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Screening and Evaluation of Agronomically useful Somaclonal Variation in Strawberry (Fragaria x ananassa Duch.)

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SCREENING AND EVALUATION OF AGRONOMICALLY USEFUL SOMACLONAL VARIATION IN STRAWBERRY (*Fragaria* × *ananassa* Duch.)

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Dedicated
In Memory of My Late Father
Amal Krishna Roy

DECLARATION

I hereby declare that the whole work submitted as a thesis entitled **"SCREENING AND EVALUATION OF AGRONOMICALLY USEFUL SOMACLONAL VARIATION IN STRAWBERRY (*Fragaria* × *ananassa* Duch.)"** in the Plant Breeding and Gene Engineering Laboratory, Department of Botany, Rajshahi University, Rajshahi-6205, Bangladesh for the degree of **Master of Philosophy** is the result of my own investigation and was carried out under the supervision of Dr. M. Monzur Hossain, Professor, Department of Botany, Rajshahi University, Rajshahi-6205, Bangladesh. The thesis has not been submitted in the substance for any degree or qualification of this or any other university or other institutions of learning.

Date..21.12.2011.

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CERTIFICATE

I hereby certify that **Uthpal Krishna Roy** has been working on his thesis entitled **"SCREENING AND EVALUATION OF AGRONOMICALLY USEFUL SOMACLONAL VARIATION IN STRAWBERRY (*Fragaria* × *ananassa* Duch.)"** under my supervision, which is the record of bonafide research carried out at the Plant Breeding and Gene Engineering Lab., Dept. of Botany, University of Rajshahi, Rajshahi-6205, Bangladesh. The results of the investigation, which are embodied are original and have not been submitted before in substance for any degree or diploma of this University. He has fulfilled all the requirements of regulations relating to the nature and prescribed period of research for submission of thesis for the award of M. Phil degree. I am pleased to forward him for getting fund for this research from the **Dutch-Bangla Bank Limited.**

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21|12|2011

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Professor Dr. M. Monzur Hossain
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The Author

ABSTRACT

As a prerequisite to induce somaclonal variation, methods and techniques for optimizing the condition for meristem culture, callus induction and subsequent plant regeneration from the callus tissue and somatic embryogenesis were evaluated. The meristems were inoculated with different concentration of plant growth regulators. Various concentrations and combinations of plant growth regulators remarkably influenced in resuming growth of meristem. Among the various concentrations of hormonal formulation in MS medium GA_3 + BA were found effective in growth, rejuvenation and subsequent shoot development. Among the different treatments, MS medium fortified with 0.5 mg/l each of GA_3 and BA was found to be the most effective in increasing the growth of meristems.

For callus induction, leaf explants from *in vitro* grown plants were cultured on to MS medium supplemented with different concentrations and combinations of plant growth regulators (2,4-D, NAA and BA) and the culture were incubated in dark at 25 ± 1^0C. Among the different PGR formulations 2.0 mg/l NAA with 0.5 mg/l BA was found to be the most effective media formulation in terms of % of explants induced to develop callus and the degree of callus growth. In present study, indirect shoot regeneration was obtained by subculturing the calli onto regeneration medium and incubating the culture at 25 ± 1^0C in 16h/8h light/dark environment. The calli formed in auxin (2,4-D and NAA) supplemented MS callus inducing media were hardly induced shoot regeneration. These calli failed to regenerate or even perpetuate when the regeneration media contained only BA. On the other hand, only a few calli derived from auxin fortified callusing media were induced to develop shoots when the regeneration contained higher proportion of BA and lower proportion of NAA. The calli proliferated in NAA – BA fortified callusing media showed the highest response to indirect regeneration when regeneration contained 1.5 mg/l BA and 0.5 mg/l NAA.

For somatic embryogenesis, the calli were repeatedly sub-cultured in the same medium formulation either 2,4-D or NAA alone and or in combination with BA and proline. It was observed that proline promoted the formation of embryogenic callus. High frequency somatic embryogenesis was recorded on the MS medium supplemented with 1.0 mg/l 2, 4-D, 0.5 mg/l BAP and 50% proline in dark condition. Embryogenic calli were observed under light microscope and found to bear somatic embryos at various developmental stages. The cotyledonary stage embryos were isolated and cultured on MS medium supplemented with different PGR formulations. Among the various plant growth regulators supplements used, the best response towards embryo germination was observed on MS medium supplemented with 0.1 mg/l GA_3 + 0.1 mg/l IBA.

Occurrence of somaclonal variation was evaluated by planting the regenerants in the field. Present study shows that the high incidence of somaclonal variations among the plants regenerated through callus phase that could be used in breeding programmes for improvement of strawberry. The results also revealed that some of the regenerated plants were more vigorous than control plants, and which could prove to be useful in order to survive in the climatic conditions in Bangladesh. Single fruit weight, fruit size and in many of the cases percent of summer survival plant was higher than control. These somaclones were distinct from each other in terms of fruit and other horticultural characters, and have potential for commercial cultivation in Bangladesh.

The RAPD profiles were generated through the amplification of the genomic DNA, extracted from the leaf samples of three selected variants and control plants, with fifteen random primers. The RAPD profiles generated with random primer AL-04 and O-05 showed distinctive plant specific polymorphic banding patterns that could be used for the detection of somaclonal variation in Strawberry.

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INTRODUCTION

1.1. ORIGIN AND DISTRIBUTION

The strawberry has an interesting history. Although they grew wild in many parts of the world, the large, beautiful, wonderful tasting strawberries of today (*Frangaria* × *ananassa* Duch.) are the result of hybridization. The cultivated strawberry comes from the cross of two wild American strawberries: the Eastern Meadow Strawberry (*Fragaria virginiana*) and the Beach Strawberry (*Fragaria chiloensis*). The name *"Strawberry"* comes from the Anglo-Saxons who called them strawberries because of the way the plants grow. The runners strew or spread along the ground. Charles Linneaus gave strawberry the scientific species name of *Fragaria*.

In the 1500s, many species and subspecies of *Fragaria* were discovered in Europe. Early colonists in North America cultivated their native strawberry, *Fragaria virginiana*. In the early 1600s, *Fragaria virginiana* was imported to Europe from North America. In the 1700s, French explorers found a wild strawberry in Chile. The European importation of the Chilean strawberry, *Fragaria chiloensis*, in 1714 was the most important event in the history of the large-fruited strawberry grown today (Darrow, 1966). A French army officer and botanist named Amedee Francois Frezier transplanted the Beach Strawberry from its native home, in Chile, to France. When the Beach (Chilean) and Eastern Meadow (North American) strawberries were planted in the same garden, a natural cross pollination occurred. The result was a new superior breed of strawberries that bore large delicious fruits. The hybrid quickly spread

through Europe. Frenchman, Antoine Nicolas Duchesne is credited with identification of the natural hybrid of the *Fragaria* × *ananassa.* With rare exceptions, every commercial strawberry on the market today arose from the hybrid cross of these two wild types.

1.2. TAXONOMY AND BIOLOGY

There are about 20 recognized species of strawberries in five chromosome groups (x = 7): ten diploids, four tetraploids, one pentaploid, one hexaploid and four octoploids (Staudt 1999; Jiajun *et al.,* 2005). Cultivated strawberry (*Fragaria* × *ananassa* Duch.) as an octoploid species (2n = 8x = 56) belonging to the genus Fragaria of the family Rosaceae. It is a dicotyledonous, perennial and low-growing herb grown in most arable regions of the World.

The *Fragaria* × *ananassa* arises from a crown of meristematic tissue or compressed stem tissue. Leaves, stems, runners, axillary crowns, inflorescences, and roots all arise from the crown. The plant has trifoliate leaves which spiral around the crown, with buds in the leaf axils giving rise to the runners technically stolons, which form roots where a node touches the ground. Strawberry blossoms contain many pistils, each with its own style and stigma attached to the receptacle. When fertilization occurs the receptacle develops into a fleshy fruit. The seeds are arranged on the outside of the receptacle tissue. The fruit is called an achene, which contains the seeds. In a strict botanical sense, the strawberry fruit is not a true fruit, but is term a pseudocarp.

1.3. ECOLOGY

Strawberry is cultivated in plains as well as in the hills up to an elevation of 3,000 m in humid or dry regions (Darrow and Walgo, 1934).

Strawberry plants need about 20~26°C (68~79°F) in Daytime, and 12~16°C (54~60°F) in Night time temperature to produce high yields of high-quality fruits. If Strawberry grown in Greenhouse, Day/Night temperature of 24°C/13°C (75°F/55°F) is preferred. Strawberries grow best in a location receiving at least 8 hours+ of direct Sunlight per day. Short-day/SD Strawberry variety initiates flowers when photoperiods (Day light) are less than 14 hours. In general, Runners and leaf formation is encouraged under warm long days (September~October in Bangladesh), while Crown formation increases under cool short days (November~February in Bangladesh), regardless of genotype. In order to produce Strawberry in Bangladesh, Short-day/SD variety of Strawberry plants are recommended for winter cultivation. The cool nights and short days of winter stimulate the plant to produce flowers. Flowers are present on plants in production areas continuously from shortly after planting until the end of harvest and flower to fruit takes around one month.

1.4. CULTIVATION AND PROPAGATION

Site selection is important for successful Strawberry production. Strawberry likes Sandy loam are adaptable to raised beds slightly acidic soil and are adaptable to raised beds. Plants do best on well-drained soils with a pH of 6.0 to 6.5, which will help reduce disease problems. Plants should be set at the same depth at which they were grown in the nursery, about mid-point in the crown.

After fruiting, the strawberry produces runners. Strawberry is mostly propagated by runners in our country. To keep a strawberry bed productive for more than one season, the runners should be removed as they are produced and the mother plants should be heavily mulched to

help them survive high summer temperatures. Production of propagules through runner has been reported to contribute 90% of total Dutch strawberry production (Dijkstra, 1993). Micropropagation of strawberry has been successfully used to increase the speedy production of disease and virus free plant material (Boxus, 1976). Furthermore, the tissue culture produced plants generate more runners as compared to conventional propagation methods (Swartz *et al.*, 1981).

1.5. IMPORTANCE OF STRAWBERRY

Strawberries are produced in 71 countries worldwide on 506,000 acres and are among the highest-yielding fruit crops (Husaini and Abdin, 2008). The United States is the largest strawberry producer in the world, providing approximately 27% of the world's strawberries. The strawberry is unique in that it is the only fruit with seeds on the outside rather than the inside. Flavourful and nutritious, strawberries are enjoyed by millions of people in all climates, including temperate, Mediterranean, sub-tropical and taiga zones (Hancock *et al.*, 1991) and are predominantly used as fresh fruit. Their use in processed forms such as cooked and sweetened preserves, jams or jellies and frozen whole berries or sweetened juice extracts or flavorings. Eight medium strawberries contain more vitamin C than an orange, 20% of the recommended daily allowance for folic acid, no fat, no cholesterol and are considered high in fiber (Driscoll's, 2004).

Today scientists know strawberries contain a phenolic compound called ellagic acid that is known to have anticarcinogenic activity. The berry is valued for its low-calorie carbohydrate and high fiber contents. Strawberries are good sources of natural antioxidants (Wang *et al.*, 1996;

Heinonen *et al.*, 1998) including carotenoids, vitamins, phenols, flavonoids, dietary glutathionine, and endogenous metabolites (Larson, 1988) and exhibit a high level of antioxidant capacity against free radical species: superoxide radicals, hydrogen peroxide, hydroxyl radicals and singlet oxygen (Wang and Jiao, 2000). These qualities have ensured that the economic importance of this crop has increased throughout the world and, now-a-days, it remains as a crop of primary interest for both research and fruit production (Mercado *et al.*, 2007).

1.6. SOMACLONAL VARIATION AND ITS IMPORTANCE IN PLANT IMPROVEMENT

Somaclonal variation can be defined as variation among plants regenerated from *in vitro* culture (Larkin and Scowcroft, 1981) and is a common phenomenon in plant tissue culture (Skirvin *et al.*, 1993). Somaclonal variation is caused by changes in chromosome number (polyploidy or aneuploidy), damage to chromosomes (insertions, deletions, translocations, mutations, etc.), or changes in methylation of chromatin (Evans *et al.*, 1984; Kaeppler and Phillips, 1993; Peschke and Phillips, 1992; Phillips *et al.*, 1994). Tissue culture activation of retrotransposons has also been demonstrated in *Oryza sativa* to induce somaclonal variation (Hirochika *et al.*, 1996). The variation observed in tissue cultured clones are of two types, epigenetic and genetic. The success in applying somaclonal variation in plant breeding is therefore dependent on the genetic stability of the selected somaclones.

Somaclonal variation has been used as a useful tool in micropropagated bananas (Hwang and Ko, 1987; Sahijram *et al.*, 2003). It was also observed in apple (Chevreau *et al.*, 1998), blackberries (McPheeters and Skirvin, 1989) and peach (Hammerschlag and

Ognianov, 1990) amongst other fruit crops. Strawberries too are amenable to *in vitro* somaclonal variation (Battistini and Rosati, 1991; Kaushal *et al.*, 2004).

There are concerns about genetic changes resulting from strawberry micropropagation. Discrete morphological variants have been observed in micropropagated strawberry plants, e.g., leaf variegation consisting of a narrow white streak in the leaf blade (Swartz *et al.*, 1981), chlorosis of the leaves (Swartz *et al.*, 1981), and growth changes including dwarfs, compact trusses, lack of runner production, and female sterility (Swartz *et al.*, 1981). Moore *et al.*, (1991) observed variability among micropropagated subclones of 'Olympus' which were most likely transient responses to the micropropagation environment, not genetic. Generally, micropropagated plants have greater vigor, runner production, and yields than runner-propagated plants (Swartz *et al.*, 1981). However, not all cultivars exhibit a yield increase (Cameron *et al.*, 1985). Genetic stability during micropropagation is controlled by numerous factors including genotype, presence of chimeral tissue, explant type and origin, media type, types and concentrations of growth regulators, culture conditions (temperature, light, etc.) and duration of culture (Graham, 2005). Previous studies suggested that the concentrations of auxin in culture media and also number of subcultures are important factors for induction of somaclonal variation in an *in vitro* system (Gaafar and Saker, 2006). Neither somatic embryogenesis nor shoot organogenesis is widely used in commercial strawberry micropropagation as adventitiously regenerated plants may give rise to somaclonal variation. Somaclonal variations can be distinguished by their morphological, biochemical, physiological and genetic characteristics.

Although in many cases somaclonal variation is not desirable for commercial micropropagation, it is a valuable tool in plant breeding wherein variation in tissue culture-regenerated plants from somatic cells can be used in the development of crops with novel traits. By applying selection pressure during tissue culture it is possible to develop somaclones resistance to biotic and abiotic stress (Jain, 2001).

1.7. THE USE OF MOLECULAR MARKERS

Markers have been used over the years for the classification of plants. There are two types of genetic markers, respectively: morphological markers or naked eye polymorphism and non-morphological markers or molecular markers. Morphological markers are those traits that are scored visually, or morphological markers are those genetic markers whose inheritance can be followed with the naked eye.

Molecular markers are any kind of molecule indicating the existence of a chemical or a physical process. Molecular markers include biochemical constituents (e.g. secondary metabolites in plants) and macromolecules (e.g. proteins and deoxyribonucleic acid) (Joshi *et al.*, 1999). Strauss *et al.* (1992) distinguished the molecular markers into two classes. Biochemical molecular markers derived from the chemical products of gene expression i.e. protein based markers and molecular genetic markers derived from direct analysis of polymorphism in DNA sequences i.e. DNA based markers.

The different molecular marker technologies that are available today can be classified into two broad categories: based on molecular hybridization and based on Polymerase Chain Reaction (DNA amplification). Restriction Fragment Length Polymorphism (RFLP) is the

first marker system that was conceived and developed by Botstein *et al.* (1993) and is based on southern blotting hybridization technique.

The discovery of polymerase chain reaction (PCR) method of DNA amplification by Mullis *et al.* (1986) is an important milestone in molecular biological research. PCR is an *in vitro* method for enzymatic amplification of a specific DNA segment from the genomic DNA. PCR based markers developed by Williams *et al.* (1990). Random Amplified Polymorphic DNA (RAPD) markers are DNA fragments from PCR amplification of random segments of genomic DNA with single primer of arbitrary nucleotide sequence. In this technique primers are designed. This process starts by extracting the genomic double stranded DNA which is made single stranded by heating at 920°C for a minute. At this stage primer hybridizes with the homologous genomic DNA and then a new strand is synthesized using enzyme taq polymerase. Separation of reaction products is achieved on standard agarose gel which is then visualized with ethedium bromide staining. Few examples of RAPD techniques in fruit crops are mentioned in **Table 1**.

Table 1. Successful examples of RAPD techniques in fruit crops.

Sl. No.	Plant Species	Work Done	References
1.	Peach	Identification of peach cultivars	Lu *et al.* (1996)
2.	Peach	Comparison of genetic diversity	Warburton *et al.* (1996)
3.	Almond	Genetic relatedness among cultivars and breeding lines	Bartolozzi *et al.* (1998)
4.	Date-Palm	Genetic fingerprint of some KSA Date-palm	El-Tarras *et al.* (2007)

1.8. RATIONALE AND OBJECTIVES OF PRESENT STUDY

Variation in plant phenotype is determined by genetic and epigenetic factors. Phenotypic and DNA variation among putative plant clones is termed somaclonal variation. Use of somaclonal variations is one possible strategy to breed vegetatively propagated crops (Heinz and Mee, 1971). Selection of somaclonal variants has successfully been used to generate cultivars in a number of plants, including apple (Donovan *et al.*, 1994), banana (Cote *et al.*, 1993), celery (Heath-Pagliuso *et al.*, 1988), cucumber (Burza and Malepszy, 1995; Filipecki *et al.*, 2005), garlic (Novak *et al.*, 1982), lettuce (Engler and Grogan, 1984), peach (Hammerschlag, 1990), strawberry (Swartz *et al.*, 1981; Toyoda *et al.*, 1991; Takahashi *et al.*, 1992; Hammerschlag *et al.*, 2006), and tomato (Evans and Sharp, 1983; Barden *et al.*, 1986).

Plant tissue culture is the science or art of growing plant cells, tissues or organs on artificial media by isolating them from the mother plant (George, 1993). The most important kinds of organ cultures used for micropropagation are meristem cultures, shoot cultures, embryo cultures and isolated root cultures. Callus cultures, suspension or cell cultures, protoplast cultures or anther cultures are grouped as unorganized tissue cultures. The technique of plant tissue culture occupies a key role in the second green revolution in which gene modification and biotechnology are being used to improve crop yield and quality. By using plant tissue culture techniques, complete new plants can be obtained from different explants through meristem culture, organogenesis and through somatic embryogenesis. Although phenotypic variants might occur, even among plants regenerated from meristem (Sansavini *et al.*, 1989), it is generally emphasized that genetic variation is associated with regeneration

from callus culture (Popescu *et al.*, 1997). Popescu *et al.*, (1997) observed useful variation in plant and fruit characteristics in strawberry plants regenerated from leaf and petiole-derived callus. Both genotype and type of explants strongly influence the occurrence of somaclonal variation. Organogenesis can follow either of the two paths: Direct organogenesis is the production of shoots from explants without passing through callus (unorganized tissue) phase while refers to induction of shoots through callus phase. The resultant plants through indirect organogenesis may show variations due to the involvement of the callus phase and the regenerants through direct organogenesis may be identical to the parent plant (Christianson and Warnick, 1985).

Embryos are not classified as organs because these structures have an independent existence that is embryos do not have vascular connections with the parent plant body (John and Lorin, 1995). The term somatic is an adjective meaning of the body, but is broadly applied to dividing (living) tissue. Embryogenesis refers to the generation of embryos. Somatic embryogenesis may then be interpreted as the generation of embryos from plant organs or tissue. Although plant regeneration through direct and indirect organogenesis has been reported in strawberry (Monticelli *et al.*, 1995; Boxus, 1999; Jones *et al.*, 1988; Kaushal *et al.*, 2004), only a few studies on somatic embryo induction in this plant have been conducted (Wang *et al.*, 1984; Biswas *et al.*, 2007).

A majority of the strawberry cultivars are grown in the temperate parts of the world, although a few day-neutral cultivars can be grown in the sub-tropical regions. However, commercial cultivation is not popular in Bangladesh due to lack of proper cultivars. *In vitro* culture of strawberry is well applied but somaclonal variation has not been explored

for strawberry improvement. The main objective of this study was to develop a new strawberry cultivar from *Fragaria* × *ananassa* Duch. through the induction of somaclonal variation and selection of stable variants which could be suitable for commercial cultivation. In this study different tissue culture techniques were applied to induce somaclonal variation.

In order to evaluate genetic variability, follow phylogenetic origin and extent of ecologically distinguished species or subspecies and to develop efficient crop breeding systems, plant breeders need to have a definitive identification both of cultivars and selections of crop plants. Reliable methods of identification are also required for the establishment of plant variety rights (Kjeldgaard and Marsh, 1994). Unambiguous identification is especially important in a clonally-propagated crop such as strawberry (Kester, 1983). Commercial cultivators need to be sure that they are investing their time and money in propagating the specific cultivar that they have chosen on the basis of yield, harvest time, size and shape.

Presently, there are various methods available which can be used to detect and monitor tissue culture-derived plants and cultivar identification. The most reliable methods are the molecular marker techniques that identify the variance depending on the plant proteins, which are expressed from defined regions of DNA, or DNA polymorphisms. RAPD (random amplified polymorphic DNA) is a powerful technique for identification of genetic variation (Welsh and McClelland, 1990). It has the distinct advantage of being technically simple and quick to perform, requiring only small amounts of DNA.

Keeping in mind the above-mentioned facts the present investigation was under taken with the following specific objectives:

1. Standardization of culture media for primary in vitro establishment of isolated apical meristem of strawberry.
2. Standardization of culture media formulation for the induction of organogenic callus from *in vitro* grown leaf explants.
3. Standardization of media composition for the maximization of plant regeneration from the leaf explant derived callus.
4. Standardization of suitable media formulation for callus establishment from leaf segment and internodal explant of strawberry and plant regeneration through somatic embryogenesis.
5. To find out the effect of some regulators and supplements on the growth of shoots and roots of these regenerated plants.
6. Establishment of *in vitro* grown plants into field and evaluation on morphological characters for the assessment of somaclonal variations among the regenerated plants.
7. Investigation of genetic variability of somaclones through RAPD analysis.
8. Effort to select suitable plant type with improved characters.

Chapter II

MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Plant Materials

Runner tips of strawberry (Clone RABI-3 developed by Plant Breeding and Gene Engineering lab, Department of Botany, University of Rajshahi, Bangladesh) were collected from nursery stock plants. Terminal buds were separated and meristem were isolated and cultured for shoot induction. Besides, leaf segments from *in vitro* grown shoots were cultured to induce callus, indirect organogenesis, subsequent somatic embryogenesis and regeneration of plantlets from somatic embryos. Fully expanded young leaves of three somaclones of strawberry were used for investigation at molecular level.

2.1.2 Basal Nutrient Media

In all the experiments, the explants were cultured on semisolid MS (Murashige and Skoog, 1962) basal medium supplemented with 3% sucrose and 0.8% agar. The pH was adjusted to 5.8. This basal medium was used in subsequent experiments with addition of appropriate plant growth regulators.

2.1.3. Chemicals

The following chemical compounds were used in the present investigation:

1) Plant growth regulators (PGRs)

The following PGRs were used either individually or in various combinations to the nutrient media. Auxins and cytokinin were added to

the nutrient media before autoclaving. However, GA_3 was used in filter-sterilized condition after autoclaving the nutrient medium.

i) Auxins:

Following 3 types of auxins were used in the present study.

α-naphthalene acetic acid (NAA).

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

Indole-3 butyric acid (IBA).

Indole-3 acetic acid (IAA).

ii) Cytokinins:

The following 2 types were used to fulfill the experimental purpose.

6-Benzyladenine (BA).

6-Furfuryl amino purine (Kin).

iii) Gibberellins:

Gibberellic acid (GA_3).

2) Sterilant and surfactant

In this experiment, mercuric chloride ($HgCl_2$) was used as surface sterilizing agent and Tween-80 and Savlon (ACI Pharma, Bangladesh) were used as surfactant cum detergent.

3) Nutrient basal salts

For plants nutrient basal salts were used which content macronutrients, micronutrients and vitamins.

Macronutrients: $MgSO_4.7H_2O$, KH_2PO_4, $NaH_2PO_4.H_2O$, KNO_3, NH_4NO_3, $CaCl_2.2H_2O$, $(NH_4)_2SO_4$.

Micronutrients: H_3BO_3, $MnSO_4.H_2O$, $ZnSO_4.7H_2O$, $NaMo_4.2H_2O$, $CuSO_4.5H_2O$, $CoCl_2.6H_2O$, KI, $FeSO_4.7H_2O$, Na_2. EDTA.

Vitamins: Thiamine HCL, Pyridoxine HCL, Nicotinic acid, Myo-Inositol.

Amino acid: Proline

All chemical compounds including macro and micro nutrients, organic acids and inorganic acids, sugar, agar, KOH, $HgCl_2$, ethanol etc. used in the present study were the reagent grade products of either BDH, England or MERCK, India. The vitamins, amino acids, growth regulators were mostly products of Sigma Chemical Company; USA and Phytotec (USA) and a small portion of thiamine was a product of BDH, England.

2.1.4. Carbon Sources

Plants cell and tissues in the culture medium lack autotrophic ability and therefore, need external carbon for energy. The most preferred carbon source in the plant tissue culture is sucrose. In present investigation, sugar was used for the sources of carbon. Normally 3% sucrose was used in culture media.

2.1.5. Gelling and Solidifying Agents

Gelling and solidifying agents are commonly used for preparing semisolid or solid tissue culture media. Agar, a polysaccharide obtained from seaweed can be used as a gelling agent in the most nutrient media. In present investigation, 0.8% agar (BDH, England) was used as a gelling agent.

2.1.6. Laboratory Equipments

i) For media preparation: Different types of glass vessels including test tubes, culture bottles, conical flasks, measuring cylinders, separating funnels, pipettes, micro-pipetter, magnetic stirrer, micro-wave oven, pH meter, electronic balance, squijar etc.

ii) For aseptic transfer: Autoclave, laminar air flow machine, spirit lamp, filter paper disc, marker pens, forceps of various sizes, fire box, needles, scissors, surgical scalpel holder with disposable blades, filter paper disc, petridishes etc.

iii) For incubation: Racks with light arrangement (16h light and 8h dark) and controlled temperature 25±1°C maintained with air cooler.

2.2. METHODS

The methods involved in the present investigation are discussed under the following headings:

2.2.1. Plant Tissue Culture Media

MS (Murashige and Skoog, 1962) medium was used in the present investigation. The compositions of MS medium are given in **Appendix I.** In the different experiments MS medium supplemented with different growth regulators were used for various purposes. A control experiment using MS medium supplemented with 3% sucrose without growth regulators was also included whenever necessary.

2.2.1.1. *Primary establishment of meristem culture*

Liquid MS medium containing different concentrations of BA, KIN, NAA, IAA and GA_3.

2.2.1.2. *Shoot differentiation*

MS semisolid medium containing different concentrations of BA.

2.2.1.3. *Callus induction and maintenance*

MS semisolid medium containing different concentrations of 2,4-D alone and combination with BA or different concentrations of NAA alone and combination with BA were used.

2.2.1.4. *Indirect organogenesis*

MS semisolid medium containing different concentrations of BA alone and combination with NAA.

2.2.1.5. *Induction of Somatic embryogenesis*

MS semisolid medium containing different concentrations of 2,4-D alone and combination with BA and proline or different concentrations of NAA alone and combination with BA and proline were used.

2.2.1.6. *Embryo germination*

MS semisolid medium containing different concentrations of GA_3 alone and combination with NAA and IBA or different concentrations of KIN alone and combination with NAA and IBA were used.

2.2.1.7. *Multiple shoots regeneration*

MS semisolid medium containing different concentrations of NAA and BA.

2.2.1.8. *Root formation and elongation*

- MS semisolid medium without any growth regulators.
- ½ MS semisolid medium without any growth regulators.
- MS semisolid medium containing different concentrations of NAA.

- MS semisolid medium containing different concentrations of IBA.
- ½ MS semisolid medium containing different concentrations of NAA.
- ½ MS semisolid medium containing different concentrations of IBA.

2.2.2. Preparation of Stock Solutions for Culture Media

In the first step of the preparation of MS culture medium, stock solutions were made. Various constituents of the respective nutrient medium were prepared into stock solutions for ready use during the preparation of the media for different experiments. As different constituents were required in different concentrations, stock solutions for macronutrients, micronutrients, plant growth regulators (PGRs), organic compound (vitamins and amino acids) etc. was prepared separately.

2.2.2.1. *Stock solution A: Macronutrients*

This stock solution was made in such a way that its strength was 10 times more than the final strength of the medium in 500 ml distilled water. For this purpose, 10 times of the weight of different salts required for 1 liter of medium were weighted accurately. Then the salts were sequentially dissolved one after another in a 500 ml volumetric flask with 350 ml of distilled water. The final volume of the solution was made up to 500 ml by further addition of distilled water. The solution was filtered with Whatman`s No.1 filter paper to remove all the solid contaminants like the dust, cotton etc. and was poured into a clean plastic container. After labeling, the solution was stored in a freeze at 4°C for several weeks.

2.2.2.2. *Stock solution B: Micronutrients*

For these constituents of the medium, two separate stock solutions were prepared:

This part of the stock solution was made with the micronutrients except $FeSO_4.7H_2O$ and Na_2-EDTA. It was made 100 times the final strength of necessary components in 500 ml of distilled water as described for the stock solution A. The solution was filtered and stored at 4°C for several weeks.

The second solution was also made 100 times the final strength of $FeSO_4.7H_2O$ and Na_2-EDTA in 500 ml distilled water in conical flask and heated slowly at low temperature until the salts dissolved completely. Finally, the solutions was filtered and stored in a fridge at 4°C for several weeks.

2.2.2.3. *Stock solution C: Vitamins and amino acids*

Stock solution C was also made 100 times the final strength of the medium in 500 ml of distilled water as described for stock solution A. The solution was also filtered and stored at 4°C for several weeks.

2.2.2.4. *Stock solutions for plant growth regulators (PGR)*

The following growth regulators were used in the present investigation. Stock solution of those regulators was prepared separately. Details of the preparation method of stock solution are given in **Appendix 2**.

To prepare any one of the previously mentioned PGR stock solution, 10 ml of the respective PGR was placed on clean plastic weighing boat and dissolved in 1 or 2 ml of respective solvent. The mixer was then washed off with distilled water and collected in 100 ml measuring cylinder. It was then made up to 100 ml with the addition of distilled water. The solution was then filtered, poured into a clean plastic container and stored in a fridge at 4°C for several weeks.

2.2.3. Preparation of 1 Liter Culture Medium

Following steps were followed to prepare 1 liter MS medium:

1) Assembling of the medium components

For preparing 1 liter MS medium, 20 ml of 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, 2 ml of stock solution-VII were added in another 1 liter flask containing 200 ml distilled water and were mixed well. 30 gm of sucrose was dissolved well in 500 ml of distilled water and filtered in a 1000 ml volumetric flask. The sucrose solution was added to the stock solution and the final volume of the mixture was then made up to 1000 ml (1 liter) with further addition of distilled water.

2) Addition of growth regulators

Stock solution of growth regulators was added in appropriate concentrations and combinations in above solutions and was mixed. For preparing MS0 medium, no growth regulators were added.

3) pH of the medium

The pH of the medium was adjusted to 5.8 using pH meter (TOA, Japan) with the help of 0.1N HCl or 0.1N NaOH (whichever as necessary) before addition of agar.

4) Addition of agar

The semi-solidified medium was prepared with agar. In all cases 8-10 gm of agar (BDH chemical Ltd.) was added to 1 liter of medium on the basis of its quality and the whole mixture was then gently heated in a microwave oven (National, Japan) till complete dissolution of agar.

5) Medium dispensing to culture vessels

Ten to twenty ml of the prepared melted medium was disposed into culture vessels like test tubes or culture bottle through separating funnel. The culture vessels were plugged with absorbent cotton plugs, or with plastic caps, which were inserted tightly at the mouth of culture vessels.

6) Sterilization

Finally, the culture vessels with medium were then autoclaved at 15 lb/inch^2 pressure and at the temperature of 120^0-121^0 C for 20 minutes to ensure sterilization. Then the culture vessels with the medium were allowed to cool and then marked with a glass marker to indicate specific hormone supplements and stored in the culture room for ready use.

2.2.4. Culture Techniques

The following techniques were used for primary establishment of meristem culture, indirect organogenesis and somatic embryogenesis.

2.2.4.1. *Collection of the field grown explants and surface sterilization of the explants*

Runner tips of the healthy disease free and young strawberry plants were collected in a conical flask from the 45-60 days old field grown plants. Then, washed thoroughly under tap water to reduce dust and surface contaminants. The runner tips were cut into convenient sizes and taken in a conical flasks containing distilled water. The materials were then surface sterilized with 2-3 drops of Tween-80 and a few drop of Savlon for about 5-9 minutes with constant shacking. The materials were then washed 4-5 times with distilled water for complete removal of sterilizing agents and taken under running laminar air-flow cabinet and transferred to 500 ml sterilized conical flask. Surface sterilization was

carried out in the laminar air-flow cabinet. The explants were taken into one, two or more sterile conical flaks and suspended into 0.1% $HgCl_2$ solution by gently shaking for 5-6 minutes. To remove $HgCl_2$, the materials were then washed 3-4 times with sterilized distilled water.

2.2.4.2. *Inoculation techniques*

Isolated meristems (0.3–0.5 mm) devoid of immature leaves and leaf primordia were excised using a binocular stereoscopic microscope. The meristems were subsequently transferred onto a filter paper bridge in test tubes containing liquid MS medium supplemented with different plant growth regulators. During inoculation special care was taken that the explants much touch the medium equally and do not dip into the medium.

All inoculations and aseptic manipulations were carried out in a running laminar-air flow cabinet. The cabinet was switched on half an hour before use and cleaned with 90% ethyl alcohol to reduce the chances of contamination. The instruments like forceps, scalpels, and requirements like petri-dishes, filter paper, empty beaker or conical flasks, cotton, distilled water were covered with brown-paper and autoclaved by steam sterilization method. These were brought to the laminar airflow cabinet. During working time, the dissecting instruments like forceps, scalpels, etc. were again sterilized by an alcoholic dip and flamed over a spirit lamp for several times. Before starting inoculation, hands were also made sterile so far it was possible by spraying rectified spirit. After inoculation, the culture tubes were labeled by glass marker and the culture tubes were ready for incubation.

2.2.4.3. *Incubation*

The inoculated culture tubes or bottles 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. It may be mentioned specially that, all cultures were grown in the growth chamber illuminated by 40 watts cool–white fluorescent tubes fitted at a distance of 30-40 cm. from the culture shelves. The cultures were maintained at 25±1°C with light intensity varied from 2000-3000 lux (50-70μ $E.m^{-2}s^{-1}$) the photoperiod maintained generally 16 hours light and 8 hours dark. For the induction of callus, the culture vessels were incubated in dark chamber but for organogenesis, the culture vessels were kept in above photoperiod.

2.2.4.4. *Shoot differentiation*

After 3–4 weeks of primary culture establishment, shoots derived through meristem were subcultured onto semisolid MS medium supplemented with BA.

2.2.4.5. *Callus culture and subsequent plant regeneration*

Young, fully expanded leaves segments were collected from 4-weeks old *in vitro* plants. The leaves were sliced into 1.0 cm^2 square pieces. Cut leaves were placed on MS basal medium supplemented with 2,4-D or NAA singly or combination with BA. When the calli attained a size of about 10-15 mm in diameter, these were rescued aseptically on a sterile petridish and were cut into convenient sizes by a sterile scalpel and again set on same or different PGRs supplemented media for maintenance of callus. The calli were placed on shoot regeneration medium supplemented with various concentrations and combinations of PGR

(MS media supplemented with different concentration of BA and NAA) for enhancing organogenesis.

2.2.4.6. *Somatic embryogenesis*

Somatic embryos were induced from leaves derived callus. Cut leaves were placed on MS basal medium for callus induction. Three weeks old leaves and internode derived calli were transferred onto somatic embryogenesis medium and placed in dark. These medium consisted of 2,4-D or NAA alone or with different concentration of BA and proline. Somatic embryos were isolated and cultured individually onto embryo germination medium and incubated under 16/8 h light/dark cycle at 25 ± 1^0C.

2.2.4.7. *Subculture of primary established explants for shoot multiplication*

After few days the explants derived through meristem culture, leaf organogenesis and somatic embryogenesis were developed with 2–3 leaf containing plantlets and then the plantlets were rescued aseptically from culture vessels and transferred into a freshly prepared semi-solid medium supplemented with different growth regulator combinations for shoot multiplication. After three weeks of culture, inoculated shoots were produces multiple shoots.

2.2.4.8. *Rooting and plantlet formation*

When the regenerated shoot apices were reached 4-5 cm in length with 5-6 well developed leaves, they were rescued aseptically from the culture vessels and were separated from each other. Micro-cutting were prepared from these shoots by snapping off the basal leaves and cultured them individually in tubes containing 20 ml of rooting medium (half strength of MS media) with different combinations of auxins and kept in growth chamber for microplantlet development.

2.2.5. Acclimatization of *In vitro* Grown Plants

After sufficient growth of plantlets with good roots and shoots they were considered ready to transfer soil. Prior to transfer, acclimatization is necessary for *in vitro* plantlets. At first the culture tube caps were removed and open culture vessels were kept inside the growth chamber. Then they were taken out from the controlled environment of growth chamber and kept in room temperature to bring them contact with normal temperature for acclimatization. After hardening, the plantlets were taken out of the culture vessels, washed thoroughly under running tap water to remove medium. The plantlets were dipped in 0.1% Bavistin solution, a fungicide, (BASF Aktiengesellschaft, Germany) for ten to fifteen minutes to kill any microbes attached to the roots and transferred to plastic pots filled with mixture of sun sterilized sand, soil and humus (1:2:1). Initially, the pots were placed under shady place and covered with polythene sheet to maintain high humidity around the juvenile plants.

2.2.6. Field Preparation and Plantlets Transplantation

The field was thoroughly prepared by ploughing and harrowing followed by laddering. All the stubbles and weed removed from the field. Then well-decomposed cow dung was applied (5 ton/acre). Field was pulverized and 12′ × 3′ size and 6′ raised beds were prepared. The beds were treated with 1% formaldehyde solution to prevent the soil borne pathogen and covered with polythene sheets for 3-5 days. The potted plants were transplanted on the raised beds during the first week of November. Row to row was 14′ and plant to plant distance was 12′. The planting was done within the first week of January. Necessary intercultural operations i.e. irrigation followed by mulching, spading and weeding were done.

In this study, plants regenerated from a particular tissue culture technique considered as a population. As a result I induced three populations those were arising from three tissue culture techniques. Runner derived explants of *Fragaria* × *ananassa* Duch. was used as control plants in the field experiments. The experimental field design was a completely randomized block with three replications. Each replication consists of one population arising from a particular tissue culture technique. In order to screen somaclonal variants from the three regenerated population, quantitative and qualitative phenotypic changes were considered.

2.2.7. Propagation of Putative Somaclones and Selection of Desirable Somaclone

Twenty two somaclonal variant were identified from the three population based on the improved traits over the control. For the selection of those somaclone, the different agronomic traits such as, number of flower cluster per plant, number of flower per cluster, number of fruit per cluster, average fruit weight, number of runner production, fruit shape were considered. Putative somaclones were propagated by runner and maintained in nursery up to next cropping season. Runner derived plantlets were sown in field. The experiment was laid out in the randomized complete block design with three replications. Field performance was evaluated up to three clonal generations. Based on the field performances of three clonal generations, I identified three potential somaclones for commercial cultivation with improved agronomic traits.

2.2.8. Micropropagation of Selected Somaclone

Runner tips collected from selected somaclones were micropropagated as previously described. These plants were again hardened and re-evaluated under field condition to evaluated stability and performance.

2.2.9. Data Recording

Data on different parameters from different treatments were recorded as follows:

2.2.9.1. *Data recording on in vitro parameters*

i) For meristem culture

Percentage of explants induced to shoot

Data recorded on percentage of explants induced to develop adventitious shoots were calculated using following formula.

$$\text{Percentage of explants induced shoot} = \frac{\text{Number of cultures induced shoot}}{\text{Total number of cultured explants}} \times 100$$

ii) For organogenesis

Percentage of explants induced to callus

Explants were cultured in test tubes containing medium with different concentrations of growth regulators for callus induction. Among the culture tubes, in which explants remained fresh and resumed new growth were counted after required days of culture. Percentage of explants induced callus was calculated using following formula:

$$\text{Percentage of explants induced callus} = \frac{\text{Number of explants induced callus}}{\text{Total number of cultured explants}} \times 100$$

Percentage of calli induced shoots:

The number of calli that produced shoots was expressed as % and data were recorded after 2-5 weeks of sub-culture.

$$\text{Percentage of callus formed shoots} = \frac{\text{Number of callus formed shoot}}{\text{Total number of callus cultured}} \times 100$$

iii) For somatic embryogenesis

Number of explant with callus

Number of explant with callus was recorded after required days of culture through mean number and standard error (SE). Mean number of explants and SE was calculated using the following formula:

$$\overline{X} = \frac{\sum X_i}{N}$$

where $\overline{X}$ = Average number of explants

$\sum$ = Summation

X_i = Total number of explant

N = Number of observation

$$SE = \frac{S}{\sqrt{N}}$$

Where $$S = \sqrt{\frac{\sum X_i^2 - \frac{(\sum X_i)^2}{N}}{N-1}}$$

$\sum$ = Summation

X_i = Total number of explant

N = Number of observation

Percentage of somatic embryogenesis

Percentage of calli induced to develop somatic embryos were calculated somatic embryo using following formula:

$$\text{Percentage of calli induced somatic embryo} = \frac{\text{Number of induced somatic embryos}}{\text{Total number of cultured calli}} \times 100$$

Number of embryos/explant

Number of embryos per explant was calculated after the required days of culture. Mean number and SE of embryos per explant were calculated by using the mentioned for Number of explant with callus.

Number of planlets/callus

Mean number of planlets per callus were calculated by using the above mentioned formula.

Length of the longest shoot

Length of the longest shoot and root was measured in cm. scale for each explant. Average length of shoot or root was calculated by using the above mentioned formula.

iv) For root induction

Percentage of shoots induced to develop roots:

Percentage of shoots induced to develop roots was calculated by using the following formula:

$$\text{Percentage of shoots developed roots} = \frac{\text{Number of cultures induced root}}{\text{Total number of shoots cultured}} \times 100$$

v) Numbers of roots per shoot:

Number of roots per shoot was calculated after required days of culture. Mean number and SE of roots per shoot were calculated by using the above mentioned formula.

2.2.9.2. *Data recording on field grown plants*

To evaluate field performance and somaclonal variation among the callus derived plants data on various morphological and agronomical characters were recorded different stage of plant growth.

A. Data on different qualitative characters *viz.* flowering habit, runner/stolon habit, crown formation, fruit shape, fruit colour, degree of attractiveness of the fruits, test, summer overcoming potential and disease susceptibility were recorded on different duration after planting.

B. Data on following quantitative characters were recorded after 45, 75 and 110 days of transplantation.

i. Plant height: Crown height was measured from the bottom to the top using a meter scale.
ii. Nos. of leaves/plant: No. leaves were counted on individual plant basis.
iii. Petiole length: Petiole length of at least five leaves/plant was measured.
iv. Nos. of runner/plant: No. of runners were counted on individual plant basis.
v. Nos. nodes/runner: No. of nodes/runner were counted from at least three stolon of individual plant.
vi. No. of crowns/plant: No. of crowns/plant were counted on individual plant basis
vii. No. of flower clusters/plant: No. of flower clusters/plant were counted on individual plant basis

viii. Length of flower cluster: Length of flower cluster was measured from three flowers/plant.

ix. No. of flowers/cluster: No. of flowers were counted from three clusters/Plant.

x. Canopy size: Canopy size was determined by measuring the length and the breadth of individual plant basis.

xi. No. of fruits/plant: No. of fruits/plant were counted at different time interval

xii. No. of fruits/cluster: No. of fruits/cluster were counted from three flower cluster/plant.

xiii. Days to flowering: Days to flowering was counted from the plantation date to first flower opening.

xiv. Days to fruit harvest: Days to first fruit harvest was counted from the plantation date to first fruit harvest.

xv. Average fruit wt.: At least 10-fruits/plant was weighted and mean value was calculated.

xvi. Fruit wt./plant: All fruits of the selected plant were sequentially harvested and weighted.

2.2.10. Data Analysis

In order to evaluate the field performance of somaclonal variant, plant characteristics, such as: Plant height, Nos. of leaves/plant, Petiole length, Nos. of runner/plant, Nos. nodes/runner, No. of crowns/plant, No. of flower clusters/plant, Length of flower cluster, No. of flowers/cluster, Canopy size, No. of fruits/plant, No. of fruits/cluster, Days to flowering, Days to fruit harvest, Average fruit wt., Fruit wt./plant were recorded by quantitative measurement. Quantitative traits were measured from ten randomly selected plants from each replication. Qualitative traits such as

fruit shape, fruit texture, leaves color, leaf shape were measured by visual observation. Quantitative data are subjected to the statistical analysis. Statistical parameters like percentage, mean, standard error were estimated by using the Microsoft Office Excel program and Duncan's multiple range test were estimated using SAS version 6.11 (SAS Institute, Cary, U.S.A.).

2.3. DETECTION OF GENETIC VARIATION WITH RAPD

Three somaclones of strawberry were used for investigation at molecular level. Selected somaclones exhibit some morphological variation in terms of characters such as leaf shape, leaf size, fruit shape and size, no. of flowers per plant, no. of fruits per plant and fruit weight etc.

2.3.1. Extraction and Quantification of Genomic DNA

Experiments for extraction and quantification of genomic DNA were carried out at the Plant Breeding and Gene Engineering Laboratory, Dept. of Botany, University of Rajshahi. Genomic DNA was isolated from the fully expanded young leaves of three somaclones by using modified CTAB method (Doyle and Doyle, 1990).

Strict hygiene condition was maintained throughout the DNA extraction process by autoclaving all glassware, micropipette tips, microfuse tubes, distilled water and buffer solutions to avoid contamination. Scissors, forceps, glass rods etc were sterilized using 70% ethnol. Fully expanded cleaned young leaves (0.1g) was grinded into fine paste in presence of 10 volume of isolation buffer [10% PEG, 0.35M Sorbitol, 0.1M Tris (pH 8.0), 0.5% β-mercaptoethnol] by pestle and mortar. The paste was collected in a microfuse tube (2ml).The homogenate was centrifuged at 10,000 rpm for 15 min at 4°C. The pellet

was suspended in 5 volume of lysis buffer [0.35M Sorbitol, 0.1M Tris (pH 8.0), 0.5% β-mercaptoethnol] and the mixture was incubated for 30 minutes at room temperature. An equal volume of preheated CTAB buffer [2% CTBT, 0.1M Tris, 20mM EDTA, 1.4M NaCl, 1% PVP) was added, mixed gently and incubated at 65° C for 30 minutes. An equal volume of chloroform: isoamylalcohol (24:1) was added and mixed gently. After centrifugation at 10,000 rpm at 20° C for 15 minutes, the aqueous phase was collected in a clean microfuse tube (15µl) and DNA was precipitated by adding 0.8 volume of chilled 2-isopropanol. The precipitated DNA was recovered by centrifugation at 10,000 rpm for 10 minutes at 4°C. The pellet was then washed with chilled 70% ethanol containing 3M sodium acetate with gentle agitation. After centrifugation at 10,000 rpm for 10 minutes at 4° C the supernatant was discarded and DNA pellet was air dried and dissolved in 50 µl of TAE buffer [10mM Tris (pH 8.0),1mM EDTA]. Two microlitre of RNase (10mg/ml) was added and incubated at 37°C for an hour. Finally, DNA sample was stored at -20°C until further use.

2.3.2. Primer Selection

Fifteen random primers were evaluated using polymerase chain reaction (PCR) as detailed in **Table 2.**

Table 2. List of primer used in this study.

SL	Primer ID	Nucleotide sequence (5′–3′)
1	A-03	AGTCAGCCAC
2	A-15	TTCCGAACCC
3	AA-01	AGACGGCTCC
4	AA-19	TGAGGCGTGT
5	AF-06	CCGCAGTCTG
6	AL-04	ACAACGGTCC
7	D-16	AGGGCGTAAG
8	E-12	TTATCGCCCC
9	G-01	GGGAATTCGG
10	G-10	CCGATATCCC
11	H-14	ACCAGGTTGG
12	I-04	CCGCCTAGTC
13	O-05	CCCAGTCACT
14	T-04	GTCCTCAACG
15	J-19	GGACACCACT

2.3.3. PCR Amplification

Genomic DNA polymorphism was determined by the random polymorphic DNA (RAPD) method (Williams *et al.*, 1990, Tingey and Del Tufo, 1993). 15 primers were used for DNA amplification (Table- 2). Amplification was performed in sterile 0.2 ml Eppendrof tubes in 10 µl reaction mixture containing PCR buffer [10 mM Tris-HCl; pH 8.5; 40 mM KCl; 0.1% Tween 20; 2 mM gelatin and 1.5mM $MgCl_2$]; additional 1.5 mM $MgCl_2$ was also added; 200 µM each of dATP, dCTP, dGTP; dTTP; 0.004 µM primer; 2 units of Taq DNA polymerage (Bioneer Corporation, Korea); and 4 ng template DNA. The PCR reaction mix

contained 25 ng genomic DNA as template. PCR cycle conditions were: 1 cycle at 92°C for 3 min, 45 cycles of 92°C for 30 s, 35°C for 1 min, 72°C for 2 min with a final cycle of 10 min at 72°C.

2.3.4. Separation of PCR Products

Amplified DNA products were separated by electrophoresis in a 1.5% (w/v) agarose gels in 0.5 X TAE buffer, stained with 0.5 μgml^{-1} of ethidium bromide (EtBr) and photographed under exposure to UV light. For this experiment, gel loading dye (6 µl) [10 mM Tris-HCl (pH 7.6), 0.3% bromophenol blue, 0.3% xylene cyanol FF, 30% glycerol, 60 mM EDTA] was added to each tube. Amplified products were analyzed by electrophoresis in 1.5% agarose gel at 50V for 1 hr in 1X TAE buffer. The gel was stained with ethidium bromide (0.5 µg/ml in 1X TAE buffer) and visualized under UV transilluminator.

2.3.5. Data Analysis

RAPD bands were observed and analyzed to find out the polymorphism among three somaclone in comparison to the control. Polymorphism was scored for the presence of band (1) or absence of band (0) of a particular amplification product.

Chapter III

RESULTS

The main objective of this study was to develop a new strawberry cultivar from *Fragaria* × *ananassa* Duch. through the induction of somaclonal variation and selection of stable variants which could be suitable for commercial cultivation. In order to achieve the objectives of the present study several experiments were conducted. The results of these experiments are described below under different headings.

3.1. PRIMARY ESTABLISHMENT OF MERISTEM

Isolated meristems from the runner tip of 30-40 days old field grown plants of strawberry were placed on 'M' shaped filter paper bridge in culture tubes (125×25 mm) containing liquid MS medium supplemented with various concentrations and combination of different PGR. Meristem showed their first growth response by increase in size and became in green colour. They continued their growth and developed shoots only without roots. When a meristem attained in this condition, they were considered to be primarily established. Data on days to response, percentage of meristems responded and morphogenic responses were recorded 21 days after culture and are presented in **Table 3 and 4**. Different stages of the *in vitro* growth of cultured meristem are shown in **Plate 01 (Figures A - F)**.

The culture media were fortified with different kinds of PGR. Days taken to resume growth were influenced by the type of cytokinin, auxin and GA_3 singly or in combination as well as their different concentrations used. In all the culture media formulations, certain number of explants was found to resume new growth. However, the growth of cultured

explants and morphogenic response were found to vary with culture media formulations.

3.1.1. Effect of BA, KIN and GA_3 on Meristem Culture

The meristems were inoculated with five different concentration of BA (**Table 3**). Among the five different concentrations in liquid medium, the highest 53% explants resumed new growth at 1.5 mg/l BA. The percentage of explants displayed their initial response was the lowest in 0.1 mg/l BA supplemented medium.

Isolated meristem showed variation in growth responses in all of the tested formulations of KIN (**Table 3**). Among the different concentrations of KIN, the highest 53% explants responded to resume new growth in MS liquid medium supplemented with 1.0 mg/l KIN. Whereas, the lowest 13% explants responded to resume new growth in liquid medium at the level of 0.1 mg/l KIN.

The meristems were cultured with five different concentrations of GA_3 showed variation in growth responses. Among the five concentrations, the highest 73% explants resumed new growth at 0.5 mg/l GA_3 (**Table 3**). Whereas, the percentage of explants resumed new growth was the lowest in 0.1 mg/l GA_3 supplemented media.

3.1.2. Effect of BA in combination with NAA, IAA and KIN on Meristem Culture

Isolated meristems were cultured in MS liquid medium supplemented with BA with NAA. Results on the influence of various concentration and combination of BA with NAA as primary establishment of meristems are given in **Table 4.** The combinations of BA with NAA were found more effective than when BA was used alone.

Table 3: Effect of different combination of BA, KIN and GA_3 in liquid MS medium on primary establishment of meristem cultures. Data were recorded 21 days after culture inoculation. Each treatment consisted of 15 explant.

Culture medium composition (mg/l)	Days to response	% of explant responded	Degree of shoot growth	Nature of response of cultured meristem	
				Shoot	Root
BA					
0.1	7-13	33	+	S	-
0.25	7-12	40	+	S	-
0.5	7-12	40	+	S	-
1.0	7-10	47	++	S	-
1.5	7-9	53	++	S	-
KIN					
0.1	8-13	13	+	S	-
0.25	8-13	20	+	S	-
0.5	8-12	33	+	S	-
1.0	7-12	53	++	S	-
1.5	7-12	47	+	S	-
GA3					
0.1	6-8	40	+	S	-
0.25	6-8	40	+	S	-
0.5	5-8	73	+++	S	-
1.0	5-8	66	++	S	-
1.5	5-9	53	++	S	-

\+ = Low
++ = Medium
+++ = High
S = Presence of shoot
– = Absence of root

Table 4: Effect of different concentration and combination of auxin, cytokinin and gibberellin in liquid MS medium on primary establishment of meristem cultures. Data were recorded 21 days after culture inoculation. Each treatment consisted of 15 explant.

Culture medium composition (mg/l)	Days to response	% of explant responded	Degree of shoot growth	Nature of response of cultured meristem	
				Shoot	Root
BA+NAA					
1.5+0.1	9-11	46	++	S	-
1.5+0.5	8-10	53	++	S	-
1.5+1.0	8-9	60	++	S	-
1.5+1.5	9-11	53	++	S	-
BA+IAA					
1.5+0.1	7-11	40	++	S	-
1.5+0.5	7-9	60	++	S	-
1.5+1.0	7-10	53	++	S	-
1.5+1.5	7-10	53	++	S	-
BA+KIN					
1.5+0.1	7-10	46	++	S	-
1.5+0.5	6-8	66	++	S	-
1.5+1.0	6-8	60	++	S	-
1.5+1.5	7-9	53	++	S	-
KIN+NAA					
1.0+0.1	9-11	30	+	S	-
1.0+0.5	8-10	33	+	S	-
1.0+1.0	8-9	53	++	S	-
1.0+1.5	9-11	40	++	S	-
KIN+IAA					
1.0+0.1	10-11	27	+	S	-
1.0+0.5	9-12	46	++	S	-
1.0+1.0	9-12	53	++	S	-
1.0+1.5	10-13	40	++	S	-
GA3+BA					
0.5+0.1	6-7	66	++	S	-
0.5+0.5	5-7	80	+++	S	-
0.5+1.0	5-7	73	+++	S	-
0.5+1.5	6-8	53	++	S	-
GA3+KIN					
0.5+0.1	8-9	53	++	S	-
0.5+0.5	7-9	60	++	S	-
0.5+1.0	7-9	60	++	S	-
0.5+1.5	8-10	53	++	S	-

S = Presence of shoot, – = Absence of root

In BA with NAA combinations, the highest 60% explants showed their first response by increasing in their size of the liquid medium supplemented with 1.5 mg/l BA + 1.0 mg/l NAA. On the other hand, the lowest 46% of the explants showed their first response in medium containing 1.5 mg/l BA with 0.1 mg/l NAA.

In the entire supplemented BA with IAA combinations, the excised meristems displayed their initial response **(Table 4)**. In media with combination of 1.5 mg/l BA with 0.5 mg/l IAA, the highest 60% explants resumed. Whereas, the lowest 40% explants resumed new growth was observed in liquid medium at the level of 1.5 mg/l BA + 0.1 mg/l IAA.

Isolated meristems commenced their initial growth in all the combination of BA and KIN supplemented medium. 66% explants induced to develop shoot were found in liquid medium combination with 1.5 mg/l BA + 0.5 mg/l KIN and the lowest 46% shoots growth was occurred in medium supplemented with 1.5 mg/l BA + 0.1 mg/l KIN.

3.1.3. Effect of KIN in Combination with NAA and IAA on Meristem Culture

The meristem explants were cultured in MS liquid medium supplemented with KIN with different combinations of NAA and IAA to see their growth response. The effects in these experiments on growth response are shown in **Table 4**.

Among the different combinations in KIN with NAA, the highest percentage (53%) of explants showed new growth was recorded in liquid medium at the level of 1.0 mg/l KIN + 1.0 mg/l NAA. Whereas, the percentage of explant resumed new growth was the lowest both in

medium containing 1.0 mg/l KIN + 0.1 mg/l NAA and 1.0 mg/l KIN + 0.5 mg/l NAA.

Meristems were cultured with KIN with different concentrations of IAA showed variation in growth responses. In KIN and IAA combinations, the highest responding explants were 53% in medium containing 1.0 mg/l KIN + 1.0 mg/l IAA. On the other hand, the lowest 27% initiation was found supplemented with medium with 1.0 mg/l KIN + 0.1 mg/l IAA.

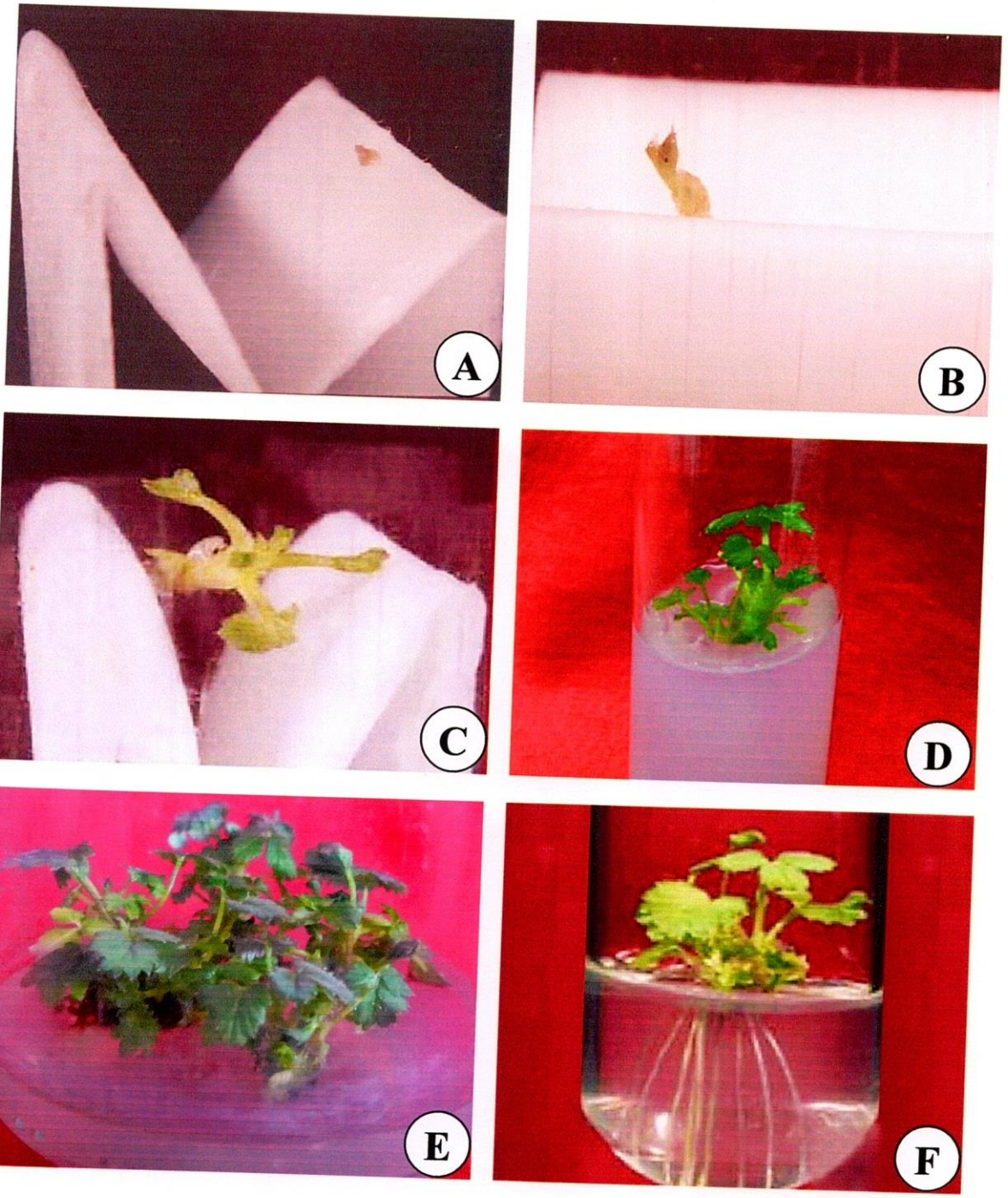
3.1.4. Effect of GA_3 with BA and KIN on Meristem Culture

In this experiments, excised meristems were cultured in MS liquid medium supplemented with four various concentrations viz. 0.1, 0.5, 1.0 and 1.5 mg/l BA and Kin with 0.5 mg/l GA_3 to see their effect in growth responses of on culture meristem (**Table 4**).

The highest 80% explants commenced to new growth in liquid medium having 0.5 mg/l GA_3 + 0.5 mg/l BA (**Plate 01, Figures A - C**). However, the lowest 53% growth response was observed in medium supplemented with 0.5 mg/l GA_3 + 1.5 mg/l BA.

Growth responses were noticed from meristematic explants in all the GA_3+KIN supplemented medium. Among the different combinations of GA_3 with KIN, the highest percentage (60%) of explants resumed new growth was found in both the medium containing 0.5 mg/l GA_3 + 0.5 mg/l KIN and 0.5 mg/l GA_3 + 1.0 mg/l KIN.

PLATE 1



3.2. INDUCTION OF CALLUS FROM LEAF EXPLANTS

Callus induction is a prerequisite on the way to induce somaclonal variability in plants. In order to induce callus, leaf segments derived from *in vitro* grown plantlets were cultured on to semi-solid MS (Murashige and Skoog, 1962) medium supplemented with different concentrations and combinations of auxin and cytokinin. All the cultures were incubated in dark in growth cabinet at 25°C for 4 weeks. Different parameters were taken in to consideration for finding out the most effective culture media formulation for the development of callus with high potential for plant regeneration. The results on induction and development of callus are discussed below on the basis of PGR type.

3.2.1. Effect of 2,4-D in MS Medium on Callus Induction

Leaf explants of strawberry were incubated onto MS culture medium containing different concentrations (1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mg/l) of 2,4-D alone for callus induction. The effect of 2,4-D on callus development from leaf explant was evaluated by recording data on different parameters such as % of explants induced callus, degree of callus development, colour and nature of calli and adventitious shoot regeneration, and the results are presented in **Table 5**.

The maximum number (86%) of leaf segments were induced callus formation when cultured on to medium with 3.5 mg/l 2,4-D. Whereas, the lowest 13% explants develop callus in medium supplemented with 1.0 mg/l 2,4-D. Moderate callusing response was recorded in media having 2.5, 3.0 and 4.0 mg/l 2,4-D. The calli induced in 2,4-D fortified were prolific in growth, creamy or light creamy in colour and almost all calli were compact in nature, few are loosely compact. Adventitious shoot

formation from leaf segments derived callus was not observed in any of the 2,4-D concentrations tested in primary culture.

3.2.2. Effect of 2,4-D in Combination with BA in MS Medium on Callus Induction

The leaf explant of strawberry was cultured in MS medium containing different concentration and combinations of 2,4-D and BA in order to induce callus from leaf explants. The combine effect of 2,4-D and BA on callus development from leaf explant in terms of % of explants induced callus, degree of callus development, colour and nature of calli and adventitious shoot regeneration are shown in **Table 5**.

Callus proliferation from leaf explant was also noticed in all 2,4-D – BA combinations. However, the combine effect of all the 2,4-D – BA formulations was not same. Among the different 2,4-D – BA formulation, the maximum 87% explants showed callus development in media having 3.0 mg/l 2,4-D and 0.5 mg/l BA. Whereas, only 20% explants showed callus induction when the culture medium was supplemented with 4.0 mg/l 2,4-D and 2.0 mg/l BA combination.

The calli developed in 2,4-D – BA media formulations were white to creamy in colour and loosely compact in nature. Moderate callusing was recorded in media having 3.0 mg/l 2,4-D and 1.0 mg/l BA, 3.0 mg/l 2,4-D and 1.5 mg/l BA, 4.0 mg/l 2,4-D and 0.5mg/l BA, 4.0 mg/l 2,4-D and 1.0mg/l BA combination. Adventitious shoot formation from leaf segment derived callus was not observed in all the 2,4-D – BA combination tested in primary culture.

Table 5: Effect of different concentrations of 2 4-D alone and combination with BA in MS medium on callus induction from *in vitro* grown strawberry leaf segments. At least 15 explants were incubated in the culture medium and the data were recorded after four weeks incubation in dark.

Culture medium composition	% of explants induced callus development	Degree of induced callusing	Colour of callus	Nature of callus
2,4-D				
1.0	13	+	Cre	C
1.5	20	+	Cre	C
2.0	40	+	Cre	C
2.5	53	++	LCre	C
3.0	73	++	LCre	LC
3.5	80	++	LCre	LC
4.0	67	++	Cre	C
2,4-D+BA				
3.0+0.5	87	+++	WCre	LC
3.0+1.0	67	++	WCre	LC
3.0+1.5	53	++	WCre	LC
3.0+2.0	33	+	WCre	LC
2,4-D+BA				
4.0+0.5	60	++	WCre	LC
4.0+1.0	73	++	WCre	LC
4.0+1.5	40	+	WCre	LC
4.0+2.0	20	+	WCre	LC
2,4-D+BA				
5.0+0.5	47	+	WCre	LC
5.0+1.0	40	+	WCre	LC
5.0+1.5	33	+	WCre	LC
5.0+2.0	33	+	WCre	LC

\+ = Little callusing (0-50 %)
++ = Moderate callusing (51-80 %)
+++ = Highly callusing (81-100 %)
Cre = Creamy

LCre = Light Creamy
WCre = White Creamy
C = Compact
Lc = Loosely Compact

3.2.3. Effect of NAA in MS Medium on Callus Induction

MS medium supplemented with different concentration of NAA were also tested to induce callus development from leaf segments of strawberry. The effect of different concentration of NAA on % of explants induced callus, degree of callus development, colour and nature of calli and adventitious shoot regeneration are shown in **Table 6**.

Among the different doses of NAA, the highest 87% explant showed callus proliferation medium supplemented with 3.0 mg/l NAA. On the contrary, the lowest 27% explants responded to callus formation in medium supplemented with 1.0 mg/l NAA. High callusing response of the leaf explant was also recorded in media having 3.5 mg/l NAA. The calli proliferated in NAA supplemented culture media also showed prolific and massive growth. Almost all calli were light creamy and loosely compact, few are creamy and compact. Like 2,4-D adventitious shoot formation from leaf segment derived callus was not observed in all of the NAA combinations tested in primary culture.

3.2.4. Effect of NAA in Combination with BA in MS Medium on Callus Induction

In the present investigation, the effect of different concentrations and combinations of NAA and BA on callus induction from leaf segments was also tested. The results on the effect of NAA and BA media formulations on callus induction are tabulated in **Table 6**.

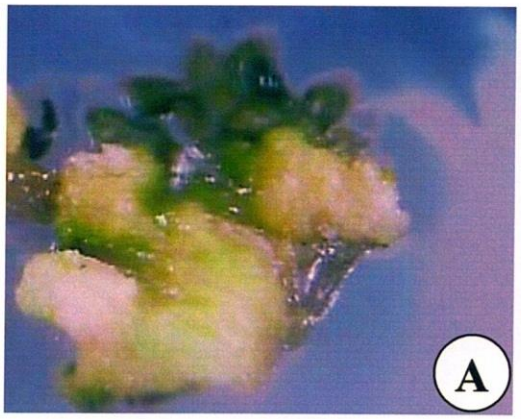
Table 6: Effect of different concentrations of NAA alone and combination with BA on callus induction from *in vitro* grown strawberry leaf segments. In each treatment 15 explants were inoculated and data were recorded after 4 weeks of culture incubation in dark.

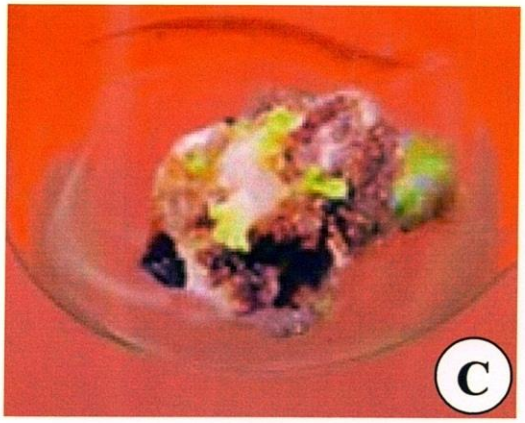
Culture medium composition mg/l	% of explants induced callusing	Degree of induced callusing	Colour of callus	Nature of callus
NAA				
1.0	27	+	LCre	LC
1.5	33	+	LCre	LC
2.0	60	++	LCre	LC
2.5	67	++	LCre	LC
3.0	87	+++	LCre	LC
3.5	80	++	Cre	C
4.0	73	++	Cre	C
NAA + BA				
3.0 + 0.5	93	+++	WBr	C
3.0 + 1.0	87	+++	WBr	C
3.0 + 1.5	80	++	WBr	C
3.0 + 2.0	67	++	WBr	C
NAA + BA				
4.0+ 0.5	80	++	WBr	C
4.0+ 1.0	67	++	WBr	C
4.0+ 1.5	60	++	WBr	C
4.0+ 2.0	60	++	WBr	C
NAA + BA				
5.0 + 0.5	67	++	WBr	C
5.0 + 1.0	60	++	WBr	C
5.0 + 1.5	47	+	WBr	C
5.0 + 2.0	33	+	WBr	C

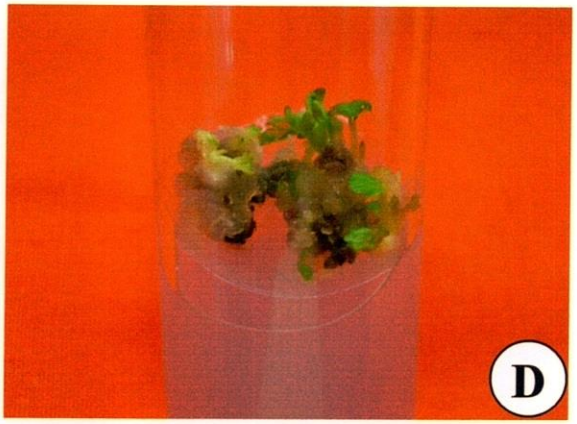
\+ = Little callusing (0-50 %)
++ = Moderate callusing (51-80 %)
+++ = Highly callusing (81-100 %)
LCre = Light Creamy
Cre = Creamy
WBr = White Brown
C = Compact
LC = Loosely Compact

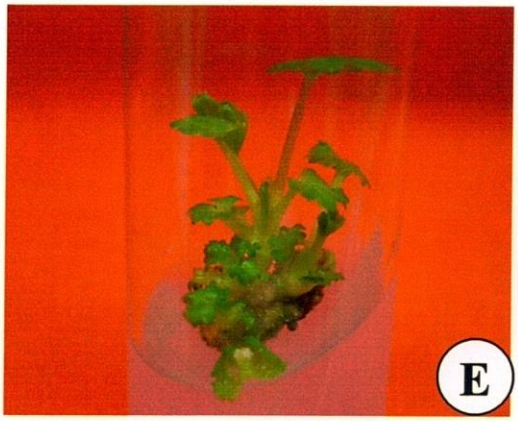
The combination of NAA and BA in MS medium also found effective in callus induction from the leaf explant of strawberry. Callus induction was noticed in all NAA and BA formulation although a certain degree of variation in terms of was observed % of explants induced callus, degree of callus development, colour and nature of calli and adventitious shoot regeneration. The highest (93%) explant induced callus formation in the culture medium containing 3.0 mg/l NAA and 0.5 BA (**Plate 02, Figures A & B**). Whereas, only 33% explants developed callus when the explants were cultured on medium containing 5.0 mg/l NAA and 2.0 mg/l BA. Moderate degree of callusing was recorded in almost all combinations. Callus growth was less prolific in NAA and BA supplemented culture medium in comparison to 2,4-D and BA formulations. The calli developed in NAA – BA media formulations were white-brown in colour and compact in nature. Adventitious shoot formation from leaf segment derived callus was not observed in all the NAA – BA combination tested in primary culture.

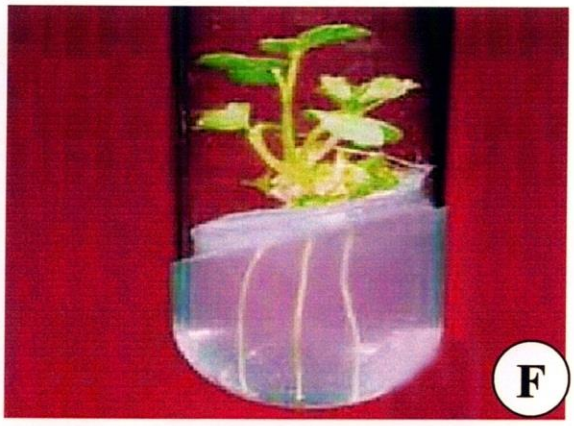
PLATE 2











3.3. PLANT REGENERATION THROUGH ORGANOGENESIS

The calli developed from leaf segments in different culture media formulations were subcultured for plant regeneration. The calli were rescued aseptically on a sterile petridish and cut into convenient sizes by sterile scalpel. The callus pieces were sub-cultured onto MS medium supplemented with different concentrations of BA either singly or in combination with NAA (considered as regeneration media) and the cultures were incubated in light for 8 weeks. The calli proliferated in different callus induction media were subcultured separately on to regeneration media. Data recorded on morphogenic dedifferentiation of the calli in regeneration media, 8-weeks after sub-culture. The results on the effect of both callus induction and shoot regeneration media on % of calli induced shoot regeneration and no. of multiple shoots/callus are presented in **Tables 7, 8, 9** and **10**.

PGR formulations in both preculture and subculture media showed pronounced effect on shoot organogenesis from strawberry leaf derived callus. The calli developed from leaf tissues in 2,4-D supplemented callus induction media showed very little organogenic response (**Table 7**). The calli proliferated in 2,4-D when subcultured on to regeneration medium having only BA, they were failed to survive and senesced within 20-25 days of culture. The calli in BA supplemented regeneration medium were gradually become brownish in colour and finally became necrotic and died. It was observed that the culture calli were survived and continued to new tissue proliferation on to the regeneration medium contained both BA – NAA. Adventitious shoot formation was only observed in regeneration media having 0.5 mg/l BA with 0.1 mg/l NAA and 1.5 mg/l BA with 0.5 mg/l NAA. In regeneration media contained 1.5 mg/l BA

Table 7: Effect of callus induction and regeneration media on shoot regeneration from leaf derived strawberry callus (in each treatment 15 calli were subcultured on to regeneration media).

PGR supplements in callus induction medium	PGR supplements in shoot regeneration medium (mg /l)	Morphogenic response after 5-weeks of subculture	
		% of calli induced shoot regeneration	No. of multiple shoots/callus
2,4-D	BA		
	0.1	—	—
	0.5	—	—
	1.0	—	—
	1.5	—	—
	2.0	—	—
	BA+NAA		
	0.5 + 0.1	7	3.8
	0.5 + 0.5	—	+
	0.5 + 1.0	—	+
	0.5 + 1.5	—	+
	0.5 + 2.0	—	+
	BA+NAA		
	1.0 + 0.1	—	+
	1.0 + 0.5	—	+
	1.0 + 1.0	—	+
	1.0 + 1.5	—	+
	1.0 + 2.0	—	+
	BA+NAA		
	1.5 + 0.1	—	+
	1.5 + 0.5	13	2.5
	1.5 + 1.0	—	+
	1.5 + 1.5	—	+
	1.5 + 2.0	—	+
	BA+NAA		
	2.0 + 0.1	—	+
	2.0 + 0.5	—	+
	2.0 + 1.0	—	+
	2.0 + 1.5	—	+
	2.0 + 2.0	—	+

— = No response

+ = New callus tissue proliferation

Table 8: Effect of callus induction and regeneration media on shoot regeneration from leaf derived strawberry callus (in each treatment 15 calli were subcultured on to regeneration media).

PGR supplements in callus induction medium	PGR supplements in shoot regeneration medium (mg /l)	Morphogenic response after 5-weeks of subculture	
		% of calli induced shoot regeneration	No. of multiple shoots/ callus
2,4-D+BA	BA		
	0.1	—	—
	0.5	—	—
	1.0	—	—
	1.5	—	—
	2.0	—	—
	BA+NAA		
	0.5 + 0.1	7	2.6
	0.5 + 0.5	—	+
	0.5 + 1.0	—	+
	0.5 + 1.5	—	+
	0.5 + 2.0	—	+
	BA+NAA		
	1.0 + 0.1	27	3.5
	1.0 + 0.5	13	3.8
	1.0 + 1.0	—	+
	1.0 + 1.5	—	+
	1.0 + 2.00	—	+
	BA+NAA		
	1.5 + 0.1	20	1.8
	1.5 + 0.5	20	3.4
	1.5 + 1.0	—	+
	1.5 + 1.5	—	+
	1.5 + 2.0	—	+
	BA+NAA		
	2.0 + 0.1	—	+
	2.0 + 0.5	—	+
	2.0 + 1.0	—	+
	2.0 + 1.5	—	+
	2.0 + 2.0	—	+

— = No response
+ = Proliferation of new callus tissues

with 0.5 mg/l NAA, 13% of the subcultured calli induced to develop adventitious shoots. In rest of the BA-NAA supplemented regeneration media, no shoot formation was observed other than new callus tissue development.

Organogenic potential of those calli proliferated in 2,4-D – BA supplemented callus induction medium were also determined by culturing them in BA – NAA containing regeneration media (**Table 8**). It was observed that regeneration potential of the calli proliferated in callusing media containing 2,4-D – BA was also very low. The calli when subcultured on to only BA supplemented, regeneration were failed to perpetuate and senesced within 2 – 3 weeks of culture. However, the cultured calli were induced to proliferate shoots in those regeneration media containing both BA and NAA and concentration of BA always higher than NAA. In regeneration media having either equal or higher concentration of NAA than BA, the cultured calli were induced to proliferate new callus tissues without showing any sign of organogenesis. Organogenic response of the calli developed in 2,4-D – BA supplemented callusing media were induced to develop adventitious shoots when cultured on to regeneration media supplemented with 0.5 + 0.1, 1.0 + 0.1, 1.0 + 0.5, 1.5 + 0.1 and 1.5 + 0.5 mg/l BA + NAA. Among these BA – NAA formulations the highest 27% calli induced adventitious shoot proliferation. Average no. of shoots/callus was low (Range: 1.8–3.8 shoots/callus). Somatic embryo formation or root development was not observed in any of the regeneration media formulations.

The calli, proliferated in NAA supplemented callus induction media were separately subcultured on to BA – NAA fortified regeneration media (**Table 9**). The calli in regeneration media contained

Table 9: Effect of callus induction and regeneration media on shoot regeneration from leaf derived strawberry callus (in each treatment 15 calli were subcultured on to regeneration media).

PGR supplements in callus induction medium	PGR supplements in shoot regeneration medium (mg /l)	Morphogenic response after 5-weeks of subculture	
		% of calli induced shoot regeneration	No. of multiple shoots/ callus
NAA	BA		
	0.1	—	—
	0.5	—	—
	1.0	—	—
	1.5	—	—
	2.0	—	—
	BA+NAA		
	0.5 + 0.1	7	3.2
	0.5 + 0.5	—	+
	0.5 + 1.0	—	+
	0.5 + 1.5	—	+
	0.5 + 2.0	—	+
	BA+NAA		
	1.0 + 0.1	13	4.8
	1.0 + 0.5	13	2.7
	1.0 + 1.0	—	+
	1.0 + 1.5	—	+
	1.0 + 2.0	—	+
	BA+NAA		
	1.5 + 0.1	33	5.4
	1.5 + 0.5	27	4.5
	1.5 + 1.0	—	+
	1.5 + 1.5	—	+
	1.5 + 2.0	—	+
	BA+NAA		
	2.0 + 0.1	—	+
	2.0 + 0.5	—	+
	2.0 + 1.0	7	2.3
	2.0 + 1.5	—	+
	2.0 + 2.0	—	+

— = No response
+ = Proliferation of new callus tissues

Table 10: Effect of callus induction and regeneration media on shoot regeneration from leaf derived strawberry callus (in each treatment 15 calli were subcultured on to regeneration media).

PGR supplements in callus induction medium	PGR supplements in shoot regeneration medium (mg /l)	Morphogenic response after 5-weeks of subculture	
		% of calli induced shoot regeneration	No. of multiple shoots/ callus
NAA+BA	BA		
	0.1	33	1.9
	0.5	40	1.5
	1.0	26	1.1
	1.5	—	—
	2.0	—	—
	BA+NAA		
	0.5 + 0.1	20	5.8
	0.5 + 0.5	—	+
	0.5 + 1.0	—	+
	0.5 + 1.5	—	+
	0.5 + 2.0	—	+
	BA+NAA		
	1.0 + 0.1	27	6.2
	1.0 + 0.5	33	7.6
	1.0 + 1.0	—	+
	1.0 + 1.5	—	+
	1.0 + 2.0	—	+
	BA+NAA		
	1.5 + 0.1	60	12.4
	1.5 + 0.5	73	14.3
	1.5 + 1.0	53	3.5
	1.5 + 1.5	—	+
	1.5 + 2.0	—	+
	BA+NAA		
	2.0 + 0.1	40	6.2
	2.0 + 0.5	33	4.8
	2.0 + 1.0	47	8.5
	2.0 + 1.5	—	+
	2.0 + 2.0	—	+

— = No response
\+ = Proliferation of new callus tissues

0.1 – 2.0 mg/l BA ware failed to survive and to show any morphogenic response. The calli proliferated in NAA supplemented callusing media, when subculture on to BA – NAA supplemented regeneration media were continued new tissue proliferation along with organogenic dedifferentiation. It was observed that the calli under went shoot organogenesis in the regeneration media contained higher amount of BA than NAA. Regeneration media contained equal concentration of BA – NAA or higher concentration NAA induced the calli to proliferate new callus tissues without any organogenesis. Among 20 BA-NAA combinations adventitious shoot proliferation was noticed in 0.5+0.1, 1.0+0.1, 1.0+0.5, 1.5+0.1, 1.5+0.5 and 2.0+1.0 mg/l BA – NAA formulations. Among these formulations the highest 33% subcultured calli under went shoot organogenesis. Shoot proliferation per callus was ranged from 2.3 – 5.4 shoots/calli.

The calli proliferated in NAA – BA supplemented callusing media were also transferred on to same regeneration media for plant regeneration (**Table 10**). The calli developed in NAA – BA supplemented callusing media showed higher degree of organogenic potential than those developed in other callusing media formulations. The regeneration media contained low concentration of BA alone (0.1 – 1.0 mg/l) were induced the cultured calli to proliferate adventurous shoots. Higher concentrations of BA (1.5 and 2.0 mg/l) were found unsuitable for the proliferation of adventitious shoots and callus growth. Adventitious shoot regeneration was also noticed in the regeneration media contained both BA – NAA but BA – NAA ratio was also found to be critical for organogenic dedifferentiation. The regeneration media contained higher concentration of BA than NAA induced the calli to develop adventitious shoots, whereas, the cultured calli induced new callus tissues

when the regeneration media contained higher concentration of NAA than BA. Among the different regeneration media formulations tested here 1.5 mg/l BA with 0.5 mg/l NAA was the most effective formulation where the highest 73% calli were induced adventitious shoot proliferation (**Plate 02, Figure C & D**). The highest average numbers of 14.3 shoots/callus was also recorded in this medium formulation. The regeneration medium having 1.5 mg/l BA with 0.1 mg/l NAA was also observed effective in shoot regeneration from the calli developed in BA – NAA supplemented callusing media. 60% calli showed shoot regeneration with 12.4 shoots /callus.

3.4. PLANT REGENERATION THROUGH SOMATIC EMBRYOGENESIS

Somatic embryogenesis was achieved from the callus from leaf segments of strawberry. Somatic embryogenesis was noticed from after repeated sub-culture of the calli onto either corresponding same callus indication media or different media formulation. Data were recorded on the morphogenic differentiation of the incubated calli in media formulations are given in **Table 11** and **12**.

3.4.1. Effect of 2,4-D in MS Medium on Somatic Embryo Induction

The effect of 2,4-D alone on somatic embryogenesis from leaf segment derived calli were studied. No somatic embryo formation was noticed when the leaf segment derived calli were repeated subculture with different combination of 2,4-D.The cultured calli were continued to new tissue proliferation (**Table 11**).

Table 11: Effect of different concentration of 2,4-D either alone or combination with BA and Proline in MS medium on somatic embryogenesis. Data were recorded 20 weeks after culture inoculation.

Culture media composition (mg/l)	% of calli induced somatic embryos	No. of embryos/explant (Mean ± SE)
2,4-D		
0.5	–	–
1.0	–	–
1.5	–	–
2.0	–	–
2,4-D+BA		
1.0+0.5	13	2.0±0.99
1.0+1.0	–	–
1.0+1.5	–	–
1.0+2.0	–	–
2,4-D+BA+Proline		
1.0+0.5+5%	20	3.6±0.56
1.0+0.5+10%	40	6.0±0.55
1.0+0.5+25%	67	10.3±0.8
1.0+0.5+50%	80	13.6±1.0

3.4.2. Effect of 2,4-D with BA in MS medium on Somatic Embryo Induction

Somatic embryo formation was noticed when the leaf and internode segment derived calli were repeated subculture with 1.0 mg/l 2,4-D with different combinations of BA. Data were recorded 20 weeks after 1st inoculation and the results were represented in **Table 11**.

Among the different combination of 2, 4-D with BA, only 13% calli were found to became embryogenic in 1.0 mg/l 2,4-D + 0.5 mg/l BA from leaf segments derived callus. 2.0 ± 0.99 embryos/callus were recorded in this medium.

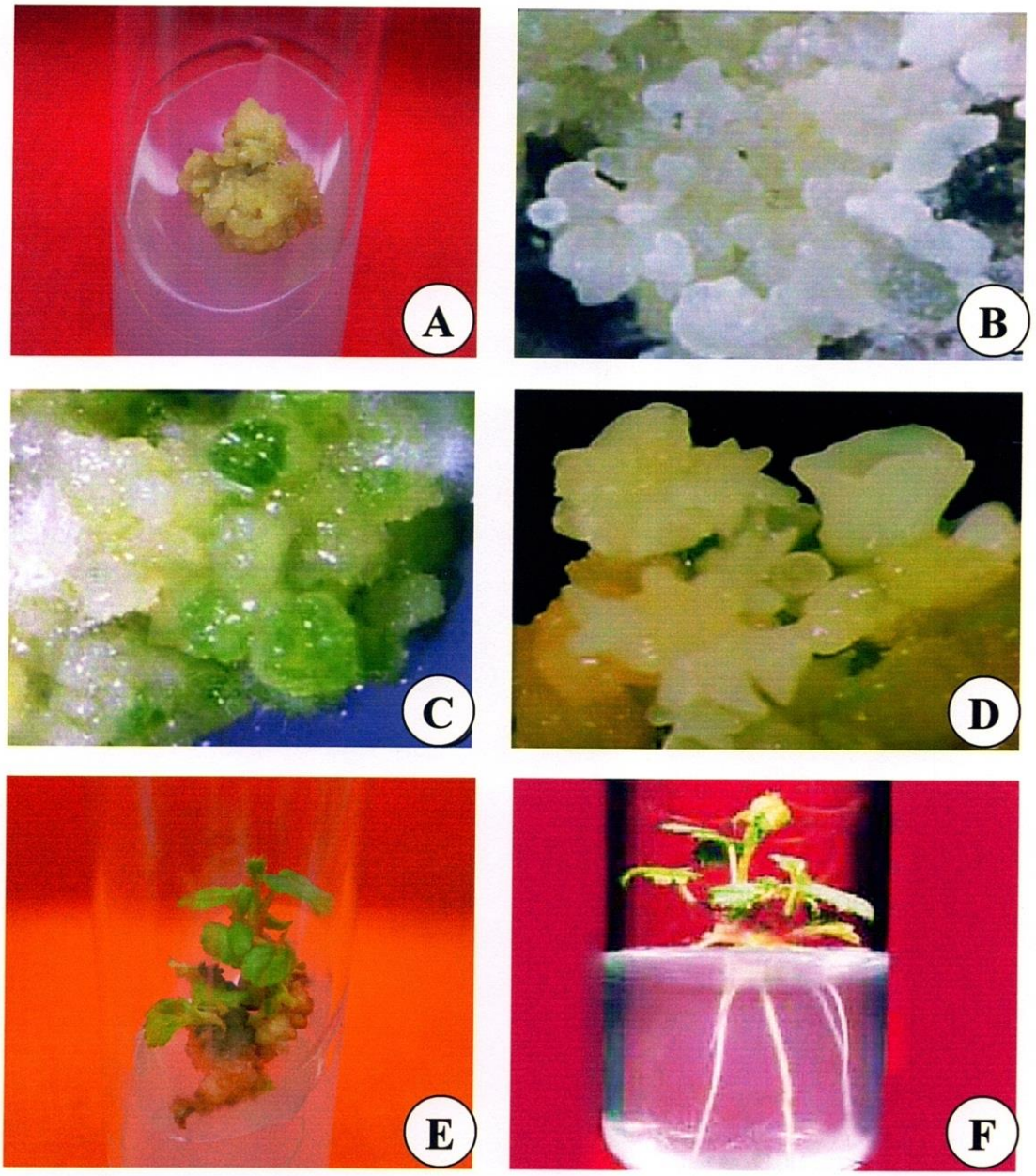
3.4.3. Effect of 2,4-D with BA and Proline on Somatic Embryo Induction

For embryo formation, leaf segment derived calli were sub culture with different concentration of proline in combination with 2,4-D and BA. The results on the morphogenic differentiation of the incubated calli are given in **Table 11**.

When leaf segment derived calli were sub cultured, the highest 80% calli were found became embryogenic in 1.0 mg/l 2,4-D + 0.5 mg/l BA + 50% proline (**Plate 03, Figure B**). Whereas the lowest 20% calli induced to became embryogenic with 1.0 mg/l 2,4-D + 0.5 mg/l BA + 5% proline supplemented medium.

The numbers of embryos were varied with different concentrations of proline. Among the different concentration of proline, the highest 13.6 ±1.0 embryos/called were found in 1.0 mg/l 2,4-D + 0.5 mg/l BA + 50% proline supplemented medium.

PLATE 3



3.4.4. Effect of NAA in MS Medium on Somatic Embryo Induction

The effect of NAA alone on somatic embryogenesis from leaf and internode segment derived calli were also studied (**Table 12**). No somatic embryo formation was noticed when the leaf segment derived calli were repeated subculture with different combination of NAA.

3.4.5. Effect of NAA with BA in MS medium on Somatic Embryo Induction

The calli proliferated in NAA when subcultured on to medium having NAA with different concentration of BA. The cultured calli were continued to new tissue proliferation. No somatic embryo formation was noticed when the leaf segment derived calli were repeated subculture with 1.0 mg/l NAA with different combination of BA (**Table 12**).

3.4.6. Effect of NAA with BA and Proline on Somatic Embryo Induction

Leaf segment derived calli were subcultured with the different concentrations of proline combination with 1.0 mg/l NAA + 0.5 mg/l BA to see their effect for somatic embryo induction. Data were recorded 20 weeks after 1st culture inoculation and the response of leaf and internode segment explants of these medium are shown in **Table 12**.

Among the different concentration of proline with NAA + BA, the highest 60% leaf segment derive calli were found to became embryogenic in 1.0 mg/l NAA + 0.5 mg/l BA + 50% proline supplemented medium. Whereas, the lowest 13% calli were become embryogenic in 1.0 mg/l NAA + 0.5 mg/l BA +5% proline. The highest 8.0 ±0.60 embryos/callus was recorded at 1.0 mg/l NAA + 0.5 mg/l BA + 50% proline.

Table 12: Effect of different concentration of NAA either alone or combination with BA and Proline in MS medium on somatic embryogenesis. Data were recorded 20 weeks after 1st culture inoculation.

Culture media composition (mg/l)	% of calli induced somatic embryos	No. of embryos/explant (Mean ± SE)
NAA		
0.5	–	–
1.0	–	–
1.5	–	–
2.0	–	–
NAA+BA		
1.0+0.5	–	–
1.0+1.0	–	–
1.0+1.5	–	–
1.0+2.0	–	–
NAA+BA+Proline		
1.0+0.5+5%	13	4.0±0.31
1.0+0.5+10%	33	5.3±0.33
1.0+0.5+25%	47	6.0±0.39
1.0+0.5+50%	67	8.0±0.60

– = No response

3.5. EMBRYO GERMINATION AND PLANTLET DEVELOPMENT

All the calli with induced embryos were subcultured onto MS medium with GA_3 and KIN either singly or with combination of various concentrations of auxin for germination of embryos. Observation were made on the different types of morphogenic response of embryos were recorded after 6 weeks of culture and the result are shown in **Table 13 and Plate 03 (Figure C & D)**.

The embryos were started to germinate with in 2-3 weeks after transfer. The germination was characterized by gradual greening and enlargement of cotyledons. The percentage of calli formed plantlets, number of plantlets per callus and length of the longest shoot and root were recorded after 4 weeks of culture on germination medium.

3.5.1. Effect of GA_3 on Embryo Germination

In semi-solid medium embryos were cultured with three different concentrations of GA_3 to see their effect for plantlet formation (**Table 13**). Among the different concentrations of GA_3, the highest 5.3 number of plantlets, the length of the longest shoot and root were 7.2 and 0.7 cm found at 0.1 mg/l GA_3 supplemented medium.

3.5.2. Effect of GA_3 with NAA and IBA on Embryo Germination

The developmental sequence of embryo into plantlets was strongly influenced by the GA_3 concentrations in MS medium. Plantlets were appeared from embryos in all the GA_3 + NAA combination. The response of embryos on these medium are shown in **Table 13**. In GA_3 and NAA combinations, the highest number of plantlets/callus was 6.0 in medium supplemented with 0.1 mg/l GA_3 + 0.1 mg/l NAA. Whereas, the lowest number of plantlets per callus were observed in medium with 0.1 mg/l

GA_3 + 1.0 mg/l NAA. Besides this, the highest length of the longest shoot and root 7.3 and 1.7 cm were noticed in medium supplemented with 0.1 mg/l GA_3 + 0.1 mg/l NAA.

The experiment was conducted to investigate the effect of different combination of IBA with GA_3 in MS semi solid medium on embryo germination and plantlets formation. Results on the effect of these formulations are presented in **Table 13**. Among the different combinations of GA_3 + IBA the highest 7.9 number of plantlets/callus was appeared in medium containing with 0.1 mg/l GA_3 + 0.1 mg/l IBA. Whereas, the lowest number of plantlets/ callus was noticed in medium supplemented with 0.1 mg/l GA_3 + 1.0 mg/l IBA (**Plate 03, Figure E**). The optimum length of the longest shoot and root were found 8.3 and 2.1 cm in medium supplemented with 0.1 mg/l GA_3 + 1.0 mg/l IBA.

3.5.3. Effect of KIN on Embryo Germination

The average number of plantlets formation per culture was also varied in different concentrations of KIN medium also. Observation were made after 28 days and the morphogenic response of embryos are tabulated in **Table 13**.

Among the different concentrations of KIN, the highest (4.9) number of plantlets/callus and the highest length of the longest shoot and root 5.1 and 0.6 were obtained at 0.5 mg/l KIN. Whereas, the lowest number of plantlets per callus and the lowest length of the shoot and root 4.0 and 0.8 cm were found in 1.0 mg/l KIN supplemented medium.

Table 13: Effect of different concentration and combination of GA_3, KIN, NAA and IBA in MS medium on embryo germination and plantlet formation. Data were recorded 6 weeks after culture inoculation.

culture medium composition(mg/l)	Nos. of plantlet /callus	Length of the longest shoot	Length of the longest root
GA3			
0.1	5.3	7.2	0.7
0.5	4.8	5.8	0.4
1.0	4.5	5.3	0.6
KIN			
0.1	4.4	4.8	0.8
0.5	4.9	5.1	0.6
1.0	3.5	4.0	0.5
GA3+NAA			
0.1+0.1	6.0	7.3	1.7
0.1+0.5	5.1	7.0	1.8
0.1+1.0	4.7	6.4	1.4
GA3+IBA			
0.1+0.1	7.9	8.3	2.1
0.1+0.5	6.4	7.9	1.8
0.1+ 1.0	6.1	7.3	1.8
KIN+NAA			
0.5+0.1	5.8	5.2	1.5
0.5+0.5	5.6	4.8	1.2
0.5+1.0	3.8	4.1	0. 8
KIN+IBA			
0.5+0.1	4.8	4.9	0.9
0.5+0.5	4.5	4.5	0.6
0.5+1.0	3.5	3.0	0.7

3.5.4. Effect of KIN with NAA and IBA on Embryo Germination

Embryos germination and plantlet formation were noticed when all kinds of embryos were cultured in MS semi-solid medium supplemented with KIN plus different concentrations of NAA. The highest number of plantlets, length of the longest shoot and root were occurred 5.8, 5.2 cm and 1.5 cm respectively in 0.5 mg/l KIN + 0.1 mg/l NAA supplemented medium. Whereas, the lowest number of plantlets, length of the shoot and root were noticed in medium containing with 0.5 mg/l KIN + 1.0 mg/l NAA.

Embryo germination and plantlet formation was also appeared from embryos in all the combination of KIN + IBA culture medium. Morphological changes in germinating embryos are shown in **Table 13**. Among the different combination of KIN with IBA, the highest 4.8 number of plantlets/callus was found in medium supplemented with 0.5 mg/l KIN + 0.1 mg/l IBA. Whereas, the lowest number of plantlet/callus were 3.5 in medium containing with 0.5 mg/l KIN + 1.0 mg/l IBA. The highest length of the longest shoot and root was found in medium at the level of 0.5 mg/l KIN with 0.1 mg/l IBA.

3.6. SHOOT MULTIPLICATION FROM THE REGENERATED SHOOT THROUGH MERISTEM CULTURE, ORGANOGENESIS AND SOMATIC EMBRYOGENESIS

In this experiment the regenerated shoots were sub-cultured onto MS agar gelled semi-solidified medium with different combination and concentration of Auxin and Cytokinin. The percentage of multiple shoot forming explants, average number of longest shoot per explant and shoot length were considered as parameters for evaluating the results and data on these parameters were recorded after 21 and 42 days of culture are shown in **Table 14**.

In BA with NAA combination, 50% explants were responded to develop multiple shoots when they were subcultered in medium containing at the level of 1.5mg/l BA+1.0mg/lNAA. Maximum numbers of shoots/culture was 4.0±0.96 after 21 days and 4.5±0.95 after 42 days in this medium. Besides, the highest length of the longest shoot was observed 6.5±0.41 after 21days and7.8±0.43 after 42 days in this medium.

Among the different concentration of KIN with BA, the highest 80% explants were responded to develop multiple shoots in medium with 1.5mg/l BA+0.5 mg/l KIN **(Plate 01, Figure D & E; Plate 02, Figure E)**. The highest number of shoots/culture was 9.8±1.2 after 21 days and11.5±1.8 after 42 days of culture and the maximum length of the longest shoot was observed 8.9±0.71 after 21days and 10.8±0.63 after 42 days in this medium.

In medium with combination of 1.0mg/l KIN+0.5 mg/l NAA and 1.0mg/l KIN+1.0 mg/l NAA, the highest 40% explants formed multiple shoots but the maximum number of shoots/culture was 4.8±0.94 after 21 days and 4.6±1.0 after 42 days of culture, the highest length of the longest shoot was recorded 5.4±0.54 after 21days and 6.0±0.67 after 42 days in this medium.

Among the different concentration of 2,4-D with BA, 60% explants were responded to develop multiple shoots in medium with 1.0mg/l 2,4-D +0.5 mg/l BA and. The highest length of the longest shoot was 6.0±0.43 after 21days and 7.0±0.47 after 42 days and in medium at 1.0mg/l 2,4-D +1.0 mg/l BA, number of shoots/culture was observed 4.8±1.0 and 5.8±1.1 after 21 days, after 42 days of culture.

Table 14: Effect of different types and concentration of cytokinin and auxin on multiple shoot proliferation from regenerated shoot through meristem culture, organogenesis and somatic embryogenesis. Data were recorded 21 and 42 days after culture inoculation.

Culture medium composition (mg/l)	% of multiple shoot forming explant	No. of shoots/explant		Length of the longest shoot	
		After 21 days Mean ± SE	After 42 days Mean ± SE	After 21 days Mean ± SE	After 42 days Mean ± SE
BA+NAA					
1.5+0.1	40	2.0±0.57	2.3±0.66	4.2±0.34	5.3±0.69
1.5+0.5	30	2.3±1.3	3.0±1.2	4.0±0.32	5.1±0.33
1.5+1.0	50	4.0±0.96	4.5±0.95	6.5±0.41	7.8±0.43
1.5+1.5	30	2.0±0.57	3.0±0.1	3.0±0.12	4.3±0.08
1.5+2.0	20	2.0±0.7	3.6±0.86	4.9±0.53	5.6±0.39
KIN+NAA					
1.0+0.1	30	3.0±0.91	2.8±1.1	4.4±0.66	5.2±0.68
1.0+0.5	40	4.8±0.94	4.6±1.0	5.4± 0.54	6.0±0.67
1.0+1.0	40	3.4±1.0	4.4±1.0	3.7±1.2	4.8±1.2
1.0+1.5	30	3.3±1.1	4.3±1.0	4.4±0.66	5.2±0.39
1.0+2.0	30	3.8±1.0	4.8±1.2	4.5±0.72	5.5±0.42
BA+KIN					
1.5+0.1	60	6.3±0.96	7.6±1.2	8.4±0.34	9.7±0.23
1.5+0.5	80	9.8±1.2	11.5±1.8	8.9±0.71	10.8±0.63
1.5+1.0	70	8.0±1.2	9.6±1.2	7.2±0.22	9.3±0.47
1.5+1.5	60	5.8±1.1	6.3±1.1	6.6±0.40	8.2±0.47
1.5+2.0	60	5.1±0.66	6.0±0.86	5.5±0.49	7.0±0.43
2,4-D+BA					
1.0+0.1	50	4.5±0.95	5.5±0.64	5.1±0.60	6.5±0.53
1.0+0.5	60	4.8±1.0	5.8±1.1	5.6±0.94	6.2±0.94
1.0+1.0	60	5.3±0.75	5.7±1.0	6.0±0.43	7.0±0.47
1.0+1.5	50	2.3±0.66	3.3±1.1	5.7±0.90	6.2±0.92
1.0+2.0	40	2.6±1.0	4.0±1.3	5.6±0.78	6.0±0.53
2,4-D+KIN					
1.0+0.1	30	2.5±1.0	3.5±0.5	4.3±0.53	5.0±0.38
1.0+0.5	30	2.8±0.01	4.8±0.25	4.5±0.53	5.2±0.42
1.0+1.0	50	4.3±1.1	5.1±1.1	4.7±0.66	5.4±0.39
1.0+1.5	60	5.3±1.0	5.5±0.64	5.4±0.63	6.1±0.53
1.0+2.0	40	3.8±1.0	4.2±1.2	4.3±0.72	4.9±0.42

In medium with combination of 1.0mg/l 2,4-D +1.5 mg/l KIN, the highest 60% explants formed multiple shoots and the maximum numbers of shoots/culture was 5.3±1.0 after 21 days, 5.5±0.64 after 42 days. The highest length of the longest shoot was recorded 5.4±0.63 after 21days and 6.1±0.53 after 42 days in this medium. In all of the tested formulations, 1.5mg/l BA +0.5mg/l KIN was the best formulation for shoot multiplication.

3.7. ROOT INDUCTION

Regenerated shoot needed root induction to grow into plantlets and to establish them into soil. To induce root, individual shoots proliferated in regeneration media from the calli developed in different callusing media, were excised and cultured in full strength MS and half strength MS (½MS) media with or without different PGR formulation (IBA and NAA). Data were recorded on percentage (%) of shoots induced root development, percentage (%) of shoots induce root and callus development and number of roots/shoot after 4 weeks of culture, and the results are presented in **Table 15**. For all the cases, 3 - 4 cm. long shoots obtained from various experiments were used for *in vitro* rooting experiments.

In vitro regenerated micro shoots of strawberry were inoculated in MS and ½MS media without plant growth regulators (MS_0 and $½MS_0$). Cent percent cultured shoot induce to develop roots when cultured in MS_0 rooting medium within 7-12 days of inoculation. Whereas, 93% shoots were induced root development in $½MS_0$ rooting medium. The highest mean number (13.0±2.3) of roots/shoot was found after 42 days of culture in MS_0 media. No callus initiation with root was observed in these rooting media formulation.

Micro shoots were inoculated in full strength MS supplemented with each of NAA and IBA alone (0.1 mg/l, 0.5 mg/l and 1.0 mg/l) [**Table 15**]. Among the different formulations of NAA and IBA tested, 87% cultured shoots induce to develop root when inoculated in full strength MS containing 0.1 mg/l IBA. However, 40% shoots along with root also induced callus formation in this rooting media formulation. Root formation along with callus proliferation was also noticed in other NAA and IBA supplemented rooting media.

Micro shoots were also cultured in half strength MS medium supplemented with each of NAA and IBA alone (0.1 mg/l, 0.5 mg/l and 1.0 mg/l) [**Table 15**]. Among the different NAA and IBA formulation the highest 83% of shoots induced to develop roots with 0.1 mg/l IBA. The highest number of roots/shoot (8.4±1.0) was recorded after 42 days of culture in the same media formulation. It was observed that IBA showed better effect on rooting of micro shoots than NAA.

From the rooting experiment, it was observed that in all the medium rooting response was well, but highest result was observed in growth regulator free full strength MS medium **(Plate 01, Figure F; Plate 02, Figure F; Plate 03, Figure F)**.

Table 15: Effect of rooting media formulation on root induction of *in vitro* regenerated strawberry shoots. Data were recorded 21 and 42 days after culture inoculation.

Medium composition	PGR formulation (mg/l)	% of root forming explants	No. of roots/explants		Length of the longest root	
			After 21 days Mean ± SE	After 42 days Mean ± SE	After 21 days Mean ± SE	After 42 days Mean ± SE
MS	–	100	7.8±1.0	13.0±2.3	2.5±0.53	2.9±0.56
1/2MS	–	93	7.6±1.5	12.8±1.4	2.0±0.18	2.5±0.16
MS+NAA	0.1	80	5.8±1.1	8.6±1.9	1.9±0.18	2.0±0.33
	0.5	73	5.1±0.93	7.3±1.8	1.9±0.5	2.3±0.30
	1.0	67	4.2±0.83	5.8±0.95	1.0±0.41	1.3±0.30
MS+IBA	0.1	87	4.9±0.83	8.2±1.3	1.4±0.23	1.8±0.5
	0.5	80	4.4±0.70	7.6±1.2	1.5±0.32	2.0±0.15
	1.0	67	3.5±0.88	6.0±1.8	1.2±0.96	1.9±0.5
1/2MS+NAA	0.1	80	5.8±1.2	7.0±1.7	1.5±0.37	1.9±0.35
	0.5	73	5.7±0.95	7.4±1.8	1.3±0.43	1.5±0.37
	1.0	60	4.4±0.96	4.5±1.5	1.0±0.95	1.4±0.34
1/2MS+IBA	0.1	83	6.0±1.7	8.4±1.0	2.1±1.3	2.3±0.55
	0.5	73	5.8±1.2	7.4±0.95	1.7±1.5	2.1±0.32
	1.0	73	4.9±2.3	7.1±1.5	1.5±1.0	2.0±0.40

– = Absence of PGR

3.8. FIELD EVALUATION

In order to evaluate the field performance of somaclonal variant, Strawberry plantlets (developed from meristem culture, leaf organogenesis and somatic embryogenesis) were gradually acclimatized in outdoor condition and finally transplanted on a specially prepared field. Data were recorded on different morphological and agronomical characters such as: plant height, nos. of leaves/plant, petiole length, nos. of runners/plant, nos. of nodes / runners, nos. of crowns/plant, canopy size, days to flowering, nos. of clusters/plant, inflorescence length (cm), nos. of flowers/cluster, nos. of fruits/plant, nos. of fruits/cluster, days to fruit harvest, average fruit wt. (gm) and fruit wt./plant (gm) at different stages of plant growth. Data recorded on plant height, nos. of leaves/plant, petiole length, nos. of runners/plant, nos. of nodes/ runners, runner length, nos. of crowns/plant and canopy size after 45-days, 75-days and 110 days of transplantation of the in vitro grown plants in the field. Variation exhibited by a particular parameter among the randomly selected 250 plants was evaluated using simple statistics of range, standard error and coefficient variability as % of mean (CV %). The results of these data are summarized in **Table 16 - Table 19.**

The results presented in **Table 16 - Table 19** reveal wide range of variation for most of the parameters. The range of data with CV% for some of the characters was increased with plant age. The range of variations as observed for the parameters plant height, no. of leaves/plant,

Table 16: Morphological characters of strawberry plant derived through meristem culture

Characters	After 45 days			After 75 days			After 110 days		
	Range	Mean ± SE	CV%	Range	Mean ± SE	CV%	Range	Mean ± SE	CV%
Plant height (cm)	3.8-9.3	6.29±0.59	29.87	5.9-16.2	9.61±1.07	35.07	13.3-39.5	26.42±2.88	34.42
No. of leaves/plant	4.1-10.7	7.22±0.71	31.19	8.5-26.8	17.63±2.06	36.96	16.2-41.4	28.29±2.69	30.03
Petiole length (cm)	2.3-8.1	4.66±0.66	44.82	3.9-14.2	8.79±1.11	39.88	5.9-17.8	12.65±1.37	34.35
No. of runners/plant	0.0-2.8	1.75±0.26	46.99	0.0-4.8	4.62±0.72	49.28	0.0-12.2	6.69±1.15	53.48
No. of Nodes/runner	1.3-1.8	1.56±0.05	10.98	1.7-4.6	3.25±0.32	31.19	2.6-7.8	5.1±0.59	36.32
Runner length (cm)	9.8-54.7	32.21±5.33	52.32	11.7-77.2	43.45±7.16	52.16	19.2-158.2	85.83±16.17	59.56
No. of crowns/plant	1.3-2.7	1.94±0.15	24.68	2.2-4.7	3.53±0.26	23.21	2.8-8.5	5.47±0.64	37.14
Canopy size (cm^2)	123.4-275.0	201.48±16.14	25.33	178.3-320.2	241.03±15.93	20.89	198.4-450.5	321.37±28.82	28.35

Table 17: Morphological characters of strawberry plant derived through organogenesis

Characters	After 45 days			After 75 days			After 110 days		
	Range	Mean ± SE	CV%	Range	Mean ± SE	CV%	Range	Mean ± SE	CV%
Plant height (cm)	2.6-7.9	4.79±0.59	37.22	4.9-14.7	9.61±1.12	36.75	15.9-28.3	21.79±1.29	18.66
No. of leaves /plant	4.2-9.8	6.41±0.61	29.86	8.8-25.8	16.83±1.78	33.48	15.9-38.5	27.19±2.34	27.27
Petiole length (cm)	2.5-7.2	4.76±0.52	34.42	3.4-13.13	8.11±1.07	41.73	4.8-17.2	11.84±1.34	35.91
No. of runners/plant	0.0-2.6	1.67±0.23	44.37	1.0-7.3	4.4±0.62	44.37	4.0-15.3	8.61±1.33	48.69
No. of nodes/runners	1.2-2.0	1.55±0.09	19.05	1.8-4.5	3.19±0.30	29.76	2.2- 7.1	4.82±0.45	29.30
Runner length (cm)	9.2-48.9	24.83±4.21	53.65	11.6-75.3	39.24±5.60	45.12	29.8-154.7	80.46±13.74	54.02
No. of crowns/plant	1.6-2.9	2.21±0.14	20.51	1.9-4.2	2.90±0.25	26.71	2.5-8.9	5.58±0.67	37.81
Canopy size (cm^2)	131.5-265.0	187.76±15.78	26.57	166.4-329.4	245.73±19.07	24.54	188.3-447.3	331.6±33.23	31.69

Table 18: Morphological characters of strawberry plant derived through somatic embryogenesis

Characters	After 45 days			After 75 days			After 110 days		
	Range	Mean ± SE	CV%	Range	Mean ± SE	CV%	Range	Mean ± SE	CV%
Plant height (cm)	2.9-7.3	5.32±0.47	27.76	5.9-13.7	9.03±0.83	36.75	16.4-29.2	22.68±1.40	19.52
No. of leaves /plant	4.8-10.0	7.5±0.59	24.86	7.9-25.0	16.63±1.96	33.48	16.3-36.1	26.01±2.29	27.79
Petiole length (cm)	2.9-7.6	4.79±0.48	31.56	4.5-14.2	9.83±1.0	41.73	6.3-18.5	11.99±1.23	32.35
No. of runners/plant	0.0-2.7	1.78±0.25	45.00	0.0-7.6	4.0±0.69	44.37	1.00-16.3	8.98±1.93	51.05
No. of nodes/runner	1.0-1.8	1.39±0.09	21.30	1.9-4.4	3.2±0.28	29.76	2.0-7.5	4.41±0.63	45.38
Runner length (cm)	10.4-52.6	34.72±4.59	41.76	12.3-78.9	48.48±6.59	45.12	18.4-163.9	85.7±13.83	68.07
No. of crowns/ plant	1.4-2.6	2.03±0.13	20.25	2.0-4.5	3.34±0.26	26.71	2.5-8.0	5.48±0.68	39.25
Canopy size (cm^2)	129.7-282.0	223.94±18.46	26.07	182.5-310.4	242.44±13.56	24.54	192.4-444.6	324.81±25.59	24.91

petiole length, no. of nodes/runner and no. of crowns/plant and canopy size recorded after 45, 75 and 110 days of plantation were not very high. The presence of noticeable range of variations was observed for no. of stolons/plant. Many of the plants did not produce any stolon at all. The highest range of variations with the highest CV% was for the parameter stolon length recorded on 45-days, 75-days and 110 days after transplantation. Plants regenerated via somatic organogenesis had a bigger stolon length while nos. of stolons was more in plants derived from meristem culture.

In case of nos. of crowns/plant, the highest CV (39.25%) was recorded in plants derived from somatic embryogenesis after 110 days of plantation. The highest CV (30.03%) of the parameter nos. of leaves/plant was found in meristem derived plants and for the parameter canopy size, the highest CV (31.69%) was recorded in the plants derived through leaf organogenesis.

Data on yield with yield contributing characters of the somaclones were recorded on 250 flowering plants and the results as standard error, range with corresponding CV% are presented in **Table 19**. Wide range of variations was also noticed for most of the parameters. The highest range (10.3-29.4) of variation was observed for average fruit weight/plant with the highest CV (57.93%) in plants derived from leaf organogenesis. Remarkable range of variations for the characters inflorescence length in meristem derived plants (range 3.9-19.6; CV- 50.26%) and in somatic embryogenesis derived plants (range 4.9-20.8; CV- 49.15%) were also recorded. Variations among the plants as observed for the characters days to flowering and days to fruit harvest did not exhibited noticeable CV%. The regenerated plantlets were subsequently evaluated for somaclonal

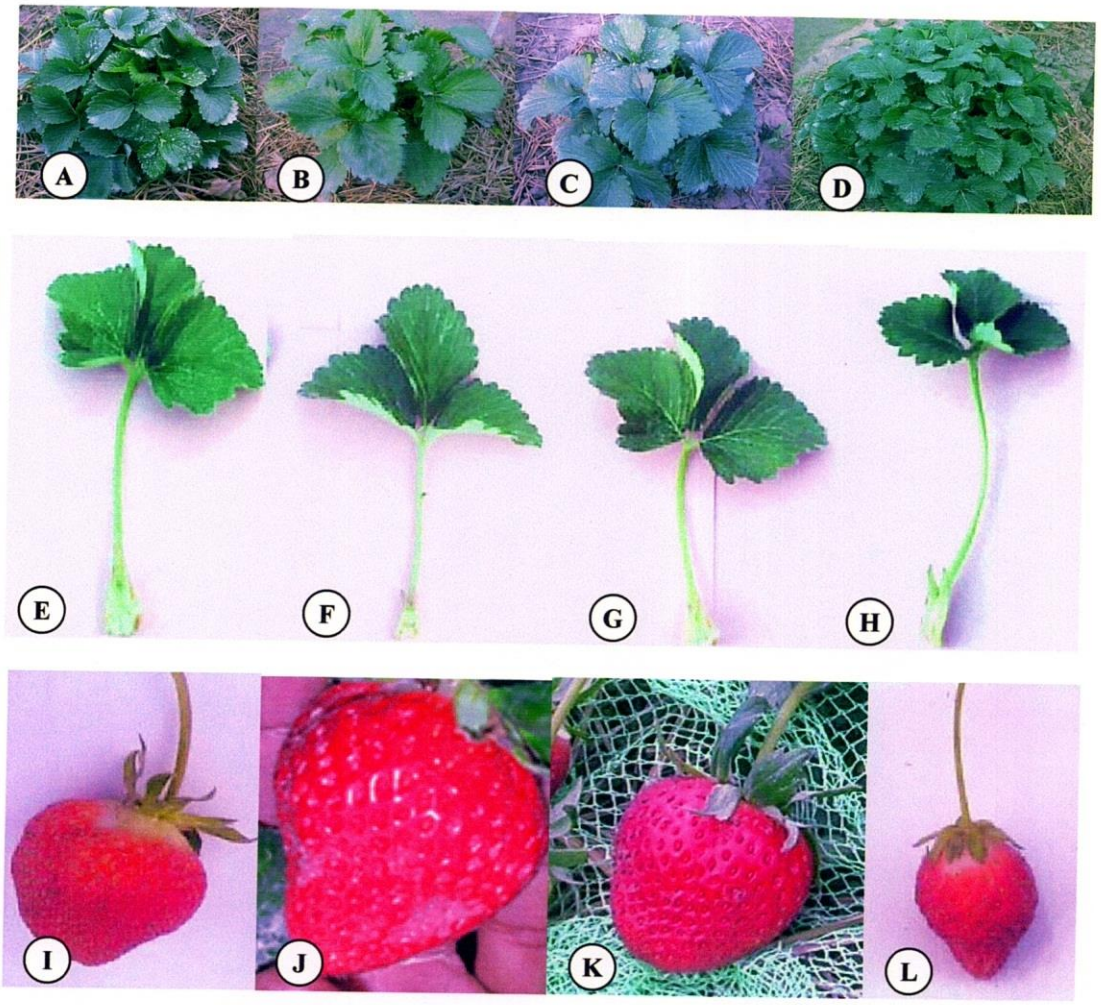
Table 19: Performance of *in vitro* produced plants derived from meristem culture, leaf organogenesis and somatic embryogenesis on the basis of fruit yield and yield contributing characters.

Characters	Methods to obtain somaclone								
	meristem culture			organogenesis			somatic embryogenesis		
	Range	Mean ± SE	CV%	Range	Mean ± SE	CV%	Range	Mean ± SE	CV%
Days to flowering	42.5-60.4	52.29±1.88	11.34	39.2-57.5	47.89±2.09	13.78	41.5-64.3	49.74±2.39	15.19
Inflorescence length (cm)	3.9-19.6	10.68±1.70	50.26	4.2-18.7	10.59±1.59	47.54	4.9-20.8	11.5±1.79	49.15
No. of flower clusters/plant	1.6-8.3	4.78±0.71	47.30	2.2-8.9	5.53±0.71	40.83	2.6-8.7	5.08±0.59	36.94
No. of flowers/cluster	2.6-7.5	4.97±0.51	32.50	1.8-8.2	5.4±0.73	43.02	2.8-7.8	5.17±0.51	30.94
No. of fruits/cluster	2.0-8.6	5.36±0.69	40.80	2.5-9.2	6.0±0.70	36.82	2.3-8.4	5.58±0.66	37.41
No. of fruits/plant	3.6-16.5	9.53±0.56	36.82	3.4-15.9	8.83±0.95	37.41	3.2-15.5	8.50±0.85	40.80
Average fruit weight/plant	10.1-25.3	18.45±2.11	44.40	10.3-29.4	19.82±1.82	57.93	10.5-25.6	19.74±2.43	47.40
Days to fruit harvest	60.6-84.1	72.17±2.26	9.92	58.2-84.5	72.29±2.65	11.60	59.3-83.2	70.01±2.40	10.84

variation under field conditions. Qualitative and quantitative traits observed in the putative somaclones. In most cases somaclones were more vigorous than control. However, different morphological characters differed in the different populations and there was no set trend. It was observed that several phenotypic differences in the *in vitro* derived plants such as leaf shape in most plants regenerated from the meristem culture, callus culture or somatic embryogenesis **(Plate-4, Figures E-H)**. Leaf petiole was shorter and thicker and lamina was comparatively bigger than control. Most of the leaves were lighter green and number of leaves was less than control. Control Plants had a significantly bigger canopy size **(Plate- 4, Figure C)** while numbers of runners were more in plants derived from meristem culture. All the *in vitro* derived plants were late to flower when compared to control. However, most of the in vitro plants had significantly more flowers per plant and number of fruits. A clear variation was observed for fruit shape among the putative somaclones **(Plate- 4, Figures I - L)**. More distinguishable variation was found in somatic embryogenesis derived somaclones, which produced bigger fruit than other somaclones and control. Also, most of the somaclones produced fruits with a different texture when compared to control fruits. Numbers of fruit from *in vitro* meristem derived plants were statistically similar to control. The average single fruit weight was also higher in these plants when compared to control.

Frequency of variant phenotypes in regenerated plant population is presented in **Table 20**. Most of the plants were similar to control. The results reveal that frequency of leaf structure variation was highest in somatic embryo derived somaclones. Flower cluster as well as fruit size variation was also highest in the plants regenerated from somatic

PLATE 4



embryogenesis. Based on the field performance initially 22 putative somaclones from the regenerated populations (**Table 20**) were selected for evaluation of their performance under field conditions. Those somaclones were selected considering the important agronomical traits such as number of flower cluster per plant, number of flower per cluster, number of fruit per cluster, average fruit weight, number of runner production, fruit shape. I decided to evaluate these somaclones for three clonal generations. The putative clonal lines were propagated through rooted runners in the nursery. The results of the field performance of selected lines revealed that 23% (5 out of 22) putative somaclonal lines did not survive in the field under varying climatic conditions. Also, 36% (8 out of 22) clonal lines had poor horticultural performance in subsequent generations and 41% (9 out of 22) reverted back to the original phenotype. On the basis of field performance three distinct somaclonal lines were selected. These selected somaclones were micropropagated to evaluate their genetic stability and subsequently re-evaluated for field performance. To examine the extent of stability of variation of the selected three clonal lines, these lines were propagated by rooted runners and evaluated for a further two successive clonal generations. Data were collected for the three important agronomic traits from the two successive clonal generations. Mean performance and analysis of variance were estimated for three agronomic important traits of three somaclonal variants and results are presented in **Table 21**. Significant differences were found among the studied colons in regards to yield contributing traits such as number of flower per plant, number of fruits per plant and average fruit weight. Mean was significantly different in the traits number of flower per plant, number of fruits per plant and average fruit weight among the studied somaclonal lines. Analysis of

Table 20: Frequency of variant phenotypes in regenerated (R_0) plants population.

Methods to obtain somaclone	Total number of regenerated plants	Leaves structure variant	Flower cluster variant	Fruit size variant	Total variant	Number of selected putative somaclones
Meristem culture	568	15(2.64)	4(0.70)	33(5.81)	52(9.15)	5
Organogenesis	673	11(1.63)	7(1.04)	34(5.05)	52(7.73)	7
Somatic embryogenesis	745	19(2.55)	11(1.48)	49(6.58)	79(10.60)	10
Total	1986	45(2.26)	22(1.11)	116(5.84)	183(9.21)	22

Numbers in parenthesis are percentage of somaclonal variants

Table 21: Field performance of selected three somaclones from three populations.

Clone	Clone derived from	No. of flower / plant	No. of fruit / plant	Average fruit weight
Variant 1	Meristem culture	18[a]	11[b]	12.18[c]
Variant 2		16[b]	10[b]	18.63[b]
Variant 3	Leaf organogenesis	12[c]	8[c]	21.29[a]
Control	Somatic embryogenesis	20[a]	13[a]	10.87[c]
Analysis of variance				
Somaclonal variants(S)		**	**	***
Clonal generation(C)		NS	NS	NS
S × C		**	**	**

Means with same letter in a column are not significantly different at $P \geq 0.05$ based on DMRT test. *** and ** significant at 0.1% and 1.0% levels, respectively; NS = non significant.

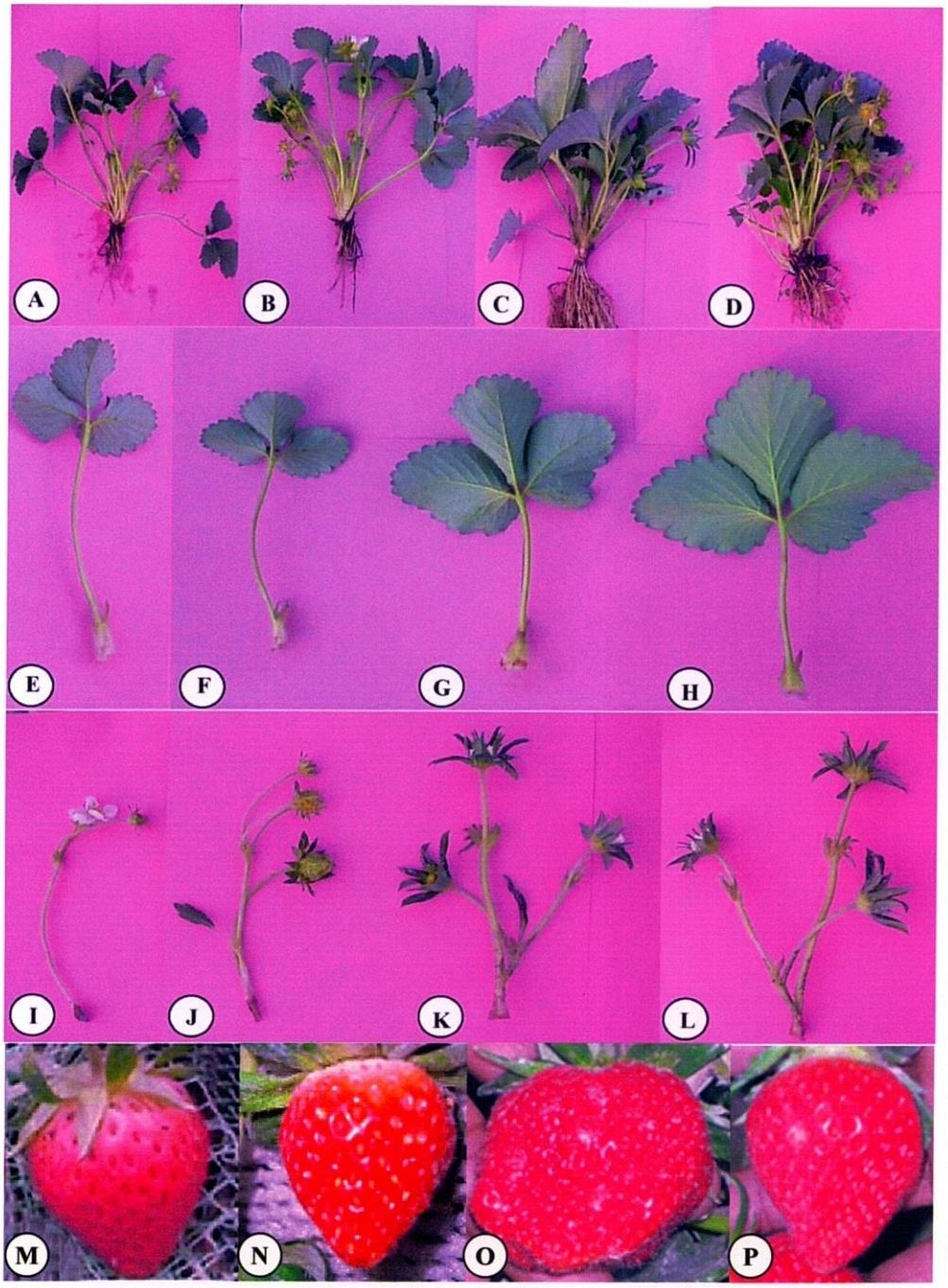
variance revealed that the somaclonal lines (S) showed highly significant difference in all the traits (**Table 21**). The clonal generations (C) had no significant difference for the studied traits. On the other hand interaction of S × C showed significant differences among all the study traits. Stable phenotypic variation was clearly observed in the leaf morphology, flower cluster branching and fruit shape (**Plate 05, Figures E - P**).

3.9. DETECTION OF GENETIC VARIATION WITH RAPD OF SELECTED SOMACLONES

RAPD is an important tool which has been extensively used to identify polymorphism among the genotypes. RAPD offers great potential for generating large number of markers representing a random sample of genome and has efficiently been used to give reliable and reproducible results for estimating the genetic variation.

In this study, RAPD was performed with three strawberry somaclones, a control and fifteen random primers, which were selected in terms of intensity and reproducibility of RAPD bands. All the primers were pretested (data not shown). Weak bands having trifling intensity and smear bands were excluded from the final analysis. DNA banding pattern following RAPD, differences were observed in the somaclonal variation among three selected somaclonal lines. Out of 15 random primers tested only eight primers successfully produced scoreable RAPD bands for all somaclonal lines. Among all fifteen primers, polymorphic banding pattern was observed with five primer and was most prominent with primers AL-04 and O-05 as indicated in **(Plate 06, Figure A & B)**. It was also observed that RAPD banding pattern of two primers (AL-04 and O-05) was something dissimilar. Bands obtained from the PCR amplified products with the primers AL-04 and O-05, are confirmed for the presence of genomic DNA in the sample.

PLATE 5



Among all bands observed in the **Figure A** (PCR amplification with the primer AL-04), band of variant 2, variant 1 and control were fairly different from the band of variant 3. Prominent and distinct bands were found at the lower portion for variant 1 and 2, which is absent in variant 3. On the other hand, at the upper position of RAPD profile, distinct and prominent band was observed at the same position of variant 3, variant 2, variant 1 and control. Although there were some bands at the same position and size, diverse bands were also found among the three variant which made them distinct from each other **(Figure A)**. Furthermore, banding pattern of the PCR amplified products of the selected clones with the primer O-05 showed that not a single band was observed at the very lower position prominent bands were documented for variant 2, variant 1 and control but for variant 3. At the same time as unique and most intense band was observed for the variant 3 which is absent in variant 2, variant 1 and control **(Figure B)**. So equivalent to the figure A, it was also noticeable from the figure B that diverse bands were also found among the three variant which made them distinct from each other. Due to the presence of genomic DNA bands in the figure A & B (amplified with primer AL-04 and O-05, respectively); it was evident that the bands of the variants were distinct to some extent among three variants. So it could be alleged that very small number of RAPD primers are sufficient to prepare genotype specific banding patterns for the identification of variants. The variation found among the studied three somaclones in terms of phenotypic characteristics were recorded as significant through morphological and statistical analysis and results obtained in the RAPD profile confirm the effectiveness and aptness of RAPD for strawberry cultivar identification and as well as researchers property rights protection.

PLATE 6

RAPD profile of primer AL-04 and O-05

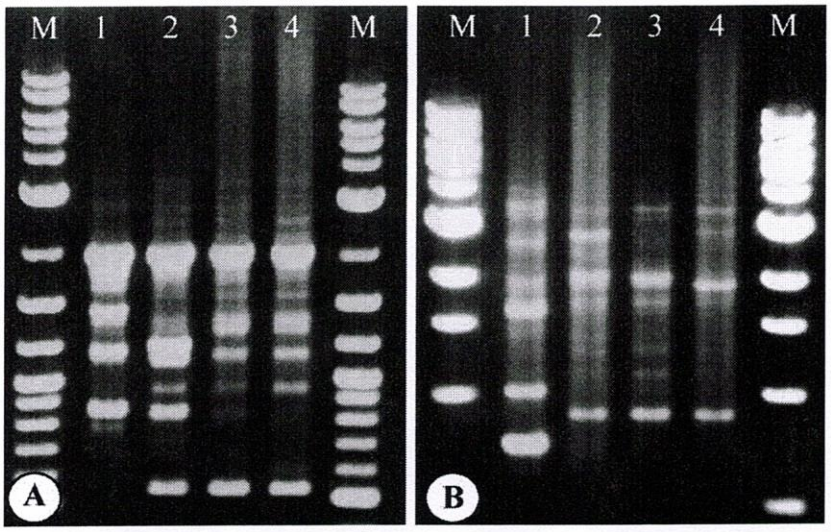


Figure A: RAPD profile generated by primer AL-04.
Figure B: RAPD profile generated by primer O-05.
M: Size marker, 1: variant 3, 2: variant 2, 3: variant 1 and 4: control.

In order to ensure the high RAPD profile reproducibility, all the experiments were carried out very consciously because RAPD does not require the DNA sequence information in advance but the DNA banding pattern might be influenced by any discrepancy during DNA extraction, purification, primer selection or PCR condition. In this study, it was observed that somaclones are distinct from each other in terms of fruit size, shape and other horticultural characters, which have also been confirmed with the DNA banding pattern of the RAPD.

Chapter IV

DISCUSSION

The strawberry (*Fragaria* × *ananassa* Duch.) is an important and popular fruit produced in temperate and sub-tropical climates and is well liked due to its fragrance, taste and nutritional properties. There are more than twenty *Fragaria* species and numerous cultivars commercially cultivated in almost thirty seven countries (Gaafar and Saker, 2006). The cultivars vary remarkably in size, color, flavor, shape, degree of fertility, season of ripening, liability to disease and constitution of plant.

Some cultivars of strawberry have been cultivated in Bangladesh but strawberry cultivation has not been flourishing in Bangladesh because there is no suitable cultivar adaptive to local agro climatic condition. The main objective of this study is to develop new cultivars adaptive to the climatic condition in Bangladesh. In the present investigation, somaclonal variation was induced via different tissue culture methods in strawberry.

In vitro tissue culture techniques have been known to be an important tool in the induction of variation leading to the development of new plant genotype (Larkin and Scowcroft, 1981; Kuksova *et al.*, 1997). This source of variability is considered as a useful tool for geneticists and plant breeders (Amzad *et al.*, 2003). In several crop species somaclonal variation was documented based on evaluation of phenotypic variation and RAPD profiles (Watanabe *et al.*, 1998; Jayanthi and Mandal, 2001; Martins *et al.*, 2004; Saker *et al.*, 2005). In strawberry, earlier studies demonstrated that somaclones could be regenerated from leaf derived callus culture (Popescu *et al.*, 1997), somatic embryogenesis (Donnoli

et al., 2001) and callus culture of leaves and petiole irradiated with gamma rays (Kaushal *et al.*, 2004). In the present investigation, somaclonal variation is induced using different tissue culture techniques, i.e. meristem culture, indirect organogenesis from leaves through callus culture and somatic embryogenesis. Three somaclones are identified on the basis of the variation of phenotypic polymorphic characters when compared to control.

Meristem culture is one of the important methods to produce virus-free stock plants (Morel and Martin, 1952; Wang and Hu, 1982; Martin *et al.*, 1955; Ahsan *et al.*, 2003). Morel (1960) was the pioneer for meristem culture. Meristem-tip culture alone or in combination with heat treatment (Yoshino and Hashimoto 1975) is widely used to obtain virus and fungus-free strawberry plants (Molot *et al.*, 1972). Posnette (1953) originally developed the technique of hot air therapy to eliminate viruses, and Belkengren and Miller (1962) began the practice of excising heat-treated meristems for placement on tissue culture media. Most commonly used explant for strawberry micropropagation is the meristem from the tip of runners (Sowik *et al.*, 2001). In the present investigation runner tips were used as explants for meristem isolation. Runner tips were collected from 45-60 days old plant. Meristems were isolated from these runner tips and cultured on MS (Murashige and Skoge, 1962) liquid culture media with different types of plant growth regulators like BA, KIN, NAA, IAA, GA3 either singly or in combinations for the establishment of primary meristem culture. Many workers got satisfactory results with MS liquid medium in different crops (White, 1943, 1968; Heller, 1963; Stone, 1963; Goodwin, 1966; Vine, 1968; Meller and Smith, 1969; Pennazio and

Redolfi, 1973; Rahaman, 1998; Steward *et al.*, 1969; Yee *et al.*, 2001; Islam *et al.*, 2003; Alam *et al.*, 2003).

Various concentrations and combinations of plant growth regulators remarkably influenced in resuming growth of meristem. Among the different treatments, MS medium fortified with 0.5 mg/l GA_3+ 0.5 mg/l BA was found to be most effective in increasing the growth of meristems. The cultured meristem commenced their initial growth by increasing in size and gradually changed to light green colour within 5-13 days. MS liquid medium supplemented with GA_3 showed better response and highest of 73% responded was found within 5-8 days. Effective role of GA_3 for establishing meristem culture was also reported by others (Ahmed *et al.*, 2000; Nagib *et al.*, 2003; Foxe *et al.*, 1986; Poleyava *et al.*, 1988; and Vasanthi *et al.*, 2001).

Leaf tissue has been studied and shown to have the greatest regeneration capacity of strawberry plant tissues (Jones *et al.,* 1988; Liu and Sanford, 1988; Nehra and Stushnoff, 1989; Nehra, *et al.,* 1990; Jelenkovic *et al.,* 1991; Popescu *et al.,* 1997 and Passey; *et al.,* 2003). Callus production is also more prolific from the leaf tissue. In addition, leaf-derived callus produces more shoots than petiole-derived callus (Popescu *et al.,* 1997). In the present study, leaf explants from *in vitro* grown plants were cultured on to MS medium supplemented with different concentrations and combinations of plant growth regulators (2,4-D, NAA and BA) and the culture were incubated in dark for callus induction. Among the different PGR formulations 2.0 mg/l NAA with 0.5 mg/l BA was found to be the most effective media formulation in terms of % of explants induced to develop callus and the degree of callus development. Auxin (NAA and 2,4 -D) alone at 3.0 - 4.0 mg/l were also

found very effective PGR formulations for callus development from strawberry leaf explant.

Previous studies have shown that PGR concentrations and selections are vital to strawberry callus induction and regeneration. Various formulations of BA, IBA, 2,4-D, CH, and KNO_3 have all been reported used in callus induction and plant regeneration studies in strawberry (Liu and Sanford, 1988; Nehra *et al.,* 1990 and Goffreda *et al.,* 1995). Liu and Sanford (1988) reported using casein hydrolysate (CH) and potassium nitrate on leaf explants of 'Allstar' strawberry. Both stimulated the production of callus and shoot and reportedly had an additive effect. Best callus and shoot production in their study was achieved with a combination of BA, IBA, CH, and KNO_3. Kartha *et al.* (1980) successfully regenerated 'Redcoat' using a combination of BA, IBA and GA_3 as a precursor to a cryopreservation study of the cultivar. In the present investigation auxin in combination of cytokinin (NAA-BA) was found the most effective for callus induction, which is concomitant with most of the previous report.

In present study, indirect shoot regeneration was obtained by subculturing the calli onto regeneration medium and incubating the culture in light. To do so the calli proliferated in dark were subcultured either onto corresponding callus initiation media or onto regeneration media consisted of MS medium supplemented with different concentrations of BA alone or in combination with NAA (considered as regeneration media) and the cultures were incubated in light for 8 weeks. It was observed that PGR formulations present in both callus induction and regeneration media showed profound effect on shoot induction. The calli induced in auxin (2,4-D and NAA) supplemented MS callus

inducing media were hardly induced to regenerate shoot. These calli failed to regenerate or even perpetuate when the regeneration media contained only BA. On the other hand, only a few calli derived from auxin fortified callusing media were induced to develop shoots when the regeneration contained higher proportion of BA (0.5 – 1.5 mg) and lower proportion of NAA (0.1 – 0.5 mg/l). Whereas, the calli proliferated in NAA – BA supplemented callusing media have very high regeneration potential. The calli proliferated in NAA – BA fortified callusing media showed the highest response to indirect regeneration when regeneration contained 1.5 mg/l BA and 0.5 mg/l NAA. It is evident from the present study that PGR formulations in callus induction as well as regeneration media are critical factors for successful indirect regeneration of strawberry through callus.

Indirect regeneration from leaf-derived callus of strawberry has also reported previously. In 1989, Nehra and Stushnoff, reported successful regeneration using greenhouse grown stock plants with surface sterilized leaves excised and cut into strips avoiding the midrib and placed adaxial side down onto the media. Nehra and Stushnoff (1989) discovered that leaf disks from young plants had a higher regeneration rate than older plants.

Plantlet regeneration via organogenesis in leaf cultures of *Fragaria* × *ananassa* has been studied extensively and is well documented (Yonghua *et al.*, 2005; Zhao *et al.*, 2004; Passey *et al.*, 2003; Schaart *et al.*, 2002; Barcelo *et al.*, 1998). Only a few studies however, have so far focused on somatic embryogenesis in strawberry (Donnoli *et al.*, 2001; Lis 1987; Wang *et al.*, 1984). These studies have primarily demonstrated the importance of various plant growth regulators and

growth media in achieving regeneration via somatic embryogenesis. Other regeneration methods tested include use of embryos. Wang *et al.* (1984) used embryos excised from strawberry achenes. The tiny strawberry embryos were excised from surface sterile achenes under a microscope and placed on hormone modified regeneration media. In a previous study, Husaini and Abdin (2007) published the first report where shoot regeneration in strawberry was achieved simultaneously through both somatic embryogenesis and shoot bud formation. The study focused on shoot induction and morphogenetic response of cultured leaf explants under complex environmental conditions of light, temperature, and TDZ, and identified some developmental constraints that affect somatic embryogenesis in strawberry. The present study was carried out for developing a reliable, reproducible, and highly efficient somatic embryogenesis system for strawberry.

In this study for somatic embryogenesis, the calli were repeatedly sub-cultured in the same medium formulation of 2,4-D or NAA alone and in combination with BA and proline. The calli obtained from leaf segment explants induced somatic embryogenesis in culture media supplemented with 2,4-D + BA; 2,4-D + BA + Proline and NAA + BA + Proline. None were developed on media with NAA + BA. On these media the callus became brown and rarely regenerated a shoot after 12 weeks of culture. The presence of amino acid in the medium is generally essential for embryo initiation. In this experiment proline promoted the formation of embryogenic callus. High frequency somatic embryogenesis was recorded on the MS medium supplemented with 1.0 mg/l 2, 4-D, 0.5 mg/l BA and 50% proline under dark treatment. The highest 80% embryogenic calli were recorded from leaf segment derived calli when incubated with

1.0 mg/l 2,4-D + 0.5 mg/l BA + 50% proline supplemented medium and the highest 13.6 ± 1.0 embryos/callus were recorded in this medium. By repeated sub-culture of the calli in the same medium formulations, embryo formation occurred 20 weeks after primary inoculation.

The first observations of *in vitro* somatic embryogenesis were made in *Daucus carota* (Cassells and Curry, 2001; Gallego *et al.*, 1997; Olmos *et al.*, 2002; Salvi *et al.*, 2001; Steward *et al.*, 1958; Reinert, 1959). Since then induction of embryoids has been obtain in a large number of species (Raghavan, 1976; Bhojwani and Rajdan, 1983). Somatic embryogenesis has been reported in a wide variety of monocots, including cereals such as rice, wheat, maize, rye, barley, several grass species and other monocots such as sugarcane, banana, lily, garlic and onion (Vasil, 1985, 1987; Novak *et al.*, 1986; Ahloowalia, 1990; Linacero *et al.*, 2000; Al-Zahim *et al.*, 1999). These studies have shown that callus gave rise to somatic embryos was usually compact and nodular and that embryogenic callus could be microscopically distinguished from non-embryogenic callus (Bouman and Klerk, 2001; Heinze and Schmidt 1995; Vasil, 1985; Nabors *et al.*, 1983).

A high frequency somatic embryogenesis and plant regeneration were observed from leaf explants. Embryogenic calli were observed under light microscope and found to bear somatic embryos at various developmental stages. Photoperiod is one of the most important factors for somatic embryogenesis of strawberry. To assess the effects of photoperiod on somatic embryogenesis of strawberry, induced calli were sub cultured on embryogenesis medium containing 1.0 mg/l 2,4-D, 0.5 mg/l BA and 50% proline and incubate under different photoperiods. Continuous dark treatments produced the highest percent of embryogenic

callus with daily light periods of more than six hours reducing embryogenesis. Somatic embryogenesis was also reported by Donnoli *et al.* (2001) from callus culture of strawberry leaf in continuous dark treatment.

Somatic embryos in the present study were identified by the simultaneous development of the root shoot poles. The germinating embryos in the present study showed a bipolar development. The cotyledonary stage embryos were isolated and cultured on MS medium supplemented with different PGR formulations. Among the various plant growth regulators supplements used, the best response towards embryo germination was observed on MS medium supplemented with 0.1 mg/l GA_3 + 0.1 mg/l IBA. After four weeks of culture the cotyledonary embryos had developed into plantlets. Globular somatic embryos appeared on the surface of the calli within 4 weeks. During subculture, these globular embryos further developed into cotyledonary stage embryos and attained maturity on the same medium. The available information from different studies suggests that optimal concentrations of different amino acids may be species or genotype-dependent, which needs to be determined before recommending its use (Chukwuemeka *et al.*, 2005; Homhuana *et al.,* 2008; Han *et al.,* 2009). Thus, the present study determined the effective concentration of amino acid to improve somatic embryogenesis of strawberry cultivars.

The effect of amino acids (proline, glutamine and alanine) on somatic embryogenesis induction, development and maturation of leaf explants of strawberry cultivars (Camarosa, Paros and Kurdistan) was studied. Stimulation of embryogenesis and embryo development was strictly dependent on the type and concentration of amino acid in the

medium. Ella and Zapata (1993) reported that proline may facilitate cell division and formation of secondary calli. Proline has been proposed to act as a compatible solute that adjusts the osmotic potential in the cytoplasm (Porgali and Yurekli, 2005; Caballero *et al.*, 2005; Bartels and Sunkar, 2006). Proline is considered to play an important role in defense mechanisms of stressed cells. Shetty and Mckersie (1993) and Girija *et al.* (2000) reported that medium supplemented with proline was found to be beneficial for somatic embryogenesis in soybean and green gram. Glutamine and proline have been reported to improve somatic embryogenesis by providing the nutrition (Garin *et al.*, 2000; Mujib and Samaj, 2005) or functions as the storage of nutritional nitrogen and carbon reserves, and finally enhancing somatic embryo maturation (Manoj *et al.*, 2009; Moghaddam *et al.*, 2000). Proline is known to stimulate auxin-induced somatic embryogenesis and elongates plant somatic embryos in a hormone-free medium. This may be due to improved cell signaling as proline is always associated with various signal transduction pathways in plants (Mujib and Samaj, 2005).

According to the results, medium supplemented with proline proved the best medium for the high levels of somatic embryogenesis induction. Similarly, medium supplemented with proline was also found to be favorable for somatic embryogenesis in *Oryza sativa* (Saharan *et al.*, 2004) and guava (Manoj *et al.*, 2009). At low concentrations (50 mg/l), the amino acids tested revealed to be inefficient for embryogenesis induction as well as for somatic embryos growth. The rise in proline concentration to 200 mg/l reduced the number of somatic embryogenesis induction. There are some reports that reveal that high concentrations of proline may be harmful to

plants, including inhibitory effects on growth or deleterious effects on cellular metabolisms (Nanjo *et al.*, 2003).

To evaluate the best media formulation for shoot multiplication of strawberry clone were tested on different treatments. The result revealed that tested different media formulations were statistically different from each other for number of shoots production. Among the various plant growth regulators supplements used, the best response towards multiple shoot regeneration was observed on MS medium supplemented with 1.5 mg/l BA+0.5 mg/l KIN. This combination showed the best performance of shoot proliferation. The highest number of shoots per culture was also recorded from this medium.

Indra and Uppeandra (2000) reported multiple shoot regeneration from Indian wild strawberry using MS supplemented with 4.0 mg/l BA and 0.1mg/l NAA. Some workers also reported shoot regeneration in strawberry using MS medium containing BA also of in combination with Kin (Lee and de Fossard, 1977; James and Newton, 1977; Sobczykiewicz, 1980; Lis, 1990; Boxus, 1999; Neeru *et al.*, 2000; Mereti *et al.*, 2003). However, the results of the present investigation slightly differed with that of the previous works. This result indicated that, low concentrations of BA alone or with KIN were found suitable for shoot initiation and further multiplication. This difference may be attributed by the difference of genotype and physiological condition of the explants.

Regenerated shoot needed root induction to grow into plantlets and to establish them into soil. To induce root individual shoots proliferated in regeneration media from the calli developed in different callusing media, were excised and cultured in MS and half MS media with or

without different PGR formulations (IBA and NAA). The microshoots of strawberry were inoculated in MS and ½MS media without plant growth regulators (MS_0 and $½MS_0$) were induced develop root without developing any callus at their base. Cent percent cultured shoot induce to develop roots when cultured in MS_0 rooting medium within 7-12 days of inoculation. Whereas, 93% shoots were induced root development in $½MS_0$ rooting medium. Addition of auxin in rooting media accentuates rooting but also micro-cuttings developed callus at their base that hamper their field establishment. Similar results on the rooting and subsequent field establishment were also reported by Boxus (1974), Owen and Miller (1996), Jimenez-Bermudez and Redondo-Nevado (2002).

Tissue culture generates a wide range of genetic variation in plant species, which can be incorporated in plant breeding programmes. Regenerated plants from calli, cells and protoplasts in different crops including strawberry (Heinz *et al.*, 1977; Larkin and Scowcroft, 1983; Ramos Leal and Maribona, 1991; Kaushal *et al.,* 2004) have been shown to exhibit great variability in agronomic traits.

In the present study, it was observed that regenerated strawberry plants grown in the field were not identical to their parent plants. Wide ranges of variations for different quantitative and qualitative characters were noticed among the regenerated plant populations. Among the different quantitative parameters, nos. of runner/plant, runner length, nos. of crown/plant, inflorescence length, days to flowering, nos. of fruit/plant, average fruit wt. (gm)/plant showed the very wide range of variations with very high coefficient of variability.

A strawberry plant normally develops a numbers of crowns during their growth. However, many of the plant as observed in the present study did not develop axillary crowns. In addition to these, many of the plant also failed to flowering, many of the plants lost their capacity to develop runner. Moreover, similar sorts of variations were also recoded among the plants for other characters such as: fruit shape colour, attractiveness and test, summer overcoming potential and the degree of disease tolerance. Plant off-types, i.e. non true-to-type and genetically not identical to the mother plant, may simply be the result from a change in the genetic make-up of the resulting plants.

In the present study the occurrence of novel variations in some the plants were also noticed. These plants were exhibited some traits such as: plant height, nos. of leaves/plant, canopy size, days to flowering, average fruit wt. (g), fruit wt./plant (g), degree of runner development, crown formation, fruit shape colour, attractiveness and test, summer overcoming potential and the degree of disease tolerance, seemed to be superior to mother plants.

Somaclonal variation has been successful in identification of new varieties in sugarcane, sorghum, tomato, wheat, celery, flax and *Pelargonium* (Skirvin and Janick, 1976; Compton and Veilleux, 1991; Sears *et al.,* 1992; Duncan *et al.,* 1995; Karp, 1995). Variation in morphological characters among callus regenerated plants were observed in rice for grain size, tiller number, leaf number, maturity (Sun and Zheng, 1990), panicle number, seed weight, mature plant height and culm height (Lal and Lal, 1990) and in potato for maturation time, shape, size, number and colour of tubers, leaf shape and size and yield (Karp, 1990; Lal and Lal, 1990).

Strawberry plants derived through organogenesis exhibited very wide range of variations in fruit wt. /plant. Even some of the somaclones exhibited better yield performances than those of micropropagated mother plants. This results elucidated that genetic variation in strawberry could be widen further through the use of indirect plants regeneration that could open an opportunity for selection of elite clones. At the same time it opens an opportunity for selection and improvement of new cultivars with better agronomic characters.

In the present investigation, different tissue culture techniques such as meristem culture, indirect organogenesis from leaves and somatic embryogenesis were used to induce somaclonal variation and three somaclones were identified on the basis of the variation of phenotypic polymorphic characters. Therefore, RAPD was carried out to confirm the genetic variation of the three somaclones at molecular level. Out of 15 random primers tested, only eight primers successfully produced scoreable RAPD bands for all three somaclonal lines and most prominent bands were observed with two primers, AL-04 and O-05. Though very few distinct and prominent bands were observed at the same position of all somaclones, diverse bands were also found among the three variants which made them distinct from each other. In the RAPD profile, the presence of genomic DNA bands (amplified with primer AL-04 and O-05) confirmed that bands of the variants were distinct to some extent. However, it could be alleged from the findings that very small number of RAPD primers are sufficient to prepare genotype specific banding patterns for the identification of variants. Sharifani and Jackson (2000) employed random 10-mer primers to identify 16 Pear species, cultivars and few progenies using RAPD technique. El-Tarras *et al.* (2001a) employed random 10-mer (RAPD) primers to identify 4 cultivars of Olive.

Somaclones under this investigation were assumed as distinct from each other in terms of fruit and other horticultural characters through morphological and statistical analysis and results obtained in the RAPD profile confirm the genetic difference among three somaclones. Moreover, this molecular analysis established the effectiveness and aptness of RAPD for strawberry cultivar identification and as well as researchers property rights protection. This finding is similar to the findings of El-Tarras *et al.* (2001b) where RAPD methodology was utilized for the genetic fingerprinting of five new strawberry cultivars and documented that using 11 random primers, 204 polymorphic DNA fragments with a high potential differentiate strawberry genotypes could be produced. Some other researchers documented that strawberries have been extensively analyzed using randomly generated markers for clone identification and diversity studies (Gidoni *et al.*, 1994; Levi *et al.*, 1994; Graham *et al.*, 1996; Landry *et al.*, 1997; Degani *et al.*, 1998; Degani *et al.*, 2001). The results indicated that the RAPD technique is effective to develop genotype-specific banding patterns valuable for cultivar identification. These results are in agreement with Òebrowska and Tyrka (2003), who used RAPD markers to identify and assess the level of genetic diversity among nine strawberry cultivars differing in their response to photoperiod. They have confirmed the value of RAPD markers for strawberry cultivars identification and author's property rights protection as well as selection of parents suitable for creating of mapping population. The results found in this study demonstrated that, the variation which was observed in tissue culture derived clones were genetic. Such genetic variants, merit detailed horticultural investigation for evaluation of their potential as a new cultivar for Bangladesh.

Chapter V

SUMMARY

In the present study *in vitro* propagation techniques of strawberry including various culture aspects of meristem culture, organogenesis and somatic embryogenesis leading to plant regeneration were studied. The main goal of this study was to select and characterize several somaclonal lines in strawberry populations. As a prerequisite to induce somaclonal variation, methods and techniques for optimizing the condition for meristem culture, callus induction and subsequent plant regeneration from the callus tissue through organogenesis and embryogenesis were evaluated. Occurrence of somaclonal variation was evaluated by planting the regenerants in the field.

The cultured meristem commenced their initial growth by increasing in size and gradually changed to light green colour within 5-13 days. It was observed that IBA, KIN and GA_3 when used alone, found less effective. In these cases, the cultured meristems resumed new growth within 7-13 days and the percentage of meristem responding ranged from 13%-73%. However of the two cytokinins used alone, BA was found to be better than KIN. When BA was combined with auxin or GA_3 the range of percentage of response increased to 27-80%. Out of five PGRs used, GA_3 + BA combination was found to be the best for the primary establishment of meristem culture. Among the different treatments, highest percentage (80%) of response was recorded in 0.5 mg/l GA_3 + 0.5 mg/l BA. The meristems also resumed their initial growth rapidly (5-8 days) in these combinations.

In order to induce callus leaf segments derived from *in vitro* grown plantlets of strawberry were cultured on to MS medium supplemented with either 2,4-D or NAA alone or in combination with BA and the cultures was incubated in dark in growth cabinet at 25°C for 4 weeks. The cultured leaf explants were induced to develop callus in most of media formulations but degree of effectiveness on callusing of these formulations were different. Among the different callusing media formulations 3.0 mg/l NAA with 0.5 mg/l BA was found to be the most effective media formulation in terms of % explants induced to develop callus and the degree of callus development. 2,4-D and NAA alone at 2.0 - 3.0 mg/l were also found very effective PGR formulations for callus development from strawberry leaf explant.

The calli developed from leaf segments in different culture media formulations were subcultured onto regeneration medium (MS formulation) supplemented with different concentration and combination of BA and NAA, and the cultures was incubated in light (16h). It was observed that growth regulator formulations present in both callus induction and regeneration media had remarkable effect on shoot induction. The calli induced in 2,4-D and NAA alone were rarely induced to regenerate shoot. These calli failed to regenerate or even perpetuate when the regeneration media contained BA alone. Only a few calli derived from auxin fortified callusing media were induced to develop shoots when the regeneration contained higher proportion of BA (0.5 – 2.0 mg) and lower proportion of NAA (0.1 – 1.0mg/l). Whereas, the calli proliferated in NAA – BA supplemented callusing media very high regeneration potential. The calli proliferated in NAA – BA fortified callusing media showed the highest response to indirect regeneration when regeneration contained 1.5 mg/l

BA and 0.5 mg/l NAA. It is evident from the present study that cytokinin-auxin ratio present in callus induction as well as regeneration media are critical factors for successful indirect regeneration of strawberry.

Somatic embryogenesis was noticed from after repeated sub-culture of the calli onto either corresponding same callus indication media or different media formulation. In this study, various combinations of plant growth regulators were tested for their potential to induced somatic embryogenesis in strawberry. None were developed on media with only 2,4-D or NAA and NAA+BA. On these media, the callus became brown and rarely regenerated a shoot after 12 weeks of culture. The presence of amino acid in the medium is generally essential for embryo initiation. Proline promoted the formation of embryogenic callus. High frequency somatic embryogenesis was recorded on the MS medium supplemented with 1.0 mg/l 2, 4-D, 0.5 mg/l BAP and 50% proline under dark treatment.

In order to evaluate the somaclonal variation among the plants, data were recorded on different morphological and agronomical characters. It was observed that regenerated plants grown in the field were not identical to their mother plants. Wide ranges of variations for different quantitative and qualitative characters were noticed among the regenerated plant populations. Significant differences were found among the studied colons in regards to yield contributing traits such as number of flower per plant, number of fruits per plant and average fruit weight.

Present study revealed that regenerated somaclones were more vigorous than control plants, and which could prove to be useful in order to survive the semi arid conditions in certain parts of Bangladesh. Single

fruit weight, fruit size and in many of the cases percent of summer survival was higher than control. These somaclones were distinct from each other in terms of fruit and other horticultural characters, and have potential for commercial cultivation in Bangladesh.

Present study also demonstrated that the variation which was observed in tissue culture derived clones were genetic. Here, RAPD has been extensively used to identify polymorphism among the genotypes. Stable phenotypic variation was clearly observed in the leaf morphology, flower cluster branching and fruit shape. These phenotypic changes with changes in the DNA banding pattern following RAPD as differences were observed in the selected three clonal lines. Somaclonal variation was examined among the selected three somaclonal lines by RAPD using 15 random primers. Out of 15 random primers tested only eight primers successfully produced scoreable RAPD bands for all three clonal lines. Polymorphic banding pattern was observed with five primer and was most prominent with the primers AL-04 and O-05. On the basis of these results, it may be concluded that RAPD is a useful technology to detect variation in strawberry somaclones.

Chapter VI

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APPENDICES

APPENDIX I

Composition of MS (Murashige and Skoog 1962) basal medium.

Components	Concentrations (mg/l)
Macro-nutrients	
KNO_3	1900
$NH_4 NO_3$	1650
$CaCl_2.2H_2o$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170
Micro-Nutrients	
KI	0.83
H_3BO_4	6.20
$MnSO_4.4H_2O$	22.30
$ZnSO_4.7H_2O$	8.60
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
$Na_2EDTA.\ 2H_2O$	37.3
$FeSO_4.\ 7H_2O$	27.8
Vitamins and organics	
Myoinositol	100.0
Nicotinic acid	0.50
Pyridoxine-HCI	0.50
Thaimine-HCI	0.10
Glycine	2.0
Sucrose	30g
pH	5.8

APPENDIX II

PREPARATION OF STOCK SOLUTION OF MS (MURASHIGE & SKOOG, 1962 BASAL MEDIUM)

Components	Amount (mg/l)	Strength of stock solution	Volume of stock solution (ml)	Amount for stock solution (mg)	Amount (ml) for 1 liter medium
Stock solution I					
1. NH_4NO_3	1650	20x	1000	33000	50
2. KHO_2	1900	20x		38000	
3. KH_2PO_4	170	20x		3400	
Stock solution II					
1. $MgSO_4.7H_2O$	370	20x	1000	7400	50
Stock solution III					
1. $CaCl_2.2H_2o$	440	20x	1000	8800	50
Stock solution IV					
1. $FeSO_4.7H_2O$	27.8	20x	1000	556	50
2. $Na_2EDTA.2H_2O$	37.3	20x		746	
Stock solution V					
1. $MnSO_4.4H_2O$	22.3	20x	1000	446	50
2. H_3BO_4	6.2	20x		124	
3. $ZnSO_4.7H_2O$	8.6	20x		172	
Stock solution VI					
1. KI	0.83	1000x	1000	830	1
2. $CuSO_4.5H_2O$	0.025	1000x		25	
3. $Na_2MoO_4.2H_2O$	0.25	1000x		250	
4. $CoCl_2.6H_2O$	0.025	1000x		25	
Stock solution VII					
1. Myoinositol	100	100x	200	10000	2
2. Nicotinic acid	0.5	100x		50	
3. Pyridoxine-HCI	0.5	100x		50	
4. Thaimine-HCI	0.5	100x		50	
5. Glycine	2.0	100x		200	

$FeSO_4/7H_2O$ and Na_2EDTA $2H_2O$ were dissolved separately in 400 ml DW by heating and constant stirring. The two solutions were then mixed, the pH was adjusted to 5.5. DW was added to make the final volume to 1000 ml.

ABBREVIATIONS

The following abbreviations have been used throughout the text:

%	: Percentage
µ	: Micron
µM	: Micro mole
m	: Meter
cm	: Centimeter
mg	: Milligram
mg/l	: Milligram per liter
ml	: Milliliter
mM	: Milli mole
gm	: Gram
mm	: Millimeter
nm	: Nanno-meter
ng	: Nanno-gram
etc.	: Etcetera = And others
e.g.	: Exampli gratia, for example
et al.	: et alile = Other people
MS	: Murashige and Skoog (1962) medium
½ MS	: MS with half strength of major salts only
MS_0	: MS Medium without plant growth regulators
2,4-D	: 2,4-dichlorophenoxy acitic acid
BA	: 6 – Benzyl adenine
IBA	: Indole-3 butyric acid
IAA	: Indol-3 acitic acid
KIN	: 6-furfuryl amino purine (Kinetin)
GA_3	: Gibberelic acid
NAA	: α-napthalene acetic acid

CV	: Coefficient of variability
wt.	: Weight
Sec.	: Second
No.	: Number
bp	: Base pair
Var.	: Variety
Viz.	: Videlicet = namely
PGR	: Plant Growth regulator
pH	: Negative logarithm of hydrogen ion (H^+) concentration
i.e.	: Id est = that is
Fig.	: Figure
min	: Minute
R_0	: Regenerated plants
sp.	: Species
PCR	: Polymerase Chain Reaction
RAPD	: Randomly Amplified Polymorphic DNA
TDZ	: Thidiazuron
^0C	: Degree Celsius
^0F	: Degree Fahrenheit
0.1N	: 0.1 Normal