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Micropropagation and Improvement of Garlic (Alliiun Sativum L.) Through in Vitro Techniques

Roksana, Mst. Rubayat

University of Rajshahi

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M. Phil. THESIS

BY

MST. RUBAYAT ROKSANA

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DEPARTMENT OF BOTANY
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BANGLADESH

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M. Phil. THESIS

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IN
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BY

MST. RUBAYAT ROKSANA

PLANT BREEDING AND GENE ENGINEERING LABORATORY
DEPARTMENT OF BOTANY
UNIVERSITY OF RAJSHAHI
RAJSHAHI- 6205, BANGLADESH
JUNE 2007

DEDICATED TO THE MEMORY
OF MY
DEPARTED PARENTS

DECLARATION

I hereby declare that the whole work submitted as a thesis entitled "**Micropropagation and improvement of garlic (*Allium sativum* L.) through *in vitro* techniques**" in the Plant Breeding and Gene Engineering Laboratory, Department of Botany, Rajshahi University, Rajshahi, Bangladesh for the degree of **Master of Philosophy** is the result of my own investigation and was carried out under the supervision of Professors Dr. A. K. M. Rafiul Islam, and Dr. M. Firoz Alam, Department of Botany, Rajshahi University, Rajshahi. I further declare that this work has not been submitted anywhere for any academic degree.

Mst. Rubayat Roksana
28. 06. 07

June 2007

(Mst. Rubayat Roksana)
Department of Botany
University of Rajshahi
Rajshahi- 6205, Bangladesh

CERTIFICATE

It is our pleasure to certify that the research work embodying the results reported in this thesis entitled "**Micropropagation and improvement of garlic (*Allium sativum* L.) through *in vitro* techniques**" submitted by Mst. Rubayat Roksana has been carried out under our guidance in the Plant Breeding and Gene Engineering Laboratory of Department of Botany, University of Rajshahi for the degree of **Master of Philosophy**. It is further certified that the research work presented here is original and suitable for submission for the award of **M.Phil.** degree.

Rafiul Islam 28/06/07.

(Professor Dr. A.K.M. Rafiul Islam)
Principal Supervisor
Department of Botany
University of Rajshahi
Rajshahi- 6205, Bangladesh

28-6-2007

(Professor Dr. M. Firoz Alam)
Co-supervisor
Department of Botany
University of Rajshahi
Rajshahi- 6205, Bangladesh

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The author

ABSTRACT

Different aspects of micropropagation through meristem culture for the production of virus free plants, callus induction, somatic embryogenesis followed by subsequent plant regeneration, *in vitro* bulblet formation and field evaluation of the *in vitro* regenerated plants were studied on five commercial cultivars of garlic *(Allium sativum* L.) viz., GC002, GC008, GC009, GC0025 and Indian variety.

For micropropagation, shoot tip of field grown mature garlic bulbs of five cultivars were used for meristem isolation. Meristems were cultured on to filter paper bridge in liquid MS medium. Among the different media formulations MS + 1.0 mg/l 2ip + 0.5 mg/l NAA was found to be the best for primary establishment of meristems and for maximum shoot proliferation.

Protocol was also developed to induce *in vitro* bulblets from meristem derived shoot clumps and among various concentrations of sucrose, 12% sucrose was found to be the best for *in vitro* bulblet formation for all the four cultivars. Same result was also found in callus culture.

Rooted plantlets with bulblets of four garlic cultivars were gradually acclimatized and successfully established in the field. Visual evaluation of the morphological traits of the meristem derived plants showed that all plants were normal and free from virus diseases. Substantial yield increase was observed from meristem derived plants over their source plants. Among the four cultivars cv. GC0025 was found to be highly responsive for *in vitro* shoot proliferation and bulblet formation.

For callus induction and somatic embryogenesis shoot tip, leaf base, leaf primordia and bulbil from field grown mature garlic bulbs of five cultivars viz., GC002, GC008, GC009, GC0025 and Indian variety were used. The explants were cultured in MS medium supplemented with different concentrations of 2,4-D, and 2.0 mg/l was found to be the most effective medium formulation for callus induction. When plant growth regulators was used in MS medium somatic embryogenesis did not occur. Among different strengths of MS medium without any growth regulators, ½ MS was found to be the most effective for somatic embryogenesis. Plantlet regeneration from somatic embryos was obtained in KIN supplemented MS media, and among various concentrations, 5.0 mg/l KIN was proved to be the most effective.

Among different explants, shoot tip and leaf base were most effective for callus induction, somatic embryogenesis, plantlets regeneration and *in vitro* bulblet formation.

Among different genotypes, cv. GC002 was found to be highly responsive for callus induction, somatic embryogenesis, plantlet regeneration and *in vitro* bulblet formation.

After proper acclimatization rooted callus derived plantlets with bulblets were transplanted in the field and somaclonal variation were found in plant height, number of leaves/plant, bulb diameter/plant, and bulb weight/plant.

ABBREVIATIONS

PGR	Plant growth regulators
BAP	6-benzyl amino purine
NAA	α-napthalene acetic acid
IBA	Indole - 3-butyric acid
°C	Celsius/ Centigrade (in degree)
cm	Centimeter
e. g.	Exempli gratia, for example
et al.	et alii and others
etc.	et cetera; and the rest
Fig/s	Figure/ Figures
g	Gram
g/l	Gram per litre
HCl	Hydrochloric acid
C_2H_5OH	Ethyl alcohol
i.e	Id est (That is)
KOH	Potassium Hydroxide
m	Meter
mg	Milligram
mg/l	Milligram per litre
min(s)	Minutes(s)
ml	Millilitre
MS	Murashigue and Skoog
NaOH	Sodium Hydroxide
No.	Number
0.1N	0.1 Normal
pH	Negative logarithm of hydrogen ion
sp/spp	Species
viz.	Videli = Namely
%	Percentage
$Na_2FeEDTA$	Sodium salt of ferric ethylenediamine tetra acetic acid
ELS	Embryo like structures
Sec.	Second
CV.	Cultivar
LSD	Test of least significant difference

CONTENTS

CHAPTER 1

INTRODUCTION

CHAPTER 1

INTRODUCTION

1.1 OUTLINE ABOUT GARLIC : AS A SPICE

Garlic (*Allium sativum* L.) is an economically important spice crop of Bangladesh and all over the world. It is cultivated in winter season. The propagation rate of garlic in the field is approximately five to ten tons per year and therefore, it takes many years to produce a sufficient number of seed bulbs for practical cultivation of a new variety. Garlic is the second most widely used cultivated *Allium* after onion. It is known to be thermo and photo sensitive. Its vegetative growth and bulb development are greatly influenced by growing environment (Jones and Mann, 1963). Exposure of cloves or young plants to a cool temperature (0-10°C) for 1or 2 months hastens bulbing. Plant that are never exposed to temperature below 20°C may fail to form bulb (Jones and Mann, 1963). Bangladesh has a short cool season, which starts after the hot-humid days and ends with sudden rise in temperature. Planting of garlic should, therefore, be carefully done in Bangladesh so that the crop can take best advantage of the available cool period.

Garlic is an important spices crop in Bangladesh. It has long been recognized all over the world as a valuable spice for foods and a popular remedy for various ailments and physiological disorders. It is used practically all over the world for flavoring various dishes. In America, about 50% of the entire output of fresh garlic is dehydrated and sold to food processors. Spray-dried garlic products are also available for some years (Pruthi, 1979). Garlic has been considered as a rich source of carbohydrate, proteins and phosphorus. Ascorbic acid content was also reported to be very high (Pradan *et al.* 1977). The uninjured bulb contains a colorless, oduorless water-soluble amino acid "allin". On crushing the garlic bulb, the enzyme allinase breaks down allin to produce allicin of which the principal ingredient is the odoriferous dialyll disulfide. Most of the available

cultivars of garlic are white colored, but sometimes pink or red types are also found. The anthocyanin pattern of garlic and onion were very similar and major pigment in each is cyanidin-3-glucoside. An antibiotic allicin is the active principle in garlic which is an enzymatic cleavage product from its precursor allin, naturally present in garlic. The precursor alliin does not possess bactericidal properties.

Garlic is endowed with several medicinal properties. According to the Unani and Ayurvedic systems as practiced in India, garlic is carminative and absorption of food. Garlic has a hypocholesterolaemic action, is present in the aqueous extract of garlic and reduces the cholesterol concentration in human food. The inhalation of garlic oil or garlic juice has generally been recommended by doctors in cases of pulmonary tuberculosis, rheumatism, sterility, impotency, cough and red eyes (Pruthi, 1979). The bulb is pungent, heating, oleagious, tonic aphrodisiac, fattening, digestive, anthelmintic improves appetite, voice, complexion, useful in diseases of the eye and heart, low fevers, bronchitis, inflammanation, piles, leucoderma, asthma, "vata" lumbago, tumours, epileptic fits, thirst earche (Ayurvenda) (Block *et al.*1984; Borida *et al.* 1996; Fujiwara and Natata, 1967; Pai and Plant, 1995). Garlic therapy in the treatment of leprosy significantly alters the bacteriological index and improves the clinical condition of the patients.

1.2 ORIGIN AND DISTRIBUTION

Alliums are among the oldest cultivated plant species. References to edible onion, garlic can be found in the Quran, Bible and in the inscriptions of ancient civilizations of Egypt, Rome, Greek and China. They are mentioned as a source of food for the builders of the great pyramid of king Cheops and the Israelites. Wondering in the desert after the exodus Egypt bemoaned the lack of appetizing onion and garlic. Original abode of garlic is said to be central Asia and Southern Europe, especially Mediterranean region (Tompson and Kelli, 1957). Garlic has

long been known as a cultivated plant. It is among the most ancient of cultivated vegetables giving pungency of the genus *Allium*. Some authorities consider that *Allium longicuspis* Regel, which is endemic to central Asia is the wild ancestor and spread in ancient times to the Mediterranean region. It was known in Egypt in predynastic time, before 3000 B.C. and also to ancient Greeks and Romans. It has long been grown in India and China. Garlic was carried to the western Hemisphere by the Spanish and French (Purseglove, 1975). Garlic was known to ancients and is said to have been disliked by Romans on account of strong odour and hence it was fed to their laborers and soldiers. It was used in England and as early as the first half of the sixteenth century.

Alliums are widely distributed through out the temperate northern hemisphere of the globe. In the new world, there are 80 species mostly in Western States, extending to Mexico and Guatemala. The old world species are abundant in Europe, especially in USSR. *Allium cepa* and *A. sativum* originated in the region of central Asia (Pakistan, Afganistan, Iran, Southern USSR., Western China) and *A. sativum* near Eastern Saudia Arabia, Iraq, Turkey, Georgia, USSR. They further identified the secondary centre (Mediterranean) for *A. cepa* and *A. sativum*. The world production of garlic bulbs was reported to be around 2.8 million tons and the major share to the extent of 1.8 million tons being that of Asia (Anonymous, 1984). The important garlic producing countries in the world are China, Turkey, Spain, India, Thailand, Korea Republic, Egypt, etc. Although China ranks first in garlic production, India has the largest area under garlic after Turkey in the world. In India, major garlic growing states are Gujrat, Orissa, Madhyaprodesh, Rajsthan, Uttraprodesh and Maharashtra.

Garlic is one of the major spice crops of Bangladesh. It is being cultivated on an area of 13077 hectare with a total production of 42805 tons, the average yield being 3.27 tons per hectare (Anonymous, 1984). The yield of garlic per unit area is very low in Bangladesh as compared to other major garlic growing countries of the world like China, (9.6 t/ha), Pakistan (7.9 t/ha), Thailand (7.8 t/ha) and Korea (5.0 t/ha).

1.3 BOTANICAL ASPECTS

The garlic is perennial herbs with bulbous and a monocot plant. Plants are 30-100 cm in height with narrow flat leaves. It is vegetatively propagated by means of bulbs. The bulb is enlarged underground-modified stem. The bulb is composed of a disc like stem, thin dry scales which are the bares of foliage leaves and smaller bulbs. The cloves are enclosed by the dry outer scales. Each clove consists of a protective cylindered sheath, a single thickened storage leaf sheath and a small central bud. It has no node and internode. Shoot has no branches and leafy. Leaf is simple radical and alternate. An incomplete flower was shown only the species of wild type. Root system is adventitious, fibrous and hairy.

Garlic belongs to the family Lilliaceae under the genus *Allium*. It is well known species and widely cultivated in Bangladesh and many other countries of the world. Family Lilliaceae has 250 genera and about 350 species. (Jones and Mann, 1963) have indicated that the basic no. of chromosome in the genus *Allium* is 7 (Acernum) or 8 with few species having 9 chromosomes. The species that are cultivated for food all have the basic chromosome number no. n= 8, *A. sativum* L. is known only as diploid (2n= 16).

Garlic is frost hardy plant requiring cool and moist period during growth and relatively dry period during maturity of bulbs. Garlic can be grown under a wide range of climate conditions but it succeeds best in mild climates without extremes of heat or cold. A cool and humid period during growth and a relatively dry period during maturation are best for the cultivation of garlic.

Garlic can be grown on wide variety of soils. It is cultivated in garden lands and wet lands. It requires a rich well drained clay loam to grow well. The land is well ploughed to a fine tilth and beds and channels made. The optimum spacing for garlic is 15.0 cm between rows and 7.5 cm between plants. Garlic is planted during Oct-Nov. in plains and during Feb-March in the hills under Bangladesh condition. The cloves or segments of bulbs are separated by trampling under feet or pressing with planks. Germination takes about a fortnight from the date of sowing.

1.4 DISEASES OF GARLIC

Garlic is considered as one of the most important spices crop of the world but its productivity is hampered due to attack of a number of pest and diseases. Garlic bulbs, particularly in storage, are subject to a number of diseases, through the crop is comparatively free from the havoc caused by fungi, insects, bacteria and virus.

A leaf blight of garlic was reported to be caused by *Cladosporium echinulatum.* Black mould caused by *Aspergillus niger*, is normally found on mature bulbs, charaterised by black powdery mass between the bulb scale and the surface of the bulbs.

A white rot disease of garlic bulbs in storage, caused by *Sclerotium cepivorum* Berk., results in brown, shrunken and hard cloves. The fungus *Macrophomina phaseoli* reduces the edible portion of stored garlic bulbs to a hard, brown mass without the characteristic smell. The best control of these disease of garlic with various fungicides.

Stored bulbs of garlic are found disfigured due to infection with *Rhizopus oryzae.* The disease is externally spread by the bulbs. Other diseases of garlic in storage include neck rot caused by *Botrytis spp.*, dry rot caused by *Fusarium spp.*, and smudge caused by *Macrosporium colletotrichum.* A mildew resulting from the infection of *Oidiopsis taurica* (Lev.) salm and *Erysiphe taurica* Lev., which occurs on chillies and bringals, is also found on garlic.

A mosaic disease has been observed (Lee *et al.,* 1979) on 'Bhutani' variety of garlic in the Darjeeling hills, resulting in the production of smaller bulbs. The virus is easily transmitted by the aphids, *Myzus persicae* and *Aphis gossypii* to garlic only.

1.5 GARLIC BREEDING: SCOPE AND LIMITATION

Garlic is vegetatively propagated which has resulted intrusion and spread of viral diseases (Walkey *et al.*, 1987). Elimination of such infection has been pursued through meristem tip culture (AboEl-Nil, 1977; Walkey *et al.*, 1987), however this may not be a cost effective propagation tool for some horticultural crops. An increase in the frequency and number of *in vitro* regenerated shoots and reduction of some subculture steps would reduce the cost of these regenerated plants.

Garlic is a sterile plant which does not form seeds. It is, therefore, propagated almost exclusively asexually by means of underground cloves. So, crop improvement by means of cross fertilization is precluded. Somatic hybridization or DNA transfer through *in vitro* technique may provide a way to overcome this problem (Bajay, 1990; Barandiaran *et al.*, 1999; Buiteveld, 1998). Using *in vitro* culture for multiplication of hybrid parent lines this disadvantage could be reduced. Thus breeding in this species is restricted to the selection of spontaneous mutants and their vegetative propagation *in vivo*. *In vitro* culture would allow efficient mutation induction and mass propagation of selected genotypes (Bayliss, 1980; Bettel *et al.*, 1986). Existing *in vitro* multiplication techniques in *Allium* species are summarized (Novak *et al.* 1986). Some authors have put forwarded the hypothesis that mycoplasma or *rickettsia*-like organisms may cause a disease of the garlic tapetum interfering with sexual reproducibility (Konvica, 1973). Further more, vegetative reproduction of garlic may led to chronic contamination with viruses and other micro-organisms (Havranek, 1972; Bos, 1983; Hwang *et al.*, 1983). Garlic is known to contain some antibacterial substances (Takagi, 1990). To avoid such problems with *in vitro* cultures of the genus *Allium,* Fellner (1994) proposed to use antibiotics as growth inhibiting supplements to the plant medium.

Single shoot regeneration and production of multiple shoots with bulblets of garlic have been possible through *in vitro* culture. Effects have been made on

rapid propagation through meristem culture in garlic. For garlic, process has been standardized for clonal propagation through meristem culture. Mass propagation through *in vitro* culture of garlic is also in progress. Experiments on somaclonal variation are new progressing well in different somaclones obtained for different garlic varieties. Somaclonal variants with useful properties have been produced in garlic (Karp and Bright, 1985; Larkin and Scowcroft, 1983).

There are many limitation of using conventional breeding method for improvement of garlic. To overcome this problem non-conventional breeding methods such as somatic hybridization and somaclonal variation may be used. Among the non-conventional breeding method mutation breeding is commonly using for improvement of crop species (Johri *et al.,* 1980; Carlson, 1975). Chimera formation during mutation breeding shows the limitation of this method in some cases of garlic improvement.

1.6 THE NEEDS OF BIOTECHNOLOGY FOR THE IMPROVEMENT OF GARLIC

Plant biotechnology has been developed as a new technology to put forward as a potential way of propagation as well as increasing genetic variability for plant improvement. It has important application in garlic breeding and production of garlic varieties. For many years tissue culture has been applied to improve garlic production by means of micropropagation, pathogen elimination and germplasm conservation (Moriaki, 1988; Moriaki, *et al.*, 1989; Keller and Senula, 2003).

The techniques of garlic biotechnology can be divided into two categories.

i) The propagation, storage and dissemination of virus free plants.

ii) The widening of genetic variability and induction of better traits into garlic by somaclonal and protoclonal variation, ploidy manipulations, somatic embryogenesis, genetic transformation and somatic hybridization.

While progress in the area of biotechnology provides new opportunities for garlic breeding, most of experts agree that biotechnology based methods will always supplements, but not replace traditional breeding methods (Novak, 1980; Ayabe and Sumi, 1998).

Cultivated garlic is sexually sterile, vegetative propagation imposes low rate of multiplication, more chance to viral infection and it is not applicable for its improvement. For these reasons, garlic improvement has focused on tissue culture techniques such as production of virus-free plants, *in vitro* propagation through shoot proliferation and callus culture (Novak, 1990). However, an efficient protocol for regeneration to facilitate rapid propagation with sufficiently high co-efficient of multiplication and genetic transformation has yet to be developed. Somatic embryogenesis has a tremendous potential for large scale production of plant material and is considered as an effective aid to genetic transformation study. It represents an alternative for massive clonal propagation, and also appears to be a potential solution to the problem of field propagation, especially in areas with frequent disease transmission and maintenance of cultivars that have been selected for their important genetic characteristics (Ayabe and Sumi, 1998; Kahane *et al.*, 1992; Kondo *et al.*, 2000).

The improvement of yield potential of cultivated crop like garlic has been obtained through conventional breeding programme, some progress has also been possible through improve culture practices. However, conventional breeding has potential limitation for garlic improvement. Recent advances in the field of genetics and genomics provide a more unifield understanding of the biotechnology.

Recent advances in the field of biotechnology as well as molecular genetic and manipulation of the plants during the recent year have been provided convincing evidence of the usefulness of these powerful technologies in complementing and supplementing plant breeding programme for genetic as well as nutritional improvement of important crop species. The most promising applications of

modern biotechnology are to improve resistance and nutritional quality of foods crop. Since the conventional interventions have not been quite successful in eliminating those constrains, the genetic engineering approach have been reported on the incorporation of disease resistance as well as quality through biotechnology.

During last two decades plant cell, tissue and organ culture have developed rapidly and become a major biotechnological role in agriculture, forestry and industry. For many years tissue culture has been applied on improve garlic production by means of micropropagation, virus elimination and germplasm conservation (Scowcroft, 1977; Shirvin *et al.* 1993; Vine, 1968; Le *et al.*, 1988). However, some of these techniques are still being refined and improved.

Enhanced resistance through intrinsic genetic constitutions manipulated by genetic engineering may be a preferable solution to reduce crop loss from various biotic and abiotic stress. There are many reports on such genetic transformation of garlic that have enormous potential to improve garlic production (Barandiaran *et al.,* 1999; Maggioni *et al.*, 1989; Buiteveld, 1998).

1.7 ROLE OF MICROPROPAGATION FOR THE IMPROVEMENT OF GARLIC

Micropropagation also is a clonal multiplication method, however once virus free starting material is secured, the maintenance of such material is a rather trivial task. This method is used mainly for seed bulb production and for collecting and distributing of germplasm throughout the world or far places. The micropropagation methods used are aimed at producing a large number of desired disease free plantlets in the shortest time possible and multiplication can take place in a small space within strictly controlled environment. Therefore, round the year production is possible.

1.7.1 Meristem culture of garlic

Garlic is vegetatively propagated because it is sexually sterile. Most commercial garlic varieties are virus infected. Further, the plant is sexually sterile and propagated strictly by cloves and bulbs. As a result, virus infections are always transferred by this vegetative propagation. The garlic mosaic virus (GMV) causes a poor quality product and reduces the plant production. Garlic mosaic virus causes visible mosaic symptoms and reduces the clove yield from 3 to 45% (Le *et al.* 1987). Meristem and callus culture is a means by which virus-free plants could be produced. So, *in vitro* methods are suitable for overcoming the problems connected with conventional propagation by cloves, at low coefficient of multiplication and uncontrolled spread of viral disease. Description of the garlic viruses and their elimination by thermotherapy and meristem culture have been described frequently (AboEl-Nil, 1977; Ayuso and Pena-Iglesias, 1981; Conci and Nome, 1991; Van Dijk *et al.*, 1991; Walkey *et al.*, 1987; Senula *et al.*, 2003; Ramawat, 2000). Garlic once infected, viruses are transmitted through seed bulbs. Two flexuous-rod viruses, garlic mosaic virus (GMV) and garlic latent virus (GLV), have been identified in the cultivars of Japan (Lee *et al.* 1979). Infection by these viruses caused a 50-70% yield reduction in the southern district and a 10-20% reduction plants in the northern districts of Japan. Virus-free garlic plants produced through shoot tip culture are useful for improving yield and quality of the crop (Bhojwani, 1980; Bhojwani *et al.* 1982/83). *In vitro* bulblet formation of garlic has been reported (AboEl-Nil, 1977; Bhojwani *et al.* 1982/83; Matsubara and Chen 1989; Moriconi *et al.*, 1990). Meristem culture provides a means for virus-free plant production and mass clonal propagation.

1.7.2 Somatic embryogenesis and selection of somaclonal variants

Somatic embryogenesis is the process of a single cell or a group of cell initiating the developmental path way that leads to reproduce regeneration of non-zygotic embryos capable of non-zygotic embryos plants. Somatic

embryogenesis has a tremendous potential for large scale production of plant materials (Amirato and Styer, 1985) and is considered as an effective aid to genetic transformation study. It represents an alternative for massive clonal propagation, specially in areas with frequent disease transmission and maintenance of cultivars that have been selected for their important genetic characteristics (De Garcia and Martinez, 1995). Unfortunately, there have been very few reports of somatic embryogenesis in garlic, although considerable improvements have been made in other *Alliums*. Hansen *et al.* (1996) reported that embryogenic callus formed somatic embryos in four *Allium sp*, *A. cepa, A. fistulosum, A. porrum,* and *A. ampeloprasum.* However regeneration from sporadic somatic embryogenesis in garlic was first reported by AboEl-Nil (1977) from calli of bulb leaf discs and stem tips on AZ medium supplemented with p-CPA (p-Cholorophenoxy acetic acid), 2,4-D and KIN. Somatic embryogenesis was also reported from receptacle, pedicle and flower buds (Suh and Park, 1986). In these cases the efficiency of plantlet regeneration was very low. Lastly, efficient plant regeneration in garlic through somatic embryogenesis was reported by Haque *et al.* (1998) and Fereol *et al.* (2002) from root tip explants.

In vitro mutant induction and selection can provide a rapid method for developing new products. Tissue culture is a very unnatural way of inducing plant growth. Permanent genetic changes that are occasionally seen in tissue culture derived plants. Variants selected in tissue cultures have been referred to as 'calliclones' (From callus cultures, Shepared *et al.*, 1980). According to (Larkin and Scowcroft, 1981) "somaclonal variation is the genetic variability which is generated during tissue culture.

The somaclonal variation may be attributed to either

i) Pre-existing variation in the somatic cells of the explants (genetic)

ii) Variation generated during tissue culture (epigenetic.)

Often both factors may contribute.

The cause of epigenetic variation is mostly attributed to changes in the chromosome number and structure (Kaeppler et al., 2000). Recent investigation has revealed that long time cultured cells or tissues undergo frequent genetic changes (Polyploidy, aneuploidy, chromosomal breakage, deletion, duplication, translocation, gene amplifications and mutations) and that these are also expressed at biochemical or molecular levels (Maggioni *et al.*, 1980; Novak, 1980; Kahane *et al.*, 1992; Choi *et al.*, 2000).

1.8 AIM AND OBJECTIVES

Micropropagated garlic mericlones serve as the initial source of planting material in foundation programms. Clean seed derived from mericlones may be economically feasible for growers compared to conventional propagation methods. Callus culture followed by plant regeneration offers somaclonal variation and a way of genetic transformation.

Keeping in mind the above-mentioned aspects of garlic the present investigation was undertaken on *in vitro* culture of garlic (*Allium sativum* L.) with the following objectives.

1. Standardization of culture media for primary *in vitro* establishment of isolated meristems from different garlic genotypes.
2. Standardization of suitable nutrient media composition and selection of suitable hormonal supplements for rapid shoot induction, elongation and multiplication from the cultured meristems.
3. Development of standard method for the production of virus-free propagules through meristem culture and production of *in vitro* bulblets through proper manipulation of plant growth regulators and carbon sources.
4. Callus induction for developing somaclonal variants through somatic embryogenesis.
5. Acclimatization and transplantation of *in vitro* grown bulblets.
6. Field evaluation of meristem and callus derived plantlets.

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

The present investigation was planned under two main heads: (i) meristem culture for disease elimination, (ii) callus culture for induction of somaclonal variation.

2.1.1 Plant materials

Shoot tips of five garlic (*Allium stivum* L.) cultivars (GC002, GC008, GC009, GC0025 and one Indian variety) were used as sources for meristem culture.

The bulbs were collected from field grown mature garlic plants from Spice Research Centre, Shibgonj, Bogra. The cloves of the bulbs were separated and meristems were isolated and were cultured for shoot induction, multiplication and bulblet formation. Besides, shoot tip, leaf primordia, leaf base and bulbil were cultured to induce callus following somatic embryogenesis, plantlet regeneration and bulblet formation.

2.2 CHEMICALS

2.2.1 Sterilant and surfactant

In the present investigation 95% ethyl alcohol was used as surface sterilizing agent.

2.2.2 Nutrient medium

In the present investigation MS basal salt formulation (Murashige and Skoog, 1962) was used to conduct all of the experiments.

2.2.3 Plant growth regulators

The basal medium was supplemented with various concentrations of different plant growth regulators. The following plant growth regulators were employed for the present investigation.

Auxins : Auxins promote cell enlargement, root induction and callus induction

α-napthalene acetic acid (NAA)

Indole- 3-butyric acid (IBA)

Indole-3-acetic acid (IAA)

2, 4-dichlorophenoxy acetic acid (2, 4-D)

Cytokinins: Cytokinins promote cell division and shoot initiation

2 - Isopentyl adenine (2ip)

6 -Benzyl amino purine (BAP)

6 - Furfuryl amino purine (KIN)

2.2.4 Equipments

The culture vessels such as test tubes (150×25 mm), bottles (12×5 cm), measuring cylinders, conical flasks (250 ml, 500 ml, 1000 ml), separating funnel, pipettes, pipette pump, parafilm, cotton plugs, rubber bands, aluminum foils, cotton, fire box, marker pen, spirit lamp, needle, sharp blade, stereo-microscope, scissors, various size of forceps, electronic balance, magnetic stirrer, autoclave, pH meter, laminar airflow machine etc. were used. On the other hand field materials such as plough, spade, smoothing plane, hoe, plough share etc. were also used in the experiment.

2.3 METHODS

Experimental methods used for carrying out this investigation were accomplished through the following steps.

2.3.1 Preparation of stock solution for culture media

The first step of the preparation of MS (Murashige and Skoog, 1962) culture medium was the preparation of stock solutions. The various constituents of the respective nutrient medium were prepared into stock solutions for ready use

during the preparation of media for different experiments. As different constituents were required in different concentrations, separate stock solutions of macronutrients, micronutrients, organic compounds (vitamins and amino acids) and growth regulators (cytokinin and auxin) etc. were prepared separately.

2.3.1.1 Stock solution (I-III): Macronutrients

This stock solution was made as 20 times of final strength of the medium in 1000 ml of DW. At first 20 times the weight of each of the major salts required for 1 liter of medium was weighted accurately, dissolved once at a time and sequentially in 750 ml of DW and then final volume was made up to 1000 ml by further addition of DW. The stock solution was filtered through Whatman No. 1 (Whatman Ltd. England) filter paper, to remove all the solid contaminants and solid particles like cellulose dust, cotton etc. The stock was then poured in a clean plastic bottle and was labeled by stock solution 1 (20×), stock solution 2 (20×), stock solution 3 (20×) and stored in a refrigerator at 4-6°C.

2.3.1.2 Stock solution (IV-VI) : Micronutrients

The stock solution of IV and V were prepared at 20X like stock solution of macronutrients as described earlier (2.3.1.1.). In case of stock solution IV, requisite amount of $FeSO_4$. $7H_2O$ and Na_2EDTA. $2H_2O$ Were taken and dissolved separately in 400 ml DW by heating and constant stirring. The two solutions were mixed, the pH was adjusted to 5.5 and DW was added to make up the final volume to 1000 ml and stored in refrigerator at 4-6°C. This solution must be kept in an amber colored bottle or covered by black paper.

The rest of the micronutrients (solution VI) were made at 1000X in 1000 ml DW. All components of solution VI were weighed separately and dissolved in 800 ml of DW. Finally, the volume of the solution was adjusted to 1000 ml and after filtering stored at 4-6°C in a plastic bottle.

2.3.1.3 Stock solution (VII): Vitamins and amino acids

The stock solution was prepared at 100X, dissolved in DW to make the volume 200 ml. After being filtered was poured into clean plastic bottle and stored at 0°C in refrigerator.

2.3.2 Preparation of stock solution for growth regulators

In addition to the nutrients, it is generally necessary to add one or more growth regulators such as cytokinin and auxin to the media to support good growth of tissues and organs (Bhojwani and Razdan, 1983). These growth regulators were dissolved in appropriate solvent as shown against each of them.

Plant Growth regulators	Amount taken (mg)	Dissolving solvent (ml)	Final volume of the stock solution with DDW (ml)	Strength of the stock solution (mg/ml)
Auxins				
IAA	10	80% ethanol 1ml	100	0.1
IBA	10	70% ethanol 1ml	100	0.1
2,4-D	10	70% ethanol 1ml	100	0.1
NAA	10	0.1 N KOH 1ml	100	0.1
Cytokinins				
2iP	10	0.1 N NaOH 1ml	100	0.1
BAP	10	0.1 N KOH 1ml	100	0.1
KIN	10	0.1 N NaOH 1ml	100	0.1

To prepare stock solution 10 mg of any of the growth regulators was taken in a clean test tube and dissolved in required volume of appropriate solvent. Final volume of the solution was made up to 100 ml by adding double distilled water. Thus stock solutions of all the growth regulators were prepared and stored at 4°C in refrigerator.

2.3.3 Preparation of culture media

The following steps were followed to prepare one liter MS medium according to Appendix 1.

2.3.3.1 Assembling of the media components

For preparing 1 liter MS medium, 20 ml stock solution I, 20 ml of stock solution II, 20 ml of stock solution III, 20 ml of stock solution IV, 20 ml of stock solution V, 2 ml of stock solution VI and 2 ml of stock solution VII in 1 liter flask containing 500 ml distilled water and mixed well.

2.3.3.2 Addition of growth regulators

Different concentrations of hormonal supplements were added either singly or in different combinations to the above solution and were mixed thoroughly. Since each hormonal stock solution contained 10 g of the chemical in 10 ml of solution, the addition of 10 ml of any hormonal stock solution to made one liter of medium results in 1.0 mg/l concentrations of that hormonal supplement. For preparing hormonless MS_0 medium no growth regulators were added.

2.3.3.3 pH of the medium

The pH of the medium was generally adjusted 5.8 using pH meter with the help of 0.1 N KOH or 0.1 N HCl whichever as necessary.

2.3.3.4 Sucrose

After adjustment of pH, 30 g sucrose was added and the final volume of the mixture was made 1 liter by adding distilled water. For *in vitro* bulblet formation different concentrations of sucrose (3, 6, 9, 12, 15 and 18%) were used.

2.3.3.5 Agar

The nutrient media used were of two types, liquid and semi-solid. The liquid nutrient medium was made up without addition of any agar and using filter paper

bridge in the test tube for supporting the plant material (specially in meristem culture). The semi-solid media were prepared with agar for shoot multiplications, root induction and callus formation. In the present investigation 7-8 g of agar (Carolina Biological Supply Co.) was added for one liter of medium. Then the whole mixture was gently heated in micro-oven for 5 minutes, the agar was melted completely and making a clear solution. Care was taken at the time of heating that the solution not be evaporated any way.

2.3.3.6 Medium dispensing to culture vessels

Fixed volume of above hot melted medium was transferred in to culture vessels like test tubes (150×25 mm) and conical flask (250 ml) through separating funnel. The culture vessels were plugged with non-absorbent cotton, which were inserted tightly at the mouth of culture vessels. The culture vessels were marked to designate specific hormonal supplements.

2.3.3.7 Sterilization

Finally the culture vessels containing medium were autoclaved at 15 lb/sq inch pressure and at the temperature 120-121°C for 20 minutes to ensure sterilization. Then the vessels with the medium were allowed to cool as vertically and marked with a glass marker pen to indicate specific hormonal supplements and stored in the culture room for ready use.

2.4 CULTURE TECHNIQUE

The following methods were employed in the present investigation for (i) meristem culture and micropropagation of meristem derived plantlets, (ii) callus culture followed by plantlet regeneration, bulblet formation through somatic embryogenesis, (iii) field establishment of plantlets derived from meristem culture and callus culture.

2.4.1 Explant collection and preparation

The bulbs of five garlic cultivars, each of them containing 10-15 cloves were collected from 90 days old field grown garlic plants and taken to the laboratory. The excess unnecessary part like roots, over mature leaves or shoots were removed, cloves were separated from the bulbs and were used in the experiments.

2.4.2 Inoculation techniques

All inoculations and aseptic manipulations were carried out in a running laminar airflow cabinet. The cabinet was switched on half an hour before use and cleaned with 90% ethyl alcohol to reduce the chances of contamination. All instruments like scalpels needles, forceps, tiles, petridishes etc. were covered with aluminium foil paper and sterilized by steam sterilization method. A stereoscopic dissection microscope was used for meristem isolation. The stage of the microscope was wiped with cotton wool containing 70% ethanol after isolating each meristem from sterile garlic cloves. To ensure complete aseptic condition, both hands were also wiped by 70% ethyl alcohol.

2.4.3 Surface sterilization of explants

Surface sterilization of cloves was carried out inside the running laminar airflow cabinet. The outer cover of cloves were removed and transferred to 200 ml sterilized beaker. Then each clove was soaked in 95% ethanol solution for 30 seconds. The soaked cloves were washed with sterile distilled water to remove ethanol from clove surface.

2.4.4 Isolation of meristem and culture

After sterilization the separated cloves were placed on sterile tiles using sterile forceps and were cut vertically and horizontally with the help of forceps and scalpel. Leaf primordia were snapped off with slight pressure and the exposed meristems tips which appeared as a shiny dome were gently isolated with a sharp blade. After deplugging of culture tubes the excised meristem tips were carefully

placed on the filter paper bridge on liquid MS medium. The neck of the tube was flamed with spirit lamp and then plugged. After inoculation, the culture tubes were labeled by glass marker and the culture tubes were ready for incubation.

2.4.5 Culture incubation

All the inoculated culture tubes were incubated in a growth chamber providing a special culture environment. The tubes were placed on the shelves of a culture rack in the growth chamber. All cultures were grown in the growth chamber illuminated by 40 watts white fluorescent tubes fitted at a distance of 30-40 cm from the culture shelves. The cultures were maintained at 25±2°C with light intensity varied from 2000-3000 lux. The photoperiod was maintained generally 16 hours light and 8 hours dark. The culture tubes were checked daily to note the morphogenic response of cultured explants in different experiments conducted in the present investigation.

2.4.6 Subculture

After 3 weeks of inoculation of meristem, the primary established meristems those showed morphogenic response were removed aseptically from the culture tubes and transferred into tubes containing agar gelled MS medium supplemented with different growth regulators. During inoculation special care was taken so that the explants must touch the medium equally and do not deep into medium. After 25-30 days of culture initiation, the primary established meristem formed shoot and root and well established in the medium.

2.4.7 Subculture for shoot multiplication and bulblet formation

After 3 weeks on the initiation medium, the explants which initiated shoots and increased in size 10-20 fold, were rescued aseptically from culture tubes and placed on sterile petridish and transferred into freshly prepared semi-solid medium supplemented with the same or different hormonal combination for shoot multiplication. Multiple shoots developed within 30 days. For *in vitro*

bulblet formation, multiple shoot clumps or mericlones were subcultured in media with different concentrations of sucrose.

Data on frequency (%) of shoot multiplication, shoot and root number per explant and length of the longest shoot and root were recorded 21 days after subculture from liquid MS media.

Besides, percentage of shoots formed bulblets, number of bulblets per culture, length of bulblets, diameter of bulblets, and bulblets weight were also recorded and analyzed.

2.5 MICROPROPAGATION

After 4 weeks of incubation when the plantlets attained a height of 7-9 cm, micropropagation was started. The plantlets were removed carefully from the test tube over a petridish using a pair of forceps. In the petridish the leaves from the stem were carefully removed and cut into single plantlet with a bulb.

The isolated plantlets were transferred to the culture tubes for multiplication. The plantlet was placed on the medium in such a way that it should not be pushed below the surface of the medium. Single plantlet with a bulblet culturing after incubation at 25±2°C temperature with 16 hour photoperiods per day grew rapidly and developed into new plantlets. The plantlets were subcultured as required after every 4 weeks.

2.6 ACCLIMATIZATION AND TRANSPLANTATION OF MERISTEM DERIVED PLANTLETS

When the regenerated plantlets with bulblets produced sufficient roots and attained a height 10-15 cm were considered ready to transfer in soil. The plantlets grow inside the tubes were brought out from the growth chamber. After depluging of the culture tubes they were kept in room temperature for 5-7 days to bring them in contact to normal temperature. The plantlets were then rescued very carefully from the test tubes. Agar attached to the roots was gently and

carefully washed out under running tap water. The plantlets were firstly transferred to small ploy bags or pots containing soil and sand in ratio of 1 : 1. The pots with the plantlets were kept in shady place and covered with polythene sheet to prevent sudden desiccation. The soil in pots was sprayed with water everyday to maintain high humidity. The polythene bag was gradually perforated to expose the plants to outer environment. After 15-25 days plantlets were finally transplanted to field, where they eventually developed into mature plants.

2.7 CALLUS CULTURE AND PLANTLET REGENERATION

2.7.1 Choice of explants

Explant is a great factor for callus induction. For callus initiation shoot tip, leaf base, leaf primordia and bulbils were used as explants. These explants were isolated from field grown mature garlic bulbs of used five cultivars.

2.7.2 Induction of callus

To induce callus, explants (shoot tip, leaf base, leaf primordia and bulbil) were cultured in MS (Murashige and Skoog, 1962) medium supplemented with different callus inducing substances and incubated in light of 25±2°C for 3-6 weeks. MS medium was supplemented with different concentrations of 2, 4-D, alone or in combination with IAA for massive callus induction. After 21 days of incubation in the light, the callus induction frequency was determined and the degree of callus growth, color and nature of callus were recorded after 3-6 weeks of culture.

2.7.3 Induction of somatic embryos

After callus induction from the explants, the calli were transferred to fresh medium without any growth regulators for further proliferation, maintenance and embryo formation. The embryogenic callus was selected for plant regeneration.

2.7.4 Embryo germination and plantlet regeneration

After about 2 months on the callus inoculation medium, calli with somatic embryos on their surfaces were placed on germination medium consisting of MS salts and vitamins with different concentrations of (1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 mg/l) kinetin and 3% sucrose. Depending on size, each callus with somatic embryo was divided into 3-5 parts and each part was cultured individually on regeneration medium. After about two months, regenerated plantlets were transferred to MS medium with different concentrations of sucrose for *in vitro* bulblet formation.

2.7.5 *In vitro* bulblet formation and transplantation

Regenerated multiple shoot clumps were transferred to MS medium with different concentrations of sucrose (3-18%) for bulblet formation. Percentage of culture induced bulblets, number of bulblets, length of bulblet, diameter of bulblet and weight of bulblet per culture were recorded after 3 weeks of culture. The developed bulblets were stored or transplanted on sterile soil (using formalin) soil and sand (1 : 1). A single bulblet was transplanted in a (98×6 cm) ploy bag in net house covered by large polythene sheet for maintaining shade and humidity. Necessary cultural practices were done for good growth of the plants.

2.8 DATA COLLECTION AND ANALYSIS

2.8.1 Data collection for *in vitro* grown meristem derived plantlets

Data were collected using the following parameters and the methods of data collection are given below.

i) Days to shoot initiation

ii) Percentage of response: Percentage of explants responded was calculated using following formula

$$\% \text{ of explants responded} = \frac{\text{Number of explants responded}}{\text{Total number of explants cultured}} \times 100$$

iii) Number of shoots/explant: Number of shoots was counted for each culture after 28 days of incubation and average shoot number was calculated and noted.

iv) Shoot length: After 28 days of culture shoot length was measured in cm scales for each plantlet and average shoot length was calculated and recorded.

2.8.1.1 Data collection for field grown plants

Data on the following characters were recorded from ten randomly selected plants of each variety.

i) % of survivability

ii) Plant height

iii) Number of leaves/plant

iv) Bulb diameter/plant

vi) Bulb weight/ plant

The number of plantlets that survived in the field were expressed as percentage. At the time of harvest plant height, number of leaves/ plant, diameter of bulb/plant, weight of bulb/plant were recorded. Weight of bulb was taken with a weighing balance.

2.8.1.2 Data analysis

LSD analysis and subsequent significant test were done according to Gomez and Gomez, 1984.

2.8.2 Data recording for callus derived plantlets

Data were collected using the following parameters and the methods for data collection given below.

2.8.2.1 For callus induction

1. Percentages of explants induced callus: Explants were cultured in 25×150 mm culture tubes containing media with different concentrations of plant growth regulators for callus induction. After required days of culture percentage of callus induction was calculated using the following formula.

$$\% \text{ of callus induction} = \frac{\text{Number of explants induced callus}}{\text{Number of cultured explants}} \times 100$$

2. Qualitative assessment of callus: After few days of culture the explants started to develop callus in medium with proper growth regulators but color of callus varied in respect of growth regulators supplements. For this reason, different symbols were used to denote the different color of callus as given below:

Description of callus color	Symbols
White	Wh
Cream	Cr
Watery	Wa

The different degrees of callus growth were assessed using different symbols as given below:

Description of callus formation	Symbols
No callus formation	-
Poor callus formation	+
Moderate callus formation	++
Massive callus formation	+++

2.8.2.2 For shoot induction

a) Percentage of calli induced to develop shoots: The percentage of calli induced to develop adventitious shoots were calculated using the following formula:

$$\% \text{ of calli induced shoots} = \frac{\text{Number of calli induced shoots}}{\text{Total no. of calli cultured}} \times 100$$

b) Number of embryos/shoots/roots/bulblets per culture: Number of embryos/shoots/roots/bulblets per culture was recorded after required days of culture. Mean number of these organs per culture was calculated using following formula.

$$\overline{X} = \frac{\Sigma Xi}{N}$$

Where, $\overline{X}$ = Average number of shoots

Σ = Summation

Xi = Total number of shoots

N = Number of observation

c) Length of the longest shoot: Length of the longest shoot and root was measured in cm scales for each explant after 30 days of inoculation. Average length of the longest shoot was calculated by using above mentioned formula.

2.8.2.3 Data recording for field grown plant

Data on the following characters were recorded from randomly 5 selected plants of each variety.

i) Plant height

ii) Number of leaves/ plant

iii) Bulb diameter/plant

iv) Bulb weight/plant

All these parameters were recorded 90 days after plantation.

2.8.2.4 Data analysis

The data recorded for different observations were analyzed using the statistical methods described by Gomez and Gomez, 1984.

2.9 TRANSPLANTATION OF CALLUS DERIVED PLANTLETS

The plantlets with bulblets were transplanted in rows keeping 7.5 cm space between two plants. The distance between two rows was 15 cm. Care was taken to avoid damage to the roots and to ensure good contact between roots and soil. The plantlets were kept in an environment with high relative humidity for the first few days following transplantation by covering the beds with polythene paper and regular spraying of water. In order to prevent viral vectors the entire field was covered with nylon nets. Interim care was taken by periodic irrigation, weeding, mulching and spraying insecticide.

2.9.1 Harvesting

The time of harvest is very important in garlic. After plantation of tissue culture plantlets, garlics were collected from the field within 90-100 days. When haulms of garlic plant and leaves started yellowing and falling on the ground, it was proper time for garlic harvest. There is no useful scientific machine or tools in Bangladesh to pluck the garlic from the field, for this reason garlics were plucked by hands. After harvesting, the garlics were stored at well ventilated dry place.

CHAPTER 3

RESULTS

CHAPTER 3

RESULTS

3.1 ESTABLISHMENT OF MERISTEM CULTURE OF CV. GC002

For primary establishment of *in vitro* cultures from field grown explants surface sterilization was essential before inoculation of explants because of the presence of loose contaminants attached to the surface of the garlic cloves. Surface sterilization was carried out by a trial and error experiments. Ethyl alcohol was used as surface sterilant for disinfecting garlic cloves by soaking for different time duration (10, 20, 30, 40, 50 and 60 sec.) with various concentration (75%, 80%, 85%, 90%, 95% and 100%). The soaked cloves were then washed with sterile distilled water to remove alcohol from the clove surface. After one week of inoculation, it was observed that among the different treatments, 100% explants were found contamination free, when the materials were treated for 30 seconds in 95% ethanol (data not shown). This treatment was considered to be ideal to get contamination free cultures. On the other hand when the garlic cloves treated for short duration (10, 20 sec) with low concentrations (75%-90%) of ethanol, they were contaminated with different microorganisms. However, when they were treated for long duration with high concentration of ethanol (95-100%), survival rates decreased but contamination was not found. Therefore, for surface sterilization of garlic cloves treatment with 95% ethanol for 30 seconds duration period was found most effective. No varietal difference was observed to sensitivity to ethyl alcohol.

For primary establishment of meristems (0.3-0.5 mm) isolated from cloves of field grown mature garlic bulbs of cv. GC002 was placed on "M" shaped filter paper bridge in culture tubes containing liquid MS medium supplemented with various concentrations and combinations of different plant growth regulators. Meristems showed their first growth response by increase in size and became green in color. They continued their growth and finally developed into shoot.

When a meristem attained in this condition, they were supposed to be established. The meristems grew further, elongated and became dark green in color and developed into plantlets.

3.1.1 Effect of different concentrations of cytokinins singly

This experiment was conducted to see the effect of different concentrations of cytokinins singly on shoot formation from the cultured meristems. The excised meristems (from sterile cloves) were cultured in MS liquid medium supplemented with six different concentration (0.1, 0.5, 1.0, 1.5, 2.0 and 3.0 mg/l) of cytokinins singly. Three types of cytokinins tested in this experiment were 2ip, BAP and KIN.

Data on days to response, percentage of meristems responded, morphogenic response and degree of shoot growth were recorded 21 days after culture and are presented in Table 1.

Days taken to resume growth were influenced by the type of cytokinins as well as their different concentrations used. The cultured meristems started their initial growth by increasing in size and gradually changed to light green in color within 4-10 days. Among the three cytokinins early response (4-8 days) was recorded in 2ip. In BAP and KIN containing media the explants took 6-10 days to response.

In all media formulations the meristems produced primary shoot but no root formation was observed in any of the formulations used. Among all the treatments most of the cultured meristems showed excellent shoot growth but in few media formulations showed moderate shoot growth and these media were 0.1 mg/l 2ip, 3.0 mg//l 2ip, 0.1 mg/l BAP, 3.0 mg/l BAP, 0.1 mg/l KIN and 3.0 mg/l KIN.

The percentage of meristems formed shoots ranged from 38.8-86.4% in 2ip. 34.1-72.7% in BAP and 21.4-58.3% in KIN. Among different concentrations of 2ip, highest percentage (86.4%) response was recorded in 1.0 mg/l 2ip

(Plate 1A) followed by 71.2% in 1.5 mg/l 2ip and the lowest response (38.8%) was noted in 3.0 mg/l 2ip. When the media were supplemented with BAP the highest (72.7%) meristems initiated shoots in 1.5 mg/l BAP followed by 64.2% in 1.0 mg/l BAP and the lowest response was recorded (34.1%) in 3.0 mg/l BAP. When the media were employed with KIN concentrations the highest percentage 58.3% of response was recorded in 1.5 mg/l KIN followed by 49.6% in 2.0 mg/l KIN and the lowest response (21.4%) was noted in 0.1 mg/l KIN.

On average among the three cytokinins tested, the highest percentage 59.6% of response was recorded in 2ip followed by 52.5% in BAP and 42.1% in KIN.

Therefore, 2ip was considered to be the best for primary response of the isolated meristems and among the different concentrations of 2ip, 1.0 mg/l 2ip was observed to be the best media formulation where maximum response was achieved and the meristems resumed their initial growth rapidly.

Table 1. Effect of different concentrations of cytokinins for primary establishment of meristem of genotype GC002. In each treatment 10-15 explants were cultured. Data were recorded after 21 days of culture and the experiment was repeated thrice.

Growth regulators (mg/l)	Primary response of explants on MS liquid medium			
	Days to response	Frequency of explants responded (%)	Morphogenic response	
			Shoot	Root
2ip				
0.1	4-6	47.9	++	-
0.5	4-6	57.7	+++	-
1.0	4-6	86.4	+++	-
1.5	5-8	71.2	+++	-
2.0	5-8	55.8	+++	-
3.0	5-8	38.8	++	-
Mean		**59.6**		
BAP				
0.1	8-10	40.5	++	-
0.5	7-10	53.8	++	-
1.0	6-10	64.2	+++	-
1.5	6-10	72.7	+++	-
2.0	7-10	50.0	+++	-
3.0	8-10	34.1	++	-
Mean		**52.5**		
KIN				
0.1	8-10	21.4	++	-
0.5	7-10	44.1	+++	-
1.0	6-10	46.1	+++	-
1.5	6-10	58.3	+++	-
2.0	8-10	49.6	+++	-
3.0	8-10	33.3	++	-
Mean		**42.1**		

- = No growth
+ = Poor shoot growth
++ = Moderate shoot growth
+++ = Massive shoot growth

3.1.2 Effect of different concentrations and combinations of cytokinins with auxins

This experiment was conducted to see the effect of different combinations of cytokinins with auxins on shoot formation from the cultured meristems. The isolated meristems were cultured in MS liquid medium supplemented with three selected concentrations of cytokinins (1.0 mg/l 2ip, 1.5 mg/l BAP and 1.5 mg/l KIN) along with various concentrations such as 0.25, 0.5 and 1.0 mg/l of auxins. The three types of auxins used were NAA, IBA and IAA. Data on days to response, percentage of meristems responded, morphogenic response and degree of shoot growth were recorded after 21 days of culture and are presented in Table 2.

Days taken to resume growth, frequency of response and shoot growth were influenced by the type of cytokinins with auxins as well as their different combinations used. The cultured meristems started their initial growth by increasing in size and gradually changed to light green in color within 3-9 days. Among all the combinations early response (3-6 days) was recorded in 2ip with auxins. In BAP with auxins and in KIN with auxins containing media the explants took 4-9 days to response.

In all the treatments the cultured meristems produced primary shoots along with roots except in media with 1.5 mg/l KIN + 0.25 mg/l IAA. In this experiment meristems showed excellent shoot growth in most cases.

The percentage of meristems responding ranged from 48.8-91.6% in 2ip with auxins, 47.6-85.4% in BAP with auxins and 46.4-73.3% in KIN with auxins.

When the media were supplemented with 2ip and auxins (NAA, IBA and IAA), the highest percentage (91.6%) of response was recorded in 1.0 mg/l 2ip + 0.5 mg/l NAA (Plate 1B) followed by 84.1% in 1.0 mg/l 2ip + 0.25 mg/l NAA and 78.4% in 1.0 mg/l 2ip + 0.5 mg/l IBA. The lowest response was recorded 48.8% in 1.0 mg/l 2ip + 1.0 mg/l IAA.

In cases of BAP with auxins (NAA, IBA and IAA) combination, the highest percentage (85.4%) of explants induced primary response in media having 1.5 mg/l BAP + 0.5 mg/l NAA followed by 81.6% in 1.5 mg/l BAP + 0.5 mg/l IBA and 75.2% in 1.5 mg/l BAP + 0.5 mg/l IAA. The lowest response was recorded 47.6% in 1.5 mg/l BAP + 1.0 mg/l IAA.

When the media were employed with KIN in combination with auxins (NAA, IBA and IAA), the highest percentage (73.3%) of response was recorded in 1.5 mg/l KIN + 0.5 mg/l NAA followed by 71.5% in 1.5 mg/l KIN + 0.5 mg/l IBA. The lowest response (46.4%) was noted in 1.5 mg/l KIN + 1.0 mg/l IAA.

From this experiment it is observed that a combination of cytokinin and auxin was found to be more effective than when cytokinin was used alone as found in previous experiment. Among all the tested combinations, the media with 1.0 mg/l 2ip in combination with different concentrations of an auxin were found to be more suitable for primary response of meristems than other combinations and of the three auxins NAA was found to be most effective.

On average among all the treatments the media fortified with 1.0 mg/l 2ip with NAA, 1.0 mg/l 2ip + 0.5 mg/l NAA were observed to be the best media formulation, where meristems resumed their initial growth rapidly (3-6 days) and highest percentage (91.6%) of response was observed.

Table 2. Effect of different concentrations and combinations of cytokinins with auxins in liquid MS medium on primary response of meristem culture of genotype GC002. In each treatment 10-15 explants were used and the experiment was repeated thrice. Data were recorded after 21 days of culture.

Growth regulators	Primary response of explants on MS liquid medium				
	(mg/l)	Days to response	Frequency of explants responded (%)	Morphogenic response	
				Shoot	Root
2ip + NAA	1.0+0.25	4-6	84.1	+++	+++
	1.0+0.5	3-6	91.6	+++	+++
	1.0+1.0	5-6	70.8	+++	+++
Mean			**82.1**		
2ip +IBA	1.0+0.25	5-6	68.1	+++	+++
	1.0+0.5	5-6	78.4	+++	+++
	1.0+1.0	5-6	62.8	+++	+++
Mean			**69.8**		
2ip +IAA	1.0+0.25	5-6	69.1	+++	+++
	1.0+0.5	5-6	72.2	+++	+++
	1.0+1.0	5-6	48.8	+++	+++
Mean			**63.4**		
BAP+NAA	1.0+0.25	6-9	71.5	+++	+++
	1.0+0.5	4-9	85.4	+++	+++
	1.0+1.0	5-9	59.2	+++	+++
Mean			**72.0**		
BAP+IBA	1.5+0.25	5-9	72.5	+++	+++
	1.5+0.5	5-9	81.6	+++	+++
	1.5+1.0	6-9	58.8	+++	+++
Mean			**70.9**		
BAP+IAA	1.5+0.25	5-9	66.2	+++	+++
	1.5+0.5	5-9	75.2	+++	+++
	1.5+1.0	6-9	47.6	+++	+++
Mean			**63.0**		
KIN+NAA	1.5+0.25	5-9	64.4	+++	+++
	1.5+0.5	4-9	73.3	+++	+++
	1.5+1.0	6-9	56.6	+++	+++
Mean			**64.8**		
KIN+IBA	1.5+0.25	6-9	57.4	+++	+++
	1.5+0.5	6-9	71.5	+++	+++
	1.5+1.0	6-9	54.4	+++	+++
Mean			**61.1**		
KIN+IAA	1.5+0.25	6-9	58.5	+++	-
	1.5+0.5	6-9	68.4	+++	+++
	1.5+1.0	7-9	46.4	+++	+++
Mean			**57.8**		

- = No growth, + = Poor growth, ++ = Moderate growth, +++ = Massive growth

3.1.3 Genotypic effect of five garlic cultivars

In this experiment isolated meristems of five garlic varieties viz., GC002, GC008, GC009, GC0025 and Indian were cultured separately on "M" shaped filter paper bridge in culture tubes containing liquid MS medium supplemented with three selected combinations (1.0 mg/l 2ip + 0.5 mg/l NAA, 1.5 mg/l BAP + 0.5 mg/l NAA and 1.5 mg/l KIN + 0.5 mg/l NAA) of plant growth regulators to find out the response of five garlic varieties. Data on days to response, percentage of meristems responded, morphogenic response and degree of shoot growth was recorded after 21 days of culture and are presented in Table 3. The graphic representation is shown in Fig. 1.

Days to response ranged from 3-20 days and among five varieties early response (3-6 days) was recorded in cv. GC0025. GC002, GC008 and GC009 took 4-8 days, 4-8 days and 5-9 days to response, respectively. The Indian variety took maximum days (15-20 days) to respond.

From the results it was observed that percentage of meristems responding ranged from 73.3-91.6% in cv. GC002, 68.8-84.4% in cv. GC008, 64.4-76.3% in cv. GC009, 85.6-96.6% in cv. GC0025 and 20.2-26.6% in cv. Indian.

Among the five cultivars, the highest percentage (96.6%) of response was recorded in cv. GC0025 followed by 91.6% in cv. GC002, 84.4% in cv. GC008 and 76.3% in GC009. The lowest percentage (26.6%) of meristems responded to new growth was recorded in Indian variety.

The results further show that in all the tested combinations, the meristems of four varieties produced shoots with roots but Indian variety produced only shoots in all the treatments.

The degree of shoot growth was found highly satisfactory for four varieties but Indian variety showed very poor growth in all the treatments.

In this experiment it is observed that genotypic difference existed among five garlic varieties and cv. GC0025 showed the best response followed by cv. GC002, cv. GC008 and cv. GC009. Indian showed the poorest response.

Table 3. Differential genotypic response of five garlic varieties with three combinations of cytokinins and auxins in liquid MS medium on primary response of mesistems. Each treatment consisted 12-15 explants and the experiment was repeated thrice. Data were recorded after 21 days of culture.

Genotypes	Plant growth regulators (mg/l)	Days to response	Frequency of meristems responded (%)	Morphogenic response	
				Shoot	Root
GC002	1.0 2ip+0.5 NAA	4-8	91.6	+++	+++
	1.5 BAP+0.5 NAA	4-8	85.4	+++	+++
	1.5 KIN+0.5 NAA	4-8	73.3	+++	+++
Mean			**83.4**		
GC008	1.0 2ip+0.5 NAA	4-8	84.4	+++	+++
	1.5 BAP+0.5 NAA	5-8	78.8	+++	+++
	1.5 KIN+0.5 NAA	5-8	68.8	+++	+++
Mean			**77.3**		
GC009	1.0 2ip+0.5 NAA	5-9	76.3	+++	+++
	1.5 BAP+0.5 NAA	5-9	72.5	+++	+++
	1.5 KIN+0.5 NAA	6-9	64.4	+++	+++
Mean			**71.0**		
GC0025	1.0 2ip+0.5 NAA	3-6	96.6	+++	+++
	1.5 BAP+0.5 NAA	4-7	93.3	+++	+++
	1.5 KIN+0.5 NAA	4-7	85.6	+++	+++
Mean			**91.8**		
Indian	1.0 2ip+0.5 NAA	15-20	26.6	+++	-
	1.5 BAP+0.5 NAA	18-20	21.1	+++	-
	1.5 KIN+0.5 NAA	18-20	20.2	+++	-
Mean			**22.6**		

- = No growth
+ = Poor growth
++ = Moderate growth
+++ = Massive growth

3.2 SHOOT MULTIPLICATION FROM PRIMARY ESTABLISHED MERISTEMS FOR CV. GC002

In this experiment the primary established meristems were rescued aseptically and subcultured on to MS semi-solid basal media supplemented with different types of growth regulators (cytokinins, auxins) either singly or in various combination in order to find out suitable culture media for rapid shoot multiplication. Shoot multiplication was highly influenced by the type of cytokinins with or without auxins as well as their different concentrations used. Details of the experiments are described under the following heads.

3.2.1 Effect of different concentrations of cytokinins singly

Primary established meristems were subcultured in MS semi-solid media supplemented with six different concentrations (0.1, 0.5, 1.0, 1.5, 2.0 and 3.0 mg/l) of three cytokinins (2ip, BAP and KIN) for multiplication of shoots. Percentage of cultures induced multiple shoots, number of shoots per culture, length of the longest shoots per culture were considered as parameters for evaluating this experiment. Data on these parameters from different treatments were recorded after 4 weeks of subculture and are presented in Table 4.

Multiple shoot proliferation occurred in all cases. When the media were supplemented with 2ip alone, the percentage of cultures induced shoots ranged from 34.2-83.3%, number of shoots per culture ranged from 3.2-7.9 and the length of the longest shoot ranged from 4.3-8.3 cm. The highest percentage of cultures induced (83.3%) shoot proliferation was recorded in 1.0 mg/l 2ip followed by 74.5% in 1.5 mg/l 2ip. Lowest percentage (34.2%) of shoot proliferation was found in medium with 0.1 mg/l 2ip. Highest number (7.9) of shoots per culture was recorded in media with 1.0 mg/l 2ip (Plate 2A) followed by 6.8 in 1.5 mg/l 2ip. The lowest number (3.2) of shoots per culture was found in media with 0.1 mg/l 2ip. Highest length of the longest shoot (8.3 cm) was recorded in medium with 1.5 mg/l 2ip followed by 6.8 cm in 1.0 mg/l 2ip and the lowest length of shoots (4.3 cm) was observed in medium having 0.1 mg/l 2ip.

In media supplemented with BAP alone, the percentage of cultures induced shoot proliferation ranged from 28.8-76.4%, number of shoots per culture ranged from 2.8-6.9 and the length of the longest shoot ranged from 3.2-7.5 cm. The highest percentage (76.4%) of cultures induced multiple shoots and highest number (6.9) of shoots per culture were observed in media containing 1.5 mg/l BAP. The highest length (7.5 cm) of the longest shoots was recorded in medium containing 2.0 mg/l BAP. Lowest percentage (28.8%) of cultures induced shoots, lowest number (2.8) of shoots per culture and the lowest length (3.2 cm) of the longest shoots per culture were observed in medium containing 0.1 mg/l BAP.

When the media were employed with different concentrations of KIN alone, the percentage of cultures induced shoots proliferation ranged from 26.6-68.8%, the number of shoots per culture ranged from 2.3-5.6, length of the longest shoots per culture ranged from 2.7-6.9 cm. The highest percentage (68.8%) of cultures induced shoots per culture was recorded in 1.5 mg/l KIN containing medium. The highest number of shoots (5.6) per culture was found in medium with 1.0 mg/l KIN and highest length of the longest shoot (6.9 cm) per culture was recorded in 2.0 mg/l KIN containing medium. The lowest percentage (26.6%) of cultures induced shoots, lowest number (2.3) of shoots per culture and the lowest length of the longest shoots (2.7 cm) per culture were observed in 0.1 mg/l KIN containing medium.

Among three hormonal supplements, the highest average percentage (60.2%) of cultures induced multiple shoots, highest average number (5.0) of shoots per culture and the highest average length (6.0 cm) of the longest shoots per culture were recorded in 2ip.

This experiment showed that media with three cytokinins, 2ip was more effective than BAP and KIN for rapid shoot proliferation. Among different concentrations of 2ip, the medium with 1.0 mg/l 2ip was found to be the most effective and most preferred concentration for shoot proliferation from primary established meristems of garlic cv. GC002.

Table 4. Effect of different concentrations of cytokinins in MS media on shoot multiplication from primary established meristems of garlic cv. GC002. Data were recorded after 4 weeks of culture. Each treatment consisted 10-15 explants and the experiment was repeated thrice.

Growth regulators (mg/l)	Frequency of cultures induced multiple shoots (%)	Number of shoots per culture	Length of longest shoot (cm)
2ip			
0.1	34.2	3.2	4.3
0.5	62.4	4.4	6.3
1.0	83.3	7.9	6.8
1.5	74.5	6.8	8.3
2.0	64.5	4.2	5.3
3.0	42.4	3.5	4.5
Mean	**60.2**	**5.0**	**6.0**
BAP			
0.1	28.8	2.8	3.2
0.5	46.6	3.6	4.5
1.0	58.8	4.7	5.8
1.5	76.4	6.9	6.3
2.0	48.5	5.2	7.5
3.0	36.3	4.2	4.2
Mean	**49.2**	**4.6**	**5.25**
KIN			
0.1	26.6	2.3	2.7
0.5	43.3	3.7	3.8
1.0	56.4	5.6	5.8
1.5	68.8	4.3	4.6
2.0	44.4	3.3	6.9
3.0	33.3	2.5	4.1
Mean	**45.4**	**3.6**	**4.6**

3.2.2 Effect of different concentrations and combinations of cytokinins with auxins

This experiment was conducted to see the effect of different combinations of cytokinins with auxins on shoot proliferation from primary established meristems of cv. GC002. The primary established meristems were rescued aseptically and subcultured on to MS semi-solid medium supplemented with three selected concentrations of cytokinins (1.0 mg/l 2ip, 1.5 mg/l BAP and 1.5 mg/l KIN) along with various concentrations such as 0.25, 0.5 and 1.0 mg/l of auxins. The three types of auxins used were NAA, IBA and IAA. The percentage of cultures induced multiple shoots, number of shoots per culture, length of the longest shoot per culture were considered as parameters for evaluating this experiment. Data on these parameters from different treatments were recorded after 4 weeks of subculture and are presented in Table 5.

Shoot proliferation was highly influenced by the type of cytokinins with auxins as well as their different concentrations and combinations used. Multiple shoot proliferation occurred in all cases. The cytokinins with auxins in most of the combinations were found to be comparatively more effective in proliferating shoots than while cytokinins were used alone. In all the treatments the cultured meristems produced shoots along with roots (Plate 1C, Plate 2B).

Among all the combinations the percentage of cultures induced shoots proliferation ranged from 62.6-94.4% in 2ip with auxins, 62.1-88.5% in BAP with auxins and 54.4-84.4% in KIN with auxins. The number of shoots per culture ranged from 4.8-8.6 in 2ip with auxins, 4.3-8.2 in BAP with auxins and 4.2-7.8 in KIN with auxins. The length of the longest shoot per culture ranged from 5.4 -9.1 cm in 2ip with auxins, 5.0-8.8 cm in BAP with auxins and 4.2-8.4 cm in KIN with auxins.

When the media were supplemented with 2ip and auxins (NAA, IBA and IAA), the highest percentage (94.4%) of cultures induced multiple shoots was recorded in 1.0 mg/l 2ip + 0.5 mg/l NAA followed by 84.5% in 1.0 mg/l 2ip + 0.5 mg/l

IBA and (83.6%) of cultures induced shoots proliferation was noted in 1.0 mg/l 2ip + 0.25 mg/l NAA. The lowest percentage (62.6%) of cultures induced shoots proliferation was noted 1.0 mg/l 2ip + 1.0 mg/l IAA.

The highest number (8.6) of shoots per culture was recorded in medium with 1.0 mg/l 2ip + 0.5 mg/l NAA (Plate 2B) followed by 7.7 in 1.0 mg/l 2ip + 0.5 mg/l IBA and 7.4 in 1.0 mg/l 2ip + 0.25 mg/l NAA. The lowest number (4.8) of shoots per culture was noted in medium containing 1.0 mg/l 2ip + 1.0 mg/l IAA.

The highest length (9.1cm) of the longest shoots per culture was found in medium with 1.0 mg/l 2ip + 0.5 mg/l NAA followed by 8.8 cm in 1.0 mg/l 2ip + 0.25 mg/l NAA. The lowest length (5.4 cm) of the longest shoots per culture was found in medium with 1.0 mg/l 2ip + 1.0 mg/l IAA.

When the media were employed with BAP in combination with auxins (NAA, IBA and IAA), the highest percentage (88.5%) of cultures induced shoot proliferation was observed in 1.5 mg/l BAP + 0.5 mg/NAA followed by 78.6% in 1.5 mg/l BAP + 0.25 mg/l NAA. The lowest percentage (62.1%) of shoot proliferation was found in 1.5 mg/l BAP + 1.0 mg/l IAA.

The highest number (8.2) of shoots per culture was recorded in 1.5 mg/l BAP + 0.5 mg/l NAA followed by (7.6) in 1.5 mg/l BAP + 0.5 mg/l IBA and the lowest number (4.3) of shoots per culture was observed in medium with 1.5 mg/l BAP + 1.0 mg/l IAA.

The highest length (8.8 cm) of the longest shoots were observed in medium containing 1.5 mg/l BAP + 0.5 mg/l NAA followed by (8.3 cm) in 1.5 mg/l BAP + 0.5 mg/l IBA and the lowest length (5.0 cm) of the longest shoot were found in medium with 1.5 mg/l BAP + 1.0 mg/l IAA.

In cases of KIN with auxins (NAA, IBA, IAA) combinations, the highest percentage (84.4%) of cultures induced multiple shoots was recorded in medium having 1.5 mg/l KIN + 0.5 mg/l NAA followed by 74.3% in 1.5 mg/l KIN + 0.25

mg/l NAA. The lowest percentage (54.4%) of cultures induced multiple shoots was observed in 1.5 mg/l KIN + 1.0 mg/l IAA.

The highest number (7.8) of shoots per culture was recorded in medium with 1.5 mg/l KIN + 0.5 mg/l NAA followed by (7.2) in 1.5 mg/l KIN + 0.25 mg/l NAA and the lowest number (4.2) of shoots per culture in 1.5 mg/l KIN + 1.0 mg/l IAA was observed.

The highest length (8.4 cm) of the longest shoots per culture was observed in 1.5 mg/l KIN + 0.5 mg/l NAA media containing followed by (7.8 cm) in 1.5 mg/l KIN + 0.25 mg/l NAA and the lowest length (4.2 cm) of the longest shoots per culture was observed in medium with 1.5 mg/l KIN + 1.0 mg/l IAA.

From this experiment it is observed that a combination of cytokinin with auxin was found to be more effective than when cytokinin was used alone as found in previous experiment. Among the nine tested combinations 2ip + NAA combination was found to be more suitable for shoot proliferation than other combinations and 1.0 mg/l 2ip in combination with 0.5 mg/l NAA was recorded to be most effective.

Table 5. Effect of different concentrations and combinations of cytokinins with auxins in MS semi-solid media on shoot multiplication from primary established meristems of garlic cv. GC002. Data were recorded after 4 weeks of culture. Each treatment consisted of 10-15 explants and the experiments was repeated thrice.

Growth regulators	(mg/l)	Frequency of cultures induced multiple shoots (%)	Number of shoots per culture	Length of longest shoot (cm)
2ip+NAA	1.0 +0.25	83.6	7.4	8.8
	1.0+0.5	94.4	8.6	9.1
	1.0+1.0	77.5	5.5	7.5
Mean		**85.2**	**7.2**	**8.5**
2ip+IBA	1.0+0.25	73.3	6.8	7.5
	1.0+0.5	84.5	7.7	8.7
	1.0+1.0	66.4	5.2	6.6
Mean		**74.7**	6.6	7.6
2ip+IAA	1.0+0.25	64.3	5.4	5.4
	1.0+0.5	74.4	6.4	6.4
	1.0+1.0	62.6	4.8	5.7
Mean		**67.1**	**5.5**	**5.8**
BAP+NAA	1.5+0.25	78.6	6.8	7.7
	1.5+0.5	88.5	8.2	8.8
	1.5+1.0	68.8	5.3	7.7
Mean		**78.6**	**6.8**	**8.0**
BAP+IBA	1.5+0.25	67.2	6.5	7.3
	1.5+0.5	75.7	7.6	8.3
	1.5+1.0	62.3	4.5	6.4
Mean		**68.4**	**6.2**	**7.3**
BAP+IAA	1.5+0.25	62.8	5.4	5.2
	1.5+0.5	68.9	6.6	6.3
	1.5+1.0	62.1	4.3	5.0
Mean		**64.6**	**5.4**	**5.5**
KIN+NAA	1.5+0.25	74.3	7.2	7.8
	1.5+0.5	84.4	7.8	8.4
	1.5+1.0	64.8	4.6	7.2
Mean		**74.5**	**6.5**	**7.8**
KIN+IBA	1.5+0.25	61.2	5.4	7.1
	1.5+0.5	73.2	6.6	7.3
	1.5+1.0	58.6	4.3	6.4
Mean		**64.3**	**5.4**	**6.9**
KIN+IAA	1.5+0.25	56.6	4.4	4.8
	1.5+0.5	65.6	6.4	6.1
	1.5+1.0	54.4	4.2	4.2
Mean		**58.9**	**5.0**	**5.0**

3.2.3 Genotypic effect of garlic cultivers

Primary shoots derived from the isolated meristems of five garlic varieties viz., GC002, GC008, GC009, GC0025 and Indian variety were transferred to fresh MS semi-solid medium supplemented with three selected combinations (1.0 mg/l 2ip + 0.5 mg/l NAA, 1.5 mg/l BAP + 0.5 mg/l NAA and 1.5 mg/l KIN + 0.5 mg/l NAA) of plant growth regulators to find out the response of five garlic varieties on shoot proliferation. Data on percentage of culture induced multiple shoots, number of shoots per culture and length (cm) of the longest shoots per culture were recorded after 4 weeks of transfer. The results are presented in Table 6 and graphic representation is shown in Fig. 1.

Shoot multiplication from established meristems of five garlic varieties was highly influenced by different concentrations and combinations of growth regulators. In three treatments all the tested genotypes produced multiple shoots.

The percentage of cultures induced shoots ranged from 77.5-98.2% among five garlic varieties in treatment 1, 72.5-92.3% in treatment 2 and 68.2-85.6% in treatment 3. Among the five varieties cv. GC0025 showed highest percentage of shoot proliferation in all cases followed by cv. GC002 and cv. GC008 .The lowest percentage of cultures induced shoots was recorded in GC009.

Number of shoots per culture of five varieties ranged from 6.3-9.8 in treatment 1, 6.2-9.3 in treatment 2 and 5.8-8.5 in Treatment 3. GC0025 showed highest average number (9.2) of shoots per culture, followed by GC002 (8.2), GC008 (7.3) and GC009 showed the lowest number (6.1) of shoots per culture.

The length of the longest shoots of five varieties ranged from 8.2-10.5 cm in T_1, 7.7-9.5 cm in T_2 and 6.7-8.6 cm in T_3. The average highest length (9.5 cm) of the longest shoots was noted in GC0025 followed by 8.7 cm in GC002 and 8.2 cm in GC008. The lowest length (7.5 cm) of the longest shoots per culture was noted in GC009.

On average maximum frequency of response (88.8%), number of shoots per culture (8.0) and shoot length (9.1cm) were recorded in T_1. The difference between T_2 and T_3 was not significant. Among the varieties maximum frequency of response, number of shoots per culture and shoot length were recorded in GC0025. Indian genotype failed to response in any of the treatments tested.

Table 6. Genotypic effect of five garlic varieties on shoot proliferation from primary established meristems. Data were recorded after 4 weeks of culture. Each treatment consisted 12-15 explants and the experiment was repeated thrice.

Treatments	Genotypes	Percentage of shoot proliferation	Number of shoots per culture	Length of longest shoot (cm)
T_1	GC002	94.4 a	8.6 b	9.1 ab
	GC008	85.4 b	7.5 c	8.8 b
	GC009	77.5 c	6.3 d	8.2 c
	GC0025	98.2 a	9.8 a	10.5 a
	Indian	-	-	-
T_2	GC002	88.5	8.2	8.8
	GC008	78.4	7.3	8.2
	GC009	72.5	6.2	7.7
	GC0025	92.3	9.3	9.5
	Indian	-	-	-
T_3	GC002	83.4	7.7	8.3
	GC008	75.6	7.2	7.6
	GC009	68.2	5.8	6.7
	GC0025	85.6	8.5	8.6
	Indian	-	-	-
Effect of treatment				
T_1		88.8 a	8.0 b	9.1 b
T_2		82.9 b	7.7 a	8.5 c
T_3		78.2 b	7.3 a	7.8 a
Effect of genotypes				
GC002		88.8 a	8.2 b	8.7 b
GC008		79.8 b	7.3 d	8.2 b
GC009		72.7 c	6.1 c	7.5 c
GC0025		92.0 a	9.2 a	9.5 a

Means in a column with same letters are statistically non-significant according to LSD test at 5% level.

T_1 = 1.0 2ip + 0.5 NAA, T_2 = 1.5 BAP + 0.5 NAA, T_3 = 1.5 KIN + 0.5 NAA

3.3 *IN VITRO* BULBLET FORMATION FROM MERISTEM DERIVED SHOOT CLUMPS

3.3.1 Effect of genotypes and sucrose concentrations

In this experiment four cultivars of garlic viz., GC002, GC008, GC009, GC0025 and six different concentrations of sucrose (3, 6, 9, 12, 15 and 18%) were used to find out the most effective concentration of sucrose and most responsive genotype for *in vitro* bulblet formation from meristem derived shoot clumps.

The meristem derived *in vitro* shoot clumps were used as explant source for this experiment and were cultured in MS medium to form *in vitro* bulblets. Percentage of cultures induced bulblets, number of bulblets per culture, length of bulblets, diameter of bulblets and weight of bulblets per culture were considered as parameters for evaluating this experiment. Data on these parameters from different treatments were recorded after 6 weeks of culture and the results are shown in Table 7. The graphic representation is shown in Fig. 1.

In cv. GC002 it was observed that percentage of *in vitro* bulblet formation ranged from 23.4-74.6%. The highest 74.6% of cultures induced bulblets was observed in medium containing 12% sucrose (Plate 2C&D) followed by 54.4% in 9% sucrose. Lowest 23.4% of cultures induced bulblets was recorded in medium containing 3% sucrose.

Number of bulbs per culture ranged from 0.8-4.9. The highest number (4.9) was found in medium containing 12% sucrose followed by 3.6 in 9% sucrose. Lowest (0.8) number of bulbs per culture was recorded in 3% sucrose. The length of bulbets per culture ranged from 0.6-1.4 cm, the diameter of bulblets per culture ranged from 0.8-1.6 cm and the weight of bulblets per culture ranged from 0.2-0.8 g. The highest bulb length (1.4 cm) was observed in medium containing 12%. sucrose. Lowest bulb length (0.6 cm) was observed in media with 3% sucrose containing media. The highest diameter of bulbs (1.6 cm) and

highest weight of bulbs (0.8 g) per culture were also observed in 12% sucrose containing medium. Lowest diameter of bulbs (0.8 cm) and lowest weight of bulbs (0.2 g) per culture were observed in 3% sucrose containing medium.

In cv. GC008, *in vitro* bulbing ranged from 22.5-71.1%. The highest 71.1% of cultures induced bulblets was observed in medium containing 12% sucrose. Lowest 22.5% was found in 3% sucrose. Number of bulblets per culture ranged from 0.7-4.5. The highest number (4.5) of bulblets per culture was recorded in 12% sucrose. Lowest (0.7) number of bulblet was found in 3% sucrose containing medium. The length of bulblets per culture ranged from 0.4-1.3 cm, diameter of bulblets per culture ranged from 0.8-1.5 cm and the weight of bulblets per culture ranged from 0.2-0.6 g. The highest length of bulblets (1.3 cm), highest diameter of bulblets (1.5 cm) and the highest weight of bulblets (0.6 g) per culture were also recorded in 12% sucrose containing medium. The lowest (0.7 cm) bulbs length, (0.8 cm) bulbs diameter and (0.2 g) bulbs weight per culture were also found in 3% sucrose containing medium.

Percentage of *in vitro* bulblets formation in cv. GC009 ranged form 20.7-68.4%, number of bulblet per culture ranged from 0.6-3.9, the length of bulbs per culture ranged from 0.2-1.1 cm, diameter of bulbs per culture ranged from 0.2-1.2 cm and the weight of bulbs per culture ranged from 0.1-0.4 g. The highest frequency (68.4%) of *in vitro* bulbing per culture, the highest number (3.9) of bulblets per culture, the highest length (1.1 cm) of bulblets, highest diameter (1.2 cm) of bulblets and the highest weight (0.4 g) of bulblets per culture were recorded in 12% sucrose containing medium. Lowest (20.7%) of cultures induced bulblets, lowest (0.6) number of bulbs, lowest (0.2 cm) length of bulbs, lowest (0.2 cm) diameter of bulbs and the lowest weight (0.1 g) of bulbs per culture were recorded in 3% sucrose containing medium.

In cv. GC0025, the percentage of *in vitro* bulblets formation ranged from 28.8-84.5%, number of bulblets per culture ranged from 1.0-5.2, bulblets length per culture ranged from 0.7-1.6 cm, bulblets diameter per culture ranged from 0.8-1.5 cm and the weight of bulblets per culture ranged from 0.3-1.0 g. The highest percentage (84.5%) of *in vitro* bulblets formation per culture, highest number (5.2) of bulbs, highest length (1.6 cm) of bulbs, highest diameter (1.5 cm) and highest weight (1.0 g) of bulbs per culture were recorded in medium supplemented with 12% sucrose. The lowest percentage (28.8%) of bulblets, lowest number (1.0) of bulbs, lowest length (0.7 cm), lowest diameter (0.8 cm) and the lowest weight (0.3 g) of bulblets per culture were found in 3% sucrose containing medium.

On average highest percentage (55.6%) of cultures induced bulblets was found in cv. GC0025 followed by 46.7% in cv. GC002, 44.9% in cv, GC008 and 41.5% in cv. GC009. The highest average number (2.8) of bulblets per culture was found in cv. GC0025 followed by (2.6) in cv. GC002, (2.4) in cv. GC008 and (1.8) in cv. GC009. The highest average length (1.1 cm) of bulblets per culture was recorded in cv. GC0025, followed by (0.96 cm) in cv. GC002, (0.83 cm) in cv. GC008 and (0.66 cm) in cv. GC009. The highest average diameter (1.2 cm) of bulblets per culture was recorded in cv. GC0025 followed by (1.1 cm) in cv. GC002, (1.0 cm) in cv. GC008 and (0.7 cm) in cv. GC009. The highest average weight (0.6 g) of bulblets per culture was found in cv. GC0025 followed by (0.45 g) in cv. GC002, (0.35 g) in cv. GC008 and (0.21 g) in cv. GC009.

From this experiment it is concluded that media containing 12% sucrose was found to be the best for *in vitro* bulblets formation among the six sucrose concentrations and among the four cultivars, the best performance of *in vitro* bulblets formation was observed in cv. GC0025 followed by cv. GC002, cv. GC008 and cv. GC009.

Table 7. Effect of various concentrations of sucrose in MS medium on *in vitro* bulblet formation from meristem derived shoot clumps of four garlic cultivars. Each treatment consisted 12-15 explants and the experiment was repeated thrice. Data were recorded after 6 weeks of subculture.

Genotypes	Sucrose concentrations (%)	Frequency of cultures induced bulblets (%)	Number of bulblets per culture	Length of bulblet (cm)	Diameter of bulblet (cm)	Weight of bulblet (g)
GC002	3	23.4	0.8	0.6	0.8	0.2
	6	43.5	2.4	0.9	1.0	0.4
	9	54.4	3.6	1.1	1.2	0.6
	12	74.6	4.9	1.4	1.6	0.8
	15	52.3	2.8	1.0	1.1	0.4
	18	32.2	1.0	0.8	1.0	0.3
Mean		**46.7**	**2.6**	**0.96**	**1.1**	**0.45**
GC008	3	22.5	0.7	0.4	0.8	0.2
	6	42.4	2.2	0.7	0.9	0.3
	9	53.7	3.5	1.0	1.2	0.4
	12	71.1	4.5	1.3	1.5	0.6
	15	48.8	2.5	0.8	1.0	0.3
	18	31.4	1.0	0.8	0.9	0.3
Mean		**44.9**	**2.4**	**0.83**	**1.0**	**0.35**
GC009	3	20.7	0.6	0.2	0.2	0.1
	6	38.3	1.0	0.4	0.5	0.2
	9	47.4	2.6	0.8	0.9	0.2
	12	68.4	3.9	1.1	1.2	0.4
	15	45.3	2.2	0.7	0.8	0.2
	18	28.9	1.0	0.8	0.6	0.2
Mean		**41.5**	**1.8**	**0.66**	**0.7**	**0.21**
GC0025	3	28.8	1.0	0.7	0.8	0.3
	6	53.6	2.8	1.2	1.3	0.5
	9	67.4	3.8	1.3	1.4	0.8
	12	84.5	5.2	1.6	1.5	1.0
	15	58.3	2.9	1.1	1.1	0.5
	18	41.2	1.0	0.8	0.9	0.5
Mean		**55.6**	**2.8**	**1.1**	**1.2**	**0.6**

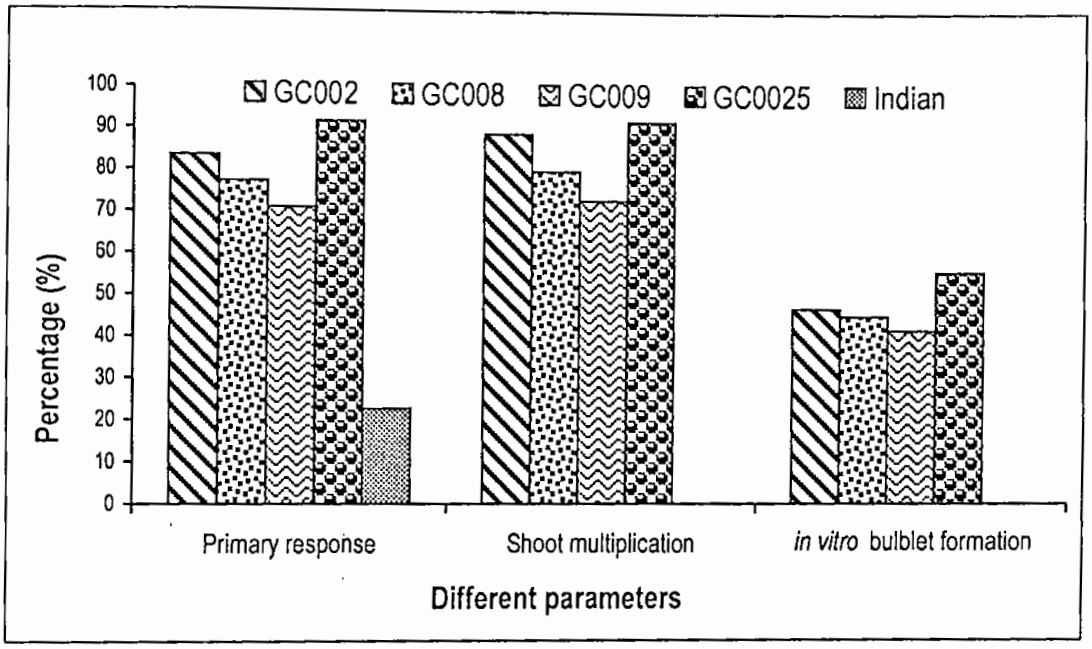

Fig. 1. Effect of genotypes on meristem culture

3.4 FIELD PERFORMANCE OF *IN VITRO* PRODUCED MERISTEM DERIVED PLANTLETS

Well developed plantlets with bulblets from some selected mericlones for each of cultivars were excised and cultured separately. The acclimatized palntlets were transplanted on to the field condition. Generally *in vitro* produced plantlets of all cultivars accliminated well in outdoor condition and grew normally to maturity and developed bulbs. The field performances of *in vitro* produced meristem derived plantlets were evaluated and data on survival percentage of transplanted plantlets, plant height, number of leaves per plant, bulb diameter and bulb weight were recorded after 90 days of transplantation. The results are presented in Table 8 and Plate 3A.

The variety GC0025 showed the highest percentage (92.4%) of plantlets that survived in the field and was closely followed by GC002 (85.6%). The lowest survival rate (68.4%) was observed in GC009.

In terms of plant height GC0025 attained the highest height (38.5 cm) and its height was significantly different from the rest of the varieties. The minimum height (31.3 cm) was recorded in GC009.

The number of leaves per plant varied with the varieties. The highest (5.8) number of leaves per plant was recorded in GC002 that differed significantly from other varieties. The lowest (3.5) number of leaves per plant was recorded in GC009.

The highest diameter of bulb per plant (4.7 cm) was recorded in cv. GC0025 which was significantly higher than the rest of the varieties. The lowest diameter of bulb per plant (3.3 cm) was recorded in cv. GC008.

The highest weight (22.2 g) of bulb per plant was recorded in GC0025 which was significantly higher than the rest of the varieties. The lowest weight of bulb per plant (16.2 g) was recorded in cv. GC009.

Table 8. Field performance of meristem derived plantlets of different garlic cultivars. Data were taken from 10 randomly selected plants of each replication. The mean values were calculated from three replications after 90 days of plantation.

Cultivars	Survival % of plantlets	Plant height (cm)	Number of leaves per plant	Bulb diameter per plant (cm)	Bulb weight per plant (g)
GC002	85.6	36.4	5.8	4.2	20.5
GC008	77.5	35.2	4.2	3.3	18.6
GC009	68.4	31.3	3.5	3.5	16.2
GC0025	92.4	38.5	4.6	4.7	22.2
Mean	**80.9**	**35.3**	**4.5**	**3.9**	**19.3**
LSD at 5% level	**7.44**	**2.00**	**0.8**	**0.5**	**2.10**

PLATE 1

Meristem culture

Fig. A. Meristem of garlic cv. GC002 cultured on filter paper bridge in MS liquid medium supplemented with 1.0 mg/l 2ip, after 3 days of inoculation.

Fig. B. Shoot development of garlic cv. GC002 cultured on filter paper bridge in MS liquid medium supplemented with 1.0 mg/l 2ip + 0.5 mg/l NAA, after 07 days of inoculation.

Fig. C. Single shoot with root from primary established meristem after subculture onto semi-solid medium supplemented with 1.0 mg/l 2ip + 0.5 mg/l NAA, after 07 days of subculture.

PLATE 1

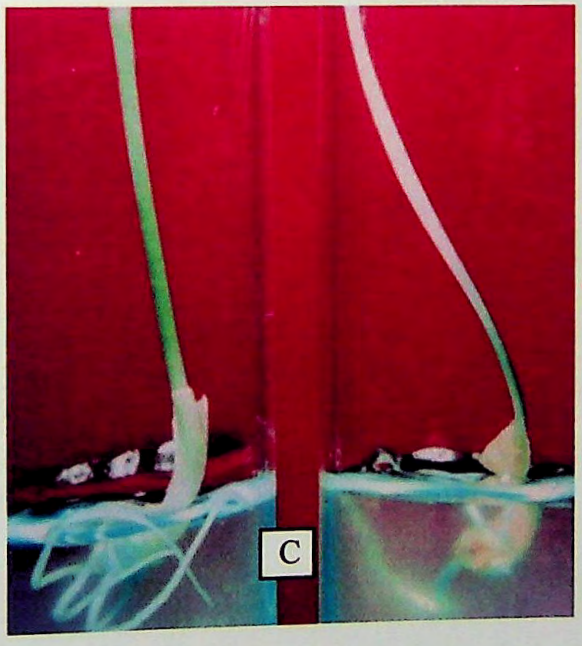

PLATE 2

Shoot multiplication and bulblet formation from primary established meristems

Fig. A. Shoots proliferated from primary established meristem in MS + 1.0 mg/l 2ip, after 15 days of subculture.

Fig. B. Multiple shoots proliferated from primary established meristem with a hairy roots in MS medium supplemented with 1.0 mg/l 2ip + 0.5 mg/l NAA, after 28 days of subculture.

Fig. C. Induction of multiple bulblets form *in vitro* grown mericlones in MS medium containing 12% sucrose. Photograph was taken, 6 weeks after culture.

Fig. D. Isolated bulblet.

PLATE 2

PLATE 3

Field evaluation of meristem derived plantlets

Fig. A. Transplanted plantlets in the field under natural condition, 3 months after plantation.

PLATE 3

3.5 INDUCTION OF CALLUS FROM DIFFERENT EXPLANTS

This part of investigation was carried out to induce indirect regeneration through callus culture of four different types of explants viz., shoot tip, leaf base, leaf primordia and bulbil from five garlic varieties. The objective of this investigation was to establish a protocol for induction of callus, somatic embryogenesis, plantlet regeneration from somatic embryos, bulblet formation and field evaluation of somaclones developed through callus culture. The results of this part of study are described under different heads.

3.5.1 Effect of 2,4-D alone or with IAA on induction of callus from shoot tip explants

This experiment was conducted to see the effect of different concentrations of 2,4-D alone or with IAA on callus induction from shoot tip explants. The explants were cultured in MS medium supplemented with seven different concentrations (0.5, 1.0, 1.5, 2.0, 3.0, 4.0 and 5.0 mg/l) of 2,4-D singly and two selected concentrations of 2,4-D (1.5 and 2.0 mg/l) along with three concentrations (0.5, 1.0 and 2.0 mg/l) of IAA in order to find out the most suitable culture media formulation to induce callus. Data on days to callus initiation, percentage of explants induced callus, degree of callus growth, color of callus and texture of callus were recorded after 4 weeks of culture and are presented in Table 9.

The cultured explants produced callus in all the treatments but the response of callus induction varied greatly with different concentrations and combinations of growth regulator formulations. In all the treatments the induced calli were white in color and the texture was friable.

Among different concentrations of 2,4-D days taken to callus initiation ranged from 7-18 days and 8-20 days in 2,4-D with IAA. Among all the concentrations

and combinations most early response (7-10 days) to callus initiation was recorded in 2.0 mg/l 2,4-D.

The percentage of explants induced callus ranged from 26.6-93.3% in 2,4-D alone and 43.3-73.3% in 2,4-D with IAA. The highest percentage (93.3%) of callus induction was obtained in media having 2.0 mg/l 2,4-D followed by 86.6% in 1.5 mg/ 2,4-D and 83.3% in 3.0 mg/l 2,4-D. The lowest frequency (26.6%) of callus formation was recorded in media containing 0.5 mg/l 2,4-D. Massive callus growth was observed in three treatments (1.5, 2.0 and 3.0 mg/l 2.4-D). Moderate callus growth was observed in two treatments (1.0 and 4.0 mg/l 2,4-D) and two treatments (0.5 and 5.0 mg/l 2,4-D) showed poor callus growth.

In cases of 2, 4-D with IAA, the highest percentage (73.3%) of callus induction was recorded in medium containing 2.0 mg/l 2,4-D + 1.0 mg/l IAA followed by (68.3%) in medium with 1.5 mg/l 2,4-D + 1.0 mg/l IAA. Degree of callus formation was moderate in all cases.

From this experiment it is observed that media with 2,4-D alone was effective for callus induction on shoot tip explants of garlic and addition of IAA along with 2,4-D was not helpful. Moderate concentrations (1.5-3.0 mg/l) of 2,4-D was more effective than high (4.0-5.0 mg/l) or low (0.5-1.0 mg/l) concentration of 2,4-D.

Table 9. Effect of different concentrations of 2,4-D alone or in combination with IAA in MS medium on callus induction from shoot tip explants of garlic cv. GC002. In each treatment 10-14 explants were used and the experiment was repeated thrice. Data were recorded after 4 weeks of culture.

Growth regulators (mg/l)	Days to callus initiation	Frequency of explants formed callus (%)	Degree of callus growth	Color of callus	Texture of callus
2,4-D					
0.5	15-18	26.6	+	Wh	Fr
1.0	13-15	36.6	++	Wh	Fr
1.5	12-15	86.6	+++	Wh	Fr
2.0	7-10	93.3	+++	Wh	Fr
3.0	12-17	83.3	+++	Wh	Fr
4.0	12-18	66.6	++	Wh	Fr
5.0	15-18	46.6	+	Wh	Fr
Mean		**62.8**			
2,4-D+IAA					
1.5+0.5	15-20	43.3	++	Wh	Fr
1.5+1.0	10-20	68.3	++	Wh	Fr
1.5+2.0	12-20	56.6	++	Wh	Fr
2.0+0.5	10-18	56.6	++	Wh	Fr
2.0+1.0	8-15	73.3	++	Wh	Fr
2.0+2.0	12-18	66.6	++	Wh	Fr
Mean		**60.7**			

+ = Poor callus
++ = Moderate callus
+++ = Massive callus

Wh = White
Fr = Friable

3.5.2 Genotypic effect on callus induction from different explants

This experiment was conducted with four different types of explants viz., shoot tip, leaf base, leaf primordia and bulbil from five garlic varieties. The explants of these five varieties were cultured separately on to semi-solid MS basal medium supplemented with 2.0 mg/l 2,4-D. This concentration was standardized from previous experiment. Objective of this investigation was to find out genotypic response on callus induction from different explants of five garlic varieties. Days taken to callus initiation, percentage of explants induced callus, color of callus, texture of callus and degree of callus growth were considered as parameters for evaluating this experiment. Data on these parameters from different treatments were recorded after 4 weeks of culture and the results are presented in Table 10 and the graphic representation is shown in Figs. 2 & 3.

In cv. GC002 days to callus initiation ranged from 5-21 days. Early response (5-10 days) was observed in leaf base explants and late response (18-21 days) was observed in leaf primordia explants. The percentage of explants induced calli ranged from 29.9-95.5%. The highest percentage (95.5%) of explants induced callus was obtained from leaf base explants (Plate 4A) followed by 93.3% from shoot tip and 66.6% from bulbil explants. The lowest percentage (29.9%) of explants induced calli was recorded in leaf primordia explants. Shoot tip, leaf base and bulbil explants produced friable white calli and leaf primordia produced watery soft calli. Degree of callus formation was massive in shoot tip, leaf base and bulbil explants while leaf primordia derived calli showed poor growth.

In cv. GC008, days to callus initiation ranged from 15-25 days. The percentage of explants induced callus ranged from 14.3-24.2%. Early response (15-22 days) to callus initiation was observed in leaf base explants. In shoot tip and bulbil explants took (18-25 days) to induce calli were observed. The highest percentage

(24.2%) of explants induced callus was obtained from leaf base explants followed by 22.4% from shoot tip explants. The lowest percentage (14.3%) of explants induced calli was recorded in bulbil explants. The calli developed in this genotype were watery in color, soft in texture and the degree of callus growth was very poor in all cases. Leaf primordia explants did not show any response for this variety.

For cv. GC009, time taken to callus initiation ranged from 8-27 days. Leaf base explants showed early response (8-12 days). Shoot tip took 10-15 days and bulbil took 15-20 days for callus initiation. Leaf primordia showed late response to callus initiation (20-27 days). The percentage of explants induced callus ranged from 22.2-62.2%. The maximum 62.2% of callus induction from leaf base explants followed by 54.4% from shoot tip and 44.5% from bulbil explants were observed. Minimum 22.2% of callus induction was obtained from leaf primordia explants. The calli developed in this genotype was white in color, friable in texture and the degree of callus formation was massive in shoot tip, leaf base and bulbil derived calli. Leaf primordia derived calli showed water color with soft texture and poor growth.

In cv. GC0025 it was observed that callus initiation time ranged from 6-25 days. Early response (6-10 days) to callus initiation was observed in leaf base explants. Shoot tips took 8-15 days and bulbil took 12-18 days to callus initiation. Late response (20-25 days) to callus initiation was observed in leaf primordia explants. The percentage of explants induced callus ranged from 25.4-86.4%. The highest percentage (86.4%) of explants induced callus from leaf base explants followed by 78.2% from shoot tip and 56.4% from bulbil explants were recorded. The lowest percentage (25.4%) of callus induction was recorded from leaf primordia explants. The calli developed from shoot tip, leaf base and bulbil were white in color, friable in texture and degree of callus formation was

massive. But leaf primordia derived calli were watery in color, soft in texture and the degree of callus formation was very poor.

In Indian variety days to callus initiation ranged from 7-25 days. Early response (7-12 days) to callus initiation was recorded in leaf base explants. Shoot tip took 8-15 days and bulbil took 15-20 days to induce of callus. Late response (20-25 days) to callus initiation was obtained from leaf primordia explants. The percentage of explants induced callus ranged from 22.6-78.6%. The highest percentage (78.6%) of callus induction was noted in leaf base explants followed by 67.4% from shoot tip explants and 48.8% from bulbil explants. The lowest explants (22.6%) of callus induction was recorded in leaf primordia explants. The calli developed in all cases were cream in color, friable in texture and the degree of callus formation was massive except in leaf primordia derived calli. It was observed that leaf primordia derived callus was soft in texture and the degree of callus growth was very poor.

From this experiment it was observed that GC002 genotype showed highest response in callus induction and among the four types of explants shoot tips and leaf bases produced high frequency of callus induction with massive callus growth. In all cases leaf primordia showed the poorest response.

Table 10. Genotypic response on callus induction from different explants. The explants were cultured in MS medium with 2.0 mg/l 2,4-D. In each treatment 12-15 explants were cultured. Data were recorded after 28 days of culture. The experiment was repeated thrice.

Genotypes	Name of explants	Days to callus initiation	Frequency of explants formed callus (%)	Degree of callus growth	Color of callus	Texture of callus
GC002	Shoot tip	7-15	93.3	+++	Wh	Fr
	Leaf base	5-10	95.5	+++	Wh	Fr
	Leaf Primordia	18-21	29.9	+	Wa	S
	Bulbil	12-18	66.6	+++	Wh	Fr
Mean			**71.3**			
GC008	Shoot tip	18-25	22.4	+	Wa	S
	Leaf base	15-22	24.2	+	Wa	S
	Leaf Primordia	-	-	-	-	-
	Bulbil	18-25	14.3	+	Wa	S
Mean			**20.3**			
GC009	Shoot tip	10-15	54.4	+++	Wh	Fr
	Leaf base	8-12	62.2	+++	Wh	Fr
	Leaf Primordia	20-27	22.2	+	Wa	S
	Bulbil	15-20	44.5	+++	Wh	Fr
Mean			**45.8**			
GC0025	Shoot tip	8-15	78.2	+++	Wh	Fr
	Leaf base	6-10	86.4	+++	Wh	Fr
	Leaf Primordia	20-25	25.4	+	Wa	S
	Bulbil	12-18	56.4	+++	Wh	Fr
Mean			**61.6**			
Indian	Shoot tip	8-15	67.4	+++	Cr	Fr
	Leaf base	7-12	78.6	+++	Cr	Fr
	Leaf Primordia	20-25	22.6	+	Cr	S
	Bulbil	15-20	48.8	+++	Cr	Fr
Mean			**54.4**			

+ = Poor callus
++ = Moderate callus
+++ = Massive callus

Wh = White
Wa = Watery
Cr = Cream

Fr = Friable
S = Soft

3.6 INDUCTION OF SOMATIC EMBRYOS

The calli developed from different explants of garlic cv. GC002 in MS medium containing 2.0 mg/l 2,4-D were aseptically taken out and cut into small pieces and subcultured on to fresh MS basal medium with or without any growth regulators for somatic embryo induction. In preliminary experiments it was observed that when the calli were subcultured to media fortified with any phytohormones singly or in combination, only proliferation of callus was observed without any embryo formation. Therefore, hormone free MS medium was always used to induce somatic embryos. The callus morphology was changed after subculture in MS_0 medium and turned to nodular structures within 4 weeks of culture (Plate 4B). Subsequently, globular embryo like structure (ELS) (Plate 4C) developed extensively on the surface of almost all the proliferated calli in the absence of phytohormones. Two experiments were conducted to induce somatic embryos and the results obtained are explained below.

3.6.1 Effect of explant source

Different explants derived calli were further subcultured on to different strength (1, ½, ¼) of MS medium without any growth regulators for induction of somatic embryos. The transferred calli continued to proliferate and turned to develop nodular structures. Subsequently these nodular structures turned into somatic embryos. Somatic embryos usually formed in clusters on the surface of callus and three distinct shapes globular, heart and torpedo (Plate 5A) like somatic embryos were observed.

Percentage of calli formed somatic embryos and number of globular, heart and torpedo shaped somatic embryos were recorded in order to observe the effect of different strength of MS (1, ½, ¼) medium. Data were recorded after 6 weeks of subculture and the results are given in Table 11.

For shoot tip explants, the percentage of calli formed somatic embryos ranged from 59.5-78.5%, number of globular shape embryos ranged from 10.6-14.7, heart shape embryos ranged from 6.7-10.5 and torpedo shape embryos ranged from 4.2-8.4. The highest percentage (78.5%) of calli formed somatic embryos was recorded in ½ strength MS medium followed by 67.4% in full MS medium. The lowest percentage (59.5%) of calli induced somatic embryos was observed in ¼ MS medium. The highest number of globular shape embryos (14.7), heart shape embryos (10.5) and torpedo shape embryos (8.4) per callus was observed in ½ strength MS medium. The lowest number of globular shape (10.6), heart shape (6.7) and torpedo shape embryos (4.2) per callus was recorded in ¼ strength MS medium.

In case of leaf base explants, the percentage of calli formed somatic embryo ranged from 64.2-85.7%, number of globular shape embryos ranged from 13.4-15.6, heart shape embryos ranged from 10.6-12.4 and torpedo shape embryos ranged from 4.7-9.2. The highest percentage (85.7%) of calli induced somatic embryos was recorded in ½ strength MS medium (Plate 4D) followed by 71.4% in 1 MS medium. The lowest percentage (64.2%) of calli induced somatic embryos was observed in ¼ MS medium. The highest number of globular shape embryos (15.6), heart shape embryos (12.4) and torpedo shape embryos (9.2) was noted in ½ strength MS medium. The lowest number of globular shape (13.4), heart shaped (10.6) and torpedo shaped embryos (4.7) per callus was noted in ¼ strength of MS medium.

In leaf primordia explants, the percentage of calli formed somatic embryos ranged from 13.3-28.8%, number of globular shape embryos ranged from 3.8-5.7, heart shape embryos ranged from 2.9-4.2 and torpedo shape embryos ranged from 2.1-3.6. The highest percentage (28.8%) of calli induced somatic embryos was recorded in ½ strength MS medium followed by 20.0 % in 1MS medium. The lowest (13.3%) of calli induced somatic embryos was noted in ¼ MS medium. The highest number of globular shape embryos (5.7), heart shape

embryos (4.2) and torpedo shape embryos (3.6) was noted in 1 MS medium. The lowest number of globular shape embryos (3.8), heart shape embryos (2.9) and the torpedo shape embryos (2.1) was recorded in ¼ MS medium.

In bulbil explants, the percentage of calli formed somatic embryos ranged from 42.8-71.4%, number of globular shape embryo ranged from 4.4-8.8, heart shape embryos ranged from 3.2-5.8 and torpedo shape embryos ranged from 2.3-4.4. The highest percentage (71.4%) of calli induced somatic embryos was recorded in ½ strength MS medium followed by 57.1% in 1 MS medium. The lowest (42.8%) of calli induced somatic embryos in ¼ MS medium. The highest number of globular shape embryos (8.8), heart shape embryos (5.8) and torpedo shape embryos (4.4) was recorded in 1 MS medium. The lowest number of globular shape embryos (4.4), heart shape embryos (3.2) and torpedo shape embryos (2.3) was recorded in ¼ MS medium.

From this experiment it is observed that leaf bases were found to be the best explant sources for somatic embryogenesis followed by shoot tip and bulbil. The embryogenic ability of leaf primordia derived calli was very low. It further is clear that ½ MS was the best for induction of somatic embryos from shoot tip, leaf base and bulbil derived calli but full MS was the best for leaf primordia.

Table 11. Effect of different strength (1, ½, ¼) of MS media without any growth regulators on induction of somatic embryos from calli derived from different explants of garlic cv. GC002. In each treatment 12-15 explants were used and the experiment was repeated thrice.

Name of explants	Preculture media	Subculture media	Frequency of calli formed somatic embryos (%)	Number of somatic embryos		
				Globular shape	Heart shape	Torpedo shape
Shoot tip	1 MS	1MS	67.4 b	12.6 b	8.5 b	5.5 b
		½ MS	78.5 a	14.7 a	10.5 a	8.4 a
		¼ MS	59.5 c	10.6 c	6.7 c	4.2 c
Mean			**68.5 B**	**12.6 B**	**8.5 B**	**6.0 A**
Leaf base	1 MS	1 MS	71.4 b	14.3 b	11.6 b	6.3 b
		½ MS	85.7 a	15.6 a	12.4 a	9.2 a
		¼ MS	64.2 c	13.4 b	10.6 b	4.7 b
Mean			**73.7 A**	**14.4 A**	**11.5 A**	**6.7 A**
Leaf primordia	1 MS	1 MS	20.0 b	5.7 a	4.2 a	3.6 a
		½ MS	28.8 a	4.2 b	3.2 ab	2.2 b
		¼ MS	13.3 c	3.8 c	2.9 b	2.1 b
Mean			**20.7 D**	**4.5 D**	**3.4 C**	**2.6 C**
Bulbil	1 MS	1 MS	57.1 b	8.8 a	5.8 a	4.4 a
		½ MS	71.4 a	6.6 b	4.2 b	3.3 b
		¼ MS	42.8 c	4.4 c	3.2 c	2.3 c
Mean			**57.1 C**	**6.6 C**	**4.4**	**3.3 B**

3.6.2 Effect of genotypes

This experiment was conducted with four different types of explants viz, shoot tip, leaf base, leaf primordia and bulbil from five garlic varieties. The calli developed from different explants of five genotypes in MS medium containing 2.0 mg/l 2,4-D were aseptically taken out and cut into small pieces and subcultured on to ½ MS basal medium without any growth regulators. This ½ MS strength was standardized in previous experiment. Objective of this investigation was to find out genotypic potentiality of somatic embryogenesis from different explant derived calli of five garlic cultivars. The percentage of calli formed somatic embryos, number of globular heart and torpedo shaped embryos were recorded after 6 weeks of subculture and the results are presented in Table 12 and the graphic representation is shown in Figs. 2 & 3.

In cv. GC002 the percentage of calli induced somatic embryo ranged from 28.8-85.7%. The highest percentage (85.7%) of calli induced somatic embryo was obtained from leaf base explants. The lowest percentage (28.8%) of calli induced somatic embryo was recorded in leaf primordia explants. The number of globular shaped embryos ranged from 4.2-15.6, heart shaped embryos ranged from 3.2-12.4 and torpedo shaped embryos ranged from 2.1-9.2. The highest number of globular shape (15.6), heart shape (12.4) and torpedo shape (9.2) was noted in leaf base explants followed by globular shape (14.7), heart shape (10.5) and torpedo shape (8.4) in shoot tip explants. The lowest number of globular shape (4.2), heart shape (3.2) and torpedo shape (2.1) was noted in leaf primordia explants. GC008 did not show any response on somatic embryogenesis from any type of callus.

In cv. GC009 the percentage of calli induced somatic embryo ranged from 18.2-68.4%. The highest percentage (68.4)% of calli induced somatic embryo was obtained from leaf base explants followed by 56.3% from shoot tip and 46.4%

from bulbil explants. The lowest percentage (18.2%) of calli induced somatic embryo was recorded in leaf primordia explants. The number of globular shaped embryos ranged from 2.2-10.5, heart shaped embryos ranged from 1.7-8.8 and torpedo shaped embryos ranged from 1.2-6.8. The highest number of globular shape (10.5), heart shape (8.8) was recorded in leaf base explants. The highest number of torpedo shape (6.8) was noted in shoot tip explants. The lowest number of globular shaped (2.2), heart shape (1.7) and torpedo shape (1.2) was recorded in leaf primordia explants.

In cv. GC0025 the percentage of calli formed somatic embryo ranged from 25.4-82.3%. The highest percentage (82.3%) of calli induced somatic embryo was obtained from leaf base explants followed by 75.4% from shoot tip explants and 67.5% from bulbil explants. The lowest percentage (25.4%) of calli induced somatic embryo was recorded in leaf primordia explants.

The number of globular shape embryos ranged from 3.2-13.3, heart shaped embryos ranged from 2.6-11.5 and torpedo shaped embryos ranged from 1.9-8.3. The highest number of globular shape (13.3), heart shape (11.5) and torpedo shape (8.3) was noted in leaf base explants followed by globular (12.4), heart shape (10.2) and torpedo shape (8.1) in shoot tip explants. The lowest number of globular shape (3.2), heart shape (2.6) and torpedo shape (1.9) was noted in leaf primordia explants.

In cv. Indian, the percentage of calli induced somatic embryo ranged from 22.1-75.6%. The highest percentage (75.6%) of calli induced somatic embryo was obtained from leaf base explants. The second highest percentage (69.2%) of calli induced somatic embryo was obtained from shoot tip explants. The number of globular shaped embryos ranged from 2.8-12.5, heart shape embryos ranged from 2.2-10.6 and torpedo shape embryos ranged from 1.5-7.4. The highest number of globular shape (12.5), heart shape (10.6) and torpedo shape (7.4) was

recorded in leaf base explants followed by globular shape (10.8), heart shape (9.9) and torpedo shape (6.8) in shoot tip explants. The lowest number of globular shape (2.8), heart shape (2.2) and torpedo shape (1.5) was noted in leaf primordia explants.

From this experiment it was observed that GC002 genotype showed highest response in respect of somatic embryo induction and among the four types of explants leaf bases and shoot tips showed high frequency of somatic embryo induction. The embryogenic ability of leaf primordia derived calli was very low.

Table 12. Genotypic effect on somatic embryogenesis from different explants derived calli in ½ strength of MS medium. In each treatment 12-15 explants were used and the experiment was repeated thrice.

Genotypes	Source of calli	Frequency of calli formed somatic embryo (%)	Number of somatic embryos		
			Globular shape	Heart shape	Torpedo shape
GC002	Shoot tip	78.5	14.7	10.5	8.4
	Leaf base	85.7	15.6	12.4	9.2
	Leaf primordia	28.8	4.2	3.2	2.1
	Bulbil	71.4	8.8	5.8	4.4
Mean		**66.1**	**10.8**	**7.9**	**6.0**
GC008	Shoot tip	-	-	-	-
	Leaf base	-	-	-	-
	Leaf primordia	-	-	-	-
	Bulbil	-	-	-	-
Mean		-	-	-	-
GC009	Shoot tip	56.3	9.6	8.1	6.8
	Leaf base	68.4	10.5	8.8	6.4
	Leaf primordia	18.2	2.2	1.7	1.2
	Bulbil	46.4	4.6	3.9	3.5
Mean		**47.3**	**6.7**	**5.6**	**4.4**
GC0025	Shoot tip	75.4	12.4	10.2	8.1
	Leaf base	82.3	13.3	11.5	8.3
	Leaf primordia	25.4	3.2	2.6	1.9
	Bulbil	67.5	6.4	5.2	4.1
Mean		**62.6**	**8.8**	**7.3**	**5.6**
Indian	Shoot tip	69.2	10.8	9.9	6.8
	Leaf base	75.6	12.5	10.6	7.4
	Leaf primordia	22.1	2.8	2.2	1.5
	Bulbil	57.5	5.7	4.8	3.9
Mean		**56.1**	**7.9**	**6.8**	**4.9**

3.7 GERMINATION OF SOMATIC EMBRYOS AND PLANTLET FORMATION

The calli with induced somatic embryos from shoot tip explants were further transferred to fresh MS basal media with different types of growth regulators to germinate the somatic embryos of garlic cv. GC002. The embryos started to germinate 2-3 weeks after transfer to MS medium containing 30 g/l sucrose, 0.8% agar and different concentrations of kinetin alone or in combination with IAA. The germination was characterized by gradual greening and enlargement of the cotyledons. The germinating embryos could be separated into a singly that exhibited a bipolar development of root and shoot. The presence of radicles in the germinating embryos enabled us to identify the somatic embryos. Green shoots were evidently observed after 4 weeks of culture. Culture for another 4 weeks led to the formation of complete plantlets. These plantlets could be easily separated from each other and also from the original callus. Not all the somatic embryos developed into plantlets. Some of them disappeared while others formed roots only. Single isolated embryos germinated but their subsequent development was poor (results not presented).

3.7.1 Effect of different concentrations of kinetin alone or in combination with IAA

This experiment was conducted to observe the effect of different concentrations of kinetin alone or in combination with IAA on germination of somatic embryo and plantlet formation from shoot tip derived embryogenic calli of garlic cv. GC002. The calli with embryods were cultured in MS medium supplemented with seven different concentrations (1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 mg/l) of KIN singly and two selected concentrations of KIN (5.0 and 6.0 mg/l) along with seven concentrations (1.0, 1.5, 2.0, 2.5, 3.0, 4.0 and 5.0 mg/l) of IAA in order to find out the most suitable culture media formulation to germinate somatic embryos. Percentage of calli with somatic embryos induced to develop plantlets, number of shoot and root per callus, length of the longest shoot and root were considered as parameters for evaluating this experiment. Data on these

parameters from different treatments were recorded after 8 weeks of culture and are presented in Table 13.

Among different concentrations of KIN, the percentage of calli produced plantlets ranged from 23.0-76.9%. The highest percentage (76.9%) of calli with somatic embryo induced to regenerate plantlets was recorded in medium containing 5.0 mg/l KIN (Plate 5B&C) followed by 63.8% in 6.0 mg/l KIN and 48.7% in 4.0 mg/l KIN. The lowest percentage (23.0%) of calli with somatic embryo developed to plantlets was recorded in medium containing 3.0 mg/l KIN. A certain percentage (15.3%) of calli were found to produce only roots in media containing 2.0 mg/l KIN. Calli failed to produced any shoots or roots in medium containing 1.0 mg/l KIN.

Number of plantlets per callus ranged from 2.2-8.3. The highest number (8.3) of plantlets per callus was observed in 5.0 mg/l KIN (Plate 6A&B) followed by 6.2 in 6.0 mg/l KIN and 4.3 in 7.0 mg/l KIN. The lowest number (2.2) of plantlets per callus was observed in 3.0 mg/l KIN.

Length of the longest shoot per callus ranged from 2.7-9.7 cm. The highest shoot length was recorded 9.7 cm in 6.0 mg/l KIN followed by 6.3 cm in 5.0 mg/l KIN. The lowest shoot length (2.7 cm) was observed in medium containing 3.0 mg/l KIN.

Number of roots per callus ranged from 1.8-4.8 and root length ranged from 1.1-2.8 cm. The highest number (4.8) of roots per callus and the highest root length (2.8 cm) were recorded in medium containing 4.0 mg/l KIN. The lowest number (1.8) of roots per callus and the lowest root length (1.1 cm) were recorded in medium containing 7.0 mg/l KIN.

In cases of KIN with IAA, the calli produced plantlets in all cases except in two combinations (5.0 mg/l KIN + 5.0 mg/l IAA and 6.0 mg/l KIN + 5.0 mg/l IAA) which produced only roots. The percentage of calli with somatic embryo

produced plantlets ranged from 37.7-68.8%. The highest percentage (68.8%) of calli produced plantlets in medium containing 5.0 mg/l KIN + 2.5 mg/l IAA, followed by 65.5% in 6.0 mg/l KIN + 2.0 mg/l IAA and 63.4% in 5.0 mg/l KIN + 2.0 mg/l IAA. The lowest 37.7% of calli was recorded to induce plantlets regeneration in media having 6.0 mg/l KIN + 4.0 mg/l IAA.

Number of plantlets per callus ranged from 2.2-7.2. The highest number (7.2) of plantlets per callus was recorded in medium containing 5.0 mg/l KIN + 2.5 mg/l IAA followed by 6.5 in 6.0 mg/l KIN + 2.0 mg/l IAA. The lowest number of plantlets 2.2 per callus was recorded in medium containing 5.0 mg/l KIN + 4.0 mg/l IAA.

Length of the longest shoot per callus ranged from 2.4-8.3 cm. The highest shoot length was recorded 8.3 cm in medium containing 5.0 mg/l KIN + 2.0 mg/l IAA followed by (7.1 cm) in 6.0 mg/l KIN + 2.0 mg/l IAA. The lowest shoot length (2.4 cm) was observed in medium containing 6.0 mg/l KIN + 4.0 mg/l IAA.

Number of roots per callus ranged from 2.2-5.4. The highest number (5.4) of roots per callus was noted in 5.0 mg/l KIN + 3.0 mg/l IAA followed by 5.1 in 6.0 mg/l KIN + 3.0 mg/l IAA. The lowest number (2.2) of roots per callus was recorded in 5.0 mg/l KIN + 1.0 mg/l IAA.

Length of root per callus ranged from 1.2-2.7 cm. The highest root length was recorded 2.7 cm in medium containing 5.0 mg/l KIN + 4.0 mg/l IAA. The lowest root length was observed 1.2 cm in medium containing 6.0 mg/ KIN + 1.0 mg/l IAA.

From this experiment it is observed that media with KIN alone was effective for germination of somatic embryo and plantlet formation from shoot tip derived embryogenic calli of garlic. Moderate concentrations (4.0-6.0 mg/l) of KIN was more effective than high (7.0 mg/l) or low (3.0 mg/l) concentrations of KIN. Addition of IAA with KIN was less effective than when KIN used alone.

Table 13. Effect of different concentrations of kinetin alone or with IAA on morphogenic differentiation of somatic embryos from shoot tip derived calli of garlic cv. GC002.

Growth regulators (mg/l)	Frequency of calli produced plantlets (%)	Morphogenic response shoot/root	Number of plantlets per callus	Length of the longest shoot (cm)	Number of roots per cullus	Length of the longest root (cm)
KIN						
1.0	-	-	-	-	-	-
2.0	15.3	R	-	-	2.4	1.2
3.0	23.0	SR	2.2	2.7	3.4	1.6
4.0	48.7	SR	3.6	4.4	4.8	2.8
5.0	76.9	SR	8.3	6.3	4.5	2.5
6.0	63.8	SR	6.2	9.7	2.8	1.3
7.0	46.1	SR	4.3	4.4	1.8	1.1
Mean	**45.6**		**4.9**	**5.5**	**3.2**	**1.7**
KIN+IAA						
5.0+1.0	43.3	SR	2.8	3.1	2.2	1.3
+1.5	52.2	SR	3.6	4.2	2.6	1.4
+2.0	63.4	SR	5.4	8.3	3.2	1.5
+2.5	68.8	SR	7.2	6.1	3.7	1.6
+3.0	58.7	SR	4.4	5.5	5.4	2.5
+4.0	46.8	SR	2.2	3.1	4.7	2.7
+5.0	20.6	R	-	-	3.4	1.4
6.0+1.0	42.2	SR	2.5	3.1	2.5	1.2
+1.5	56.7	SR	3.2	4.1	2.5	1.2
+2.0	65.5	SR	6.5	7.1	3.3	1.4
+2.5	52.4	SR	5.3	5.2	3.5	1.4
+3.0	48.8	SR	3.5	3.2	5.1	1.5
+4.0	37.7	SR	2.4	2.4	4.3	2.5
+5.0	20.2	R	-	-	3.2	1.8
Mean	**48.3**		**4.0**	**4.6**	**3.5**	**1.7**

- = No response, S = Shoot, R = Root

3.7.2 Effect of genotypes

This experiment was conducted with four different types calli source viz., shoot tip, leaf base, leaf primordia and bulbil from four garlic varieties. Different explants derived calli with somatic embryos of these four garlic varieties were cultured separately on to MS medium supplemented with 5.0 mg/l KIN. This concentration was standardized from previous experiment. The objective of this investigation was to find out genotypic potentiality on germination of somatic embryos and plantlet formation from different explants derived calli of four garlic varieties. Data on percentage of calli induced plantlets, number of plantlets per callus, length of the longest shoot, number of roots per callus and length of root per callus were recorded after 4 weeks of culture and the results are presented in Table 14 and the graphic representation is shown in Figs. 2 & 3.

In cv. GC002, percentage of calli induced plantlets ranged from 19.6-88.6%. The highest 88.6% calli induced to regenerate plantlets from leaf base derived calli followed by 76.9% from shoot tip derived calli. The lowest 19.6% calli with somatic embryos were recorded to develop plantlets from leaf primordia derived calli.

Number of plantlets per callus ranged from 2.4-10.7. The highest number (10.7) of plantlets per callus was observed in leaf base derived calli followed by 8.3 in shoot tip derived calli. The lowest number of plantlets was 2.4 per callus was recorded in leaf primordia derived calli.

Length of the longest shoots per callus ranged from 2.5-10.2 cm. The highest shoot length was recorded 10.2 cm from shoot tip derived calli followed by 9.7 cm from leaf base derived calli. The lowest shoot length (2.5 cm) was observed in leaf primordia derived calli.

Number of roots per callus ranged from 2.4-5.3. The highest number (5.3) of roots per callus was recorded in leaf base derived calli followed by 4.5 in shoot

tip derived calli. The lowest number (2.4) of roots per callus was recorded in leaf primordia derived calli.

Length of root per callus ranged from 2.1-3.2 cm. The highest root length (3.2 cm) was recorded in leaf base derived calli followed by 2.5 cm in shoot tip and bulbil. The lowest root length (2.1 cm) was recorded in leaf primordia derived calli.

In cv. GC009 percentage of calli induced plantlets ranged from 10.2-62.3%. The highest 62.3% calli induced to regenerate plantlets from shoot tip derived calli followed by 58.6% from leaf base derived calli. The lowest 10.2% calli were recorded to develop plantlets from leaf primordia.

Number of plantlets per callus ranged from 1.8-7.8. The highest number (7.8) of shoots per callus was recorded in shoot tip followed by 6.8 in leaf base derived calli. The lowest number (1.8) of shoots per callus was recorded to develop plantlets from leaf primordia derived calli.

Length of the longest shoot per callus ranged from 1.9-8.5 cm. The highest shoot length was recorded 8.5 cm from leaf base derived calli followed by 7.7 cm from shoot tip derived calli. The lowest shoot length (1.9 cm) was observed in leaf primordia derived calli.

Number of roots per callus ranged from 1.5-4.5 and the length of root per callus ranged from 1.6-3.0 cm. The highest number (4.5) of roots per callus and the highest root length (3.0 cm) per callus was recorded in leaf base derived calli. The lowest number (1.5) of roots and the lowest root length (1.6 cm) per callus was observed in leaf primordia derived calli.

In cv. GC0025, percentage of calli induced plantlets range from 15.2-77.6%. The highest 77.6% calli with somatic embryo induced to develop shoot regeneration from leaf base derived calli followed by 66.6% from shoot tip and 63.3% from bulbil derived callus. The lowest 15.2% calli induced plantlets from leaf primordia.

Number of plantlets per callus ranged from 2.3-8.9. The highest number (8.9) of plantlets was observed in shoot tip followed by 7.5 in leaf base derived calli. The lowest number (2.3) of plantlets per callus was recorded in leaf primordia.

Length of the longest shoot per callus ranged from 2.4-9.5 cm. The highest shoot length was recorded 9.5 cm from shoot tip and leaf base derived calli. The lowest shoot length (2.4 cm) was observed in leaf primordia derived calli.

Number of roots per callus ranged from 2.1-5.1 and the length of root per callus ranged from 1.9-3.2 cm. The highest number (5.1) of roots and the highest length (3.2 cm) of roots per callus was observed in leaf base derived calli. The lowest number (2.1) of roots and lowest root length (1.9 cm) per callus was recorded in leaf primordia derived callus.

In Indian variety, percentage of calli with somatic embryo induced plantlets ranged from 12.2-69.2%. The highest 69.2% calli were recorded to induce plantlets from leaf base derived calli followed by 62.4% from shoot tip derived calli. The lowest 12.2% calli were recorded to induce plantlet regeneration in leaf primordia derived calli.

Number of shoots per callus ranged from 2.1-8.5. The highest number (8.5) of shoots per callus was recorded in leaf base followed by 7.5 in shoot tip derived calli. The lowest number (2.1) of shoots per callus was recorded in leaf primordia derived calli.

Length of the longest shoot per callus ranged from 2.2-8.8 cm. The highest shoot length was recorded 8.8 cm from shoot tip followed by 8.2 cm from leaf base derived calli. The lowest shoot length (2.2 cm) was observed in leaf primordia derived calli.

Number of roots per callus ranged from 1.8-4.8 and the length of root per callus ranged from 1.7-3.2 cm. The highest number (4.8) of roots per callus and the

highest length (3.2 cm) of root per callus were recorded in leaf base derived calli. The lowest number (1.8) of roots and the lowest root length (1.7 cm) per callus were observed in leaf primordia derived calli.

From this experiment it was observed that GC002 genotype showed highest response on germination of somatic embryos and plantlet formation. Among the four types of explants shoot tips and leaf bases produced high frequency of plantlet induction with sufficient number of roots. In all cases leaf primordia derived calli with somatic embryo showed the poorest response.

Table 14. Genotypic effect on morphogenic differentiation of somatic embryos from different explant derived calli in MS medium with 5.0 mg/l KIN. In each treatment 12-15 explants were cultured. Data were recorded after 4 weeks of culture. The experiment was repeated thrice.

Genotypes	Sources of calli	Frequency of calli produced plantlets (%)	Number of plantlets per callus	Length of longest shoot (cm)	Number of roots per callus	Length of longest root (cm)
GC002	Shoot tip	76.9	8.3	10.2	4.5	2.5
	Leaf base	88.6	10.7	9.7	5.3	3.2
	Leaf Primordia	19.6	2.4	2.5	2.4	2.1
	Bulbil	65.5	7.4	7.6	3.4	2.5
Mean		**62.7**	**7.2**	**7.5**	**3.9**	**2.6**
GC009	Shoot tip	62.3	7.8	7.7	3.4	2.0
	Leaf base	58.6	6.8	8.5	4.5	3.0
	Leaf Primordia	10.2	1.8	1.9	1.5	1.6
	Bulbil	46.6	4.7	4.8	3.0	2.1
Mean		**44.4**	**5.2**	**5.7**	**3.1**	**2.2**
GC0025	Shoot tip	66.6	8.9	9.5	4.2	2.1
	Leaf base	77.6	7.5	9.5	5.1	3.2
	Leaf Primordia	15.2	2.3	2.4	2.1	1.9
	Bulbil	63.3	6.5	6.7	3.2	2.2
Mean		**55.7**	**6.3**	**7.0**	**3.7**	**2.4**
Indian	Shoot tip	62.4	7.5	8.8	3.4	2.2
	Leaf base	69.2	8.5	8.2	4.8	3.2
	Leaf Primordia	12.2	2.1	2.2	1.8	1.7
	Bulbil	58.2	5.5	5.6	3.2	2.2
Mean		**50.5**	**5.9**	**6.2**	**3.3**	**2.3**

3.8 *IN VITRO* BULBLET FORMATION

3.8.1 Effect of sucrose

This experiment was conducted to investigate *in vitro* bulblet formation from calli derived shoot clumps of garlic cv. GC002 in MS media with different sucrose concentrations. Four Different types of shoot clumps (shoot tip, leaf base, leaf primordia and bulbil derived shoot clumps) were used as explant sources for this experiment. Six different concentrations (3, 6, 9, 12, 15 and 18%) of sucrose were tested to find out the most effective concentration of sucrose in MS medium to induce *in vitro* bulblet.

In vitro bulbing was promoted in all the sucrose concentrations except in 3% sucrose. Percentage of explants formed bulblets, number of bulblets per culture, length of bulblets (cm), diameter of bulblets (cm) and weight of bulblets (g) were considered as parameters for evaluating this experiment. Data on these parameters from different treatments were recorded after 6 weeks of culture and the results are presented in Table 15.

In soot tip, it was observed that percentage of calli formed bulblets ranged from 27.4-66.7%. The highest 66.7% of cultures induced bulblets was observed in medium containing 12% sucrose followed by 53.3% in 15% sucrose. The lowest 27.4% bulblet formation was recorded in medium with 6% sucrose concentration.

Number of bulblets per callus ranged from 1.2-4.6. The highest number (4.6) of bulblets was found in medium containing 12% sucrose followed by 3.2 in 9% sucrose. The lowest number (1.2) of bulblets per culture was observed in medium with 18% sucrose.

Length of bulblet per culture ranged from 0.8-2.5 cm. The highest bulb length (2.5 cm) was observed in medium containing 12% sucrose followed by 1.3 cm in 9% sucrose. The lowest bulb length (0.8 cm) was noted in 18% sucrose.

Diameter of bulblets per culture ranged from 1.0-1.7 cm. The highest diameter (1.7 cm) of bulblets was recorded in medium with 12% sucrose and the lowest diameter (1.0 cm) of bulblets was recorded in medium containing 6% and 18% sucrose.

Weight of bulblets per culture ranged from 0.3-0.9 g. The highest bulblet weight (0.9 g) was observed in medium with 12% sucrose followed by 0.7 g in 9% sucrose. The lowest bulb weight (0.3 g) was observed in 18% sucrose containing MS medium.

In leaf base, percentage of calli induced bulblets from 32.6-68.8%. The highest 68.8% calli induced bulblet was observed in medium containing 12% sucrose followed by 57.8% in 9% sucrose. The lowest 32.6% *in vitro* bulblet formation was recorded in medium with 6% sucrose.

Number of bulblets per culture ranged from 2.4-5.2. The highest number (5.2) of bulb was found in medium containing 12% sucrose (Plate 6C) followed by 4.3 in 9% sucrose. The lowest number (2.4) of bulb per culture was observed in medium with 18% sucrose.

Length of bulb per culture ranged from 1.0-3.3 cm. The highest bulb length (3.3 cm) was observed in medium with 12% sucrose followed by 2.5 cm in 9% sucrose. The lowest bulb length (1.0 cm) was noted in 6% sucrose.

Diameter of bulblets per culture ranged from 1.2-1.9 cm. The highest diameter (1.9 cm) of bulb was recorded in medium with 12% sucrose followed by 1.4 cm in 15% sucrose and the lowest diameter (1.2 cm) of bulb per culture was recorded in medium containing 6% sucrose.

Weight of bulblets per culture ranged from 0.5-1.2 g. The highest bulblet weight (1.2 g) was observed in medium with 12% sucrose followed by 0.8 gm in 9%

sucrose. The lowest bulblets weight (0.5 g) was observed in 18% sucrose containing MS medium.

In leaf primordia, percentage of calli induced *in vitro* bulblets ranged from 10.2-25.0%, number of bulblets per culture ranged from 1.1-1.8, length of bulblet per culture ranged from 0.3-0.8 cm, diameter of bulblets per culture ranged from 0.4-0.8 cm, weight of bulblets per culture ranged from 0.2-0.6 g. The highest percentage (25%) of calli induced bulblets, highest number (1.8) of bulb, highest length (0.8 cm), highest diameter (0.8 cm) and the highest weight (0.6 g) of bulblet per culture were observed in medium containing 12% sucrose. The lowest percentage (10.2%) of calli induced bulblets, lowest number (1.1) of bulb, lowest length (0.3 cm), lowest diameter (0.4 cm) and the lowest weight (0.2 g) of bulb per culture were recorded in 15% sucrose containing medium. Three concentrations (3%, 6% and 18%) of sucrose were failed to induce any bulblet.

In bulbil, percentage of calli induced *in vitro* bulblets ranged from 25.4-58.4%. Highest percentage (58.4%) of *in vitro* bulblet formation was observed in 12% sucrose containing medium followed by 45.3% in 15% sucrose. The lowest percentage (25.4%) of bulblet formation was noted in 18% sucrose.

Number of bulblets per culture ranged from 1.2-3.8. The highest number (3.8) of bulblets per culture was found in 15% containing medium followed by 2.8 in 12% sucrose. The lowest number (1.2) of bulb was observed in 18% sucrose with medium.

Length of bulblets per culture ranged from 0.8-1.8 cm. The highest bulb length (1.8 cm) was recorded in medium with 12% and 15% sucrose. The lowest bulb length (0.8 cm) was noted in 6% sucrose containing medium.

Diameter of bulblets per culture ranged from 1.0-1.5 cm. The highest diameter (1.5 cm) of bulb was recorded in medium with 12% sucrose followed by 1.3 cm in 9% sucrose. The lowest diameter (1.0 cm) of bulb per culture was recorded in medium containing 6% and 18% sucrose.

Weight of bulblets per culture ranged from 0.2-0.8 g. The highest bulblet weight (0.8 g) was observed in medium with 12% sucrose followed by 0.6 g in 9% sucrose. The lowest bulb weight (0.2 g) was observed in 18% sucrose containing MS medium.

From this experiment it is observed that moderate concentration (9-15%) of sucrose was effective than high (18%) or low (6%) concentration of sucrose and 12% sucrose containing media were the best for *in vitro* bulblet formation.

Table 15. Effect of various concentrations of sucrose in MS medium on *in vitro* bulblet formation from calli derived plantlets. In each treatment 14-16 explants were cultured. The experiment was repeated thrice.

Sucrose concentr-ations (%)	Sources of plantlets	Frequency of culture produced bulblets (%)	Number of bulblets per culture	Length of bulblet per culture (cm)	Diameter of bulblet per culture (cm)	Weight of bulblet per culture (g)
3		-	-	-	-	-
6		27.4	2.3	1.1	1.0	0.4
9		42.3	3.2	1.3	1.2	0.7
12	Shoot tip	66.7	4.6	2.5	1.7	0.9
15		53.3	2.5	1.2	1.3	0.5
18		36.4	1.2	0.8	1.0	0.3
Mean		**45.2**	**2.8**	**1.3**	**1.2**	0.6
3		-	-	-	-	-
6		32.6	3.3	1.0	1.2	0.6
9		57.8	4.3	1.8	1.3	0.8
12	Leaf base	68.8	5.2	3.3	1.9	1.2
15		46.6	3.5	2.5	1.4	0.6
18		44.4	2.4	1.3	1.3	0.5
Mean		50.04	**3.7**	**1.9**	**1.4**	**0.7**
3		-	-	-	-	-
6		-	-	-	-	-
9		16.0	1.2	0.6	0.5	0.4
12	Leaf primordia	25.0	1.8	0.8	0.8	0.6
15		10.2	1.1	0.3	0.4	0.2
18		-	-	-	-	-
Mean		**17.0**	**1.4**	**0.6**	**0.6**	**0.4**
3		-	-	-	-	-
6		28.2	2.2	0.8	1.0	0.4
9		43.3	2.3	1.2	1.3	0.6
12	Bulbil	58.4	2.8	1.8	1.5	0.8
15		45.3	3.8	1.8	1.2	0.4
18		25.4	1.2	1.2	1.0	0.2
Mean		**40.1**	**2.5**	**1.4**	**1.2**	**0.5**

3.8.2 Effect of genotypes

This experiment was conducted with four different types of calli sources viz., shoot tip, leaf base, leaf primordia and bulbil from four garlic genotypes. Different explants derived calli with plantlets of these four genotypes were cultured separately on to MS medium supplemented with 12% sucrose concentration. This concentration was standardized from previous experiment. The objective of this investigation was to find out genotypic ability on *in vitro* bulblet formation from different explants derived calli of four garlic genotypes. Data on percentage of cultures induced bulblets, number of bulblets per culture, length of bulblet, diameter of bulblet and the weight of bulblets per culture were recorded after 6 weeks of culture and the results are presented in Table 16 and the graphic representation is shown in Figs. 2 & 3.

In cv. GC002 it was observed that percentage of *in vitro* bulblet formation ranged from 25.0-68.8%. The highest 68.8% calli induced *in vitro* bulblets was recorded from leaf base derived calli followed by 66.7% from shoot tip derived calli. The lowest 25.0% *in vitro* bulblet formation was recorded from leaf primordia derived calli.

Number of bulblets per culture ranged from 1.8-5.2. The highest number (5.2) of bulblets was found in shoot tip derived calli followed by 4.6 in leaf base derived calli and the lowest number (1.8) of bulblets was observed in leaf primordia.

Length of bulblets per culture ranged from 0.8-3.3 cm. The highest length (3.3 cm) of bulblets was observed in shoot tip followed by 2.5 cm in leaf base and the lowest length (0.8 cm) of bulblets was recorded in leaf primordia derived calli.

Diameter of bulblets per culture ranged from 0.8-1.9 cm. The highest diameter (1.9 cm) of bulblets per culture was observed in leaf base followed by 1.7 cm in shoot tip and the lowest diameter (0.8) of bulblets was recorded in leaf primordia derived calli.

Weight of bulblets per culture ranged from 0.6-1.2 g. The highest weight (1.2 g) of bulblets was observed in leaf base followed by 0.9 g in shoot tip and the lowest weight (0.6 g) of bulblets was recorded in leaf primordia derived calli.

In cv. GC009, Percentage of *in vitro* bulblet formation ranged from 18.2-52.2%. The highest 52.2% calli induced bulblets was recorded from shoot tip, 44.3% from leaf base and the lowest 18.2% bulblet formation was recorded from leaf primordia derived calli.

Number of bulblets per culture ranged from 1.2-3.7. Highest number (3.7) of bulblets per culture was found in shoot tip followed by 3.2 in leaf base. The lowest (1.2) number of bulblets per culture was observed in leaf primodia derived calli.

Length of bulblets per culture range from 0.4-1.8 cm. Highest length (1.8 cm) of bulblets was observed in shoot tip followed by 0.9 cm in leaf base derived calli and the lowest length (0.4 cm) of bulblets was recorded in leaf primordia.

Diameter of bulblets per culture ranged from 0.4-1.3 cm. The highest diameter (1.3 cm) of bulb per culture was recorded in leaf base derived calli followed by 1.0 cm in shoot tip. The lowest diameter (0.4 cm) of bulbs per culture was observed in leaf primordia.

Weight of bulblets per culture ranged from 0.5-1.0 g. Highest weight (1.0 g) of bulbs per culture was observed in leaf base derived calli followed by 0.6 g in shoot tip. The lowest weight (0.5 g) of bulblets per culture was noted in leaf primordia and bulbil derived calli.

In cv. GC0025, Percentage of *in vitro* bulblet formation ranged from 22.4-62.4%. The highest 62.4% calli with plantlets induced *in vitro* bulblets was recorded from leaf base derived calli followed by 58.4% from shoot tip. The lowest 22.4% bulblets formation was recorded from leaf primordia derived calli.

Number of bulblets per culture ranged from 1.5-4.8. The highest number (4.8) of bulblets per culture was observed in leaf base derived calli with plantlets followed by 4.1 in shoot tip. The lowest number (1.5) of bulblets was observed in leaf primordia derived calli.

Length of bulblets per culture ranged from 0.6-2.1 cm. Highest length (2.1 cm) of bulblet in leaf base derived calli followed by 1.2 cm in shoot tip derived calli. The lowest length (0.6 cm) of bulblets per culture was noted in leaf primordia derived calli.

Diameter of bulblets per culture ranged from 0.6-1.7 cm. The highest diameter (1.7 cm) of bulb per culture was recorded in shoot tip derived calli followed by 1.5 cm in leaf base derived calli. The lowest diameter (0.6 cm) of bulblets per culture was found in leaf primordia derived calli.

Weight of bulblets per culture ranged from 0.6-1.0 g. The highest weight (1.0 g) of bulblets per culture was found in leaf base derived calli followed by 0.8 g in shoot tip derived calli. The lowest (0.6 g) weight of bulb per culture was found in leaf primordia derived calli.

In Indian variety, percentage of *in vitro* bulblet formation ranged from 20.5-56.5%. The highest 56.5% calli induced *in vitro* bulblets was recorded in leaf base and the lowest 20.5% calli induced bulblets was recorded from leaf primordia derived calli.

Number of bulblets per culture ranged from 2.1-4.5. The highest number (4.5) of bulblets was found in leaf base derived calli followed by 3.5 in shoot tip and bulbil and the lowest number (2.1) of bulblets per culture was observed in leaf primordia derived calli.

Length of bulblets per culture ranged from 1.2-4.8 cm. The highest length 4.8 cm of bulbs was recorded in shoot tip derived calli followed by 3.2 cm in leaf

base derived calli. The lowest 1.2 cm length of bulbs per culture was found in leaf primordia derived calli.

Diameter of bulblets per culture ranged from 1.2-4.8 cm. Highest diameter 4.8 cm of bulbs was recorded in leaf base derived calli followed by 3.4 cm in shoot tip and the lowest diameter 1.2 cm of bulb per culture was found in leaf primordia.

Weight of bulblets per culture ranged from 1.3-2.4 g. The highest weight (2.4 g) of bulblets per culture was found in leaf base derived calli followed by 1.7 g in shoot tip and the lowest weight 1.3 g of bulblets per culture was observed in leaf primordia derived calli.

From this experiment it was observed that GC002 genotype showed highest response in respect of *in vitro* bulblet formation. Among the four types of explants shoot tips and leaf bases were more effective than other explants. In all cases leaf primordia showed the poorest response.

Table 16. Genotypic effect on *in vitro* bulblet formation from calli derived shoot clumps in MS with 12% sucrose. In each treatment 14-16 explants were used. The experiment was repeated thrice.

Genotypes	Source of plantlets	Frequency of cultures induced bulblets (%)	Number of bulblets per culture	Length of bulblet per culture (cm)	Diameter of bulblet per culture (cm)	Weight of bulblet per culture (g)
GC002	Soot tip	66.7	5.2	3.3	1.7	0.9
	Leaf base	68.8	4.6	2.5	1.9	1.2
	Leaf Primordia	25.0	1.8	0.8	0.8	0.6
	Bulbil	58.7	3.8	1.8	1.5	0.8
Mean		**54.8**	**3.9**	**2.1**	**1.5**	**0.87**
GC009	Soot tip	52.2	3.7	1.8	1.0	0.6
	Leaf base	44.3	3.2	0.9	1.3	1.0
	Leaf Primordia	18.2	1.2	0.4	0.4	0.5
	Bulbil	38.3	2.5	0.8	0.9	0.5
Mean		**38.3**	**2.7**	**0.97**	**0.90**	**0.65**
GC0025	Soot tip	58.4	4.1	1.2	1.7	0.8
	Leaf base	62.4	4.8	2.1	1.5	1.0
	Leaf Primordia	22.4	1.5	0.6	0.6	0.6
	Bulbil	54.2	3.2	1.0	1.2	0.6
Mean		**49.4**	**3.4**	**1.2**	**1.3**	**0.75**
Indian	Soot tip	49.3	3.5	4.8	3.4	1.7
	Leaf base	56.5	4.5	3.2	4.8	2.4
	Leaf Primordia	20.5	2.1	1.2	1.2	1.3
	Bulbil	45.4	3.5	2.6	2.2	1.6
Mean		**42.9**	**3.4**	**2.9**	**2.9**	**1.8**

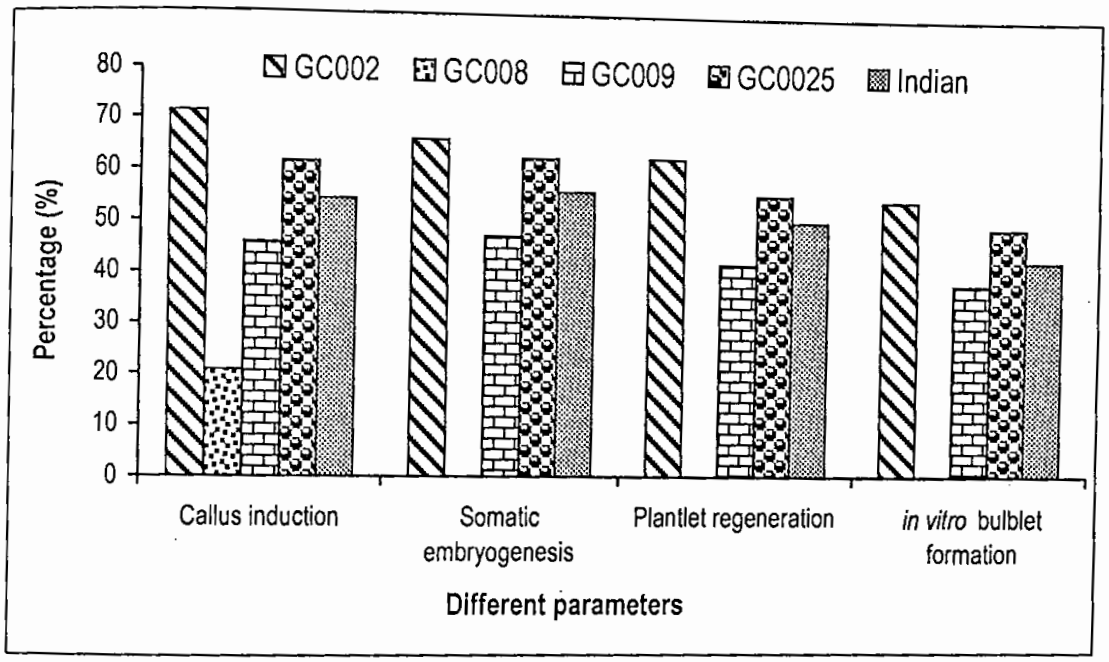

Fig. 2 . Effect of genotypes on callus induction, somatic embryogenesis, plantlet regeneration and *in vitro* bulblet formation.

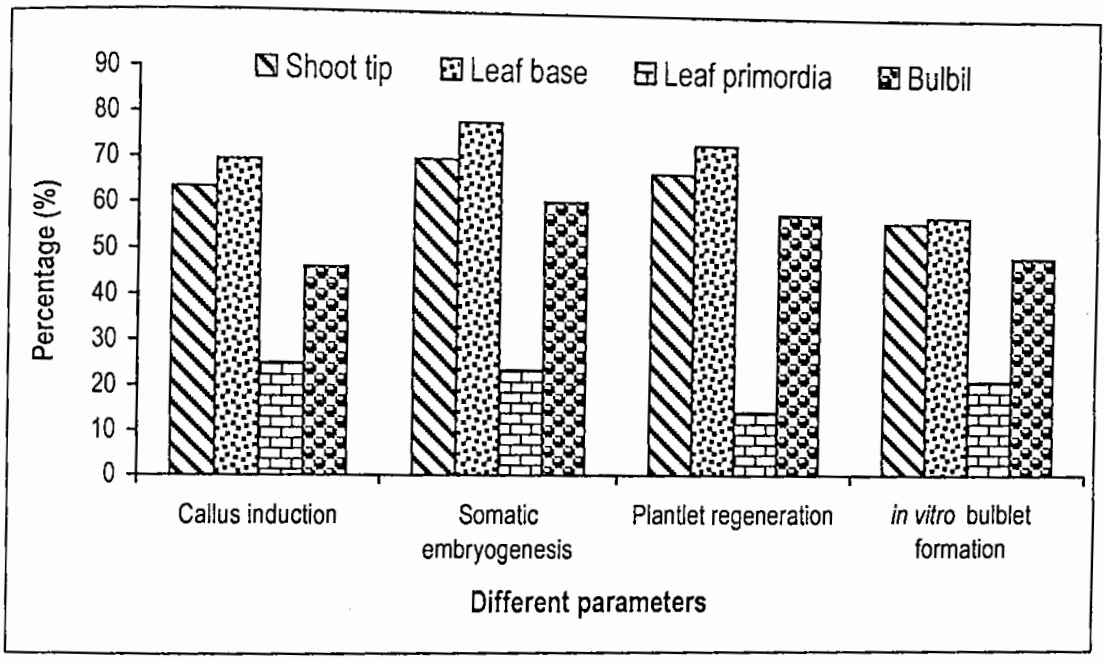

Fig. 3 . Effect of explants on callus induction, somatic embryogenesis, plantlet regeneration and *in vitro* bulblet formation.

3.9 FIELD EVALUATION OF CALLUS DERIVED PLANTLETS

Well developed plantlets with bulblets from the selected calli for each of cultivars were excised and transplanted separately. Accession of somaclones was named and maintained as A_1–A_5, B_1–B_5, C_1–C_5 and D_1–D_5 for GC002, GC009, GC0025 and Indian variety respectively. These plantlets along with the induced bulb grown inside the test tubes were brought out from the growth chamber. After depluging the culture tubes were kept in the room temperature for 4-5 days to bring them in contact to normal temperature. After 3-5 days of hardening the plantlets were taken out from test tubes carefully. The acclimatized plantlets were transplanted on the specially prepared field. Data were collected from 5 randomly selected plants of each somaclonal line and mean values were calculated.

For evaluation of somaclonal variations among the somaclones, data on plant height, number of leaves per plant, bulb diameter and bulb weight per plant were recorded and the results are presented in Table 17 and Plate 7A.

Considerable variation was noticed among the plants of the 5 callus lines of cv. GC002 for all the characters. Among the different somaclones A_4 showed highest plant height (44.2 cm) and lowest plant height (34.7 cm) was recorded in A_1 callus line. The highest number of leaves per plant was recorded (6.4) for the plants of A_3 callus line and lowest (3.5) for the plants for A_2. Similarly the highest diameter of bulbs per plant (4.7 cm) was recorded for the plants belonged to callus line A_2 and lowest diameter (3.2 cm) was recorded for A_3 callus line. Bulb weight per plant (28.6 g) was recorded highest for callus line A_2 and the lowest (17.5 g) for callus line A_5. Analysis of variance followed by subsequent LSD test for these attributes supported real difference among the different callus lines.

Somaclonal variation among different callus lines of cv. GC009 was also observed for all characters under study. Plants developed from the callus line B_2

displayed the highest (42.5 cm) plant height, highest number of leaves (5.4) per plant and highest bulb diameter (5.5 cm) per plants. Whereas, highest weight (22.3 g) of bulb per plant was recorded for the plants grown from the callus line B_1. On the other hand the lowest plant height (28.7 cm) was observed n B_4, lowest number of leaves per plant (2.7) and lowest bulb diameter per plant (2.3 cm) were recorded in B_5 callus line. The lowest bulb weight per plant (13.2 g) was observed in B_3 callus line. Statistical analysis of the individual characters supported the existence of significant variation among the different callus lines.

Variation was also recorded among the plants of 5 callus lines of cv. GC0025. Plants developed from the callus line C_1 displayed the highest plant height (46.4 cm). Highest number of the leaves per plant (5.3) was noted in C_2 callus line and the highest diameter of bulb per plant was recorded 5.2 cm in C_4 callus line. The highest weight of bulb (23.6 g) per plant was found in callus line C_4. On the other hand, the lowest plant height (25.5 cm), lowest number of leaves (3.6), lowest diameter of bulb (2.4 cm) and lowest weight of bulb per plant (14.0 g) were recorded for the plants belonging to the callus lines C_2, C_4, C_1 and C_5 respectively. Statistical analysis for this character also supported the presence of significant variation.

In Indian variety variations among different callus lines were observed for all the characters under study. Plants developed from the callus line D_4 displayed the highest plant height (45.4 cm). Highest number of leaves per plant (6.3) was noted in D_1 callus line. Highest diameter of bulb per plant (6.3 cm) was found in callus line D_4 and highest weight of bulb per plant (45.4 g) was found in callus line D_4. On the other hand the lowest plant height (34.5 cm) and lowest number of leaves per plant (2.8) were recorded in D_3 callus line. The lowest bulb diameter (4.4 cm) and lowest bulb weight (29.4 g) were recorded in D_5 callus line.

Table 17. Somaclonal variation among 5 somaclones of four garlic cultivars raised from leaf base derived calli. Data on plant height (cm), number of leaves per plant, bulb diameter (cm) and bulb weight (g) per plant were recorded after 90 days of plantation.

Plant accession	Plant height (cm)	Number of leaves per plant	Bulb diameter (cm)	Bulb weight (g)
A_1	34.7	6.2	4.6	18.4
A_2	36.5	3.5	4.7	28.6
A_3	35.4	6.4	3.2	25.5
A_4	44.2	5.6	3.5	19.4
A_5	41.3	4.5	4.2	17.5
Mean	**38.42**	**5.24**	**4.o4**	**21.88**
LSD at 5% level	**3.57**	**0.8**	**1.03**	**1.97**
B_1	38.4	3.7	4.2	22.3
B_2	42.5	5.4	5.5	15.6
B_3	39.2	5.3	3.5	13.2
B_4	28.7	3.5	3.2	18.8
B_5	35.5	2.7	2.3	18.3
Mean	**36.86**	**4.12**	**3.74**	**17.6**
LSD at 5% level	**5.44**	**1.01**	**0.72**	**2.67**
C_1	46.4	4.4	2.4	16.4
C_2	25.5	5.3	4.7	15.5
C_3	33.2	4.8	3.3	22.2
C_4	42.4	3.6	5.2	23.6
C_5	37.3	4.6	3.5	14.0
Mean	**36.96**	**4.54**	**3.82**	**18.34**
LSD at 5% level	**4.98**	**0.25**	**0.37**	**1.59**
D_1	38.5	6.3	6.2	38.3
D_2	43.6	3.2	5.8	42.4
D_3	34.5	2.8	5.4	36.2
D_4	45.4	4.8	6.3	45.4
D_5	40.3	5.7	4.4	29.4
Mean	**40.46**	**4.56**	**5.62**	**38.4**
LSD at 5% levels	**6.00**	**7.3**	**0.45**	**2.11**

GC002=A_1-A_5, GC009=B_1-B_5, GC0025=C_1-C_2, Indian=D_1-D_5

PLATE 4

Callus induction and somatic embryogenesis from leaf base explants of garlic cv. GC002

Fig. A. Callus tissue developed from leaf base explant cultured in MS medium supplemented with 2.0 mg/l 2,4-D, after 4 weeks of culture.

Fig. B. Development of nodular callus in MS_0 medium, after 4 weeks of culture.

Fig. C. Formation of "somatic embryo like structures" (ELS) in MS_0 medium after 4 weeks of culture;

Fig. D. Formation of somatic embryos in clusters in ½ MS medium, after 8 weeks of culture.

PLATE 4

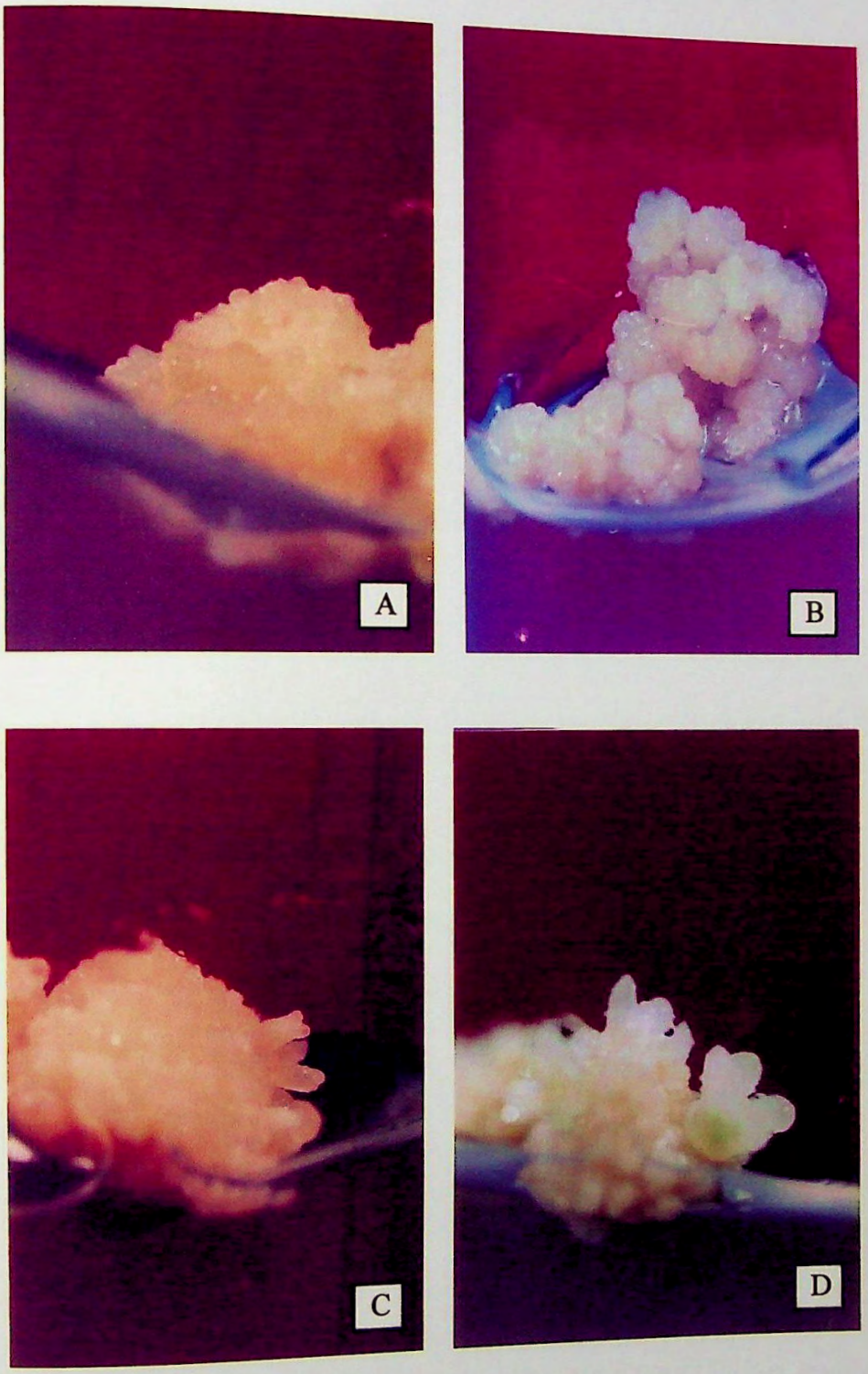

PLATE 5

Germination of somatic embryos

Fig. A. Isolated globular heart and torpedo shaped somatic embryos in ½ MS medium.

Fig. B. Germination of somatic embryos in MS medium supplemented with 5.0 mg/l KIN, 3 weeks after culture.

Fig. C. Germination of somatic embryos in MS medium supplemented with 5.0 mg/l KIN, 5 weeks after culture.

PLATE 5

PLATE 6

Plantlet regeneration and bulblet formation from somatic embryos

Fig. A. Plantlet regenerated from leaf base derived somatic embryos in MS medium supplemented with 5.0 mg/l KIN, 4 weeks after subculture.

Fig. B. Multiple shoots regenerated from leaf base derived somatic embryos in MS medium supplemented with 5.0 mg/l KIN, 8 weeks after subculture;

Fig. C. Isolated bulblets in MS medium supplemented with 12% sucrose.

PLATE 6

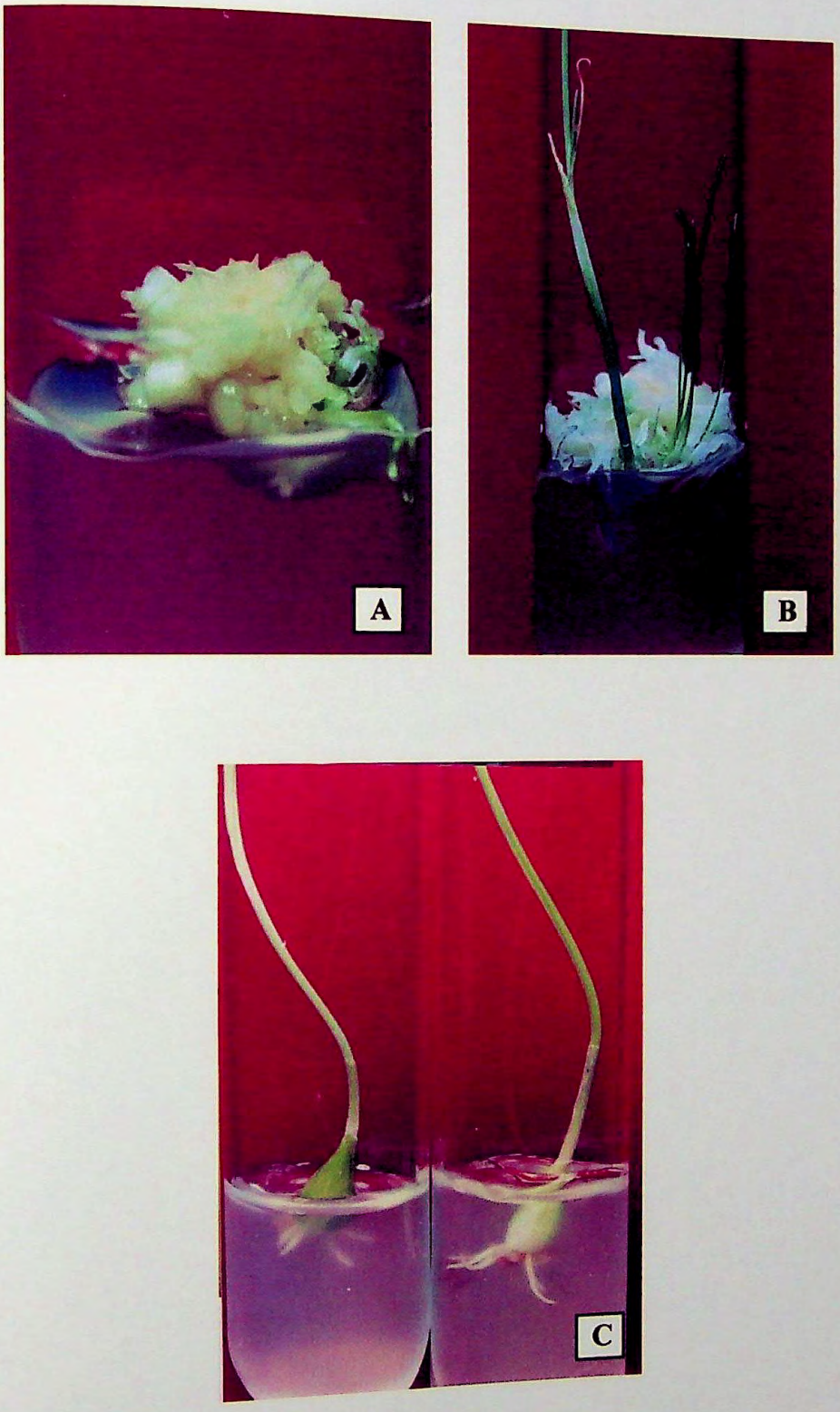

PLATE 7

Field performance of calli derived plantlets of four garlic cultivars

Fig. A. Transplanted plantlets in the field under natural condition, 3 months after plantation.

PLATE 7

CHAPTER 4

DISCUSSION

CHAPTER 4

DISCUSSION

4.1 MERISTEM CULTURE

Garlic (*Allium sativum* L.) is usually vegetatively propagated and very much susceptible to the number of viral disease, which causes remarkable decrease in yield. Meristem culture is one of the important methods to produce virus-free stock plants (Morel and Martin, 1952, 1955; Wang and Hu, 1982; Ahsan *et al.*, 2003). Whereas, micropropagation does not permit the microclones to be free from viruses. The shoots of an apical meristem and first set of primordial leaves are generally not connected to the vascular system of the plant and therefore, are not contaminated by viruses that travel through the vascular system (Prakash and Pierik, 1993; Rahaman, 1998). If this explant is carefully excised so as not to contaminate it with sap from more mature leaves or stem tissue and it is placed in culture tube a virus-free plant can be established. Many important horticultural crops (e.g. citrus, strawberries, potato, orchids, ladys finger etc) are routinely produced free of viral contamination by using this procedure (Prakash and Pierik, 1993; Ali, 1998; Ahsan *et al.*, 2003; Islam *et al.*, 2003).

Generally to established a virus-free plant one can culture the apical dome plus two or four subjacent primordial leaves. This technique is also valuable for the maintenance of carefully defined stock of specific varieties in disease free state. The size of the meristem explant is critical for virus eradication. Often so-called meristem tip cultures have failed to eliminate virus infection because the explant contains shoot apices with vascular tissue instead of true meristem (Adams, 1975 and Rahman, 1998).

In the present investigation initially meristems were isolated from field grown mature garlic bulbs of cv. GC002 and cultured on MS (Murashige and Skoog, 1962) liquid media with different types of plant growth regulators like 2ip, BAP,

KIN, NAA, IBA and IAA either singly or in combination in order to find out the best culture media formulation for primary establishment of meristem cultures. For meristem culture many workers also got satisfactory results with MS liquid medium in different crops (White, 1943; Heller, 1963; Stone, 1963; Good win, 1966; Vine, 1968; Miller and Smith, 1969; Pennazio and Redolfi, 1973; Rahman, 1998; Ali, 1998; Steward *et al.,* 1969; Walkey, 1986; Yee *et al.,* 2001; Islam *et al.,* 2003; Alam *et al.*, 2003; Ayabe and Sumi, 2001).

Various concentrations and combinations of plant growth regulators remarkably influenced the cultured meristems in resuming growth. Among the different treatments in MS medium 1.0 mg/l 2ip + 0.5 mg/l NAA was found to be the best media formulation for primary establishment of meristem culture in garlic. The media with 1.5 mg/l BAP + 0.5 mg/l NAA also showed better performance to enhance the growth of the meristem. 2ip was the most effective cytokinin commonly used in meristem culture in some herbaceous plant (Ravnikar *et al.*, 1993; Park and Lee, 1980).

In this study the primary explants produced only shoots in media with cytokinins singly, and the explants produced both shoots and roots in media with cytokinins and auxins, but did not show any callus formation at the base of the explants.

In order to find out genotypic response, five garlic varieties viz., GC002, GC008, GC009, GC0025 and Indian were cultured separately in MS liquid medium supplemented with three selected combinations (1.0 mg/l 2ip + 0.5 mg/l NAA, 1.5 mg/l BAP + 0.5 mg/l NAA and 1.5 mg/l KIN + 0.5 mg/l NAA) of plant growth regulators. In this experiment it was observed that genotypic difference existed among five garlic varieties, and cv. GC0025 showed the best response followed by GC002, GC008 and GC009. Indian variety showed the poorest response.

For shoot multiplication, established meristems were subcultured on to MS semi-solid media supplemented with different concentrations of cytokinins and

auxins either singly or in combination in order to find out suitable growth regulator formulations for maximum shoot proliferation. Multiple shoot induction and shoot elongation were found to be highly influenced by the type and concentrations of growth regulators present in shoot proliferation medium. Among all the concentrations and combinations of cytokinins and auxins 2ip with NAA showed the best performance for shoot multiplication. The highest number of shoots and length of the longest shoot were recorded in media having 1.0 mg/l 2ip + 0.5 mg/l NAA. A number of workers such as Choi *et al.* (1992), Roksana *et al.* (2002) also reported that 2ip and NAA combination performed better than BAP + NAA combination. Proliferation efficiency of the meristem was also influenced variously by concentrations and combinations of BAP with NAA (Nagasawa and Finar, 1988; Novak and Havel, 1986; Mohamed Yassen & Splittostoesser, 1992; Nieuwkerk *et al*, 1986; and Mohammed-Yasseen & Splittostoesser, 1994).

Generally, during shoot multiplication development of sufficient roots was also observed. Therefore it, was not necessary to conduct separate experiment for adventitious root induction on the regenerated shoots. Shoot regeneration occurred without a callus phase. This is an important criterion in micropropagation industry to maintain genetic stability of garlic (Novak *et al.*, 1986; Maggioni *et al.*, 1989; Bovo and Morginshi, 1985; Tapai, 1987).

In this study primary shoots derived from the isolated meristems of five garlic varieties viz., GC002, GC008, GC009, GC0025 and Indian varieties were transferred to fresh MS semi-solid medium supplemented with three selected combinations (1.0 mg/l 2ip + 0.5 mg/l NAA, 1.5 mg/l BAP + 0.5 mg/l NAA and 1.5 mg/l KIN + 0.5 mg/l NAA) of plant growth regulators to find out the response of five garlic varieties on shoot proliferation. Among the varieties maximum frequency of response, number of shoots per culture and shoot length were recorded in GC0025. Indian genotype failed to response in any of the treatments tested.

In the present study four cultivars of garlic viz., GC002, GC008, GC009, GC0025 and six different concentrations of sucrose (3, 6, 9, 12, 15 and 18%) were used to find out the most effective concentration of sucrose and most responsive genotype for *in vitro* bulblet formation from meristem derived shoot clumps. The mericlones were cultured in MS medium with different concentrations of sucrose and among all the media compositions the highest frequency of bulblet formation was observed in media having 12% sucrose. There have been many reports of bulblet formation in high sucrose medium (AboEl-Nil, 1977; Bhojwani, 1980; Nagakubo *et al.,* 1993; Seabrook, 1994; Mohamed-Yasseen *et al.*, 1994; Zel *et al.,* 1997; Matsubara and Chen, 1990).

The result also showed that the rate of bulblet formation was low at low (3% and 6%) and high (18%) sucrose concentrations containing medium. The result further showed that the frequency of bulbing increased with the increased concentration of sucrose and 9-15% was found optimum. The concentration of sucrose above this limit decreased the bulbing ability significantly. This might be due to effect of high osmoticum (Haque *et al.*,1998; Mohamed-Yasseen *et al.*, 1994). Among four garlic varieties, cv. GC0025 showed the best response for *in vitro* bulblet formation followed by GC002, GC008 and GC009.

The bulblets were successfully transferred to the soil and 90 to 100% of them formed plants. No dormancy was observed as the bulblets sprouted immediately after harvest and even sprouted in the cultured tubes when the harvest was delayed. This finding is in contradiction with that of Nagakubo *et al.* (1993) and in agreement with the results of Mohamed Yasseen *et al.* (1994). The bulblets could be stored several months without any effect in their ability to sprout and form plant. This might be due to genotypic differences.

In the presents study five agronomic traits were screened to detect variation of the meristem derived plants of different cultivars. The wide variation of the

studied traits among the varieties was observed. GC0025 showed the highest performance of all the traits and was closely followed by GC002.

The commercially cultivated garlic is sexually sterile and propagated by seed cloves. The method described here can be effectively used for the continuous production and supply of garlic bulblets, thus can substitute the seed clove method of garlic propagation. Once culture is established, there is no need of additional explant. This technique can be proposed for the commercial micropropagation of garlic.

4.2 CALLUS CULTURE

Garlic (*Allium sativum* L.) is one of the cheapest important vegetatively propagated crops of Bangladesh. Its production is increasing day by day because of this spices value and other importance. The present work was undertaken to evaluate the potentials of the variability induction in garlic plants through *in vitro* techniques and to justify its application in garlic breeding programme (Coni *et al.*, 1987). Callus induction is a prerequisite on the way to generate somaclonal variation. To generate somaclonal variability, induction and establishment of fast growing embryonic calli are prerequisites. Because during callus division in artificial conditions different types of abnormalities occur in the genetic constituents which ultimately contributed to the regenerated plants (Scowcroft and Larkin, 1982; Shamima *et al.*, 2003). As a result a lot of variation may be found in the plants regenerated from single callus population.

Callus is an actively dividing non-organized tissue of undifferentiated and differentiated cells often developing from injury (wounding) or in tissue culture (Pierik, 1987). Callus formed during *in vitro* culture has some similar to tissue arising *in vivo* injury to plants (so called wound callus). However, there is difference in morphology, cellular structure, growth and metabolism between callus derived through tissue culture and natural wound cells. Now it has been well established that any tissue can be changed into callus, if cultured on a

suitably defined medium under controlled conditions. Skoog and Miller (1957) demonstrated hormonal control of differentiation and laid the foundation of clonal propagation of plants through tissue culture techniques. Exogenous supplies of auxin and often in combination with cytokinin to medium are essential for callus induction (Lee *et al.*, 1988; Min *et al.,* 1991). Rao and Lee (1986) reported that intermediate levels of auxin and cytokinin usually promote callusing. But many other factors such as light, temperature, humidity etc. are important for callus induction (Pierik, 1987).

Callus formation and indirect regeneration has been reported in different species of *Allium* from basal parts of leaves or basal stem plates (Kher and Schaeffer, 1976; Hussey, 1975; Sco, 1986).

In the present study callus induction was conducted with explants, from field grown mature plants of five garlic varieties. Four types of explants (shoot tip, leaf base, leaf primordia and bulbil) of five garlic varieties were used. These explants were cultured on MS medium supplemented with different concent-rations of 2,4-D and in combination with IAA in order to find out the most suitable culture media for rapid callus induction.

Among all the treatments of 2,4-D and 2,4-D with IAA, highest callusing rate was found in medium having 2.0 mg/l 2,4-D. In general 2,4-D alone was found more effective in callus induction than those with 2,4-D + IAA. Many workers observed 2,4- D as the best auxin for callus induction as common as in monocot and even in dicot (Evans *et al.*, 1984; Lu *et al.*, 1982; Ho and Vasil, 1983; Jaiswal and Narayan, 1985; Hong and Debera, 1995; Nadel *et al.,* 1989; Chee, 1990; Khatun, 2004; and Alsadon *et al.*, 2004; Novak, 1990).

In this study it was observed that GC002 genotype showed highest response in callus induction and among the four types of explants shoot tips and leaf bases produced high frequency of callus induction with massive callus growth. In all cases leaf primordia showed the poorest response.

The calli derived from the four sources of explants (shoot tip, leaf base, leaf primordia and bulbil) were cultured in plant growth regulator free MS media to form somatic embryos it was observed that presence of growth regulators (cytokinins or auxins) in medium failed to induce somatic embryogenesis in garlic callus (Roksana *et al.*, 2000; Suh and Park, 1986).

It was observed that the calli upon transfer to hormone free different strength of MS media somatic embryogenesis occurred. The highest frequency of somatic embryogenesis was obtained in half strength MS media from shoot tip, leaf base and bulbil explants derived calli. Only leaf primordia derived calli formed somatic embryos in full strength MS medium. Three distinct stages of embryos (globular, heart and torpedo shape) were observed. Many workers also observed somatic embryogenesis on calli in other *Allium* species from different explants (AboEl-Nill, 1977; Suh and Park, 1988; Xue *et al.*, 1991; Hansen *et al.*, 1996; Philips and Luteyn, 1983; Van der Valk *et al.*, 1992; Dunstan and Short, 1978; Silvertand *et al.*, 1996).

In this study it was observed that GC002 genotype showed highest response in respect of somatic embryo induction and among the four types of explants leaf bases and shoot tips showed high frequency of somatic embryo induction. The embryogenic ability of leaf primordia derived calli was very low.

The calli with induced somatic embryos from different explants were further transferred to fresh MS basal media with different concentration of kinetin alone or in combination with IAA in order to find out the most suitable culture media formulation to germinate somatic embryos and plantlet regeneration. Among different treatments the highest percentage of cultures formed plantlets and maximum number of plantlets per culture were obtained in media with 5.0 mg/l KIN (Jayati, 2003). However, the length of longest shoot was recorded in media with 6.0 mg/l KIN. The palntlet regeneration through somatic embryogenesis in garlic was first reported by Haque *et al.* (1997) from root tip explants in callus

culture. Kim and Sho, (1996) observed embryos in suspension culture and *Allium fistulosum* and seedlings of *Allium cepa* but few regeneration of plantlets was found. Previous reports showed that root tip explants of *Allium spp.* produced callus tissue but very few or no plants were produced from those calli (Mohamed-Yasseen and Splittstoesser, 1992; Shuto *et al.*, 1993; Barringer *et al.*, 1996). Garlic roots produced embryo like structures in liquid culture but no regeneration of plantlets was possible. In other *Allium*, higher 2,4-D levels suppressed (Shaheen and Kaneko, 1986; Dunstan and Short 1977, 1978; Phillips and Luteyn, 1983) shoot regeneration and lower 2,4- D levels promoted shoot regeneration (Silvertand *et al.*, 1996). No regeneration was claimed from root tip derived callus induce with BAP (Mohamed-Yasseen and Splittstoesser, 1992; Barringer *et al.*, 1996).

In vitro bulblet formation is promoted by high osmoticum mainly induced by higher concentrations of sucrose. However, there is little information on the appropriate concentrations of sucrose for bulblet formation (Haque, 1997).

In the present study various concentrations of sucrose were tested to find out the most effective media formulation for bulblet formation from calli derived shoot clumps of four garlic cultivars. The bulblet size differed among the sucrose concentrations. The best concentration of sucrose was 12% containing media for *in vitro* bulblet formation. Moderate concentrations (9-15%) of sucrose was more effective than high (18%) or low (6%) concentration of sucrose. No bulblet formed on the medium supplemented with 3% sucrose. Nagakubo *et al.* (1993), Taeb and Alderson (1990) reported that bulblets were formed after a low temperature treatment of 6 months followed by culture on LS medium with 6 to 12% sucrose. The use of mannitol and glucose in combination with a low salt concentration promoted bulblet formation in shoot cultures of other cultivars (Seabrook, 1994). Zel *et al.*, (1997) reported that sucrose significantly increased the bulblet formation and a simultaneous of Jasmonic acid also been reported for onion by Mohamed-Yasseen and Splittstoesser (1992). *In vitro* bulblet formation of garlic has been observed on wide range of media and culture conditions

(AboEl-Nil, 1997; Bhojwani, 1980; Matsubara & Chen, 1989; Moriconi *et al.*, 1990 Takaji, 1979) although little is known about factors affecting the bulbing.

The find out varietal performance of five garlic varieties viz., GC002 GC008, GC009, GC0025 and Indian variety with four different explants (shoot tip, leaf base, leaf primordia and bulbil) an experiment was carried out and the results of this experiment proved that four varieties showed good performance in all cases except GC008. GC002 was found more responsive for callus induction, somatic embryo formation, plantlet regeneration and *in vitro* bulblet formation followed by GC0025, Indian variety and GC009. GC008 was not responsive for any criteria. The differences found among the varieties was statistically significant. Among four types of explant shoot tips and leaf bases showed better performance in all cases. On the other hand leaf primordia showed very poor response in all cases. Embryogenic potentiality of this explant was very low.

Somaclonal variation among plants regenerated through callus culture was reported by Amirato *et al.*, (1985). They observed that plantlets regenerated through callus culture was not genetically stable. In present study statistically significant variation in some morphological characters was also observed among the different calliclones of four garlic varieties, which support the findings of Amirato *et al.* (1985). Somaclonal variation was also observed and reported in many other crops by many earlier workers (Shepard *et al.*, 1980; Larkin *et al.*, 1984 and Scowcroft, 1977).

Findings of the present study elucidate that plant regeneration could be possible from the calli developed from different explants (shoot tip, leaf base, leaf primordia and bulbil) of four cultivars of garlic. Somaclonal variation as predicated by the workers (Amirato and Styer, 1985; Amstrong and Phillips, 1988; Kaeppler *et al.*, 2000; Nasrin *et al.*, 2003) was also observed on yield and yield related characters among the different somaclones of garlic varieties.

The protocol described here could be used as an alternative way of conventional method of propagation and genetic improvement of garlic.

CHAPTER 5

REFERENCES

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APPENDIX

APPENDIX 1

Preparation of stock solution of MS (Murashige & Skoog, 1962) basal medium

Constituents	Amount (mg/l)	Strength of stock solution	Volume of stock solution (ml)	Amount for stock solution (mg)	Amount (ml) for 1 letter medium
Stock solution I					
1. NH_4NO_3	1650	20X	1000	33000	20
2. KNO_3	1900	20X		38000	
3. KH_2PO_4	170	20X		3400	
Stock solution II					
1. $MgSO_4.7H_2O$	370	20X	1000	7400	20
Stock solution III					
1.$CaCl_2.2H_2O$	440	20X	1000	8800	20
Stock solution IV					
1. $FeSO_4.7H_2O$	27.8	20X	1000	556	20
2. $Na_2EDTA.2H_2O$	37.3	20X		746	
Stock solution V					
1. $MnSO_4.4H_2O$	22.3	20X	1000	446	20
2. H_3BO_3	6.2	20X		124	
3. $ZnSO_4.7H_2O$	8.6	20X		172	
Stock solution VI					
1. KI	0.83	1000X	200	830	2
2. $CuSO_4.4H_2O$	0.025	1000X		25	
3. $NaMoO_4.2H_2O$	0.25	1000X		250	
4. $CoCl_2.6H_2O$	0.025	1000X		25	
Stock solution VII					
1. Myoinositol	100	100X	100	1000	2
2. Nicotinic acid	0.5	100X		5	
4. Pyridoxine HCl	0.5	100X		5	
4. Thiamine HCl	0.5	100X		5	
4. Glycine	2.0	100X		20	

Dissolve $FeSO_4.7H_2O$ and $Na_2EDTA.2H_2O$ separately in 175 ml distilled water by heating and constant stirring. Two solutions were mixed, the pH was adjusted to 5.5 and then distilled water was added to make up the final volume to 1000 ml.

APPENDIX 2

MS medium (Murashige and Skoog, 1962)

Components	Concentration (mg/l)
Macronutrients	
KNO_3	1900.00
NH_4NO_3	1650.00
KH_2PO_4	170.00
$CaCl_2.2H_2O$	440.00
$MgSO_4.7H_2O$	370.00
Micronutrient	
$FeSO_4.7H_2O$	27.80
Na_2EDTA	37.30
$MnSO_4.4H_2O$	22.30
H_3BO_3	6.20
$ZnSO_4.7H_2O$	8.60
$Na_2MoO_4.4H_2O$	0.25
KI	0.83
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Organic nutrient	
Glycine	2.00
Nicotinic acid	0.50
Pyridoxine HCl	0.50
Thiamine HCl	0.10
Inositol	100.00

pH adjusted to 5.8 before autoclaving.