2.	M1	0.5	-	-	$12.60(20.78)^{i}$	2.41 ± 0.04^{e}	1.00 ± 0.01^{bcd}	45-50	0.33 ± 0.33^{ab}
3.	M2	1.0	-	-	$20.11(26.63)^{f}$	3.26 ± 0.02^{d}	1.21 ± 0.01^{bcd}	45-50	0^{b}
4.	M3	1.5	-	-	$30.02(33.21)^{c}$	3.24 ± 0.04^{d}	1.06 ± 0.01^{bcd}	45-50	0^{b}
5.	**M4**	**2.0**	**-**	**-**	**$73.33(58.89)^{a}$**	**8.00 ± 0.01^{a}**	**6.50 ± 0.09^{a}**	**40-45**	1.0 ± 0.33^{a}
6.	M5	0	0.5	-	0^{k}	0^{h}	0^{d}	-	0^{b}
7.	M6	0.5	1.0	-	$15.71(23.34)^{gh}$	3.22 ± 0.01^{d}	2.51 ± 0.02^{bcd}	50-55	0^{b}
8.	M7	1.0	1.5	-	$56.87(48.93)^{b}$	7.82 ± 0.18^{a}	6.23 ± 0.04^{a}	50-55	0.67 ± 0.33^{ab}
9.	M8	1.5	2	-	$25.26(30.16)^{d}$	1.12 ± 0.01^{g}	0.54 ± 0.01^{cd}	55-60	0^{b}
10.	M9	2.0	2.5	-	$23.20(28.78)^{e}$	1.01 ± 0.003^{g}	0.49 ± 0.01^{cd}	80-85	0^{b}
11.	M10	-	0.5	0.1	$10.02(18.45)^{j}$	2.20 ± 0.13^{e}	2.08 ± 0.03^{bcd}	80-85	0^{b}
12.	M11	-	1.0	0.2	$10.35(18.76)^{j}$	2.01 ± 0.02^{f}	2.52 ± 0.02^{bcd}	70-75	0.33 ± 0.33^{ab}
13.	M12	-	1.5	0.3	$11.91(20.18)^{i}$	2.50 ± 0.04^{e}	2.74 ± 0.04^{bcd}	65-70	0.33 ± 0.33^{ab}
14.	M13	-	2	0.4	$14.30(22.21)^{h}$	4.60 ± 0.06^{c}	3.68 ± 0.02^{abc}	65-70	0.67 ± 0.33^{ab}
15.	M14	-	2.5	0.5	$16.51(23.96)^{g}$	4.82 ± 0.09^{b}	3.82 ± 0.05^{ab}	50-55	1.0 ± 0.33^{a}
$CD_{0.05}$					1.42 (0.88))	0.19	2.76		
SE±					0.49(0.30)	0.07	0.95		

Where, M = Media

Here, CD = Critical Difference

SE = Standard Error

Haque et al. (1997) reported direct shoots from roots on media containing 0.2 mgL^{-1} 2,4 D and 2.5 mgL^{-1} BAP. In present study, direct regeneration was observed from basal plate on MS media containing 1.0 mgL^{-1} 2,4 D and 2.0 mgL^{-1} BAP. Direct regeneration has been observed through various explants till date (roots, leaves).

4.4.2 *In vitro* induction of shoots

Although, single shoot of 0.5-1 cm was observed from basal plate in medium M4, in order to induce considerable number of shoots in the *in vitro* plantlets with induced roots, the medium was supplemented with different concentrations of auxin (2,4 D and NAA), cytokinin (BAP and 2 iP) and gibberellin (GA_3). Shoot induction was observed on all the media combinations. Highest average shoot length observed was 12.06±0.09 cm while highest average number of shoots observed were 5.21±0.11 (Table 13). SE6 medium (MS + 1.0 mgL^{-1} 2,4 D + 2.0 mgL^{-1} BAP + 1 mgL^{-1} GA_3) was observed to be the best media for highest average shoot length (Plate 3) whereas SE9 medium (MS + 1.0 mgL^{-1} 2,4 D + 2.0 mgL^{-1} BAP) was observed to be best for highest number of shoots per explant (Plate 4). Number of days taken were least in SE9 medium. A comparison was done of shoot length on both media, SE6 and SE9 (Plate 5).

Table 13: Effect of different growth regulators on shoot establishment of 'Yamuna Safed' from *in vitro* basal plates

S. No.	Media code	MS + Growth regulator						Average length of shoot (cm)	Average number of shoots per explant	Number of days taken for shoot induction
		NAA (mgL^{-1})	2,4D (mgL^{-1})	BAP (mgL^{-1})	2 iP (mgL^{-1})	JA (µL)	GA_3 (mgL^{-1})			
1.	Control	-	-	-	-	-	-	2.83±0.02^{i}	2.21±0.05^{i}	45-50
2.	SE1	0.2	-	0.5	-	-	-	2.64±0.01^{ij}	2.43±0.04^{h}	40-45
3.	SE2	0.3	-	1.0	-	-	-	3.21±0.03^{h}	3.19±0.06^{g}	40-45
4.	SE3	0.4	-	1.5	-	-	-	3.34±0.05^{gh}	3.21±0.01^{g}	35-40
5.	SE4	0.5	-	2.0	-	-	-	3.75±0.09^{f}	3.56±0.05^{e}	35-40
6.	SE5	-	0.5	1.0	-	-	0.5	5.66±0.04^{e}	4.97±0.05^{b}	60-65
7.	**SE6**	**-**	**1.0**	**2.0**	**-**	**-**	**1.0**	**12.06±0.09^{a}**	**5.18±0.06^{a}**	40-45
8.	SE7	-	1.5	3.0	-	-	1.5	6.42±0.08^{d}	5.07±0.01^{ab}	45-50
9.	SE8	-	0.5	1.0	-	-	-	2.42±0.03^{j}	2.23±0.04^{i}	45-50
10.	**SE9**	**-**	**1.0**	**2.0**	**-**	**-**	**-**	8.60±0.18^{b}	**5.21±0.11^{a}**	**35-40**
11.	SE10	-	1.5	3.0	-	-	-	7.82±0.03^{c}	4.80±0.07^{c}	45-50
12.	SE11	-	-	-	4.0	0.5	-	3.28±0.06^{h}	4.11±0.04^{d}	50-55
13.	SE12	-	-	-	6.0	1.0	-	3.51±0.03^{g}	3.46±0.05^{ef}	65-70
14.	SE13	-	-	-	8.0	1.5	-	3.21±0.04^{h}	3.34±0.04^{fg}	65-70
15.	SE14	-	-	-	10.0	2.0	-	3.42±0.01^{gh}	3.52±0.08^{e}	60-65
$CD_{0.05}$								0.20	0.16	
SE±								0.07	0.06	

Where, SE = Shoot Establishment

Here, CD = Critical Difference

SE = Standard Error

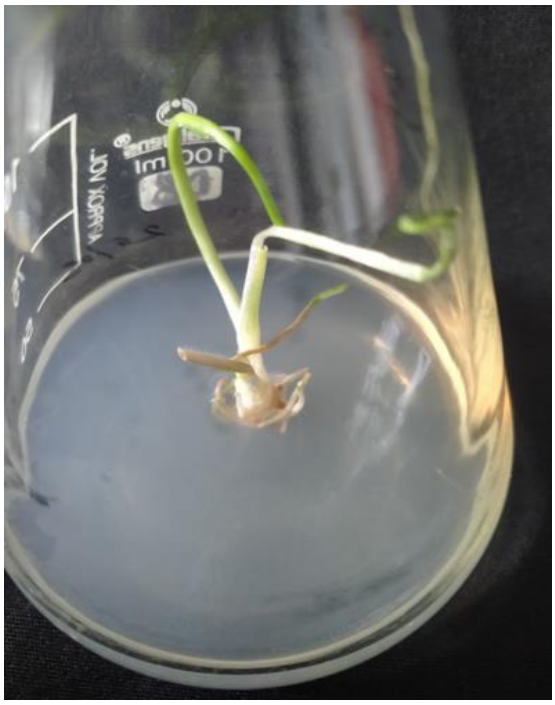
A) Day 3

B) Day 15

C) Day 25

D) Day 35

Plate 3 : *In vitro* shoot induction in 'Yamuna Safed' on SE6 medium (MS + 1 mgL^{-1} 2,4 D + 2 mgL^{-1} BAP + 1 mgL^{-1} GA3)



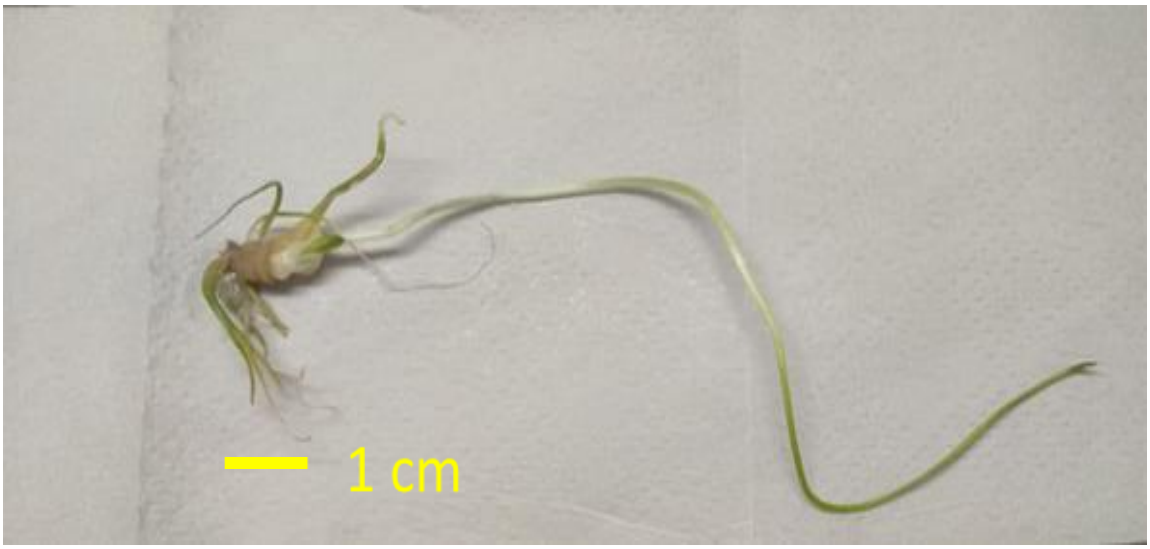
A) Day 3

B) Day 11

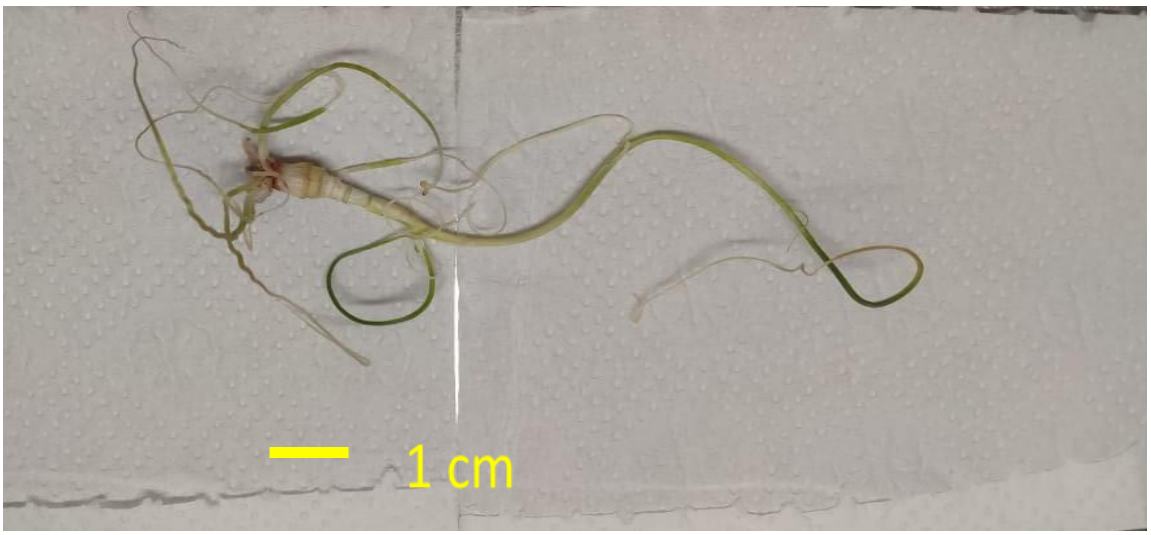
C) Day 18

D) Day 35

Plate 4 : *In vitro* shoot induction in 'Yamuna Safed' on SE9 medium (MS + 1 mgL^{-1} 2,4 D + 2 mgL^{-1} BAP)



A)



B)

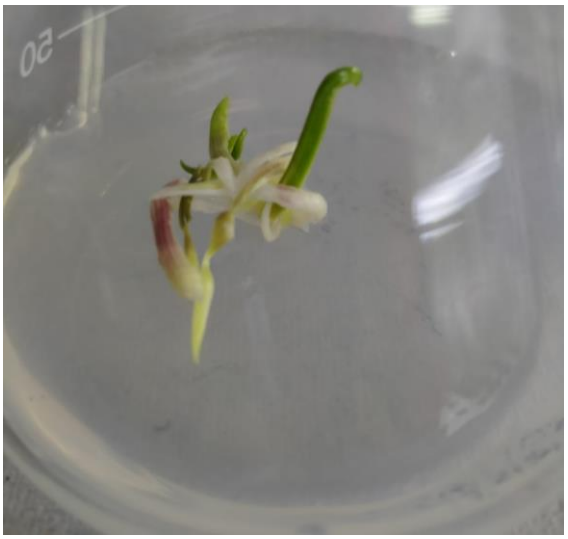
A) Shoot observed on SE6 medium (MS + 1 mgL^{-1} 2,4 D + 2 mgL^{-1} BAP + 1 mgL^{-1} GA3)

B) Shoot observed on SE9 medium (MS + 1 mgL^{-1} 2,4 D + 2 mgL^{-1} BAP)

Plate 5 : Shoots obtained on SE6 and SE9 media

A) Day 0

B) 3 days



C) 7 days

D) 21 days

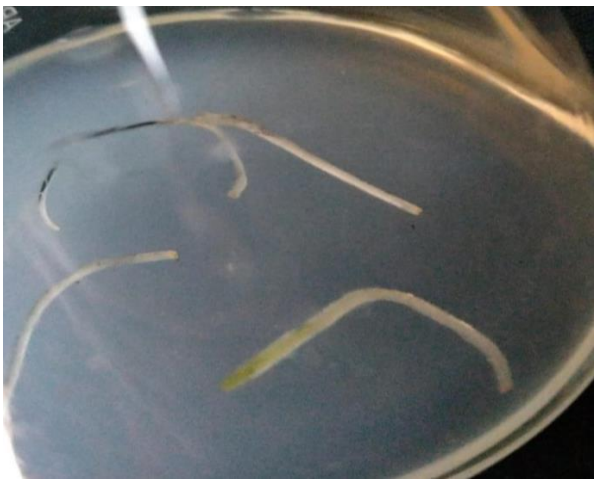
Plate 6: *In vitro* shoot induction in 'VL Garlic 1' on E5 medium (MS + 2 mgL^{-1} BAP + 0.3 mgL^{-1} GA_3 + 0.5 mgL^{-1} NAA) from shoot tip along with basal plate

A) 7 days

B) 12 days

C) 20 days

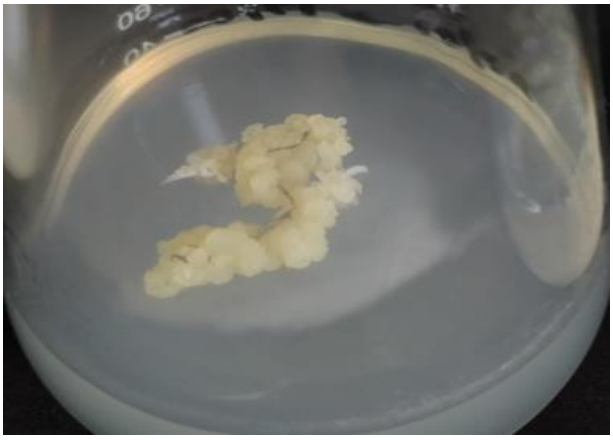
Plate 7: *In vitro* roots obtained on E11 medium (MS + 2 mgL^{-1} BAP, 0.3 mgL^{-1} GA3 + 0.5 mgL^{-1} NAA + 5 gL^{-1} activated charcoal)



A) Day 0

B) 3 weeks

C) 5 weeks

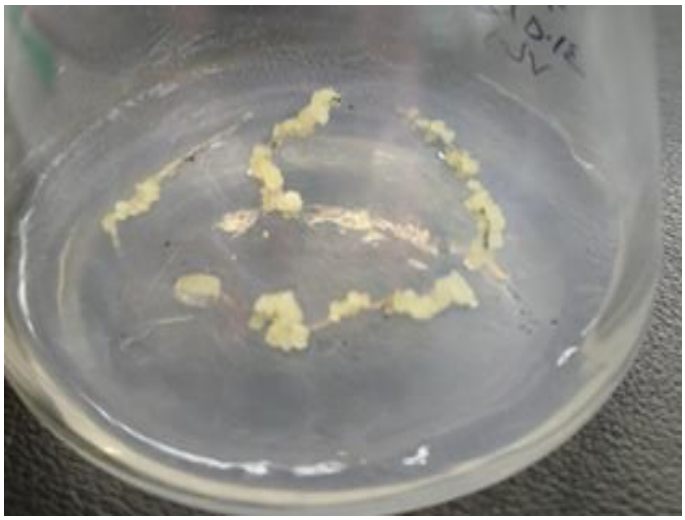


D) 17 weeks

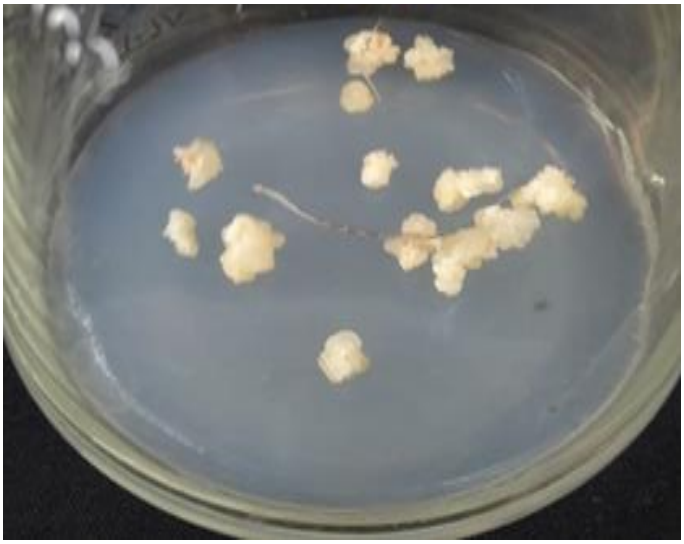
20 weeks

Plate 8: Callus induction from roots of *in vitro* raised plantlets of 'VL Garlic 1' in CFR6 medium (MS + 0.6 mgL^{-1} 2,4 D + 0.5 mgL^{-1} IAA)

A) Day 0

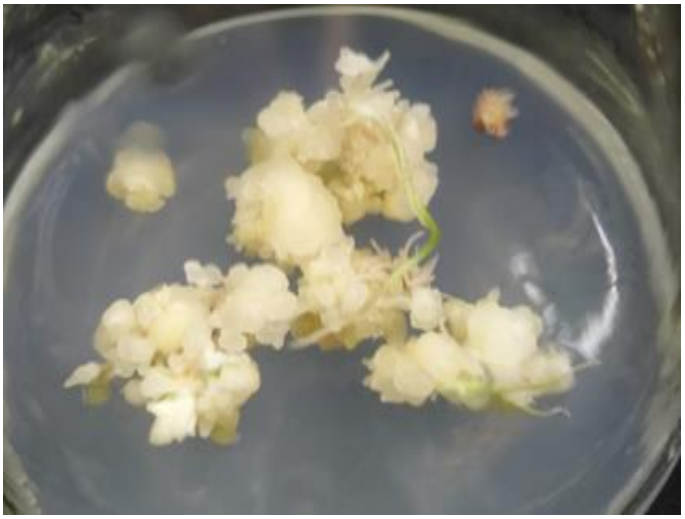


B) 3 weeks



C) 17 weeks

D) 5 weeks



20 weeks

Plate 9: Callus induction from roots of *in vitro* raised plantlets of 'VL Garlic 1' in CFR11 medium (MS + 0.6 mgL^{-1} 2,4 D + 0.5 mgL^{-1} IAA + 2.5 gL^{-1} KNO_3)

4.5 *In vitro* regeneration in 'VL Garlic 1'

4.5.1 *In vitro* establishment

Different media were attempted for root and shoot induction from shoot tips along with basal plate in 'VL Garlic 1'. Average number of shoots and shoot length were observed highest on E5 medium (MS + 2 mgL^{-1} BAP + 0.3 mgL^{-1} GA_3 + 0.5 mgL^{-1} NAA) (Plate 6). Average number of roots and root length were observed highest on E11 medium (MS + 2 mgL^{-1} BAP + 0.3 mgL^{-1} GA_3 + 0.5 mgL^{-1} NAA + 4 gL^{-1} activated charcoal) (Plate 7). Highest average shoot and root length observed were 5.61 cm and 3.79 cm respectively whereas highest average number of shoots and roots observed were 3.69 and 6.74 respectively (Table 14). The results obtained were similar to studies done on *in vitro* regeneration of 'Agrifound Parvati' and 'CITH' by Tandon (2022).

4.5.2 *In vitro* callus induction

The basal plates as well as roots obtained from *in vitro* regenerated plantlets were used to induce callus in 'VL Garlic 1'. No regeneration was observed from basal plates. However, callus was induced when roots from *in vitro* regenerated plantlets were cultured on different media compositions having varying concentrations of 2,4 D and IAA along with KNO_3 and TDZ. The best media observed for callus induction was CFR11 i.e., MS + 0.6 mgL^{-1} 2,4 D + 0.5 mgL^{-1} IAA + 2.5 gL^{-1} KNO_3 (Table 15). The number of explants producing callus were more in CFR 11 (Plate 9). However, mass of callus produced was more in CFR 6 i.e., MS + 0.6 mgL^{-1} 2,4 D + 0.5 mgL^{-1} IAA (Plate 8). The media compositions in which callus induction was observed from roots of 'VL Garlic 1' was used to induce callus from roots of 'Yamuna Safed' but no response was observed.

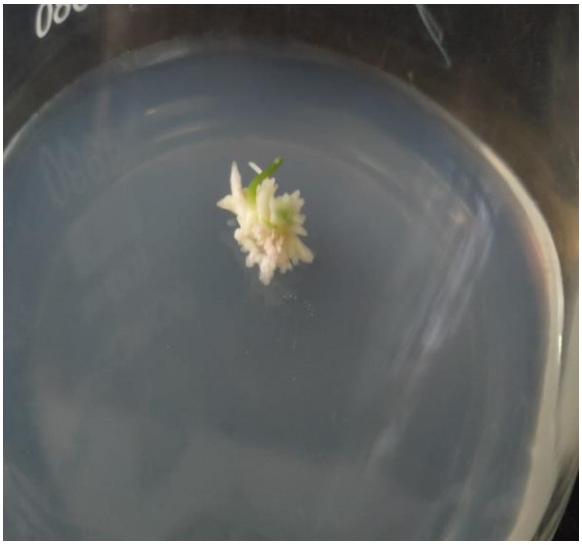
Table 14: Effect of different growth regulators on shoot and root regeneration in 'VL Garlic 1' from shoot tips along with basal plates

S. No.	Medium code	MS + Growth regulators				Average shoot length (cm)	Average number of shoots	Average root length (cm)	Average number of roots
		BAP (mgL^{-1})	GA_3 (mgL^{-1})	NAA (mgL^{-1})	Activated charcoal (gL^{-1})				
1.	Control	-	-	-	-	2.86 ± 0.05^{f}	1.09 ± 0.01^{g}	0^{f}	0^{f}
2	E1	0.5	0.1	0.1	-	2.90 ± 0.03^{f}	1.24 ± 0.01^{f}	0^{f}	0^{f}
3.	E2	0.5	0.1	0.2	-	3.26 ± 0.01^{e}	1.71 ± 0.02^{d}	0^{f}	0^{f}
4.	E3	1.0	0.2	0.3	-	4.33 ± 0.07^{d}	1.78 ± 0.02^{d}	0^{f}	0^{f}
5.	E4	1.0	0.2	0.4	-	4.96 ± 0.04^{c}	2.52 ± 0.06^{c}	0^{f}	0^{f}
6.	**E5**	**2.0**	**0.3**	**0.5**	-	**5.61 ± 0.14^{a}**	**3.69 ± 0.07^{a}**	0^{f}	0^{f}
7.	E6	2.0	0.3	0.6	-	5.22 ± 0.02^{b}	3.20 ± 0.07^{b}	0^{f}	0^{f}
8.	E7	0.5	0.1	0.1	1.0	1.63 ± 0.03^{j}	0.75 ± 0.01^{j}	2.32 ± 0.01^{e}	3.27 ± 0.01^{e}
9.	E8	0.5	0.1	0.2	2.0	1.98 ± 0.03^{i}	0.93 ± 0.01^{i}	2.43 ± 0.06^{de}	4.25 ± 0.06^{d}
10.	E9	1.0	0.2	0.3	3.0	2.09 ± 0.05^{h}	0.96 ± 0.01^{hi}	2.53 ± 0.01^{d}	4.57 ± 0.01^{c}
11.	E10	1.0	0.2	0.4	4.0	2.54 ± 0.01^{h}	1.05 ± 0.01^{gh}	3.16 ± 0.06^{c}	5.82 ± 0.06^{b}
12.	**E11**	**2.0**	**0.3**	**0.5**	**5.0**	2.68 ± 0.03^{gh}	1.15 ± 0.01^{fg}	**3.79 ± 0.09^{a}**	**6.74 ± 0.11^{a}**
13	E12	2.0	0.3	0.6	6.0	2.83 ± 0.04^{fg}	1.50 ± 0.02^{e}	3.44 ± 0.06^{b}	6.63 ± 0.17^{a}
$CD_{0.05}$						0.15	0.10	0.11	0.19
SE±						0.05	0.04	0.04	0.07

Where, E = Establishment

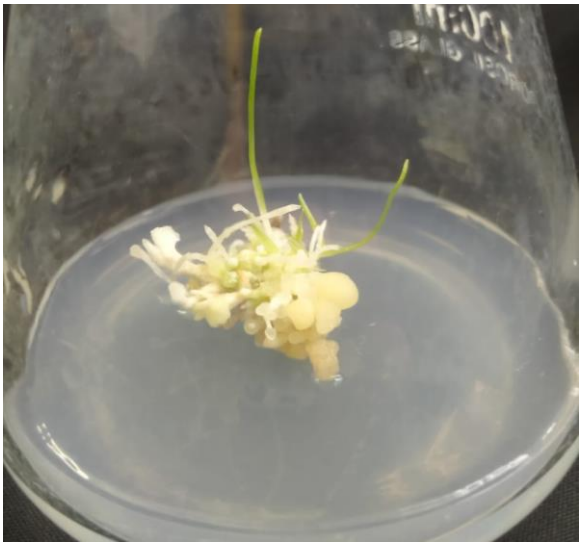
Here, CD = Critical Difference

SE = Standard Error

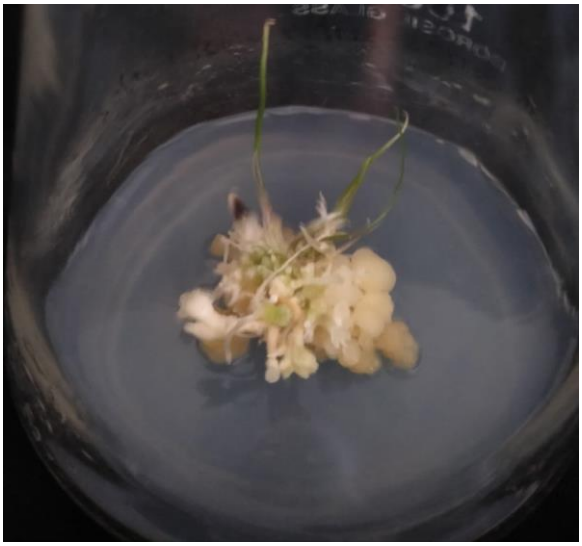


A) 15 days

B) 35 days



C) 45 days



C) 70 days

Plate 10: *In vitro* shoot induction from callus in 'VL Garlic 1' on SFC9 medium (MS + 0.6 mgL^{-1} 2,4 D + 0.5 mgL^{-1} IAA + 0.6 mgL^{-1} 2 iP)

Table 15: Effect of different concentrations of growth regulators on callus induction from *in vitro* roots in 'VL Garlic 1'

S. No.	Media code	Medium composition with MS		Medium composition with B5		KNO_3 (g)	Percent callus induction	Days taken for callus induction
		2,4 D (mgL^{-1})	IAA (mgL^{-1})	TDZ (mgL^{-1})	Picloram (mgL^{-1})			
1.	Control	-	-	-	-	-	0^{f}	-
2.	CFR1	0.1	-	-	-	-	0^{f}	-
3.	CFR2	0.2	0.1	-	-	-	0^{f}	-
4.	CFR3	0.3	0.2	-	-	-	0^{f}	-
5.	CFR4	0.4	0.3	-	-	-	0^{f}	-
6.	CFR5	0.5	0.4	-	-	-	33.24(35.19)^{e}	85-90
7.	**CFR6***	**0.6**	**0.5**	-	-	-	**78.88(62.66)^{b}**	**65-70**
8.	CFR7	0.2	0.1	-	-	0.5	33.25(35.20)^{e}	90-95
9.	CFR8	0.3	0.2	-	-	1.0	36.49(37.15)^{d}	110-115
10.	CFR9	0.4	0.3	-	-	1.5	38.20(38.16)^{cd}	80-85
11.	CFR10	0.5	0.4	-	-	2.0	40.64(39.57)^{c}	80-85
12.	**CFR11****	**0.6**	**0.5**	-	-	**2.5**	**87.33(69.17)^{a}**	**70-75**
13.	CFR12	-	-	0.5	1	-	0^{f}	-
14.	CFR13	-	-	1.0	2	-	0^{f}	-
15.	CFR14	-	-	1.5	3	-	0^{f}	-
16.	CFR15	-	-	2.0	4	-	0^{f}	-
$CD_{0.05}$							2.87(2.06)	
SE							0.95(0.68)	

Where, CFR = Callus from Root

Here**,** CD = Critical Difference

SE = Standard Error

***** The mass of callus produced per excised root was more in CFR6.

****** The number of excised roots producing callus were most in case of CFR11.

4.5.3 *In vitro* induction of shoots from callus

The obtained callus was shifted to shoot induction media after 25 days with varying concentrations of growth regulators and observations were recorded from the day of its shoot initiation (Plate 10). Medium SFC9 (MS + 0.6 mgL^{-1} 2,4 D + 0.5 mgL^{-1} IAA + 0.6 mgL^{-1} 2 iP) was observed to be best for shoot induction with highest average number of shoots 5.67±0.33 (Table 16). The results have previously been reported by

Koch et al. (1995) in which medium composition of 3 µM 2,4 D; 3 µM and 9 µM 2 iP were used.

Table 16: Effect of different concentrations of growth regulators on shoot induction from callus in 'VL Garlic 1'

S. No.	Media code	MS + Growth regulator					Average number of shoots
		2,4 D (mgL^{-1})	BAP (mgL^{-1})	GA_3 (mgL^{-1})	IAA (mgL^{-1})	2 iP (mgL^{-1})	
1.	Control	-	-	-	-	-	0^{e}
2.	SFC1	0.5	1.0	0.5	-	-	3.31 ± 0.05^{cd}
3.	SFC2	1.0	2.0	1.0	-	-	3.67 ± 0.06^{c}
4.	SFC3	1.5	3.0	1.5	-	-	3.43 ± 0.06^{cd}
5.	SFC4	2.0	4.0	2.0	-	-	3.63 ± 0.05^{c}
6.	SFC5	0.2	-	-	0.1	0.2	0^{e}
7.	SFC6	0.3	-	-	0.2	0.3	3.32 ± 0.07^{cd}
8.	SFC7	0.4	-	-	0.3	0.4	3.1 ± 0.06^{d}
9.	SFC8	0.5	-	-	0.4	0.5	4.33 ± 0.33^{b}
10.	**SFC9**	**0.6**	**-**	**-**	**0.5**	**0.6**	$\mathbf{5.67\pm0.33^{a}}$
$CD_{0.05}$							0.46
SE							0.16

Where, SFC = Shoot From Callus

Here**,** CD = Critical Difference

SE = Standard Error

4.6 Hardening of the *in vitro* raised micro plants

In vitro regenerated plantlets of garlic cultivar 'Yamuna Safed' were evaluated for their survival upon transfer to potted soil conditions. Sixty percent of plants survived. Coco peat, vermiculite and perlite were mixed in 2:1:1 ratio (Plate 11).

4.7 Biochemical analysis

4.7.1 Quantitative estimation of Total Soluble Solid content

Analysis of TSS content has been done in various garlic cultivars (Akinwande and Olatunde 2015; Algadi et al. 2014; Selen 2019; Umaretiya et al. 2019). In this study, TSS was calculated using a digital refractometer which measures TSS content

A)

B)

C)

D)

E)

A), B) *In vitro* obtained plants of 'Yamuna Safed'
C) Day 1 D) Day 7 E) Day 20

Plate 11: Hardening of 'Yamuna Safed' plantlets

using the LED to pass light through a prism in contact with the sample. TSS values of 3 samples of garlic cloves each of each variety, 'Yamuna Safed' and 'VL Garlic 1' were calculated. TSS value obtained was higher for cloves of garlic cultivar 'VL Garlic 1' i.e., 34±0.50 ºBrix than that of 'Yamuna Safed' i.e., 29.47±0.52 ºBrix (Table 17). TSS content of 'VL Garlic 1' was higher as compared to TSS content of 'Agrifound Parvati' (31.44 ºBrix) and 'CITH' (23.14 ºBrix) as reported by Tandon (2022) while TSS of 'Yamuna Safed' was less as compared to TSS of 'Agrifound Parvati' and more as compared to TSS of 'CITH'.

4.7.2 Quantitative estimation of Ascorbic Acid content

The ascorbic acid content in garlic cloves was estimated as described in section **3.8.2** of both cultivars ('Yamuna Safed' and 'VL Garlic 1'). Ascorbic acid content was determined using titration method as described by Rao and Deshpande (2006) and was calculated using following formula:

$$\text{Ascorbic acid (mg/100 g)} = \frac{\text{Titre value} \times \text{Dye factor} \times \text{Dilution} \times 100}{\text{Weight of sample} \times \text{Volume of sample}}$$

Dye factor of 3 samples of each variety was obtained to calculate ascorbic acid content by using formula:

$$\text{Dye factor} = 0.5/\ \text{Titre value}$$

Ascorbic acid has been reported in *Allium spp.* (Aydogmus et al. 2002; Benkeblia 2005; Denre et al. 2011; Rekowska and Skupien 2008). Ascorbic acid content of cloves of 'Yamuna Safed' i.e., 12.55 mg/100 g was higher than that of 'VL Garlic 1' i.e., 11.93 mg/100 g (Table 17). However, the ascorbic acid content of both the cultivars was lower than 'Agrifound Parvati' (12.66 mg/100 g) and higher than that of 'CITH' (8.35 mg/100 g) as reported by Tandon (2022).

4.7.3 Quantitative estimation of Total Phenol

Total phenols were determined as described in section **3.8.3**. Total phenol content was found higher in cloves of 'VL Garlic 1' with concentration of 36.27±0.17 mg GAE/100 mL (Table 17). Khan et al. 2016 reported phenol content of 40.80 mg GAE/100 g in garlic. Ceryova et al. (2023) analysed 7 different garlic cultivars (Novozamocky, Lumir, Mojmir,

Havran, Garpel, Eden Rose, and Bjetin) and reported highest total phenol content of 47.2 mg GAE/100 g in cultivar 'Garpel'.

Total phenol content was calculated from standard curve (Fig 2) using following equation:

$$y = 1.7877x + 0.0456$$

Where, y = Mean OD of garlic sample

x = Concentration of total phenol in garlic sample (mg mL^{-1})

4.7.4 Quantitative estimation of Flavonoids

Flavonoid estimation was done as described in section **3.8.4** to compare the flavonoid content in cloves of 'VL Garlic 1' and 'Yamuna Safed'. Higher quantity of flavonoids was found in cloves of 'VL Garlic 1' i.e., 17.73±0.06 mg/100 mL (Table 17). Flavonoid content of 4.59 mg RE/100 g has been reported in garlic by Khan et al. (2016) in local species of Faisalabad, Pakistan by using rutin as standard.

The flavonoid content was calculated from standard curve (Fig 3) using following equation:

$$y = 24.754x - 0.755$$

Where, y = Mean OD of garlic sample

x = Concentration of flavonoid content in garlic sample (mg mL^{-1})

4.7.5 Quantitative estimation of Antioxidants

Quantitative estimation of antioxidants was performed in cloves of 'VL Garlic 1' and 'Yamuna Safed' as described in section **3.8.5**. Higher antioxidant activity was found in cloves of 'VL Garlic 1' i.e., 47.86±1.17% (Table 17). Estimation of antioxidants in garlic has been done before in various studies. Kovarovic et al. (2019) analysed antioxidant activity in samples of *Allium porrum* collected from Zohor (Slovakia) and reported highest antioxidant activity of 47.40%. Lenkova et al. (2017) reported highest and lowest antioxidant activity of 20.22% and 13.61% in variety 'Mojmir' and 'Zahorsky' respectively among five garlic cultivars studied using DPPH method while Selen (2019) reported high antioxidant activity of 62.58% in Turkish variety of garlic.

Table 17: Comparison of TSS, Ascorbic acid content, Total Phenol, Flavonoids and Antioxidants in cloves of garlic cultivars

Treatments	TSS (ºBrix)	Ascorbic Acid (mg/100 g)	Total Phenol (mg GAE/100 mL)	Flavonoids (mg QE/100 mL)	Antioxidants (% DPPH)
Cloves of 'VL Garlic 1'	**34±0.50**	11.93±0.39	**36.27±0.16**	**17.73±0.29**	**47.86±0.43**
Cloves of 'Yamuna Safed'	29.47±0.52	**12.55±0.30**	33.56±0.38	17.04±0.50	42.10±0.35
t_{cal}	6.26	1.52	1.35	5.54	10.35
p value	0.0033	0.02040	0.0001	0.0052	0.0005

Chapter-5

SUMMARY AND CONCLUSION

The present research "***In vitro* propagation studies in garlic (*Allium sativum*)**" was being carried out in the Department of Biotechnology, Dr. YS Parmar University of Horticulture & Forestry, Nauni, Solan, Himachal Pradesh. The results are summarized as follows:

5.1 Morphological analysis

Bulb and clove characteristics of two garlic cultivars 'VL Garlic 1' and 'Yamuna Safed' were studied using Distinctiveness, Uniformity and Stability (DUS) Descriptor for garlic from Protection of Plant Variety and Farmer's Rights Authority (PPV F&RA) (Anonymous 2009). Out of eleven (bulb and clove) visual morphological characteristics studied, a total of four characteristics were found to be same, except for as follows: Bulb shape (longitudinal section), Bulb shape (cross section), Anthocyanin strips, Bulb colour, Colour of scale, Colour of flesh and Clove position at tip of base. Average bulb weight of 'VL Garlic 1 was more than that of 'Yamuna Safed'. Average bulb diameter was more of 'VL Garlic 1' than that of 'Yamuna Safed'. Average clove weight of 'VL Garlic 1' was more than that of 'Yamuna Safed' while cloves of 'Yamuna Safed' had greater average diameter of clove than that of 'VL Garlic 1'. Number of cloves were approximately equal.

5.2 Sterilization of explants

An efficient sterilization of explants of 'VL Garlic 1' and 'Yamuna Safed' was achieved on treatment T6 consisting 0.2% carbendazim followed by 0.1% $HgCl_2$ for 5 minutes and 3 minutes respectively. This resulted in 90.34% mean uncontamination and 91.50% mean percent survival in 'VL Garlic 1' whereas 90.50% mean uncontamination and 90.89% mean percent survival in 'Yamuna Safed'.

5.3 *In vitro* regeneration in 'Yamuna Safed'

5.3.1 *In vitro* establishment

For *in vitro* regeneration, basal plates and roots were used. The basal plates produced roots in 10 days. However, no response was observed in roots even after 20 days. Maximum

in vitro per cent establishment of 73.33% in 'Yamuna Safed' was achieved on M4 medium (MS + 2 mgL^{-1} 2,4 D) with average number of roots and average root length 8.00 and 6.50 cm respectively.

5.3.2 *In vitro* induction of shoots

The established cultures with induced roots were transferred to shoot induction media after 60 days. Maximum average shoot length of 12.06 cm was achieved on SE6 medium (MS + 1 mgL^{-1} 2,4 D + 2 mgL^{-1} BAP + 1 mgL^{-1}GA3) in 'Yamuna Safed'. However, average number of shoots per explant was found at par in both SE6 (MS + 1 mgL^{-1} 2,4 D + 2 mgL-1 BAP + 1 mgL-1 GA3) and SE9 (MS + 1 mgL^{-1} 2,4 D + 2 mgL^{-1}BAP) medium. It took least number of days for shoot induction on SE9 medium.

5.4 *In vitro* regeneration in 'VL Garlic 1'

5.4.1 *In vitro* establishment

For establishment of 'VL Garlic 1, shoot tips along with basal plate were used as explant and cultured in medium containing different concentrations of growth regulators. Highest average shoot length and number of shoots from shoot tips with basal plate was achieved on E5 medium (MS + 2 mgL^{-1} BAP + 0.3 mgL^{-1} GA_3 + 0.5 mgL^{-1} NAA) while highest average root length and number of roots was achieved on E11 medium (MS + 2 mgL^{-1} BAP + 0.3 mgL^{-1} GA_3 + 0.5 mgL^{-1} NAA + 5 gL^{-1} activated charcoal). The highest average shoot and root length observed were 5.61 cm and 3.79 cm respectively whereas highest average number of shoots and roots observed were 3.69 and 6.74 respectively.

5.4.2 *In vitro* callus induction from roots of *in vitro* plants

The roots from *in vitro* regenerated plants were used as explants to induce callus. Percent callus induction from *in vitro* roots of 'VL Garlic 1', was highest in CFR11 medium (MS + 0.6 mgL^{-1} 2,4 D + 0.5 mgL^{-1} IAA + 2.5 g KNO3) and was 87.33%. However, bulkier and more proliferating callus was observed in CFR6 medium (MS + 0.6 mgL-1 2,4 D + 0.5 mgL-1 IAA). It took less number of days in CFR6 medium to produce callus.

5.4.3 *In vitro* induction of shoots from callus

For *in vitro* shoot induction from callus in 'VL Garlic 1', SFC9 medium (MS + 0.6 mgL^{-1} 2,4 D + 0.5 mgL^{-1} IAA and 0.6 mgL^{-1} 2 iP) was found to be best and resulted in

maximum average number of shoots as well as maximum average shoot length of 5.67 and 2.81 cm respectively.

5.5 Hardening of the *in vitro* raised micro plants

In vitro plants of 'Yamuna Safed' were hardened in potting mixture with coco peat, vermiculite and perlite in ratio 2:1:1. Sixty percent of the plants survived in hardening.

5.6 Biochemical analysis

TSS value was obtained higher in cloves of 'VL Garlic 1' i.e., 34 ºBrix. Ascorbic acid content of 'Yamuna Safed' was higher than 'VL Garlic 1' i.e., 12.55 mg/100 g. Total phenol content was found higher in cloves of 'VL Garlic 1' with concentration of 36.27 mg/100 mL. Higher quantity of flavonoids was found in cloves of 'VL Garlic 1' i.e., 17.73 mg/100 mL. Higher percentage of antioxidants was found in cloves of 'VL Garlic 1' i.e., 47.86%.

CONCLUSION

Studies on *in vitro* propagation of two varieties i.e., 'VL Garlic 1' and 'Yamuna Safed' was carried out in present research. Morphological characters were studied using Distinctiveness, Uniformity and Stability (DUS) Descriptors for garlic from Protection of Plant Variety and Farmer's Rights Authority (PPV F&RA). *In vitro* direct regeneration was achieved in 'Yamuna Safed' from basal plates while *in vitro* regeneration via callus was achieved in 'VL Garlic 1' from roots. No response was observed in basal plates of 'VL Garlic 1'. Similarly, roots of 'Yamuna Safed' also did not respond at all.

The protocol standardised for callus induction and regeneration can be further used for *in vitro* selection for stress tolerance and genetic transformation studies in garlic. Attempts can be made for root induction from callus in 'VL Garlic 1' and plantlets can be subjected to hardening. Protocol for direct regeneration from basal plate was standardised in 'Yamuna Safed' which can be used for propagation of garlic for production of healthy planting material.

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APPENDIX - I

Chemical constituents used in the preparation of MS medium

Chemical constituents of the media used		MS medium (1962)
a)	**Major constituents (Stock I)**	**gL^{-1}**
	Ammonium nitrate	66.00
	Potassium nitrate	76.00
	Magnesium sulphate (Anhydrous)	14.80
	Potassium dihydrogen orthophosphate	6.80
	Calcium chloride	17.72
b)	**Major constituents (Stock II)**	
	Boric acid	6.2
	Manganese sulphate (monohydrate)	22.30
	Potassium iodide	0.80
	Zinc sulphate (heptahydrate)	8.65
	Sodium molybdate	0.25
	Copper sulphate (pentahydrate)	0.025
	Cobalt chloride (hexahydrate)	0.025
c)	**Major constituents (Stock III)**	
	Na_2 EDTA (disodium salt dehydrate)	7.46
	Ferrous sulphate (heptahydrate)	5.56
d)	**Major constituents (Stock IV)**	
	Pyridoxine	0.1
	Thymine	0.2
	Nicotinic acid	0.1
	Glycine	0.4

APPENDIX - II

Analysis of variance (ANOVA)

Analysis of variance of '*In vitro* propagation studies in garlic (*Allium sativum*)'

Analysis of variance for morphological characteristics of garlic cultivars: 'VL Garlic 1' and 'Yamuna Safed'

Descriptive Statistics

Character	Group	N	Mean	Variance	Standard deviation	Standard error
Bulb weight	1	3	30.62	0.03	0.17	0.10
	2	3	28.6	0.18	0.43	0.25
Clove weight	1	3	54.02	5.07	2.25	1.30
	2	3	53.10	1.14	1.07	0.62
Bulb diameter	1	3	54.02	4.20	2.05	1.18
	2	3	53.10	3.05	1.75	1.01
Clove diameter	1	3	22.20	0.88	0.94	0.54
	2	3	23.13	1.07	1.04	0.60

T-test

Character	Group	Assumptions	t	d.f.	Probability
Bulb weight	1&2	Equal variances assumed	7.59	4	0.0016
		Unequal variances assumed	7.59	2.62	0.0075
Clove weight	1&2	Equal variances assumed	0.64	4	0.5571
		Unequal variances assumed	0.64	2.85	0.5699
Bulb diameter	1&2	Equal variances assumed	0.59	4	0.5884
		Unequal variances assumed	0.59	3.90	0.5892
Clove diameter	1&2	Equal variances assumed	1.15	4	0.3131
		Unequal variances assumed	1.15	3.96	0.3137

Analysis of variance for effect of sterilant on *in vitro* survival of explants of 'VL Garlic 1' when treated for different time durations

i) Mean percent uncontaminated explants

Source of variation	d.f.	Sum of Squares	Mean Squares	F-calculated
Treatment	7	10,607.443	1,515.349	822.672
Error	16	29.472	1.842	
Total	23	10,636.915		

ii) Mean percent survival

Source of variation	d.f.	Sum of Squares	Mean Squares	F-calculated
Treatment	7	27,515.508	3,930.787	1,815.269
Error	16	34.646	2.165	
Total	23	27,550.155		

Analysis of variance for effect of sterilant on *in vitro* survival of explants of 'Yamuna Safed' when treated for different time durations

i) Mean percent uncontaminated explants

Source of variation	d.f.	Sum of Squares	Mean Squares	F-calculated
Treatment	7	11,586.220	1,655.174	669.350
Error	16	39.565	2.473	
Total	23	11,625.785		

ii) Mean percent survival

Source of variation	d.f.	Sum of Squares	Mean Squares	F-calculated
Treatment	7	26,778.972	3,825.567	1,011.359
Error	16	60.522	3.783	
Total	23	26,839.493		

Analysis of variance for effect of different growth regulators on excised basal plates of 'Yamuna Safed'

i) Percent establishment

Source of variation	d.f.	Sum of Squares	Mean Squares	F-calculated
Treatment	14	16,471.985	1,176.570	1,632.836
Error	30	21.617	0.721	
Total	44	16,493.602		

ii) Average number of roots

Source of variation	d.f.	Sum of Squares	Mean Squares	F-calculated
Treatment	14	245.143	17.510	1,303.484
Error	30	0.403	0.013	
Total	44	245.546		

iii) Average root length

Source of variation	d.f.	Sum of Squares	Mean Squares	F-calculated
Treatment	14	132.007	9.429	3.469
Error	30	81.549	2.718	
Total	44	213.557		

iv) Average number of shoots

Source of variation	d.f.	Sum of Squares	Mean Squares	F-calculated
Treatment	13	4.130	0.332	1.989
Error	28	4.667	0.167	
Total	41	8.976		

Analysis of variance for effect of different growth regulators on shoot induction from *in vitro* basal plate of 'Yamuna Safed'

i) Average length of shoot

Source of variation	d.f.	Sum of Squares	Mean Squares	F-calculated
Treatment	14	323.923	23.137	1,688.569
Error	30	0.411	0.014	
Total	44	324.334		

ii) Average number of shoots per explant

Source of variation	d.f.	Sum of Squares	Mean Squares	F-calculated
Treatment	14	48.137	3.438	365.860
Error	30	0.282	0.009	
Total	44	48.419		

Analysis of variance for effect of growth regulators on shoot and root regeneration in 'VL Garlic 1' from shoot tips along with basal plates

i) Average shoot length

Source of variation	d.f.	Sum of Squares	Mean Squares	F-calculated
Treatment	12	61.11	5.097	620.084
Error	26	0.214	0.008	
Total	38	61.325		

ii) Average number of shoots

Source of variation	d.f.	Sum of Squares	Mean Squares	F-calculated
Treatment	12	30.834	2.570	687.791
Error	26	0.097	0.004	
Total	38	30.931		

iii) Average root length

Source of variation	d.f.	Sum of Squares	Mean Squares	F-calculated
Treatment	12	89.604	7.467	1,750.081
Error	26	0.111	0.004	
Total	38	89.715		

iv) Average number of roots

Source of variation	d.f.	Sum of Squares	Mean Squares	F-calculated
Treatment	12	293.004	24.417	1,864.869
Error	26	0.340	0.013	
Total	38	293.345		

Analysis of variance for effect of different media compositions on callus induction from *in vitro* roots of 'VL Garlic 1'

i) Percent callus induction

Source of variation	d.f.	Sum of Squares	Mean Squares	F-calculated
Treatment	7	10,593.015	1,513.288	561.690
Error	16	43.107	2.694	
Total	23	10,636.121		

ii) *In vitro* induction of shoots from callus

Source of variation	d.f.	Sum of Squares	Mean Squares	F-calculated
Treatment	9	84.321	9.369	128.388
Error	20	1.459	0.073	
Total	29	85.780		

Analysis of variance for biochemical characteristics of garlic cultivars:

'VL Garlic 1' and 'Yamuna Safed'

Descriptive Statistics

Character	Group	N	Mean	Variance	Standard deviation	Standard error
TSS	1	3	34.00	0.76	0.87	0.50
	2	3	29.47	0.81	0.90	0.52
Ascorbic acid	1	3	11.93	0.28	0.53	0.30
	2	3	12.99	0.01	0.12	0.07
TPC	1	3	36.27	0.13	0.36	0.21
	2	3	33.56	0.44	0.66	0.38
Total flavonoids	1	3	17.73	0.08	0.29	0.17
	2	3	17.04	0.25	0.50	0.29
Antioxidant (%DPPH)	1	3	47.86	0.56	0.75	0.43
	2	3	42.10	0.37	0.61	0.35

T-test

Character	Group	Assumptions	t	d.f.	Probability
TSS	1&2	Equal variances assumed	6.26	4	0.0033
		Unequal variances assumed	6.26	4.00	0.0033
Ascorbic acid	1&2	Equal variances assumed	3.42	4	0.0268
		Unequal variances assumed	3.42	2.20	0.0663
TPC	1&2	Equal variances assumed	6.20	4	0.0034
		Unequal variances assumed	6.20	3.08	0.0078
Total flavonoids	1&2	Equal variances assumed	2.06	4	0.1090
		Unequal variances assumed	2.06	3.18	0.1269
Antioxidant (%DPPH)	1&2	Equal variances assumed	10.35	4	0.0005
		Unequal variances assumed	10.35	3.84	0.0006

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Title of Thesis	**:**	**"*In vitro* propagation studies in garlic (*Allium sativum*)"**
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ABSTRACT

Garlic (*Allium sativum*) is an important monocotyledonous aromatic bulb crop belonging to family Alliaceae. It is grown all over the world for medicinal and culinary purposes. Garlic is propagated vegetatively by cloves. It is sexually sterile and has lost its ability to flower due to repeated vegetative propagation. Also, its multiplication rate is low in field. *In vitro* propagation can alleviate these problems, improving propagation efficiency. The present research was taken up to standardize the protocol for *in vitro* regeneration in two garlic varieties 'VL Garlic 1' and 'Yamuna Safed'. Morphological analysis of clove and bulb characteristics was done in both varieties. Average bulb weight, Average bulb diameter and average clove weight were higher of 'VL Garlic 1' whereas average clove diameter was higher of 'Yamuna Safed'. Effective sterilization was achieved for explants of both varieties on treatment T6 in which explants were treated with 0.2% carbendazim and 0.1% $HgCl_2$ for 5 and 3 minutes respectively. Highest mean percent establishment, average number of roots and average root length in 'Yamuna Safed' was achieved on M4 medium (MS + 2 mgL^{-1} 2,4 D). Highest average shoot length was obtained on SE6 medium (MS + 1.0 mgL^{-1} 2,4 D + 2.0 mgL^{-1} BAP + 1 mgL^{-1} GA_3) and highest average number of shoots were at par in SE6 and SE9 medium (MS + 1.0 mgL^{-1} 2,4 D + 2.0 mgL^{-1} BAP). Highest average number of shoots and shoot length from shoot tip along with basal plate in 'VL Garlic 1' were achieved in E5 medium (MS + 2 mgL^{-1} BAP + 0.3 mgL^{-1} GA_3 + 0.5 mgL^{-1} NAA) whereas highest average number of roots and root length were observed on E11 medium (MS + 2 mgL^{-1} BAP + 0.3 mgL^{-1} GA_3 + 0.5 mgL^{-1} NAA + 4 gL^{-1} activated charcoal). Obtained *in vitro* roots were used as explants to produce callus. Percent callus induction was highest on CFR11 medium (MS + 0.6 mgL^{-1} 2,4 D + 0.5 mgL^{-1} IAA + 2.5 gL^{-1} KNO_3). Best medium for shoot induction from callus was SFC9 (MS + 0.6 mgL^{-1} 2,4 D + 0.5 mgL^{-1} IAA + 0.6 mgL^{-1} 2 iP) on which highest average number of shoots were observed.

Signature of Major Advisor **Signature of Student**

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