# Cytogenetical and Phytochemical Assays on *in vitro* and *in vivo* Plants of Indian Strawberry- *Fragaria indica* L. (*Duchesnea indica* Focke).

Thesis
submitted to the University of Calicut
for the award of the degree of
**DOCTOR OF PHILOSOPHY IN BOTANY**

*by*

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# DECLARATION

I, Umesh, B. T., hereby declare that the thesis entitled **"Cytogenetical and phytochemical assays on *in vitro* and *in vivo* plants of Indian strawberry-*Fragaria indica* L. (*Duchesnea indica* Focke)."** submitted to the University of Calicut, for the award of the degree of DOCTOR OF PHILOSOPHY is a record of original research work done by me under the supervision and guidance of Dr John E. Thoppil, Reader in Botany, University of Calicut and that it has not formed the basis for award of any degree or diploma.

Date:07-10-2008. Umesh B. T.

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Date:07-10-2008

# CERTIFICATE

This is to certify that the thesis entitled **"Cytogenetical and phytochemical assays on *in vitro* and *in vivo* plants of Indian strawberry-*Fragaria indica* L. (*Duchesnea indica* Focke)."** submitted to the University of Calicut, for the award of the degree of DOCTOR OF PHILOSOPHY is an authentic record of original research work done by Sri Umesh B. T., during the period of her study (2001 - 2008) at the Genetics and Plant Breeding Division, Department of Botany, University of Calicut under my supervision and guidance and that it has not formed the basis for award of any degree or diploma.

**Dr. JOHN E. THOPPIL**
Supervising Teacher

# INTRODUCTION

The relationship between man and plants has always been important as plants affect every aspect of our life. The role of plants in the existence of nature is beyond comparison. Since the Stone Age, human beings of all cultures and races have been incredibly innovative with regard to utilizing plants for various needs.

The Western Ghats is characterized by the presence of several minor fruit yielding plants that grow naturally and spontaneously. However, some of these fruit plants have been set aside by specialized fruticulture and the risk of losing these genetic resources is high. In addition, due to the loss of farmland, new pests, diseases, and wild fires, this risk is even higher. The several uses of these minor fruit plants (alimentary, ornamental, forestry and medicinal) as well as the socio-economic impact, justifies the need to preserve these genetic resources. Previous studies described problems related to the *in vitro* propagation of some of these minor fruit species, connected with sterilization, low adaptability to the *in vitro* artificial environment and a low multiplication rate. Establishment of *in vitro* cultures of these fruit yielding plants has shown regeneration problems, as the explants may oxidize and/or necrotize. They rarely produce callus. In addition, developing apices frequently vitrify and after a few subcultures, the explants are not enough rejuvenated.

Micropropagation provides a potential cost effective means for the mass propagation of certain plants. An important consideration of this technology is the ability to add significant economic value to the plants being propagated. Examination of the relevant literature reveals that present micropropagation protocols are in general very inefficient at inducing active

growth of plantlets *in vitro*. Efficiency is dependent on the variety of plant used but is nevertheless low (about 10%) even for the best cultivars.

Micropropagation has become a reliable and routine approach for large scale rapid plant multiplication, which is based on plant cell, tissue and organ culture on well defined tissue culture media under aseptic conditions. Many research efforts are being made to develop and refine micropropagation methods and culture media for large scale plant multiplication of several number of plant species. However, many forest and fruit yielding plants still remain recalcitrant to *in vitro* culture and require highly specific culture conditions for plant growth and development. The recent challenges on plant cell cycle regulation and the potential molecular mechanisms of recalcitrance are providing excellent background for understanding totipotency and to know more about development of micropropagation protocols. For large scale *in vitro* plant production the important attributes are the quality, cost effectiveness, maintenance of genetic fidelity, and long-term storage. The need for appropriate *in vitro* plant regeneration methods for fruit yielding plants is still overwhelming in order to overcome challenges facing micropropagation such as somaclonal variation, recalcitrant rooting, hyperhydricity, polyphenols, loss of material during hardening and quality of plant material. Moreover, micropropagation may be utilised, in basic research, in production of virus-free planting material, cryopreservation of endangered and elite species and possess applications in breeding (Mohan, 2007).

The Rosaceae or rose family is a large family of trees, shrubs and herbs with about 3,000 - 4,000 species in 100 - 120 genera. Most species have alternate leaves and stipules. These may be adnate to petiolate. You have likely heard the saying, "*a rose is a rose is a rose*," suggesting that when you have seen one, you have seen them all. The family does tend to have somewhat monotonous actinomorphic flowers, commonly with 5 - parted perianth and numerous stamens. However, closer inspection

reveals that the gynoecium varies tremendously among different species of the family. Traditionally it has been divided into four subfamilies: Rosoideae, Spiraeoideae, Maloideae, and Prunoideae. These subfamilies are primarily diagnosed by the structure of the fruits. In the subfamily Rosoideae, many apocarpous pistils mature into achenes. While in the Prunoideae, a single monocarpellate pistil matures into a drupe. In the subfamily Spiraeoideae, the gynoecium consists of two or more apocarpous pistils that mature into follicles. In all these cases, the ovary is superior and there is commonly some development of a perigynous zone. However, in the fourth subfamily, Maloideae, the ovary is compound, inferior and an epigynous zone may occur.

The plant family Rosaceae includes many important fruit, nut, ornamental, and wood crops. Members of this family provide high value nutritional foods and contribute desirable aesthetic and industrial products. Most rosaceous crops have been enhanced by human intervention through sexual hybridization, asexual propagation, and genetic improvement since ancient times; 4000 - 5000 B.C. Modern breeding programs have contributed to the selection and release of numerous cultivars having significant economic impact on the world market. In recent years, the Rosaceae community, internationally, has benefited from newfound organization and collaboration that has hastened progress in developing genetic and genomic resources for representative crops such as apple, peach, and strawberry.

Rosaceae is the third most economically important plant family in temperate regions (Dirlewanger *et al*., 2006). The family encompasses some of the most economically important temperate fruit crops, such as apple (*Malus*), pear (*Pyrus*), peach, apricot, sour cherry, sweet cherry, plum and almond (all within the *Prunus* genus), strawberry (*Fragaria* spp.), raspberry (*Rubus* spp.) and the non edible species with almost exclusively ornamental value include rose (*Rosa* spp). Other species of this family with

lower market value, cultivated as ornamentals are *Potentilla,* cotoneaster, crab apple and *Pyracantha*, fruit yielding plants such as quince, timber yielding plants like wild cherry and invasive plants such as hawthorns and shadbushes. The products of this family are in high demand for their nutritional and aesthetic values, and their cultivation drives regional economies.

During ancient times, *Malus*, *Pyrus,* and *Prunus* populated native human habitats, and fruits from these species were valuable sources of food. Subsequent centuries of selection and domestication produced the dramatic increase in fleshy fruit size that distinguishes today's commercial fruit from their wild relatives. The economic importance of edible rosaceous crops derives from their flavourful fruits and nuts that provide unique contributions to dietary choices of consumers and overall human health. Rosaceous fruits were consumed in multiple forms, including fresh, dried, juice, and processed products. The variety of flavours, textures, and levels of sweetness and acidity offered by these fruits satisfies diverse consumer tastes and choices. Rosaceae fruits are also a major human dietary source of phytochemicals, such as flavanoids and other phenolic compounds, cyanogenic glucosides, phytoestrogens (Mazur *et al*., 2000), and phenols that could potentially yield health and disease-fighting advantages (Macheix *et al*., 1991; Swanson, 1998; Selmar, 1999). Lascorbic acid, quercetin, kaempferol, myricetin, p-coumaric acid, gallic acid, and ellagic acid are well known antioxidants and/or cancer inhibiting compounds that have been identified in these fruits. Epidemiological evidence suggests that diets rich in fruit and vegetables significantly reduce cancer risk (Caragay, 1992; Ziegler *et al*., 1996). *In vitro* and *in vivo* studies with animal models provide evidence that fruit and leaf extracts from many Rosaceae species inhibit some cancers or have strong antioxidant activities (Yau *et al*., 2002). Ellagic acid, present in strawberry, red raspberry, arctic bramble, cloudberry, and other rosaceous berries (Mandal and Stoner, 1990; Hakkinen *et al*., 1998; Masuda *et al*., 1999; Hakkinen and Torronen, 2000; Harris *et al*., 2001), has been

shown to affect cell proliferation and apoptosis, suggesting a potential anticancer role. Some strawberry cultivars have the highest concentrations of L-ascorbic acid among all fruits (Haffner and Vestrheim, 1997). Other phenolic compounds belonging to distinct chemical classes, many of which are also potential antioxidants and anticancer agents (Eichholzer *et al*., 2001; Schieber *et al*., 2001), have been isolated from rosaceous fruits (Macheix *et al*., 1991).

Studies on the cultivated strawberries dates back to the mid 18$^{th}$ century. In the last five decades considerable progress has been made in breeding programmes. It is now estimated that there are approximately 500 commercially grown cultivars of strawberries throughout the world (Galletta and Maas, 1990).

Some species of strawberry are used as medicinally important plants. The genus *Fragaria* consisting of 25 species out of which four are native to India. The infusion of leaves of *F. vesca* (Alpine strawberry), is diuretic and diaphoretic. Fruits are refringent, diuretic, and sometimes laxative. The root is astringent and useful in genito-urinary diseases.

In the case of *F. virginiana* (Virgin strawberry), leaves, fruits and roots are useful. Tea prepared from leaves is used for diarrhoea. Fruits are used to treat kidney stones and gout. Roots are used to increase urine flow and to treat gonorrhoea.

Fisetin, a naturally occurring flavanoid commonly found in strawberries and other fruits and vegetables, stimulates signaling pathways that enhance long-term memory, was reported by researchers at the Salk Institute for Biological Studies, USA ([3]http).

Throughout the past century, agricultural and horticultural research has focused on increasing crop production to feed the growing human population. However, as malnutrition and infectious and nutrition-related diseases remain common, the research focus has begun to shift to improve

the dietary value of different foods and identifying novel compounds with pharmacological properties (Mazur et al., 2000).

Traditionally, new traits for improvement of crop plants have been primarily derived from their related wild species. However, genomics and bioinformatics advances over the past decade have provided new options for identifying useful compounds in plants and for manipulating the genes responsible for their production. The profitable production of desirable fruits and nuts can be challenging, as rosaceous crop producers must balance stringent quality expectations with yield requirements, cost efficiency and shipping/marketing constraints. A further challenge is to maintain crop quality following harvest while avoiding loss due to chilling injury disorders, decay, chemical contamination, and over ripening. Thus, post harvest and consumer valued qualities and traits serve as ideal targets for crop product improvement. Complementary to the inescapable economic realities of crop production and distribution, consumer perception of quality characteristics deserves recognition as a primary driving force for cultivar development and establishment of research priorities. Consumers make quality judgements based on sensory perception (including smell, taste, and appearance) as well as perceived nutritional value. The flavour quality of fruits and nuts is determined by many criteria including firmness, juiciness, sweetness, acidity, aroma, and texture.

Recent consumer interests in purchasing locally produced fruits and vegetables have highlighted the importance of rosaceous fruits and invigorated their production in areas having relative proximity to large population centers. Rosaceous crop products are valued precisely because of their nutritional and aesthetic qualities, thereby offering opportunities for varietal improvement aimed at both problem solving and market expansion. The impressive gains achieved by conventional breeding notwithstanding, the addition of *in vitro* techniques as breeding tools and broadened research

perspectives offers unprecedented opportunities for rapid and sustainable enhancement of value to all rosaceous crops (Davis *et al*., 2007).

Advanced biotechnological methods of culturing plant cells and tissues should provide new means of conserving and rapidly propagating valuable, rare, and endangered fruit yielding plants. Combinations of *in vitro* propagation techniques and cryopreservation may help in conservation of biodiversity of locally used fruit bearing plants. Cryopreservation is a reliable method for long-term storage of the germplasm of endangered species (De Verno *et al*., 1999). Several useful plant species have been successfully cryopreserved (Bajaj, 1990). *In vitro* cell and tissue culture methodology is envisaged as a mean for germplasm conservation to ensure the survival of endangered plant species, rapid mass propagation for large-scale revegetation, and for genetic manipulation studies. With deforestation, many of our fruit yielding plants are rapidly lost, such that many valuable plants are threatened with extinction.

Plant improvement through clonal propagation utilises both the natural and induced variations through various *in vitro* and *in vivo* procedures (Skirvin, 1978). Variation has been a ubiquitous phenomenon associated with tissue culture (Carlson *et al*., 1991, Carlson and Polacco, 1975; Green, 1977). Induced variation is considered as an alternative source to naturally occuring variability for crop improvement programmes, hybridization and recombination in plant breeding (Ansari and Siddiqui, 1995).

Somaclonal variation obtained through micropropagation results in improvement in yield and quality characters, which would be of utmost importance in crop improvement (Ahloowalia, 1986). Here comes the importance of the present investigation, which involves a comparison between the somatic and somaclonal genotypes of *Duchesnea indica* (Andr.) Focke commonly known as Mock strawberry or Indian strawberry.

The genus *Duchesnea* is a trailing, evergreen perennial herb. It is in leaf all year, in flower from May to October, and the seeds ripen from July to October. The flowers are hermaphrodite (have both male and female organs) and are pollinated by Insects. The plant is self-fertile. The plant prefers light (sandy), medium (loamy) and heavy (clay) soils and requires well-drained soil. The plant prefers acid, neutral and basic (alkaline) soils. It can grow in semi-shade (light woodland) or no shade. It requires moist soil.

*Duchesnea indica* is also known as the Snake Berry. Previously it was known as *Fragaria indica*. Although the foliage and fruit are quite similar, is not a true strawberry and can readily be distinguished by its yellow flowers, as opposed to the flowers of true strawberries which are white or slightly pink. It is native to eastern and southern Asia, but has been introduced to many other areas as an ornamental plant, though it is considered a noxious weed in some regions.

*Duchesnea fragiformis*, *Fragaria indica*, *Potentilla indica etc*. are the synonyms of *Duchesnea indica* (Andr.) Focke.

Originally, from Southeast Asia and the Indies, the species *Duchesnea indica*, is a perennial growing to 0.1m by 1m at a fast rate (CSIR, 1994). The stem is trailing, rooting at nodes, strigose to pilose, herbaceous multiple from the base. The leaves are alternate, petiolate, stipulate, trifoliate, roughly veined beneath, dark green, and often persisting through the winter, arising from short crowns. The plant spreads along creeping solons, rooting and producing crowns at each node. Indian strawberry produces beautiful yellow flowers, sporadically throughout the growing season. The flower is single, pedunculate, axillary, antrorse, strigose, flower is a hypanthodium with five 3 lobed bracts alternating with sepals. The very attractive bumply soft round strawberry-like fruit is edible though nearly flavorless. The fruits are red, 10 mm in diameter, with red seeds, and while juicy and edible are not sweet like the true strawberries.

These vines just do not know how to stop producing fruit! The continuous presence of yellow blooms and round red strawberries is quite striking. The berries are more decorative than tasty. The vines, flowers and fruits can appear dainty, but it is a tough and lively plant, requiring little care beyond occasional watering.

Fruit is raw (Tanaka, 1976; Kunkel, 1984), dry and insipid (Clapham *et al.*, 1962). The fruit contains about 3.4% sugar, 1.5% protein, 1.6% ash. Vitamin C is 6.3 mg per 100 ml of juice (Parmar and Kaushal, 1982).

The whole plant is used as an anticancer herb in Chinese medicine. The plant is anticoagulant, antiseptic, depurative and febrifuge. It can be used in decoction or the fresh leaves can be crushed and applied externally as a poultice. It is used in the treatment of boils and abscesses, eczema, ringworm, stomatitis, laryngitis, acute tonsillitis, snake and insect bites and traumatic injuries. A decoction of the leaves is used in the treatment of swellings. An infusion of the flowers is used to activate the blood circulation. The fruit is used to cure skin diseases. A decoction of the plant is used as a poultice for abscesses, boils, burns *etc*. (Duke and Ayensu**,** 1985).

Decline of potential for the production of fruits is a common feature associated with cultivation of fruit bearing plants and a continuous selection is required to maintain the high yield of fruits for commercially viable programmes. Therefore, selection through conventional breeding as well as through tissue culture is exploited now a days for obtaining high yielding cultivars.

Development in the technology of plant tissue culture since its pioneering experiments by White (1934) and Murashige and Skoog (1962) have contributed in establishing a strong foundation for the various applications of this versatile technology. Since the present population is sterile, they can only be propagated vegetatively, which has prevented the production of new cultivars by conventional plant breeding. One of the

alternative methods for creating new forms is to produce and select somaclonal variants through tissue culture method.

Somaclonal variation is a term coined by Larkin and Scowcroft (1981) to cover all types of variations, which occur in plants regenerated from cultured tissues. Such variation has been observed among regenerants from a large number of species (Karp, 1992; Peschke and Phillips, 1992). Tissue culture induced genetic variation is defined as the variation that arises *de novo* during the period of differentiated cell proliferation that takes place between the culture of an explant and production of regenerants (Munthali *et al*., 1996).

Although chromosomal constitution of certain plants seems to be highly stable *in vitro* (Sheridan, 1974), much of the variability found in tissue culture can directly or indirectly attributed to gross chromosomal changes and chromosomal abnormalities. Chromosomal variation is a common feature of plant tissue cultures (Patau *et al*., 1957; Mitra *et al*., 1960; Mitra and Steward, 1961; Patau and Das, 1961; Partenen, 1963; 1965; Melchers, 1965; Muir, 1965; Kunakh, 1974). The most common changes are increase in ploidy level like the production of aneuploids (Taliaferro *et al*., 1989), polyploids and mixoploids (Taliaferro *et al*., 1989; Torrey, 1959; 1961; Murashige and Nakano, 1967; Nishi *et al*., 1968; Malnassey and Ellison, 1970; Kochar *et al*., 1971; Sacristan, 1971; Collins *et al*., 1972; Kasperbauer and Collins, 1972; 1974; Geier and Kohlenbach, 1973; Maretzki and Nickell, 1973; Sharp *et al*., 1973; Cooper *et al*., 1986). Aneuploid changes, typically involving gain or loss of a few chromosomes have also been reported (De Torok and White, 1960; Muir, 1965; Halperin, 1966; Murashige and Nakano, 1965; Shimada and Tabata, 1967; Heinz *et al*., 1969; Heinz and Mee, 1969; 1971; Norstog *et al*., 1969; Sacristan and Melchers, 1969; Shimada *et al*., 1969; Kao *et al*., 1970; Sacristan and Lutz, 1970; Nickell and Maretzki, 1972; Singh *et al*., 1972; Bayliss and Gould, 1974; Singh and Harvey, 1975). Structural changes also occur resulting in lagging chromosomes, bridges

and multipolar configurations especially after repeated passages in tissue culture (Venketeswaren, 1962; 1963; Cooper *et al*., 1964; Bayliss, 1973; 1975). A number of other mutation types that are likely to be the cause of such phenotypic variations had also been described including chromosome breakage, single base changes, changes in copy numbers of repeated sequences and alteration in DNA methylation patterns.

Chromosome observation is necessary to elucidate the structure, function, organization, and evolution of strawberry plants genes and genomes. However, distinguishing strawberries chromosomes from one another using light microscopy is extremely difficult, not only because of their small size and large number, but also because current chromosome observation methods are insufficient. Ordinary karyotype analysis has provided a limited success in chromosome identification and possibility of making errors is much greater in the conventional method of measuring and characterizing of chromosomes by visual evaluation. These difficulties can be overcome by computer aided chromosome image analysis system. Suitable chromosome preparation methods are necessary to overcome these difficulties that have heretofore hindered chromosome analyses of strawberry plants. Therefore the present analysis by using the computer softwares allows an accurate chromosome analysis especially in those cases where the chromosome size is very small (Fukui and Kakkeda, 1994). By this technique, we can have a better knowledge of the cytogenetic constitution of the material under study (Fukui and Kakkeda, 1990; 1994; Fukui and Iijima, 1992; Fukui and Kamisugi, 1995).

The value of tissue culture induced or somaclonal variation to crop improvement depends on establishing a genetic basis for this variation. Efforts to describe somaclonal variation at the molecular level had revealed the amplification of highly repetitive DNA sequences in cell cultures of different plants (Larkin and Scowcroft, 1981; Durante *et al*., 1983; Phillips *et al*., 1990). Recently amplification of DNA using arbitrary 10-base

oligonucleotide primers has been described as a way to detect Random Amplified Polymorphic DNA (RAPD) in many eukaryotic organisms (Williams *et al*., 1990). RAPD markers have been used to develop genetic maps (Reiter *et al*., 1992; Chparro *et al*., 1994) and as target genetic markers of isogenic lines (Klein-Lankhorst *et al*., 1991).

Genomic analysis is a prerequisite for establishing the genetic stability and uniformity of a desired clone (Ikeda and Ono, 1967). Several strategies can be used to assess the genetic fidelity of *in vitro* derived clones but most of them have limitations and none of them is completely desirable.

Genetic level or small chromosomal rearrangements sometimes cannot be revealed through mere karyological analysis (Isabel *et al*., 1993). Amplification of the genome can be carried out through Polymerase Chain Reaction (PCR), a technique derived by Kary Mullis (Saiki *et al*., 1985), has been the basis of a growing range of newer techniques. With this technique, amplification of a specific DNA sequence can be achieved and is considered ideal for identification of plant genotypes. Amplification of a genotype specific sequence can take advantage of some of the many features of PCR like speed, simplicity, specificity, and cost effectiveness (Klein-Lankhorst *et al*., 1991). Molecular marker such as Randomly Amplified Polymorphic DNA (RAPD) (Welsh and Mc Cleand, 1990., Williams *et al*., 1990) appears to be good but when compared with RFLP, RAPD provides a better basis for genetic characterization because of the simplicity of the procedures (Biard *et al*., 1990).

The approach of using molecular markers including RAPD profiles is a powerful tool both for identification of genotypes and to quantify the extent of genetic variation in any given population. Using this method polymorphisms can be revealed, as a single nucleotide change can alter the primer binding site or an insertion or deletion within the amplified region produces a band of different length. While on one hand the approach of RAPD profiling has been useful in tissue culture methods for detection and

selection of somaclonal variants (Munthali *et al*., 1996), this molecular technique at the same time with the same logic, is directly utilisable for assessing the micropropagated clones from any given explant for genetic uniformity.

The analysis of polymorphism by means of RAPD method does not require preliminary information concerning the DNA sequence under analysis. During the reaction, the sequences of many loci of the genome are amplified. The products of the RAPD reaction are segregated according to Mendelian models; therefore, they can be successfully used as genetic markers. Michelmore *et al*. (1991) have described the method of quick identification of the DNA sequences that are associated with the well known genes in plants.

Using PCR with short primers of arbitrary sequences, RAPD markers were recently shown to be sensitive for detecting variations among individuals between and within the species (Carlson *et al*., 1991; Roy *et al.*, 1992). This is an alternative approach for finding new DNA based polymorphic markers among closely related genotypes (Welsh and Mc Clelland, 1990., Lindhout *et al*., 1999).

The RAPD analysis using PCR with arbitrary oligonucleotide primers (Williams *et al*., 1990; 1993) has the advantage of being non-radioactive, rapid and convenient assay of polymorphisms and requires only a small amount of crude DNA. Today RAPD technique has been accepted widely.

The main issues associated with the use of these techniques are the problem of ensuring reproducibility of amplification profiles. The nature of the amplification process with short primers is such that many sites in the genome are potential templates and the profile obtained may be influenced by any variation in the method used to prepare the DNA template and exact reaction composition and the conditions used in PCR technique (Muralidharan and Wakeland, 1993). Standardization of these conditions will result in obtaining constant results, even when variation in the key variables

is encountered. A key requirement for reliable and reproducible RAPD results is a consistent approach to sample preparation and DNA isolation.

Polymorphisms result from either base changes at the primer-binding site (point mutation) or chromosomal changes in the amplified regions (insertions, deletions or inversions) which alter the size or prevent the succesful amplification of target DNA.

Plants represent an unlimited source of phytochemicals produced as a product or byproduct of primary and secondary metabolism. The secondary metabolic compounds may be interesting on their different functions and wide range of biological activities. Secondary metabolites are compounds that are biosynthetically derived from the primary metabolites and their distribution in the plant kingdom is restricted. These compounds are generally detected in lower volume compared to the primary metabolites and possess significant biological activities. Hence secondary metabolites are also termed as the ‘higher value- lower volume’ products or ‘speciality chemicals’ (Roja and Rao, 1998).

Essential oils, as it indicates, are ‘oils of essence’ belong to the most vital constituents of spice crops and other aromatic as well as medicinal plants. These natural products were extensively used in pharmacology as biologically active compounts. Essential oils may be the first preservative used by man. These oils will evaporate or volatilize when they are in contact with air. They also possess pleasant taste and strong odour. Essential oils can be easily removed from plants without any changes in composition. Most of them are complex mixtures of hundreds of compounds (Robinson, 1983). Essential oils are complex mixtures of odourous and steam volatile compounds which are deposited in sub cuticular space of glandular hairs and in the cell organelles like oil bodies, idioblasts, excretory cavities and canals of heartwoods. Clinically, essential oils act in different complicated ways. Certain oil is beneficial in specific medical cases, but there is no rule that similar illness in another patient can be cured with the

same result. These effects are different from those achieved with simple chemicals, which are much more convenient for evaluation and used in chemotherapy. Therefore, individual chemicals isolated from essential oils are more often used than oils as such (Brud and Gora, 1989). So quality of oil will be enhanced by identification of components. Here comes the importance of Gas Chromatography (GC) or Gas Chromatography and Mass Spectrometry (GC - MS) to evaluate the quality of essential oils. GC is a tool used for separating the volatile components while analysis of oil depends upon retention characteristics under standard conditions. The Mass Spectrometer can be used as a detector for a gas chromatogram in which case the high degree of specificity of the mass spectrometer is an aid to the identification of the sample. With the help of GC-MS technique, it has now been possible to analyse directly the fragrances of natural or synthetic materials without the use of heat or solvents and directly by the use of headspace analysis (Thappa *et al*., 1982). MS differs from other types of spectral analysis in that the sample does not absorb radiation from the spectrum. It is highly sensitive and only a small quantity of sample is required. When coupled with separation techniques like GC or HPLC (High Performance Liquid Chromatography), it is a highly specific way to identify organic compounds (Smith and Busch, 1999).

The biogenesis of strawberry flavour is a popular area of current research bringing together diverse areas of science, including flavour chemistry, biochemistry, molecular biology and genetics. Plant breeders are interested in strawberry species as donors of primary and secondary metabolic compounds in breeding programmes because of the diversity and intensity of wild strawberry aroma and sweetness. Detection of total carbohydrate content is a suitable option to evaluate the flavour qualities of these fruit yielding plants.

The present study is an attempt on the micropropagation of *Duchesnea indica* (Rosaceae) by *in vitro* techniques and for the probable

establishment of a somaclonal variant, that differ in both quantity and quality of the fruit and in the content and composition of the leaf essential oil from parent plant and to reveal the genetic basis of variation in them by using RAPD technique and chromosomal analysis through computer aided technologies. It also compares the carbohydrate content of the fruits of both *in vitro* and *in vivo* plants.

The present study aims to fulfill the objectives like:

- To establish a protocol for *in vitro* propagation of the plant "*Duchesnea indica*".
- To analyze the variations of the *in vitro* derived plants from the parent plant by comparing them in various aspects like,
- Karyomorphology.
- Genetic polymorphism using RAPD.
- Essential oil content and composition of herbage.
- Total carbohydrate content of fruits of both *in vivo* and *in vitro* derived plants.

# REVIEW OF LITERATURE

Plant tissue culture technique which paved the way for the development of modern biotechnology has become a powerful tool for studying and solving various problems of plant biotechnology. As our traditional wealth on plant genetic resources has been decreasing tremendously, these techniques have gained greater momentum on commercial application in the field of plant propagation. A considerable account of *in vitro* studies had been undertaken both nationally and internationally in agricultural and horticultural fields. Now-a-days the method for plant micropropagation has been well developed (Paranjothy *et al*., 1990; Machey *et al*., 1995).

Rapid clonal propagation is possible through bud or shoot proliferation (Pierik, 1990), induction of bulbs or corms (Ziv, 1990), or somatic embryogenesis (Ammirato, 1989). Using culture techniques it has been possible to regenerate propagules with better qualities, greater vigor, higher yield and disease resistance. A number of physiological and morphological changes have been reported in unorganized callus tissue including habituation, changes in biochemical sensitivity and requirements, alteration of growth habit and modification of cellular constituents. Only certain cells are capable of normal embryo development (Barba and Nitchell, 1969). Somatic embryogenesis has tremendous potential for large scale production of plant material.

A general phenomenon of tissue culture is the ability to produce embryoids, organs, tissues *etc*. Murashige (1974) described plant regeneration accomplished from explants like leaves, stem, cotyledons, microsporocytes and shoot tips. According to Gamborg *et al*. (1974), most important determinant of plant multiplication and quality of regenerated plants is the initial explant used.

Micropropagated plants may or may not show variations from the parent plant. Variations in plant tissue culture also include changes in growth habit, rates, appearance and requirements. Callus cultures of *Picea glauca* (Reinert, 1956), pea (Torrey and Shigemura, 1957), tobacco (Sievert and Hildebrandt, 1965), carrot (Blakely and Steward, 1964; Muir, 1965) and wheat (Nakai and Shimada, 1975) *etc*. are some of the reports on differences in growth habit with some clones. Nickell and Maretzki (1972) and Nickell (1973) reported the difference in growth rate of yellow and white clones of sugar cane.

Intact plants isolated from tissue culture have been reported to be variant in many cases. Ibrahim (1969) obtained variants in carrot after high kinetin treatments *in vitro*. Altered phenotype in *Digitalis purpurea* plants regenerated from kinetin containing media was reported by Corduan and Spix (1974).

Geranium (*Pelargonium hortorum*) plants derived from stem and anther culture differ in variegation, number of petals and stamens and phyllotaxy (Abo El-Nil and Hildebrandt, 1972). Clonal plants obtained from callus culture of *Coptis* were reported to produce more jatrrhine than the parent plants (Ikuta *et al*., 1975).

Molecular genetics can be used as a tool for crop improvement. For the application of molecular genetics, plant tissue culture is one of the essentials. Regeneration may be achieved through organogenesis or somatic embryogenesis and progress has been made in defining the conditions required as well as the physiological and biochemical changes accompanying these types of developments (Ammirato, 1987; Christianson, 1987; Thorpe, 1990).

The suitability of sucrose as a carbon source for uptake and utilization of plants was analysed by Khuri and Moorby (1995). There are reports that during subculturing, the number of variant plantlets increases (Ziv *et al*.,1983; Leshem *et al*.,1998; Safrazbekyam *et al*., 1990).

The effect of growth hormones cannot be generalized because different plants behave differently and may have different requirements. Cytokinins are found to be very effective for both direct and indirect shoot bud initiation. BA is the most commonly used cytokinin (Moreno *et al*., 1985; Bonabdallah and Branchard, 1986; Misra and Bhatnagar, 1995). Endogenous cytokinin is known to occur in plant tissue culture and exhibit a change in concentration during cell growth (Mackenzie and Street, 1972; Ernst *et al*., 1984). Somaclonal variation for both quantitative and qualitative characters had been reported in many species of higher plants (Bajaj, 1990). Plant cells cultured *in vitro* produced wide range of primary and secondary metabolites of economic importance. Partially differentiated callus cultures of *Ruta graveolens*, chamomile, *Coriandrum* and peppermint synthesized flavor compounds. Callus cultures of saffron produced flavor and pigments (Ravishankar and Venkataraman, 1990).

Plant hormones at different combinations affect the growth of plants differently (Rojina, 1991). Agarwal *et al*. (1987) successfully developed callus using 2, 4-D. Addition of NAA and KIN to IAA containing medium resulted in initiation of organogenesis and formation of semi-differentiated callus.

Several reports indicate that plants regenerated from callus or suspension cultures may show genetic changes like phenotypic variability (Nishi *et al*., 1968; Williams and Collins, 1976), chromosomal rearrangements (Cummings *et al*., 1976), polyploidy or aneuploid plants (Murashige and Nakano, 1965; Sacristan and Melchers, 1969). But culture raised through meristem explants successfully regenerate large number of genetically uniform plants (Murashige, 1974) suggesting that significant chromosomal changes occur principally in dedifferentiated callus or suspension cultures (Malnassy and Ellison, 1970).

More variation can be induced in plants raised through florets, than those raised through leaf and stem explants (Khalid *et al*., 1989).

Suryanarayanan and Pai (1988) reported *in vitro* propagation of *Coleus forskohlii* using flowers as explants and were of the opinion that flowers are a better alternative for regeneration from callus. Khalid *et al*. (1989) reported that flowers of *Chrysanthemum* are an ideal explant for inducing somaclonal variation.

Scanning of literature reveals that several workers had conducted micropropagation studies on the fruit yielding members of Rosaceae. Pereira-Netto (1996), conducted studies on the *in vitro* propagation of *Hancornia speciosa* on Murashige and Skoog medium supplemented with growth regulators. *In vitro* studies were carried out, comparing rooting of almond, apple, plum, *Pyrus pyraster* and two hybrid rootstocks, after co-culture with *Agrobacterium rhizogenes* strain 1855, with and without the addition of hormones. Studies reveal that infection at the base of microcuttings *in vitro* can improve the rooting of these fruit species (Damiano and Monticelli, 1998).

*In vitro* culture and evaluation of development interdependence in fruits, seeds and embryos, *etc*. were made on some fruit and berry yielding members of Rosaceae like, *Ribes, Fragaria, Malus, Prunus* and *Chaenomeles* by Rugienius *et al*. (2003). Breeding of *Rosa rugosa* using different tissue culture methods was done by Jakobsone *et al*. (2006).

Wide spectrum of literature is available on the micropropagation and related aspects of strawberry plants. Successful plant regeneration from leaf explants in strawberry (*Fragaria x ananassa*) has been reported ([7]http). Plant regeneration from leaf explants *i.e*., petioles and leaf blades at various concentrations of 6-benzylaminopurine (BAP) 0; 1.6; 3.2; 6.4 mg/l in Murashige and Skoog (MS) medium were found to be effective.

*In vitro* culture work of *Fragaria* x *ananassa* was attempted by Oda (1989). He studied the effect of light intensity, temperature and $CO_2$ concentration on photosynthesis and growth of strawberry plantlets

cultivated *in vitro*. Mohamed (1990) made a detailed study of the role of various plant growth regulators in tissue culture induced rejuvenation of strawberry plants. Shoot organogenesis studies in strawberry (*Fragaria* x *ananassa*) were made by Rashid (1991). The effects of various growth regulators, explant age and size were also studied.

Study on hardening and field survival of micropropagated plants of strawberry (*Fragaria x ananassa*) was made under Punjab conditions ([8]http). The experimental plant material of cv. Chandler was raised through tissue culture on Murashige and Skoog (MS) nutrient medium using runner tips as explant. The MS medium supplemented with 6-benzyladenine (0.5 mg/l) + indole-3-butyric acid (0.10 mg/l) was used for shoot multiplication. The rooting was obtained on the same basal medium fortified with indole-3-butyric acid (1 mg/l) alone.

Scanning of literature reveals a low cost medium for the *in vitro* propagation of strawberry (*Fragaria* x *ananassa* cv. Chandler) ([9]http). Vegetative buds were cultured on Murashige and Skoog (MS) medium supplemented with low cost medium components. The major medium manipulations were made by replacing agar-agar, sucrose and distilled water with tapioca granules, table sugar and tap water, respectively. Maximum *in vitro* multiplication of shoots was obtained on MS medium supplemented with KIN 0.5 mg/l, BAP 1 mg/l and GA3 2.0 mg/l and table sugar in place of sucrose and maximum *in vitro* rooting was induced on one fourth MS medium supplemented with IBA 1 mg/l, charcoal 200 mg/l and table sugar 20 gm/l in place of sucrose. Hundred per cent survival of micropropagated plants was recorded in the field. Plants grown through cost effective micropropagated protocol showed good field adaptability.

*In vitro* studies were conducted to evaluate the performance of somaclonal strawberry (*Fragaria x ananassa* cv. Brighton) variants for susceptibility to *Phytophthora cactorum* (Battistini and Rosati, 1991). Experimental studies were conducted to evaluate the micropropagated

seedlings of strawberry (*Fragaria* spp.) for salt tolerance by Esensee *et al*. (1991). The different aspects and conditions of tissue culture regeneration of strawberry (*Fragaria vesca)* plants from leaf explants were reported by Greene and Davis (1991). Genetic transformation studies in cultivated strawberry, *Fragaria x ananassa* was conducted by Jelenkovic *et al*. (1991).

Studies on the resistant lines of strawberry (*Fragaria* x *ananassa*) to the fungal wilt disease caused by *Fusarium oxysporum* f. sp. *fragariae* were made by Toyoda *et al*. (1991). These strawberry plants were regenerated from leaf derived callus tissues. The study demonstrated the existence of somaclonal variation for disease resistance against a soil borne fungal pathogen.

*In vitro* culture of *Fragaria* x *ananassa* was performed along with various other plants by Riquelme *et al*. (1991). They also studied the preconditioning effects and acclimatization of these micropropagated plantlets in greenhouse conditions.

Photosynthesis and growth of *in vitro* cultured strawberry plantlets (*Fragaria* x *ananassa* cv. Kent) were investigated during a 4 week *in vitro* culture in a rooting medium and a 4 week *ex vitro* period by Yue *et al*. (1993). The leaves formed *in vitro* on a medium containing sucrose developed a positive photosynthetic capacity. Shoot tip culture studies of *Fragaria* x *ananassa* was done by Hdider and Desjardins (1994). They also studied changes in ribulose-1, 5-bisphosphate carboxylase/oxygenase and phosphoenolpyruvate carboxylase activitites and $^{14}CO_2$ fixation during the rooting of shoots *in vitro*.

Production of anthocyanins from strawberry cell suspension cultures was investigated by Mori and Sakurai (1994). The effect of controlling sugar concentration and ratio of ammonium : nitrate in culture medium of strawberry, *Fragaria ananassa* cv Shikinari was also analysed. Short term studies of $^{15}$NO-3 and $^{15}$NH4+ uptake by micropropagated strawberry

(*Fragaria* x *ananassa*) shoots cultured with or without $CO_2$ enrichment was made by Hdider *et al*. (1994).

Micropropagation studies of strawberry (*Fragaria* x *ananassa*) plantlets were made by Hdider and Desjardins (1995). They also studied the reduction of ribulose-1, 5-bisphosphate carboxylase/oxygenase efficiency by the presence of sucrose during the tissue culture of these plants.

Studies were conducted by Yang *et al*. (1995) on the micropropagation of *Fragaria* x *ananassa*. The effect of tonic composition and strength of culture medium on the photoautotrophic growth, transpiration and net photosynthetic rates of the strawberry plantlets were also analyzed *in vitro*.

Studies conducted by Motomori *et al*. (1995) revealed that hairy roots of *Fragaria* x *ananassa* cv. Reikou, induced with *Agrobacterium rhizogenes*, grew well in hormone free Murashige and Skoog (MS) and Gamborg B5 liquid media. Particularly, in MS medium, hairy roots showed maximum growth producing high contents of polyphenols. Polyphenol contents in the intact plant (leaf blade, petiole, calyx, receptacle and root) were also investigated.

A study was conducted by Owen and Miller (1996) to maximize plant regeneration frequencies from cultured anthers of 'Chandler', 'Honeoye', and 'Redchief' strawberries (Fragaria x ananassa). A comparison of auxins (IAA, NAA), cytokinins (BA, BAP, KIN) and carbohydrates (sucrose, glucose, maltose) in MS medium showed that the highest shoot regeneration across cultivars (8%) occurred when using a medium containing 2 mg/l IAA, 1 mg/l BA, and 0.2 M glucose.

The biosynthesis of 6-deoxy-D-fructose has been investigated by Zabetakis and Holder (1996) in order to improve the flavour of cultivated strawberries (*Fragaria* x *ananassa* cv. Elsanta). Callus cultures of strawberries have been established. Methylpentoses were found to be the

key compounds for the biosynthesis of 6-deoxy-D-fructose.

An efficient and reliable method for shoot regeneration from leaf disks of *Fragaria vesca* has been developed by El Mansouri *et al*. (1996). This protocol has been successfully employed to obtain transformed plants using *Agrobacterium tumefaciens* as gene vector. Murashige and Skoog basal medium supplemented with benzyladenine (4 mg/l) and indole-3-butyric acid (0.25 mg/l) induced maximum percentage of shoot regeneration (98%) and the highest number of shoot colonies per explant (4.6) after 8 weeks of culture. Isolated shoots would elongate and proliferate when the benzyladenine concentration was lowered to 0.5 mg/l.

Experimental work had been conducted by Irkaeva and Matveeva (1997) to find out the response of different strains of strawberry (*Fragaria vesca*) micropropagated plants to varying combinations of cytokinins.

Experimental studies were conducted to estimate the growth of *in vitro* cultured callus of musk strawberry (*Fragaria* moschata) by Infante *et al*. (1998). They have also shown that plant regeneration could be made from leaf disk and petiole derived callus sub cultured for 18 months.

An efficient method of micropropagation leading to an increased percentage survival of explants and reduced phenol induced browning in wild strawberry (*Fragaria indica*) has been developed by Bhatt and Dhar (2000). Nodal segments cultured on Murashige and Skoog medium supplemented with 6-benzyl adenine (4.0 µM) and α-naphthalene acetic acid (0.1 µM) gave the best (94.4%) explant establishment and shoot number (22.3) per explant. Of the cytokinins tested, 6-benzyl adenine was found to be more effective than kinetin and $N^6$-(γ,γ dimethylallyamino) purine. Excised shoots rooted on half strength agar gelled medium with 1.0 µM α-naphthalene acetic acid.

*In vitro* studies were conducted to find out the suitability of strawberry (*Fragaria x ananassa*) microplants to field cultivation by Zebrowska *et al*.

(2003). The method of *in vitro* selection for increased salt tolerance during seed germination and early growth phase of strawberry seedlings was proposed by Dziadczyk *et al*. (2003). Adventitious shoot regeneration studies were conducted on seven commercial strawberry cultivars of *Fragaria* x *ananassa*, *viz*. Calypso, Pegasus, Bolero, Tango, Elsanta, Eros and Emily, using a range of explant types (leaf discs, petioles, roots and stipules) and culture conditions by Passey *et al*. (2003).

The influence of inbreeding on the micropropagation process in the strawberry (*Fragaria x ananassa*) was estimated by Zebrowska (2004). The explants of this plant material were proliferated in two subcultures. The micropropagation has been described by the number and weight of microplants derived from individual explants. The results showed that inbreeding influenced on the parameters of micropropagation. Khan and Spoor (2004) conducted a study on the *in vitro* callus culture from the leaf disc explants in strawberry (*Fragaria x ananassa* cv. Tango). They reported a high percentage of regeneration and established a new protocol for the speedy micro propagation of strawberry.

Agrobacterium mediated leaf disc technique is currently the main strawberry genetic transformation method applied. Tang (2004) established a stable, efficient and renewable system of genetic transformation in strawberry.

Three strawberry (*Fragaria* spp.) cultivars (Clea, Irvine and Paros) useful for South Italy pedoclimatic conditions and several genotypes of wild strawberry, *Fragaria vesca* were tested for plant regeneration, somatic embryogenesis and genetic transformation ([12]http). High percentage of organogenesis and plant regeneration was obtained in strawberry and *Fragaria vesca* by using MS medium supplemented with 1 mg/l IBA and 1 mg/l BAP. Somatic embryogenesis events were observed when the explants were maintained in darkness during callus induction step. A sufficient number of genetic transformation events were obtained either in strawberry

or in *Fragaria vesca*. Studies on the morphogenesis in *Fragaria* tissues *in vitro* after biolistic treatment with nitrogen fixing bacteria had been conducted recently ([13]http). Both somatic embryogenesis and organogenesis occurred in high frequency on modified MS medium.

Studies on the phenotypic stability of *in vitro* regenerated plants of strawberry (*Fragaria x ananassa*) had been made by Singh *et al*. (2004). The effects of various culture conditions on the shoot growth of *Fragaria* x *ananassa* were examined by Takayama and Takizawa (2004). To multiply the shoots efficiently, modified MS medium (with the concentrations of $NH_4NO_3$, $KNO_3$ and $CaCl_{2.}$ $2H_2O$ halved) supplemented with 1 mg/l 4PU (N-(2-chloro-4-pyridyl)-N'-phenylurea), 30 gm/l sucrose and 8 gm/l agar was selected as the optimum medium. Studies on the effects of different culture conditions on regeneration of plantlets from leaf explants of strawberry (*Fragaria x ananassa cv.* Toyonoka) were made by Wu *et al*. (2004).

The influence of three photoperiods: a) 16/8 (d/n) - control, b) 4/2 (d/n) (4 cycles per 24 h), and c) 22/2 (d/n) on the growth of *in vitro* cultures of strawberry (*Fragaria* x *ananassa)* cultivars ‘Senga Sengana’ and ‘Elsanta’ were investigated by Litwinczuk and Zubel (2005). Kaushal *et al*. (2006) established a new protocol for the maintenance of callus cultures and plant regeneration in strawberry (*Fragaria x ananassa* cv. Chandler). Lucyszyn *et al*. (2006) formulated a new combination of media containing blends of agar/galactomannan for the micropropagation of strawberry (*Fragaria x ananassa* cv. Pelican).

Micropropagation studies of strawberry (*Fragaria* x *ananassa*) plants newly introduced in Bangladesh was made by Sakila *et al*. (2007). Nodal segments of strawberry gave rise to multiple shoots when cultured on MS medium supplemented with different concentration of BA with KIN or GA. The highest response of shoot multiplication was obtained in MS medium containing 1.5 mg/l BA + 0.5-0.1 mg/l KIN. The regenerated shootlets were rooted on MS basal medium with different concentrations IBA and IAA.

Studies on somatic embryogenesis from callus derived out of leaf and nodal segments of strawberry were made by Biswas *et al*. (2007). The highest percentage of cultures with somatic embryos was achieved on MS medium supplemented with 1.0 mg/l 2,4-D, 0.5 mg/l BAP and 50% proline.

Experimental works on the somaclonal variation in strawberry (*Fragaria x ananassa*) cultivars is available in literature ([10]http). A combination of BAP (0.50 mg/l) and NAA (0.75 mg/l) was found to be best for induction and multiplication of callus cultures in cv. Chandler and cv. Fern. It was 88.89% in cv. Chandler and 83.55% in cv. Fern. Plant regeneration was obtained only in cv. Chandler in MS medium containing a combination of BAP (2 mg/l), NAA (0.25 mg/l) and kinetin (0.5 mg/l).

Recently studies were conducted to reveal somaclonal variation in plants regenerated by organogenesis from callus culture of strawberry (*Fragaria x ananassa*) ([11]http). Somaclonal variation was induced in strawberry plants regenerated from leaf and petiole derived callus by increasing the intervals for subcultures to 12 weeks, and also by transferring the calli, starting with the first subculture, to media containing combinations of BA (4.4, 13.3 or 22.2 µM) and 2,4-D (4.5 µM). It was suggested that both genotype and type of explant strongly influence the occurrence of somaclonal variation. Several somaclones exhibiting useful variation in plant and fruit characteristics have been identified so far. A variant having a modified (white) colour of flesh for all fruits, induced from petiole derived callus of cv. Gorella, is reported for the first time. Since most of the useful variations affecting plant vigour, fruit yield and runnering ability occurred in strawberry somaclones regenerated from leaf derived callus, it has been suggested that the type of variation in plants regenerated by organogenesis, is related to the type of explant.

All tissue culture systems exhibit some degree of somaclonal variation during culture (Larkin and Scowcroft, 1981). Tissue culture plants are

different from the normal plants because of the environmental conditions in the tissue culture container. The water retention capacity (WRC) of the head space in the container is responsible for the different physiological response. Debergh *et al*. (1992) showed that by controlling the WRC of head space, the physiology and anatomy of the cultured plants can be improved to resemble normal plants. The rapidly dividing and differentiated cells fail to age and therefore clones obtained by vegetative propagation could be viable for an unlimited period of time (Kazaryan, 1969; Libbert, 1974).

The ordered process of chromosomal replication and division is the basis of growth and development. Any change in the process, irregularities or imperfections lead to variation and evolution. In tissue culture, as the cells are grown in artificial environment, possibilities of change in the genetic level are common. The changes include doubling of basic set of chromosome complement, 2x, 4x, 8x, 16x and so on. This phenomenon had been discussed in detail by Partenen (1963; 1965). A change in chromosome number through breakage almost invariably results from the formation of ring, dicentric or tricentric chromosomes. The occurrence of such chromosomes in tissue and cell culture was common and reported in several species (Torrey, 1959; Mitra *et al*., 1960; Mitra and Steward, 1961; Norstog *et al*., 1969). Cells growing in an artificial environment may have many genetic changes such as increased frequencies of single gene mutations, chromosome breakages, transposable element activation, quantitative trait variation and variation of normal DNA methylation patterns (Kaeppler and Phillips, 1993b; Do *et al*., 1999).

There are many reports on the instability of chromosome behavior during culture (D'Amato, 1952, 1978; Skirvin, 1978; Constantin, 1981). Variation in morphology and chromosome numbers of callus derived plants is a common observation. Changes in the ploidy level can be directly detected by chromosome counting in the cells of the strains or the regenerated plants. Karyotype analysis of metaphase chromosomes was

used to determine rearrangements of chromosomes of somaclones (Bhojwany *et al*., 1986). Chromosomal abnormalities, especially chromosomal doubling is a common feature associated with tissue culture (Morel, 1971).

Variations occurring in plant genome in response to passage of plant cells through cycles of tissue culture and regenerated plants have been mentioned by Constantin (1981). As per his view the genomic changes usually occur in the highly repetitive fraction of the genome and are limited to a specific subset of these sequences which may be localized at particular chromosomal sites.

Ghosh and Sharma (1979) reported chromosomal variation in *Vigna* and *Pisum* cultures. This type of observation was also found in *Vicia* cultures (Jha and Roy, 1982). Chromosomal variation and frequency of spontaneous mutation associated with *in vitro* cultures was assessed by Edallo *et al*. (1981). According to Morel (1971), chromosomal abnormalities, especially chromosome doubling is a common feature associated with tissue culture. Rearrangements involving chromosome breaks at heterochromatin was described by Sacristan (1971) in *Crepis capillaris*. Three callus lines of *Allium fistulosum* culture and their regenerated plantlets were observed by Lee and Ono (1999) to elucidate the relationships between chromosomal aberration and morphogenetic potential. Cytological methods have been employed to assess the somaclonal variants within garlic callus culture (Dolezel and Novak, 1985).

Cytological studies have been conducted on several fruit yielding plants of Rosaceae. Various estimates suggests that as many as 30 - 70% of flowering plants are of polyploid origin (Grant, 1971; Goldblatt, 1980). Polyploidy had been reported in several members of Rosaceae. In many genera, different species will have different ploidy levels (multiples of a base number) representing a series of polyploids. The Rosaceae family has been traditionally divided into four subfamilies, grouped by fruit type and also on

the basis of basic chromosome numbers.

The Maloideae subfamily is characterized by a distinctive fruit, the pome, and a base chromosome number (x) of 17. Most other members of the family have x = 7, 8, or 9. Subfamily Rosoideae, which contains plants such as roses, strawberries, and raspberries, have x = 7 (rarely 8). Amygdaloideae, best known for cherries, apricots, peaches, plums, and almonds, have x = 8. The fourth traditional subfamily, Spiraeoideae, or bridlewreath subfamily, is heterogeneous and has x = 9 or, in a few genera, x = 15 or 17 (Goldblatt, 1976).

Maloideae are hypothesized to have originated through an ancient polyploidization event as their base chromosome number is x = 17; all other Rosaceae are primarily x = 7, 8, or 9. Two most popular hypotheses for the origin of the Maloideae are: (1) allotetraploidization following an ancient hybridization event between Amygdaloideae (x = 8) and Spiraeoideae (x = 9) ancestors and (2) a polyploidization event (allo or auto) within the Spiraeoideae ([2]http).

The plants in the rosaceous subfamily Maloideae (*Malus, Pyrus, Photinia, Chaenomeles*, *etc*.) are believed to have originated from an ancient allopolyploid since they have *n* = 17 base chromosomes whereas plants in other rosaceous subfamilies have *n* = 8 or 9 (Rowley, 1993).

A literature survey of chromosome number counts for *Rubus* species was made by Thompson (1997). Numbers are presented for 387 species, representing about 40 percent of the total number, and including 11 of the 12 subgenera. The basic number is universally 7 and ploidy levels include 2x, 3x, 4x, 5x, 6x, 8x, 9x, 10x, 11x, 12x, 14x, and, questionably, 13x and 18x. In a few species, more than one chromosome number has been reported.

An improved technique for counting chromosomes in the root tips of almond has been developed ([6]http), where the pretreatment was effected with colchicine, fixation in 6 methanol : 3 propionic acid : 2 chloroform,

hydrolysis in 1N HCl, followed by staining in acetic acid - orcein. This technique has been successfully applied to other *Prunus* species including peach and apricot, utilizing either root tips or pollen mother cells.

Lim *et al*. (1998) reported genomic *in situ* hybridization (GISH) and fluorescent *in situ* hybridization (FISH) data for chromosomes of raspberry (*Rubus idaeus* 2n = 2x = 14), blackberry (*Rubus aggregate* subgenus *Eubatus* 2n = 2 - 12x = 14 - 84) and their allopolyploid derivatives used in fruit breeding programmes. GISH analysis of an aneuoctaploid blackberry cv. Aurora (2n = 8x = 58) showed that both whole and translocated raspberry chromosomes were present.

Somatic chromosome numbers and morphology in three species of *Spiraea* collected in China was studied by Oginuma *et al*. (1999). Karyomorphology is consistent between two diploid species, *S. japonica* (2n = 18) and *S. rosthornii* (2n = 18). Somatic chromosome number of 2n = 72 of *S. schneideriana* var. *amphidoxa* collected in high mountainous regions at an altitude of 3,800 m in Sichuan Province is octoploid with x = 9 and was found to be the highest number reported within the genus.

According to Potter *et al*. (2002), the basic chromosome data of the four subfamilies of the family Rosaceae are as follows: Rosoideae (*Rosa*, *Fragaria*, *Potentilla*, and *Rubus*; fruit = achene; x = 7, 8 or 9), Prunoideae (*Prunus*; fruit = drupe; x = 8), Spiraeoideae (*Spirea*; fruit = follicle or capsule; x = 9), and Maloideae (*Malus*, *Pyrus*, and *Cotoneaster*; fruit = pome; x = 17).

Zhao-Yang *et al*. (2002) reported a karyomorphological study on one natural population of each of eight varieties in the *Spiraea japonica* complex. The species *S. japonica* var. *japonica*, *S. japonica* var. *acuta*, *S. japonica* var. *incisa*, *S. japonica* var. *stellaris, S. japonica* var. *acuminate, S. japonica* var. *ovalifolia* and *S. japonica* var. *glabra* were found to be with 2n = 18 chromosomes. Whereas, *S. japonica* var. *fortunei* possesses 2n = 36.

The localization of ribosomal RNA genes on chromosomes of almond

(*Prunus amygdalus*, 2n = 16) was studied with the help of fluorescence *in situ* hybridization by Corredor *et al*. (2005). Hayirhoglu-Ayaz *et al*. (2006) conducted karyological analysis of 14 species of *Alchemilla* collected from SW Europe (Spain and French Pyrenees). About 75% of the analyzed species have chromosome numbers 2n = 95 – 136. A few species have lower chromosome numbers. The chromosome numbers of 13 species are presented for the first time.

Recent phylogenetic analyses of combined sequence data from six nuclear and four chloroplast loci together with the basic chromosome number data provided strong support for the division of Rosaceae into three subfamilies (Potter *et al*., 2007). These include Dryadoideae (including *Cercocarpus, Dryas,* and *Purshia*; x = 9), Rosoideae (including *Fragaria, Potentilla, Rosa*, *Rubus,* and others; x = 7), and Spiraeoideae (including *Kerria*, *Spiraea* and others; x = 8, 9, 15, or 17).

Biosystematic studies of *Sorbus meinichii* (Rosaceae) was made by Bolstad and Salvesen (2008). Morphometric and cytological data were used to test the variation within the apomictic hybrid *Sorbus meinichii* from its type locality, in the Moster area, Bomlo in Sunnhordland, Norway. Cytological studies showed *S. meinichii* to be triploid, with 2n = 51 chromosomes.

Cytological and karyotype studies had been reported on strawberry plants by several earlier workers. Yarnell (1929) revealed the somatic chromosome number of *Fragaria collina, F. maxima,* and *F. nilgerrensis,* all belonging to the diploid group with 2n = 14 somatic chromosomes. Detailed genetic and cytological studies on the various polyploid and aneuploid forms in *Fragaria had been conducted by* Kihara (1930) and also by Yarnell (1931a; 1931b). Karyological and genetic studies of some species of *Fragaria* conducted by Lilienfeld (1933) revealed the chromosome numbers of *F. nipponica* (n = 7) and *F. elatior* (n = 21). Similar studies conducted by Lilienfeld (1936), revealed that autopolyploidy might have occurred during the evolution of the strawberry, *Fragaria elatior*.

Fedorova (1934) conducted studies on the polyploid forms shown by all *Fragaria* species. He had found 16 cases of doubling of the chromosome number in sexual cells of hybrids, and 5 cases of doubling in pure species. According to him the higher polyploid series of *Fragaria*, with 14, 28, 42, 56 somatic chromosomes, may arise as a result of allopolyploidy or autopolyploidy.

Cytological studies were conducted by Scott (1951) on a colchicine-induced autotetraploid, *Fragaria vesca* and on a strawberry plant derived from crosses of the autotetraploid *F. vesca* and cultivated strawberry. The hybrid hexaploid plants when crossed with the cultivated octoploids gave seedlings with chromosome numbers of 49, 70, and 77. The 70 chromosome plants (decaploids) are new types which are relatively fertile and some have the high aroma characteristic of *F. vesca.* These plants have 14 chromosomes from *F. vesca* and 56 from the cultivated strawberry.

Reports are also available regarding the cytogenetical and karyological studies in different species of *Fragaria* (Staudt, 1952; 1955). Accessions of the cultivars two octaploid progenitor species, *F. chiloensis* and *F. virginiana*, are valued by strawberry breeders as sources of novel traits, especially pest resistance and abiotic stress tolerance. Because strawberry is a relatively new crop, dating to the 1700s (Darrow, 1966), as few as three introgressive backcrosses can yield selections of cultivar quality.

A natural nonaploid hybrid (2n = 63) from fusion of a reduced *F. vesca* male gamete with an unreduced *F. chiloensis* gamete, and a partially fertile natural hexaploid hybrid (2n = 42) from fusion of an unreduced *F. vesca* (2n = 14) male gamete with a reduced F*. chiloensis* (2n = 56) gamete were discovered in separate mixed colonies along with over 20 additional pentaploid (2n = 35) hybrids (Bringhurst and Senanayake, 1966). This justifies the postulation that other euploid levels also may occur naturally, including triploids, tetraploids, decaploids, 12 ploids, and 16 ploids.

Of these, the even multiples tetraploids, decaploids, 12 ploids, and 16 ploids should be at least partially fertile.

Studies on the relationship between production of albino berries and mixoploidy in the strawberry (*Fragaria* x *ananassa* cv. Kent) was done during summer of 1997 and 1998 ([1]http). As clonal origin seemed to be correlated with this disorder, they characterized normal and abnormal plants by RAPD and chromosome numbers. RAPD analysis did not yield polymorphism. However, chromosome counts revealed mixoploidy in 63 % of the cells evaluated in the abnormal plants when compared to 40 % in the normal plants; numbers of chromosomes was found to be varying from 44 to 106 and 51 to 105 respectively. The results suggest that there could be a critical level for the presence of mixoploid cells, which seems to be associated with the appearance of phenotypic or biochemical abnormalities like albinism.

Studies on the ecological differentiation in perennial, octoploid species of *Fragaria* (2n = 56) had been conducted by Hancock and Bringhurst (1979). It has been suggested that ecological differentiation plays a minor role in determining the eco-geographical range of allopolyploids due to the effects of polyploidy on the generation of mutational and recombinational variability, which has not been the case in *Fragaria*.

Recently karyotype and ribosomal gene mapping had been made in *Fragaria vesca* ([5]http). Fluorescent *in situ* hybridisation (FISH), with cloned probes for the ribosomal RNA genes 45S and 5S, were used for detecting the location of the rDNA gene sequences on the chromosomes of *Fragaria vesca* (2n = 2x = 14). Three pairs of 45S loci and a pair at 5S locus were identified and these provided 3 chromosomal markers for the karyotype of the seven chromosome pairs.

According to Hancock and Luby (1993) the cultivated strawberry (*Fragaria x ananassa*) is a hybrid derived from two New world species, *F. chiloensis* and *F. virginiana*, in the mid 18th century and all three species are octaploids (2n = 8x = 56).

Ahokas (1998) described a method for inducing polyploids in detached stolons using an aqueous solution of colchicine, DMSO and glycerol. About 10% of the treated stolons, starting with diploid and tetraploid strawberry, are converted, but 50% non viability is caused by the treatment. The method can be applied to some other species and shoot types, using other chemicals with chromosome doubling or reducing effect, or even mutagens.

An interspecific hybrid was obtained from the cross of *Fragaria nilgerrensis* (2n = 2x = 14), a diploid species derived from China, and two cultivars of octaploid *F. ananassa*. (2n = 8x = 56) by the means of immature embryo culture technique with the latter as the pollen parent (Ma and Chen, 2004). The hybrids were pentaploids and have poor fertility. The hybrids showed expected 2n = 35 chromosomes in somatic cells and shows abnormal meiotic chromosome associations forming aberrant microspores.

A recent work reports cytological studies on unreduced gamete formation of strawberries (*Fragaria*) ([14]http). A diploid strawberry (*Fragaria vesca*) which can naturally produce unreduced gametes (2n pollen) and doubled unreduced gametes (4n pollen) was used to study the cytological mechanism of 2n and 4n gamete formation. The result showed that the formation of 2n gamete was mainly due to the abnormal orientation of spindles at metaphase.

The phenotypical and cytogenetical characterization of intergeneric hybrids of *Fragaria* x *Potentilla* and some ornamental varieties of *Fragaria* with unknown origin were made by Sutan and Popescu (2006). The ploidy level of the intergeneric hybrids of *Fragaria* x *Potentilla* was found to be different, depending on their origin.

In strawberry the octoploids are diploidized allopolyploids that have descended from four diploid ancestors. Although the diploid ancestry of the octoploid has yet to be definitively established, the leading ancestral candidate diploids are *F. vesca* and *F. iinumae*, with *F. bucharica* and *F. mandshurica* as additional intriguing possibilities (Folta and Davis, 2006). Currently, 23 species are recognized in the genus *Fragaria,* including 13 diploids (2*n* = 2*x* = 14), four tetraploids, one hexaploid, and four octaploids

(Folta and Davis, 2006).

According to Davis *et al*. (2007), the cultivated strawberry is genomically complex due to its octaploid (2n = 8x = 56) composition. Chromosome studies conducted by Preeda *et al*. (2007) revealed somatic chromosomes in *Fragaria vesca* (2n = 14) and *Fragaria* x *ananassa* (2n = 56). They used 0.002 M 8-hydroxyquinoline as cytostatic solution, fixed using 3 : 1 absolute alcohol : glacial acetic acid for 40 min, hydrolyzed in the 1N HCl solution at room temperature for 2 h, macerated using an enzyme solution for 25 min at 42 °C, and stained in 1.5% lacto-propionic orcein solution. On the other hand, in case of DAPI staining, the macerated root tips of *F.* x *ananassa* were soaked in 60% acetic acid for 5 min. before staining. Their respective 14 (diploid) and 56 (octaploid) chromosomes were counted.

Very recently Ahokas (2008) had described a tetraploid (2n = 28) clone of *Fragaria* from SW Finland. The following alternatives are considered as possible reasons for the tetraploidy; unreduced gametes in an interspecific hybrid or hybrid derivative; hybridization followed by induction of tetraploidy either by viruses, or by industrial pollutants, *e.g*. chlorophenols and their congener compounds in the soil. A hexaploid (*F. moschata*) diploid hybrid leads directly to tetraploidy and a tetraploid derivative from an octoploid (*F.* x *ananassa*) diploid hybridization can also be obtained.

The karyotypes and chromosome associations at meiosis in two types of natural hybrids, 7x and 8x, between *Duchesnea chrysantha* (2x) and *D. indica* (12x) were investigated by Naruhashi and Iwatsubo (1991). The 7x hybrid had a haploid chromosome set from each parent plant, whereas the 8x hybrid was considered to have a full set of *D. chrysantha* and half a set of *D. indica*. In the two hybrids, the chromosomes of *D. chrysantha* and *D. indica* conjugated only slightly at meiosis. It is probable that no common genome set between the diploid *D. chrysantha* and the dodecaploid *D. indica* exists. They concluded that *D. chrysantha* and *D. indica* should be

considered to be distinct species, although they have sometimes been treated as a single species.

RAPD analysis proves to be very useful to detect the genomic changes that usually occur in the highly repetitive fraction of the genome and are limited to a specific subset of these sequences which may be localized at particular chromosomal sites. RAPD markers were used for distinguishing various plant species by several workers. Major *et al*. (1998) compared the isozyme and RAPD analysis to identify the variability among the clones of *Robinia pseudoacacia*. There are reports on the use of RAPD in determining the genetic relationship and variation in many plant species (Yu and Pauls, 1993; Liu *et al*., 1994; Swobodha and Bhalla, 1997; De Bustos *et al*., 1999). This technique had been used for genetic analysis of micropropagated plants (Rani *et al*., 1995; Shoyama *et al.,* 1997; Goto *et al*., 1998; Watanabe *et al.,* 1998). Several workers applied RAPD to detect somaclonal variation (Bohm and Zyprian, 1998; Al-Zahim *et al*., 1999; De Verno *et al*., 1999) and to identify micropropagated plants and cultivars (Ho *et al*., 1997). RAPD markers were used for distinguishing plant species such as cereals (Ko *et al*., 1994) and *Festuca* (Valles *et al*., 1993) and also for genetic mapping (Williams *et al*., 1990; Yu and Pauls, 1993). Valles *et al*. (1993) used this technique for analyzing genetic stability of tissue cultured plants. Study of DNA polymorphism, marker assisted breeding programmes *etc*. also rely upon RAPD studies. Other applications are in creating linkage maps, locating defective and disease resistant genes, identification of chromosome specific markers *etc*. According to Gallego and Martinez (1997), RAPD is found to be technically easier with low statistical errors.

There are reports on the use of RAPD based technology for studying genetic relationships and diversity in some of the members of Rosaceae. Molecular analysis of a random sample of roots obtained from almond, apple, plum, *Pyrus pyraster* and two hybrid rootstocks, after co-culture with *Agrobacterium rhizogenes*, with and without the addition of hormones was

done by (Damiano and Monticelli, 1998). Amplification of the sequences of *rolB* and *vir* genes was done using PCR.

Forty one of the major strawberry (*Fragaria* x *ananassa*) cultivars grown in the United States and Canada were examined by RAPD (randomly amplified polymorphic DNA) marker polymorphisms using 10mer primers (> 50 % GC content) by Degani *et al*. (1998). A set of 10 primers produced 15 polymorphic fragments ranging in size between 450 and 1200 bp, which were more than sufficient to distinguish among all tested cultivars. Ten of the markers derived from seven primers were absolutely required for distinguishing the cultivars. They demonstrated that RAPD markers can be used effectively for strawberry cultivar identification.

Bartish *et al*. (1999) studied genetic relatedness in *Chaenomeles* (Rosaceae) by RAPD analysis in 42 plants representing accessions of three wild species and one hybrid taxon. Amplification with 17 primers yielded a total of 156 polymorphic RAPD bands. Genetic relatedness was estimated among the different species of *Chaenomeles*, suggesting the most distantly related species, the most closely related species and which takes an intermediate position between these two.

Shimada *et al*. (1999) investigated the genetic diversity of 42 plum varieties by RAPD analysis. Twenty primers discriminated all plum varieties thereby revealing the genetic distinctness of each variety from the other plums by cluster analysis.

The combination studies of *in vitro* embryo culture with the use of molecular markers were made by Hormaza (1999). In this work the combination of those two techniques has been used to assess, with certainty, the paternity of embryos obtained after mixed pollinations with pollen from three cultivars of cherry (*Prunus avium*).

The taxonomy of the dog-roses (*Rosa* sect. *Caninae*) were analysed with the help of molecular markers by Olsson *et al*. (2000). They used a

novel combination of random amplified polymorphic DNA (RAPD) markers and elliptic Fourier analysis of leaflet shape to investigate relationships within and between the seven common dog-rose taxa in the Nordic countries.

Genetic relationships among four taxa in the genus *Chaenomeles* using isozyme analysis were studied by Garkava *et al*. (2000). The band patterns obtained with six polymorphic isozyme systems provided 108 reliable markers, which were scored as unordered multistate traits. A cluster analysis as well as a multidimensional scaling analysis grouped the taxa in agreement with previously published results obtained with RAPD (random amplified polymorphic DNA) analysis. However, the isozyme data were less efficient than the RAPD data for intraspecific grouping of the genotypes according to the origin of the plant material.

RAPD analysis was applied to reveal the genetic diversities of 4 species of subg. *Lithocerasus* within the genus *Prunus* using 40 accessions representing the subgenera *Prunophora*, *Amygdalus*, *Lithocerasus* and *Cerasus* (Takehiko *et al*., 2001).

The extent of clonality and genetic diversity in *Lyonothamnus floribundus*, or island ironwood (Rosaceae), an endemic species found on only four of the eight California Channel Islands had been analysed by Bushakra *et al*. (2003). They have used random amplified polymorphic DNA (RAPD) analysis to examine clonality and genetic diversity in *L. floribundus*.

Genomic studies of Rosaceous fruit trees conducted by Arus and Gardiner (2007) have concentrated on two species; peach (*Prunus persica*), which has served as a model for other species of the same genus, such as the stone fruits (apricot, cherry and plum) and almond; and apple (*Malus* x *domestica*), which itself is a model for other close species such as pear, quince and loquat.

Several earlier workers had conducted molecular research on the different strawberry species. Few isoenzymatic markers have been used in the identification of variants and in evaluation of the genetic diversity of strawberries. Apparently, the studies on isoenzymes are not highly effective (Arulsekar *et al*., 1981; Bringhurst *et al*., 1981).

Genetic diversity studies can be very effectively conducted with the help of RAPD, which has been proved to be successful in Chilean *Fragaria chiloensis* accessions. ([15]http). The genetic diversity of a Chilean *Fragaria chiloensis* collection (82 accessions collected between 34º and 48º S) was assessed with a genomic analysis technique known as RAPD. Out of 160 primers evaluated only 10% turned out to be informative, yielding a total of 38 polymorphic bands.

In recent years genetic markers associated with usable properties have proved particularly useful in plant breeding. Genetic markers enable observation of regrouping in strawberry genomes and permanent and relatively quick analysis of the segregation of alleles, which facilitates the selection of mixed species (Levi *et al*., 1994).

Graham *et al*. (1996) analysed genetic similarities between 8 cultivars of strawberry. Using 10 primers for their analysis, they obtained 116 bands, 79 (68%) of which were polymorphic and 37 (32%) were monomorphic.

The level of genetic diversity between 16 cultivars of strawberry (*Fragaria* x *ananassa*) and the wild species of *Fragaria virginiana* was studied on the basis of the analysis of their DNA by RAPD reaction ([16]http). Six 10-nucleotide primers generated jointly 354 bands, of which 94.8% were polymorphic and 5.2% monomorphic.

Relationships among 37 North American octaploid strawberry populations were studied by evaluating 44 morphological traits and 36 randomly amplified polymorphic DNA (RAPD) markers (Harrison *et al*., 1997). RAPD data revealed that all octaploid North American strawberries

have likely derived from a common ancestor and have differentiated into *F*. *chiloensis* and *F*. *virginiana* by adapting to moister and drier environments, respectively.

RAPD analysis had been conducted to find out the relationship of North and South American subspecies of *Fragaria chiloensis (*Porebski and Catling, 1998). To improve the intraspecific classification of *Fragaria chiloensis*, 35 plants including 5 North American subsp. *lucida*, 15 North American subsp. *pacifica*, and 15 South American subsp. *chiloensis* were analysed. From 100 primers screened, 12 provided 62 scorable polymorphic bands.

Phylogenetic studies can be very effectively done by using random amplified hybridized fragment polymorphism (RAHFPs) as has been found in the case of some *Fragaria* spp. ([17]http) with the aim to understand better the genomic structure of the cultivated strawberry and the genetic relationship among the octaploid *Fragaria* species. Out of the 92 random primers of 10bp, several DNA fragments were obtained and 84 out of 213 were utilized like probes. The hybridizations were performed either on amplification products or on digested genomic DNA fragments. The polymorphisms obtained indicated that the class of molecular marker named RAHFPs are more informative than RAPDs.

Micropropagated strawberry plants (*Fragaria* x *ananassa*) grown on 5 µM and 15 µM BA medium or cold-stored were grown in the field to examine morphological variation (Kumar *et al*., 1999). Except for plant height, morphological characteristics did not differ for field grown plants micropropagated on 5 µM and 15 µM BA medium. Cold-stored plants were less vigorous, both vegetatively and reproductively, than BA treated plants. Random amplified polymorphic DNA (RAPD) markers were used to determine if cold storage or supra-optimal levels of $N^6$-benzyladenine (BA) in the culture medium caused genetic changes leading to somaclonal variation. Analysis was made in 246

loci amplified by the 29 primers tested. Possible changes in methylation patterns of ribosomal DNA genes of strawberries were also examined.

Morphological, anatomical and molecular techniques were used to characterize wild strawberry and wild strawberry-like species in northwest Argentina (Ontivero *et al*., 2000). Characteristics of leaves, flowers, runners, achenes, and genomic DNA polymorphisms were used to analyze similarities among *Potentilla tucumanensis*, *Duchesnea indica*, and *Fragaria vesca*. Comparison of phenograms obtained by using morphological and anatomical traits or genomic DNA characters revealed similar clustering of the species. Both phenograms suggest that *D. indica* is more closely related to *P. tucumanensis* than to *F. vesca*. Using the randomly amplified polymorphic DNA (RAPD) technique with specific primers, they detected polymorphic bands that permit the identification of *P. tucumanensis*, *D. indica*, and *F. vesca*. In addition, they report new morphological and anatomical characters that can be used as diagnostic traits for better identification of species in reproductive and vegetative states.

Congiu *et al*. (2000) used random amplified polymorphic DNA (RAPD) technique to settle a lawsuit involving unauthorized commercialization of a patented strawberry variety of high economical relevance (Marmolada). Because of economical involvements, the molecular approach was added to the more traditional morphological examination in a double-blind test. All plants belonging to the patented variety were unambiguously identified (13 plants among a total of 31 plants examined). The results were accepted as evidence in the court. This study confirms that the RAPD technique is especially suitable for identification of asexually reproduced plant varieties for forensic or agricultural purposes.

A detailed work was conducted to determine the genetic diversity of a representative sample of the Chilean strawberry (*Fragaria chiloensis*) using biochemical and molecular markers (Becerra *et al*., 2001). They compared the information generated by random amplified polymorphic DNA (RAPD)

diversity within the same accessions and also to determine the feasibility of using this information for a strawberry breeding and improvement program.

Nineteen of the major strawberry (*Fragaria* x *ananassa*) cultivars grown in the United States and Canada were examined for AFLP and RAPD marker polymorphisms (Degani *et al*., 2001). The RAPD markers had been specifically selected for fingerprinting purposes because they successfully distinguish 41 strawberry cultivars, including the 19 cultivars analyzed. Separate dendrograms were constructed based on analysis of the AFLP and RAPD marker data using a neighbor-joining algorithm. A detailed comparison of genetic relationship measures in strawberry (*Fragaria x ananassa*) had been made based on AFLPs, RAPDs and pedigree data.

Kuras *et al*. (2004) used two PCR based techniques, RAPD and ISSR, for determination of genetic relationship of 24 strawberry cultivars used in breeding program at the Research Institute of Pomology and Floriculture in Poland. Polymorphism of investigated genotypes was observed in reactions with 23 out of 48 tested RAPD primers and 41 from 90 tested ISSR primers. Recently molecular characterization and genetic diversity in *Fragaria* genotypes were revealed by randomly amplified DNA polymorphisms ([18]http). The efficiency of RAPD markers for undertaking *Fragaria* DNA fingerprinting and the estimation of genetic relatedness was evaluated. Thirteen cultivars of *Fragaria* x *ananassa* and two wild species, *Fragaria indica* and *Fragaria vesca* were analyzed with twenty 10 bp primers. Only thirteen yielded scorable polymorphic amplification patterns based on discernible bands, however, no single primer out of these thirteen could produce a unique fingerprint for all the fifteen genotypes.

Gambardella *et al*. (2005) conducted studies on the molecular and morphological characterization of wild and cultivated *Fragaria* growing in southern Chile. Hokanson *et al*. (2006) conducted studies to find out the relationships among the new world octaploid strawberry species, *Fragaria virginiana* and *Fragaria chiloensis*, based on simple sequence repeat marker

analysis. Marker analysis revealed that these two new world octaploid strawberry species are closely related. Experimental studies were conducted by Sugimoto *et al*. (2006) to identify markers for the everbearing gene in strawberries (*Fragaria* x *ananassa*). Seventy one primers, which produced 89 polymorphic fragments between the two parents, were identified from a total of 175 primers.

Random amplified polymorphic DNA (RAPD) markers were effectively used to evaluate genetic variability among populations of an Italian strawberry ecotype, and to determinate genetic relationships between genotypes and their putative ancestor (Milella *et al*., 2006). A total of 222 RAPD markers were obtained using 16 decamer primers and 6 longer primers, 90.8% of the markers obtained by selected primers were turned to be polymorphic at least within analysed genotypes. The results obtained confirm that RAPD markers could be used as reliable markers to perform phylogenetic studies in *Fragaria* x *ananassa*.

One hundred and two accessions of wild and cultivated *Fragaria chiloensis*, collected in southern Chile were studied ([4]http). They were characterized using isozymes and RAPD markers. Out of the 80 Operon primers used, 17 were selected because they provided a greater number of polymorphic bands. The information was used to evaluate the genetic variability among wild clones. The results indicated that there exists a high degree of genetic variability among the different accessions and that four genetically related groups can be identified.

Cytogenetics and RAPD analysis of interspecific hybrids of two *Fragaria* spp. had been conducted by Ma *et al*. (2007). Embryo rescue obtained interspecific hybrids of *Fragaria mandschurica* and *F*. x *ananassa* were used as materials for the identification of somatic cell hybridization between the species. RAPD analysis showed that the hybrid offspring showed similarities with parents. Individual genetic heterogeneity existed among the generations were also revealed.

Previous reports reveal that the members of Rosaceae are blessed with an array of aromatic compounds, which are stored in various parts of these plants. Vollmann and Schultze (1995) studied the root essential oils of several members of the genera *Geum*, *Waldsteinia* and *Coluria*, of the subtribe *Geinae* (Rosaceae) by GC-MS. The qualitative composition of these oils was relatively similar with eugenol and some pinane derivatives, *e.g*. myrtanals, myrtenal, myrtanols and myrtenol, as characteristic constituents. The oils differed considerably concerning the quantitative composition, *e.g*. two groups of *Geum* species could be observed, one with a high (> 65%) and one with a low (< 5%) content of eugenol. In the root oil of *Waldsteinia ternata*, only traces of this phenyl propanoid were found. Headspace analysis of a root extract of *W. geoides* showed a high percentage of cinnamyl alcohol.

Volatile constituents of the essential oil of flowers of *Rosa brunonii* was studied by Kaul *et al*. (1999). The essential oil of the flowers of *Rosa brunonii* (Rosaceae) was prepared by hydrodistillation and studied by capillary GC-FID and GC-MS; 35 constituents were identified, accounting for 78.4% of the total oil. The essential oil consisted mainly of eugenol (30%), citronellol (2.65%), geraniol (10.5%) and terpinen-4-ol (13.7%) as the major compounds.

Demetzos *et al*. (2002) reported the chemical composition of the essential oils of twenty five populations of *Cistus creticus* subsp. *creticus* (Rosaceae) from the island of Crete (Greece) and their interpopulation variability were analysed in detail by GC-MS. 142 compounds were identified representing an average of 56.8 - 89.8% of the oil composition. The components are represented here by homologous series of monoterpenes, oxygenated monoterpenes, sesquiterpenes, oxygenated sesquiterpenes, diterpenes, labdane diterpenes, aldehydes, alkanes, esters, fatty acids, ketones, and others. Labdane diterpenes were detected and identified in the essential oils and have been found in high percentage composition.

Essential oil analysis of three species of “Chamae Roses” was made by Tucker *et al*. (2003).

The essential oil of *Chamaebatiaria millefolium* from California is dominated by 24.90 ± 4.46% camphor, 17.36 ± 4.23% borneol, 11.17 ± 4.26% camphene, and 10.95 ± 4.59% α-pinene. The essential oil of *Chamaebatia australis* from California is dominated by no constituent greater than 10% but contains 7.07 ± 0.97% δ-cadinene, 6.84 ± 1.47% terpinen-4-ol, and 5.46 ± 1.50% linalool. The essential oil of *Chamaebatia foliolosa* from California is dominated by 12.90 ± 3.67% unidentified sesquiterpene alcohol and 15.96 ± 6.61% viridiflorol.

Eutuxia and Loannis (2005) analysed the volatile constituents of the jam of *Crataegus azarolus* (Rosaceae) using simultaneous distillation and extraction and identified by gas chromatography - mass spectroscopy. Forty four volatile compounds were identified for the first time in this jam, while 12 peaks were not identified. The volatile compounds were quantitatively determined by the use of an internal standard. The major classes of compounds identified included aliphatic and aromatic aldehydes, ketones, alcohols, monoterpenes, sesquiterpenes and hydrocarbons. The major constituent identified was 2-furaldehyde. Physicochemical properties of the seed oils of three species of Rosaceae family, namely *Prunus armeniaca* (apricot), *P. cerasifera* (prune), and *P. persica* (peach) have been determined by Javed *et al*. (2006).

Previous reports show that considerable amount of work had been done to evaluate the chemical components present in the strawberry plants. Larsen *et al*. (1992) evaluated the aroma composition of some strawberry (*Fragaria* ananassa) cultivars by the use of odor threshold values.

Hamilton Kemp *et al*. (1993) isolated headspace compounds from detached strawberry (*Fragaria ananassa*) foliage by using both air and nitrogen as entrainment gases and trapping on the porous polymer Tenax.

Compounds were eluted from traps with hexane, analyzed by GC and GC -MS, and identified by comparison with authentic standards. The profile of volatiles entrained with nitrogen differed considerably from that obtained with air; the former yielded more aliphatic alcohols, esters, and aromatics and the latter yielded greater quantities of terpene hydrocarbons.

GC – MS studies of the essential oil composition of three strawberry genotypes, *Fragaria* x *ananassa* were made by Khanizadeh and Belanger (1993). Thirty seven compounds were detected of which sixteen were identified. The major components were linalool (16.08 - 18.80%) and nonanal (5.89 - 16.63%). Many of the other constituents were aliphatic in nature. Differences in oil composition among the three cultivars were observed.

Two isomeric methyl jasmonates were isolated from extracts of strawberry fruit (*Fragaria* x *ananassa* cv. Kent) by means of micropreparative gas chromatography (Gansser *et al*., 1997). It was found that the levels of methyl jasmonates in strawberry may affect aroma formation and further events during fruit development.

The aroma compositions of *Fragaria* x *ananassa* varieties are assessed by purge and trap high-resolution gas chromatography by Gomes da Silva and Chaves das Neves (1999). Gas chromatography - mass spectrometry and gas chromatography - Fourier transform infrared spectroscopy allow the identification of 93 components from which 21 are for the first time described as constituents of strawberry aroma.

Volatile flavour components of two strawberry (*Fragaria* x *ananassa*) varieties, Bogyojosaeng and Suhong, ere extracted by SDE (Simultaneous steam distillation and extraction) using a mixture of n-pentane and diethylether (1 : 1, v/v) as an extract solvent (Eun-Ryong *et al*., 2000). Analysis of the concentrate by capillary gas chromatography and gas chromatography - mass spectrometry led to the identification of 146 and 153 components in Bogyojosaeng and Suhong respectively. Among these, (E)-2-

hexenyl acetate (4.56%) in Bogyojosaeng and (E)-nerolidol (12.38%) in Suhong were major compounds and acetic acid, (E)-2-hexenal, hexyl acetate, ethyl acetate, ethyl butanoate, methyl butanoate, ethyl hexanoate and γ-dodecalactone were the main components in each sample, though there were several differences in composition and threshold of volatile compounds.

Studies conducted by Ruan *et al*. (2001) reveal the chemical constituents from the fruit of *Fragaria* x *ananassa*. They used chromatographic methods to isolate compounds and followed chemical and spectral methods to elucidate their structures. Three compounds, 9, 19-cyclolanost-24-en-3-ol, 14-methyl-stigmasta-7, 24(28)-dien-3-ol and beta-sitosterol were isolated from the freeze dried powder.

Total soluble phenols, soluble flavanols, (+)-catechin, ferulic acid and 1-O-feruloyl-beta-d-glucose were analyzed during the development of a strawberry (*Fragaria* x *ananassa,* cv. Chandler) callus culture by Lopez Arnaldos *et al*. (2001). The time course changes of the different phenols assayed were well correlated with callus growth and morphology.

Volatile components of strawberries (*Fragaria x ananassa* cv. Korona) kept under low oxygen conditions had been evaluated by Rosenfeld *et al*. (2003). Compounds responsible for off-flavors in fruits kept in anaerobic atmosphere have partly been identified by means of GC-sniff. Ethyl acetate, ethanol, ethyl butanoate, butyl acetate and ethyl hexanoate showed relatively high concentrations in samples stored at 0.5 - 2% of oxygen and may together with ethylene cause off-flavors like fermented flavor.

Twenty six compounds, mainly flavanoids, coumarins and phenolcarbolic acids, were observed in *F. vesca* leaves by HPLC and TLC (Bubenchicova and Drozdova, 2003). Seven of these compounds were identified for the first time. Results showed that the carbohydrate complex of the leaves consists of water soluble polysaccharides, pectins and

hemicelluloses. Pectins and hemicelluloses are isolated from *F*. *vesca* leaves for the first time.

High performance liquid chromatography combined with diode array and electro spray ionization mass spectrometric detection was used to study soluble and insoluble forms of phenolic compounds in strawberries, raspberries (red and yellow cultivated and red wild), arctic bramble, and cloudberries (Maatta-Riihinen *et al*., 2004). Hydroxy-cinnamic acids were present as free forms in cloudberries and mainly as sugar esters in the other berries. Quercetin 3-glucuronide was the typical flavanol glycoside in all of the berries studied.

Strawberry fruits (*Fragaria* x *ananassa* cv. Elsanta) were harvested at the ideal stage of maturity and their volatile compound profile was analysed using both Atmospheric Pressure Chemical Ionisation–Mass Spectrometry (APCI–MS) and Gas Chromatograph – Mass Spectrometry (GC – MS) by Modise *et al*. (2004). There was good agreement on the compound identity using the two techniques for nine out of fourteen selected volatile compounds.

The fruits of 23 strawberry cultivars (*Fragaria x ananassa*) were studied to determine the contents of soluble solids, organic acids, ascorbic acid, sugar and anthocyanins (Zmuda *et al*., 2004). The cultivars contained 7.14 (Dukat) to 9.84% (Mara des Bois) soluble solids. The sugar and total organic acid contents ranged from 4.09 (Dukat) to 6.21% (Calypso) and 0.67 (Senga Sengana) to 1.06% (Mara des Bois), respectively. Elsanta and Gerida had the highest ascorbic acid content (~60 mg). Kama and Honeoye had the highest anthocyanin content (more than 800 mg/kg fresh weight).

Aaby *et al*. (2005) measured total phenolics and total monomeric anthocyanin content in the flesh and achenes of strawberries (*Fragaria x ananassa*). Ellagic acid, ellagic acid glycosides, and ellagitannins were the main contributors to the antioxidant activities of achenes. The major anthocyanin in flesh was pelargonidin-3-glucoside, whereas achenes

consisted of nearly equal amounts of cyanidin-3-glucoside and pelargonidin-3-glucoside.

Carrasco *et al*. (2005) revealed the chemical constituents responsible for the most popular strawberry aroma. Esters and furanones are the main aroma determinants in fresh strawberries. They also identified the inheritance patterns of the aroma trait in segregating populations of strawberries.

A detailed chemical and sensory analysis of the fruits of strawberry was conducted by Lawson *et al*. (2006). They have detected diacetyl compounds in strawberry fruit extract, which contributes to the quality of strawberry juice. A review on the biosynthesis of three major classes of flavor compounds in strawberry, namely carbohydrates, esters, and furanones were made by Bood and Zabetakis (2006). They are qualitatively discussed with respect to their importance in fruit flavor, their biochemical formation, and the biochemical relationships between each other and fruit ripening.

Phenolic compounds in strawberry (*Fragaria* x *ananassa*) fruits were identified by Aaby *et al*. (2007). They characterized about 40 phenolic compounds including glycosides of quercetin, kaempferol, cyanidin, pelargonidin, and ellagic acid, together with flavanols, derivatives of p-coumaric acid and ellagitannins. Quercetin-3-malonylhexoside and a deoxyhexoside of ellagic acid were reported for the first time.

The total phenolic, flavonoid and anthocyanin content of achenes (true fruit) and thalamus (receptacle) from the native South American *Fragaria chiloensis* subsp. *chiloensis* (*F*. *patagonica* and *F*. *chiloensis*), *Fragaria vesca* and *Fragaria* x *ananassa* cv. Chandler was determined by Cheel *et al*. (2007). Highest phenolic content was found in *F*. *vesca* while lowest content was measured for white strawberry (*F*. *chiloensis* subsp. *chiloensis*). The total anthocyanin and total flavanoid contents in the samples investigated was lower for the white strawberry and higher in *F*. x

*ananassa* cv. Chandler.

The phenolics from different strawberry cultivars (Aromas, Camarosa, Diamante, Medina and Ventana) cultivated in two different soil less systems (with and without recycling nutrient solution) were quantified to assess differences in their profiles as a function of both the variety and the cultivation system (Hernanz *et al.,* 2007). Considering groups of phenols, it was found that either anthocyanins (including pelargonidin - 3 - glucoside, cyaniding - 3 - glucoside, pelargonidin – 3 - rutinoside, pelargonidin – 3 -acetylglucoside and two unidentified pelargonidin derivatives) or phenolic acids (including caffeic, ferulic, p-coumaric, p-hydroxybenzoic, and ellagic acid) were quantitatively more important than those of flavanols (quercetin and kaempferol).

Ozgen *et al*. (2007) conducted studies on the determination of total phenolics. They found that strawberry plants are rich in phenolic compounds and possess potent antioxidant activity. Ulrich *et al*. (2007) conducted prospective analysis of four accessions of four wild strawberry accessions in comparison to a standard cultivar of *Fragaria* x *ananassa* by using human sensory, gas chromatography - mass spectrometry (GC - MS) and gas chromatography – olfactometry (GCO) analyses. The wild species have higher aroma intensities compared with the cultivated one. The flavour quality differs significantly. Semi quantitative GC analysis revealed that *F.* x *ananassa* cv. ‘Elsanta’ has the lowest content of volatile compounds whereas *Fragaria moschata* cv. Cotta has the highest. The aroma impressions, measured by GCO, support the findings of GC - MS analyses.

Tung *et al.* (2007) studied the chemical constituents of the wild strawberry plant (*Duchesnea indica*) from China. By employing a variety of chromatographic techniques like the chemical constituent purification and spectral data analysis *etc*. Several compounds were identified as short leaf hematoxylin acid, short leaf hematoxylin methyl phenol, short leaf hematoxylin phenol, Hill kaempferol, kaempferol, oleic acid, oleanolic acid, β – sitosterol and 6 - β hydroxyl - 2, 4 - ethyl - cholesteric - 4 – en - 3 - ketone.

The inheritance of important aroma compounds, especially the ester methyl anthranilate in strawberry is demonstrated by the use of a model population of *Fragaria* x ananassa (Olbricht *et al*., 2008). Studies conducted by Pinto *et al*. (2008) revealed that strawberry fruits are rich in various

bioactive compounds. They quantified total ellagic acid present in the fruits of strawberries (*Fragaria x ananassa*).

Aroma components of amphiploid strawberries derived from interspecific hybrids of *Fragaria x ananassa* and diploid wild species were detected recently ([19]http). The aroma components of amphiploids of *F*. *vesca* x *F*. *x ananassa* and *F*. *x ananassa* x *F*. *nilgerrensis* method were analyzed by GC - MS. Major aroma compounds of *F*. x *ananassa* were methyl butylate, ethyl butylate, methyl butyric acid, acetic acid and 2, 5 – dimethyl - 4-hydroxy (2H) furanone. *F*. *vesca* contained a high amount of ethyl acetate, but amounts of methyl butylate, ethyl butylate, organic acids and furanones were low. *Fragaria nilgerrensis* contained a high level of ethyl acetate and furanone, but amounts of methyl butylate, ethyl butylate and organic acids were low.

Recently a detailed analysis of volatile aromatics in the fruit extracts of 'Toyonoka' strawberries (*Fragaria* x *ananassa*) was made ([20]http). 52 volatile aromatic compounds were detected, out of which 2,5-dimethyl - 4 – hydroxy - 2H – furan – 3 - one (DMHF) was found to be the prominent one.

Even though Indian strawberry is closely related to cultivated commercial strawberries (*Fragaria* spp.), reports on research work of *Duchesnea indica* is scanty. However, the wide spectrum of literature collected regarding the work done on the various aspects of some rosaceous plants and commercial strawberries may help in comparing *D. indica* with its close allies. Apart from a very few reports cited earlier (Naruhashi and Iwatsubo, 1991; Ontivero *et al*., 2000; Tung *et al.*, 2007) cytological, RAPD and GC -MS analyses of the *in vivo* and *in vitro* plants of Indian strawberry, *D. indica* had not been attempted. Hence the present investigation is carried out on all these aspects in *D. indica*.

# MATERIALS AND METHODS

The experiments performed in the present investigation included the following:-

- Micropropagation of *Duchesnea indica* (Andr.) Focke.
- Cytological analysis of callus as well as *in vivo* and *in vitro* plants.
- Genetic evaluation of *in vivo* and *in vitro* plants using RAPD.
- Herbage essential oil characterization of *in vivo* and *in vitro* plants using GC - MS analysis.
- Total carbohydrate content analysis of both *in vitro* and *in vivo* derived fruits of *Duchesnea indica*.

**A. Micropropagation**

The species *Duchesnea indica* (Andr.) Focke was collected from Ooty, Tamilnadu, South India and authenticated at Calicut University Herbarium [*Umesh B. T., 86006* (CALI)] where voucher specimens are deposited. The plants were grown at the experimental garden attached to the Department of Botany, Calicut University. Micropropagation was attempted through direct multiplication as well as callus mediated regeneration using different explants. Though different explants were tried for the study, the nodal segments (2 - 2.5 cm) were used largely. The leaves from the explants were removed, washed thoroughly in running tap water to remove the soil particles. Washing continued for about 10 minutes by using mild detergents like Labolene, Teepol *etc*. After a quick rinse in 70% ethyl alcohol, surface sterilization was carried out with 0.1% mercuric chloride for

about 3 minutes. The explants were then washed with double distilled sterile water to remove the traces of mercuric chloride. Then after removing the exposed cut surfaces, the explants were implanted on to the nutrient medium.

Murashige and Skoog (1962) basal medium (Appendix I) with 3% glucose as carbon source and Agar (8%) as solidifying agent, 100mg/l myo inositol and 0.8% agar in a pH of 5.8 was used for the preparation of culture medium. Culture initiation was done with basal medium supplemented with different concentrations and combinations of growth hormones. The pH of the medium was adjusted to 5.7 - 5.8 before dispensing to the culture tubes. Sterilization of the media was done at 122$^{o}$ C for 20 minutes. Each experiment was set up with 10 - 15 replicates and repeated twice. The cultures were grown at 25 ± 3$^{o}$ C with humidity of 55 - 60% under fluorescent light tubes emitting 2000 lux for 16/8 hours light/dark period and were sub cultured every 4 - 6 weeks.

Four to six weeks old regenerated plantlets were transferred to MS medium with different auxin concentrations for rooting. The rooted plants were carefully taken out of the tube and were potted in a sterilized mixture of soil and sand (1 : 1) after removing the traces of the medium. The plantlets were initially watered with ½ MS solution for one week. Established plantlets were transplanted to the field in earthen pots and watered regularly. These plants were used for further study.

**B. Cytological Analysis**

**a) Mitotic squash preparation:-**

Somatic chromosome spreads were prepared with the help of improved techniques (Sharma and Sharma, 1990). Young root tips were collected from both the parent plant and field transplanted *in vitro* plants at the period of peak mitotic activity (9 am - 10 am). Root tips were thoroughly washed in distilled water and treated in pre-treatment chemicals. Saturated

solution of para dichloro benzene with traces of aesculin was used for pre-treatment. Small quantity of saponin was also added to remove the oil particles from the cells. The pre treatment solution is initially chilled at 0 to 5$^{o}$C for 4 to 5 minutes and the root tips were treated at 12 to 15$^{o}$C for 2 to 3 hours. The treated root tips were then washed with distilled water and fixed in 1 : 3 acetic acid - ethanol mixture (modified Carnoy's Fluid) overnight.

The root tips were washed in distilled water followed by treatment in 1N HCl for 5 minutes at room temperature. After washing the root tips thoroughly to remove the traces of acid, they were stained with 2% aceto orcein for 3 - 4 hours. Stained root tips were washed in 45% acetic acid to remove the excess stain and squashed. The slides were scanned under Olympus microscope CX 21 and the photographs of well spread mitotic plates were taken using Olympus Camedia C - 4000 Zoom digital compact camera attached to the microscope.

**b) Karyomorphological Analysis:-**

Karyograms were generated with the aid of computer soft wares such as Adobe Photoshop, CHIAS and a data based analyzing system (Microsoft EXCEL). Photographs were scanned and stored as digital images. These digital images were converted into grey scale images using Adobe Photoshop programme. Identification numbers were allotted to each chromosome and then loaded to CHIAS for karyomorphometric analysis. The arm length of each chromosome was measured after determining the centromeric position. Then the centromeric indices were calculated. Homologous chromosomes were identified and classified on the basis of arm ratio and centromeric indices, according to Abraham and Prasad (1983). The images were reloaded to Adobe Photoshop and karyograms were generated.

Karyotype formula was calculated depending upon the length of the chromosome, position of the centromere and presence or absence of secondary constriction.

Disparity index (DI) of the chromosomes were calculated as per Mohanty *et al*. (1991) by using the formula,

$$DI = \frac{\text{Longest chromosome - shortest chromosome}}{\text{Longest chromosome + shortest chromosome}} \times 100$$

**Table 1. Details of chromosome nomenclature in relation to centromeric location based on arm ratios and centromeric indices (Abraham and Prasad, 1983).**

| Nomenclature | Notation | $R_1$ s/l | $R_2$ l/s | $I_1$ 100 s/c | $I_2$ 100 l/c |
|---|---|---|---|---|---|
| Median | M | 1.000 | 1.00 | 50.00 | 50.00 |
| Nearly median | nm | 0.99 to 0.61 | 1.01 to 1.63 | 49.99 to 38.01 | 50.01 to 61.99 |
| Nearly submedian | nsm (-) | 0.60 to 0.34 | 1.64 to 2.99 | 38.00 to 25.01 | 62.00 to 74.99 |
| Submedian | SM | 0.33 | 3.00 | 25.00 | 75.00 |
| Nearly submedian | nsm (+) | 0.32 to 0.23 | 3.01 to 4.26 | 24.99 to 18.20 | 75.01 to 81.80 |
| Nearly subterminal | nst (-) | 0.22 to 0.15 | 4.27 to 6.99 | 18.19 to 12.51 | 81.81 to 87.49 |
| Subterminal | ST | 0.14 | 7.00 | 12.50 | 87.50 |
| Nearly subterminal | nst (+) | 0.13 to 0.07 | 7.01 to 14.38 | 12.49 to 5.01 | 87.51 to 94.99 |
| Nearly terminal | nt | 0.06 to 0.01 | 14.39 to 19.99 | 5.00 to 0.01 | 95.00 to 99.99 |
| Terminal | T | 0.00 | $\infty$ | 0.00 | 100.00 |

R1 and R2 = Arm ratios
I 1 and I 2 = Centromeric indices
s - short arm length
I - long arm length

C-total chromosome length

The total Forma percentage (TF%) or mean centromeric index value was calculated after Huziwara (1962) by the formula:

$$TF\% = \frac{\text{Total sum of short arm length}}{\text{Total sum of chromosome length}} \times 100$$

**C. Random Amplified Polymorphic DNA (RAPD) Analysis**

RAPD method reveals sequence polymorphism between template DNAs based on selective amplification of DNA sequence. The template DNA can be prepared with any DNA purification protocol appropriate for the biological sample under study. Plants selected for DNA fingerprinting were the same as that used for cytological analysis.

**a) Isolation and purification of DNA:-**

DNA was extracted from young leaf tissues of *Duchesnea indica* (both *in vivo* and *in vitro* plants) following CTAB method of Ausubel *et al*. (1995). The steps involved are:-

- Grind 100 mg of fresh young leaf tissue in liquid nitrogen with a mortar and pestle. Add 900 µl of pre-heated (60$^{o}$C) CTAB buffer (Appendix II).
- Incubate the mixture at 60$^{o}$C for 10 min. on a water bath. Transferred it to the tubes and refrigerated (4$^{o}$C) for 5min.
- Add equal volume (900µl) of ice cold chloroform : isoamyl alcohol (24:1) mixture. Mix well and spin at 10,000 rpm for 10 min. at room temperature.
- Draw the clear supernatant, add 2/3$^{rd}$ volume of ice cold isopropanol and mix gently.

- Incubate the mixture at -20$^{o}$C for 30 min. for better precipitation of DNA. Centrifuge at 14,000 rpm for 10 min at 4$^{o}$C.
- Discard the supernatant. Add 50 µl 70% ethanol and rinse the pellet by spinning at 5000 rpm for 5 min.
- Discard the supernatant and dissolve the pellet in 200 µl of TE buffer (Appendix III).
- Add an equal volume of phenol : chloroform : isoamyl alcohol (25 : 24 : 1) mixture. Mix well and spin at 10,000 rpm for 10 min. at room temperature.
- To the clear supernatant, add 2/3$^{rd}$ volume of ice cold isopropanol and incubate the mixture at -20$^{o}$C for 30 min.
- Centrifuge at 14,000 rpm for 10 min at 4$^{o}$C. Discard the supernatant.
- Add 500 µl 70% ethanol and rinse the pellet by spinning at 5,000 rpm for 5 min.
- Discard the supernatant, air dry the pellet and dissolve the pellet in 200 µl TE buffer.
- Add 1 µl of RNase stock (1 mg/ml), incubate at 37$^{o}$C for 30 min.
- Add 1/10$^{th}$ volume of sodium acetate and 2.5 volume of ice cold absolute alcohol. Allow for precipitation at -20$^{o}$C for 1 hour.
- Centrifuge at 14,000 rpm for 10 min. at 4$^{o}$C. Discard the supernatant and wash the pellet in 70% ethanol.
- Air dry the pellet and dissolve in 60 µl of TE buffer and estimate the yield.

**b) Quantification of DNA:-**

The isolated DNA was quantified and estimated spectrophotometrically by using UV scanning Shimadzu Spectrophotometer. 10 µl of DNA was diluted in 3 ml of TE buffer and its optical density was read

at 260 nm wavelength. The concentration of DNA was calculated using the relation:-

DNA concentration (in micrograms) = 50 X $A_{260}$ X dilution factor.

Where an OD of 1 corresponds to ~50 µg / ml of double stranded DNA, $A_{260}$ is the OD of the sample at 260 nm and

$$\text{Dilution Factor} = \frac{\text{Total volume of the sample}}{\text{Volume of DNA used}}$$

The genomic DNA extracted was visualized on agarose gel (0.8%) for its quality and stored at -20$^{o}$C.

**c) Arbitrarily Primed Polymerase Chain Reaction (AP-PCR):-**

The Arbitrarily Primed Polymerase Chain reaction (AP-PCR) is a modification of the PCR that generates informative genomic fingerprinting. AP-PCR combines PCR and primers of arbitrary sequence to amplify genomic DNA and produce a fingerprint. For the present reaction different oligonucleotide primers of OPA, OPB, OPC and OPD series of Operon Technologies Inc. Almeda, USA was used. The sequences of all the primers which responded are given below.

- OPA 01 5’ GGGACGATGG 3’
- OPA 03 5’ TCTGTCGGTC 3’
- OPA 09 5’ GGTCACCTCA 3’
- OPB 03 5’ AGTGCGCTGA 3’
- OPB 11 5’ CCGCGTCTTG 3’
- OPB 13 5’ GAGCGCCTTG 3’
- OPC 03 5’ CCC CGATGGT 3’
- OPC 06 5’ CTCCAGCGGA 3’
- OPD 01 5’ GGCTAACCGA 3’
- OPD 03 5’ TGTGCCCGAA 3’

The amplification was carried out in a MJ Research PTC 200 Thermal Cycler.

The PCR was performed in 25 µl reaction mixture containing 2.5 µl (50-200 ng) genomic DNA, 1 µl (2.5 mM each dNTP) of dNTPs, I µl (5-10 picomoles) of primer, 0.4 µl (IU) of Taq polymerase and 2.5 µl of 10 X PCR buffer (Appendix IV). This mixture was made upto 25 µl using 17.6 µl double distilled water. Amplification was carried out in a PTC 200 thermal cycler under programmed cycling conditions. The cycling parameters for denaturation, annealing and extention were as follows:

1 cycle of 3 min. at 94$^{o}$C, 38 cycles of 1 min. at 92$^{o}$C, 2 min. at 36$^{o}$C and 2 min. at 72$^{o}$C. One last cycle of 6 min. at 72$^{o}$C was performed to complete the reaction.

The product of the amplification was subjected to electrophoresis on a 1.2% agarose gel containing 10 µl ethidium bromide (0.5 µg/ ml) in 1 X TAE buffer (Appendix V) after mixing with 5 µl gel loading dye (Appendix VI). The gels were then visualized and photographed on a UV transilluminator. 1 kb DNA ladder was used as the molecular weight standard.

**d) Band pattern analysis:-**

Polymorphism was scored on the basis of presence or absence of bands for each DNA sample. The differences in the intensity of the bands were also noted.

**D. Essential oil analysis:-**

The fresh herbage of *in vitro* and *in vivo* plants of *Duchesnea indica* (Andr..) Focke was collected from the experimental garden.

**a) Essential oil extraction:-**

Shade dried, flaked and powdered plant material was hydrodistilled separately in a Clevenger apparatus for 4 hours at 100°

C. The quantity of the essential oil was measured and isolated oil was dried over anhydrous sodium sulphate and stored in a small amber coloured bottle and kept at 4°C. The percentage of oil was calculated on a dry weight basis to avoid faulty estimation that may arise due to difference in water content of the tissues analyzed each time (Von Rudoff, 1972).

**b) Gas Chromatography- Mass spectrometry:-**

The volatiles of the essential oil were analysed by the hyphenated system, GC-MS. This was performed on HP 6890 GC/HP 5973 MSD at 70 eV and 250°C. The GC column used was: H5-5 (DB5) fused silica capillary-0.32 mm X 30 m with film thickness 0.25 µ. The carrier gas used was helium with a flow rate of 1.4 ml/min. The column temperature programme: initial temperature of 60°C for 1 min. followed by an increase of 3°C/min. to 250°C. Run time was 62 min. The components were analyzed and ascertained with the help of Wiley Library 275 combined with the analyzer.

**c) Chemotaxonomic evaluation:-**

The data obtained from the qualitative analysis of both *in vivo* and *in vitro* plants were subjected to numerical analysis to understand the chemical affinity of both by arriving at a numerical constant, the co-efficient of similitude (CS), using the following formula proposed by Sokall and Sneath (1963).

$$CS = \frac{\text{Number of similar components}}{\text{Total number of components}} \times 100$$

**E. Determination of total carbohydrate content of Fruits.**

Carbohydrates are the important components of storage and structural materials in the plants. They exist as free sugars and polysaccharides. The basic units of carbohydrates are monosaccharides which cannot be split by hydrolysis into more sugars. The carbohydrate

content can be measured by hydrolysing the polysaccharides into simple sugars by acid hydrolysis and estimating the resultant monosaccharides.

The sugar estimation was done by Anthrone method as per Hedge and Hofreiter (1962).

The steps involved are:-

- Weighed 100 mg of the sample into a boiling tube.
- Hydrolysed by keeping in a boiling water bath for three hours with 5 ml of 2.5 N HCl and cooled to room temperature.
- Then neutralised it with solid sodium carbonate until the effervescence ceases.
- Made up the volume to 100 ml and centrifuged.
- Prepared the standards by taking 0, 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard.
- The blank was set at ‘0’.
- Made up the volume to 1 ml in all the tubes including the sample tubes by adding distilled water.
- Then added 4 ml of the Anthrone reagent.
- Heat for 8 minutes in a boiling water bath.
- Cool rapidly and read the green colour at 630 nm by a spectrophotometer.
- Optical density of each tube was noted.
- Calculated the amount of carbohydrate present in mg of the sample by using the formula,

$$\frac{\text{OD of the test}}{\text{OD of the standard}} \times \frac{\text{Concentration of the standard}}{\text{Volume of the test sample}}$$

Amount of cabohydrate present in 100 mg of the sample:-

$$= \frac{\text{mg of carbohydrate}}{\text{Volume of the test sample.}} \times 100.$$

# RESULTS

**A. Micropropagation.**

The MS medium with different hormonal combination was used for initiating multiple shoots in the present study. Among the different explants tested, (leaves, nodes, internodes *etc*.), positive response was exhibited by nodal explants.

Multiple shoots were induced when the explant was inoculated in the medium with combinations of auxin and cytokinins. Nodal segments were used to initiate cultures on MS medium supplemented with various combinations of BA and KIN with IAA and NAA (Table 3). The multiple shoot initiation was noticed in the medium with 2 mg/l IAA and 2 mg/l NAA (Figs. A - F). About 75% of the culture produced 2 to 3 shoots. In most of the other combinations culture establishment was in the form of clustered shoots from proliferated callus. The combination of BA and IAA produced only callus and BA together with IAA and 2, 4-D resulted in occasional shoot elongation (Figs. G - L). Lower concentrations of BA and IAA also were used (BA 0.5 mg/l, and IAA 0.1 mg/l; BA 0.15mg/l and IAA 0.1 mg/l). No morphological variant among the regenerated plants was observed. The callus obtained from nodal cultures was used for further studies.

When 2, 4-D (0.2 mg/l) was used with IAA (0.5 mg/l), profuse callusing was observed in the medium. Subculturing this callus in MS medium fortified with IAA and NAA (2 mg/l each) showed development of multiple shoots. The clumps of multiple shoots showed rooting in the same medium when it had been kept for 4 weeks. Profuse rooting was found in the plantlets that are obtained from nodal segments (Figs. M – R).

The combination of 2, 4-D (0.2 mg/l), IAA and NAA (0.5 mg/l each) showed multiple shoot and root development. The use of other explants showed little or no response as compared to nodal explants. Some combinations showed little callus development (IAA, 0.5 mg/l with BA 0.75 mg/l). About 80% of cultures showed callus formation (Table 3). The callus obtained was with varying colors from green to pale yellow and was of the hard type. When these calli were subcultured for shoot induction, the response was unsatisfactory. Morphogenic response was better in the case of young callus. Maximum response was elicited from 2 weeks to 5 weeks old callus. The calli that remained in the culture for more than 7 weeks did not respond. Some of the calli showed rhizogenesis when retained in the medium for 7 weeks. Rhizogenesis along with callus formation was observed in plants cultured on MS medium supplemented with NAA (0.5 mg/l) alone.

**Table 2. Effect of phytohormones on shoot induction in *Duchesnea indica*.**

| IAA mg/l | NAA mg/l | % frequency of response | | Nature of response | % of soma-clones |
|---|---|---|---|---|---|
| | | Shoot | Callus | | |
| 2 | 0.2 | 15 | 70 | Axillary branches | 0 |
| 2 | 3 | 10 | 40 | Single shoot | 0 |
| 2 | 2.5 | 12 | 60 | Single shoot | 1 |
| 2 | 4 | 20 | 20 | 2-3 shoots, axillary branching, callus at cut ends | 0 |
| 1 | 3 | 10 | 10 | 2-3 shoots, rooting. | 0 |
| 1 | 0.5 | 12 | 20 | Rooting | 0 |
| 1 | 0.4 | 13 | 22 | Callus at cut ends | 0 |
| 1 | 0.6 | 10 | 12 | Callus at cut ends | 0 |
| 2 | 2 | 15 | 10 | Multiple shoots with pronounced axillary branches with roots | 12 |

| | | | | | |
|---|---|---|---|---|---|
| 0.5 | 2 | 10 | 10 | Callusing | 0 |
| 0.5 | 2.5 | 10 | 10 | 1 - 2 shoots, rooting, | 3 |

**Table 3. Effect of phytohormones on shoot multiplication from *in vitro* nodal explants of *Duchesnea indica*.**

| BA mg/l | NAA mg/l | IAA mg/l | 2,4 D mg/l | % frequency of response | | Nature of response. |
|---|---|---|---|---|---|---|
| | | | | Shoot | Callus | |
| 0.5 | - | 0.1 | - | 05 | 80 | Little callusing |
| 0.75 | 0.75 | - | - | 0 | 2 | Little callusing |
| 0.15 | - | 0.1 | - | 0 | 2 | Hard callus |
| 0.2 | - | 0.2 | 0.2 | 1 | 5 | Little callusing, occasional shoot developing |
| - | - | 0.5 | 0.2 | 10 | 25 | Profuse callusing with little shooting and rooting |
| - | 0.5 | 0.5 | 0.2 | 40 | 40 | Multiple shoots with rooting |
| | 0.5 | 0.5 | - | 30 | 30 | Callusing |
| - | 0.5 | - | - | 0 | 10 | Callus with roots. |
| - | 0.5 | 0.5 | 0.5 | 50 | 50 | Shooting with rooting |
| - | 2 | 2 | - | 80 | 20 | Multiple shooting |
| 0.75 | - | 0.5 | - | 0 | 5 | Little callusing |

Plantlets thus obtained were transferred to paper cups in a mixture of soil and sand (1:3) for a week under humid conditions and was found to be with 70 % survival efficiency. Hardened plants were then transferred to the field. No phenotypic variant was obtained in the regenerated plants.

**B. Cytological analysis**

The present study is aimed to examine the karyomorphological changes in the chromosome complement of the callus, *in vivo* and *in vitro* plants of *Duchesnea indica* and to know the cytological basis of variation. The ploidy level of *in vitro* plant, in *vivo* plant and callus were found as dodecaploid (2n = 7 x = 84). All *in vitro* plants studied showed the same number of chromosomes. No chimeral or aneuploid variations were found. Chromosome complements of parent plant, callus and somaclonal variant are analysed in detail (Plates 5 – 7; Tables 4 – 6). The number of chromosomes with secondary constrictions was found to be the same in the parent, callus and *in vitro* plant, *i.e*., 12 chromosomes.

Changes in chromosome length, disparity index and total forma percentage were noticed. The chromosomes ranged in size from 1.5323 µm to 0.3838 µm in the parent plant, from 1.5323 µm to 0.4402 µm in the variant and from 1.6191 µm to 0.3838 µm in the callus. The total chromosome length of the cells of the parent plant was noticed as 34.566 µm and that of the variant was 35.7675 µm. The total chromosome length of the callus was 33.3151 µm. The disparity indices observed in the chromosome complement of parent, variant and callus were 61.6755 and 55.3662 and 59.9394 respectively. The total forma percentages of the parent, variant and callus were estimated as 40.6758, 42.0408 and 41.5358. The total length of the chromosome complement of the variant was found to be more when compared to the parent plant and callus. Disparity index was observed to be greater in the parent plant when compared to the variant plant (61.6755 and 55.3662). Total forma percentage of the variant was found to be higher than

that of the parent (40.6758 and 42.0408). The average chromosome length of the parent plant was greater than that of the variant (0.6176 and 0.4257).

Karyotype formulae deduced for the karyotypes of the parent, *in vitro* plant and callus showed variation in the types of chromosomes.

The general description of common types of chromosomes found in the parent plant, *in vitro* plant and callus cells are: -

Type A: - Chromosome with secondary constriction ranging from 1.6191 µm to 0.8027 µm with nearly median/ nearly submedian primary constriction.

Type B: - Chromosome with length of 1.2879 µm to 0.3838 µm with nearly median primary constriction.

Type C: - Chromosome with length ranging from 1.3603 µm to 0.4402 µm with nearly sub median primary constriction.

The nomenclature of chromosome was depicted according to the system followed by Abraham and Prasad (Table 1).

The karyotype formula of the parent and the *in vitro* plant was $A_{12}$ $B_{60}$ $C_{12.}$ The karyotype formula of the callus was found to be slightly different ($A_{12}$ $B_{58}$ $C_{14}$). Detailed karyotype description, microphotographs of mitotic metaphase stages, computer images of karyotypes and idiograms (Plates 5 – 7; Tables 4 – 6) of the *in vivo* plant, *in vitro* plant and callus are given below:

**Random Amplified Polymorphic DNA (RAPD) Analysis.**

DNA was isolated from the parent plant and two micropropagated plants, designated as T1 and T2. The extracted DNA was quantified spectrophotometrically and the DNA required for the reaction mixture was calculated.

The table represents the quantity of DNA obtained from the samples.

**Table 7. Composition of the amount of DNA isolated from *in vivo* and *in vitro* (T1 and T2) plants of *Duchesnea indica*.**

| Sample | Amount of DNA present (µg / ml) |
|---|---|
| Parent plant | 6.25 |
| T1 | 5.50 |
| T2 | 5.81 |

Optical density at wavelength 260 nm of the DNA extracted from the donor plant is 0.125, T1 is 0. 110 and T2 is 0.1162. So the quantity of DNA in the sample is,

0.125 X 50 µg = 6.25 µg,

0.110 X 50 µg = 5.50 µg

0.116 X 50 µg = 5.81 µg respectively.

(Where 50 µg is the quantity of DNA that corresponds to optical density 1 at wavelength 260 nm).

20 to 30 ng of DNA is required to amplify a specific DNA segment by PCR. 3:1 of the template DNA was used for the preparation of the reaction mixture. To detect the variation in *in vitro* regenerated plant at molecular

level, RAPD analysis was carried out using 27 primers of arbitrary sequence. Only 10 primers successfully amplified the extracted DNA with consistently reproducible banding. The base sequence of primers with which the genes are amplified is given in table 8. The number of bands resolved per primer ranged from a minimum of 5 to a maximum of 28. The size of amplification product also differed from approximately 500 bp to over 10000 bp in the 10000 bp ladder.

The RAPD fingerprints of the variant, T1 differed from that of the donor plant with all the ten primers, whereas, the variant, T2 differed with only eight primers, *viz*.

OPA 01, OPA 03, OPA 09, OPB 13, OPC 03, OPC 06, OPD 01 and OPD 03.

Some bands were found to be missing in the variants and some additional bands were also detected. In the case of amplification products with OPA 01, there were no bands above 4000 bp region in T1, T2 and *in vivo* plant. T1 and T2 showed no bands below 2000 bp. This is different from the parent plant. The amplification product of T1 showed 2 bands whereas T2 showed 3 bands and *in vivo* plant showed 6 bands in between 1000 and 4000 bp region.

For OPA 03, one band appeared close to the 6000 bp ladder in both *in vitro* plants and is absent in the *in vivo* plant. Three bands present in between the region 4000 bp and 6000 bp in the parent plant was found to be absent in T1 and T2. Altogether 6 bands were scored in parent plant and 3 each in *in vitro* plants.

With primer OPA 09, one band was found to be prominent above the 10000 bp ladder in the parent, another near the 8000 bp region and a third band close to 6000 bp marker. The former 2 bands were found to be missing in both T1 and T2. Whereas the band at the 6000 bp region was present only in T2. Altogether 5 bands were scored in T1 and 7 bands in T2.

4 additional bands below 6000 bp length appeared in the amplification product of T1 and 6 in T2, but these were absent in the parent.

For the primer OPB 03, no bands were detected for both *in vitro* plants and *in vivo* plant above length of 5500 bp.above length of 700 bp. T1 sample showed 5 bands, whereas T2 and parent plant showed 4 bands each between 6000 bp and 2000 bp regions.

Using OPB 11 primer, the intensity of the bands decreased from parent through the T1 and T2. Three bands each were scored in parent and T2 plants, whereas 4 bands were scored in T1 plant.

With OPB 13 primer one band each was scored below 1000 bp region in all three samples. No band was scored above 7000 bp region in all three plants. A band near 1000 bp marker, present in parent were found to be absent in both T1 and T2 plants. One additional band each was present near 7000 bp marker in both *in vitro* plants.

With OPC 03 primer, single band just below 300 bp length was observed in the parent but absent in the *in vitro* plants. Approximately between 7000 bp and 8000 bp markers one band was found in T1, but absent in T2 and parent plant. An additional band was scored above 10000 bp marker in T1 plant alone. Altogether 9 bands were present in parent, 8 in T1 and 6 in T2 plants.

Band above 7000 bp length in T1 was not detected in parent and T2 plant with OPC 06 primer. The band below 1000 bp was present in low intensity in parent, but absent in both in vitro plants. Altogether 9 bands were scored in parent plant, 8 in T1 and 7 in T2 plants.

With OPD 01 primer, two bands were observed in between 4000 bp and 1000 bp regions in the parent plant. Only 3 bands were scored in parent and one each in the T1 and T2 plants.

With the primer OPD 03, three bands were similar to the parent for T1 where as two bands were identical with parent for T2. An additional band was present for T1 near the 10000 bp marker, which was absent in parent and T2.

Since the amplification for T1 was showing more differences than that of T2, T1 is considered as somaclonal variant and used for further analysis.

Altogether 20 bands were absent and 14 additional bands were present in T1 when compared with parent during RAPD analysis with 10 primers. However only 18 bands were absent and 8 new bands were present in T2 when compared with the parent. The reproducibility of these genomic DNA bands was consistent in successive repetitions.

**Table 8. Primers and characterization of consistent bands in parent and two tissue cultured plants of *Duchesnea indica*.**

| Primer | Sequence | No. of Bands | | |
|---|---|---|---|---|
| | | Parent | T1 | T2 |
| OPA 01 | 5’ GGGACGATGG 3’ | 6 | 2 | 3 |
| OPA 03 | 5’ TCTGTCGGTC 3’ | 6 | 3 | 3 |
| OPA 09 | 5’ GGTCACCTCA 3’ | 3 | 5 | 7 |
| OPB 03 | 5’ AGTGCGCTGA 3’ | 4 | 5 | 4 |
| OPB 11 | 5’ CCGCGTCTTG 3’ | 3 | 4 | 3 |
| OPB 13 | 5’ GAGCGCCTTG 3’ | 9 | 9 | 9 |
| OPC 03 | 5’ CCC CGATGGT 3’ | 9 | 7 | 6 |
| OPC 06 | 5’ CTCCAGCGGA 3’ | 9 | 8 | 7 |
| OPD 01 | 5’ GGCTAACCGA 3’ | 3 | 1 | 1 |
| OPD 03 | 5’ TGTGCCCGAA 3’ | 3 | 4 | 2 |

**Essential Oil analysis:-**

The field performance of the *in vitro* derived plants (T1) was examined to find out its adaptability. The karyomorphological and chemical variability may not be visible in the morphological characteristics. Screening of plants for desirable qualities like yield and composition of essential oil and total carbohydrate is significant in this aspect. So the plant which was considered as the variant (T1) was tested for its carbohydrate content and essential oil composition and yield and compared with the parent plant. The essential oil isolated from the whole plant by hydro distillation was subjected to GC - MS and compared the components.

The color of the essential oil of the parent plant was pale yellow (yield 0.1%) and that of the variant was yellow (yield 0.1%). The oil collected in an amber colored bottle was subjected to GC - MS analysis.

The results of GC-MS analysis of *in vivo* and *in vitro* (T1) plants are listed in table 9. The gas chromatogram of the *in vivo* and *in vitro* plants is shown in plates 10 and 11.

The analysis of the essential oil samples revealed a range of variation in their constituents. The major components identified from the essential oils of *in vivo* and *in vitro* plants were similar (carvacryl acetate, valencene, nona hexa contanoic acid, aristalone, dihydro aromadendrene, 2-hexa-decan-1-ol, eicosane), even though, there is marked difference in the percentage of occurrence. Considerable increase in the quantity of carvacryl acetate (30.5% and 31.2%) and valencene (7.6 % and 7.7%) and a decrease in percentage in the quantity of α- humulene (0.8% and 0.5%) was observed in the *in vitro* (T1) plant. The mass spectra of the compounds identified in the GC-MS analysis are shown in plates12- 19.

The total number of components detected by GC-MS in the *in vitro* (T1) plant was found to be 21 but in the parent plant the number of components was found to be 25. However the number of similar

components, which occur in both the plants, was 21. The co efficient of similitude between the parent plant and the callus regenerated plant (T1) was found to be 84.

**Table: 9.- Chemical composition of the leaf essential oil of *in vivo* and *in vitro* plants of *Duchesnea indica*.**

| No. | Retention time | Chemical compounds | Class of Compounds | Percentage | |
|---|---|---|---|---|---|
| | | | | Parent Plant | *In Vitro* plant (T1) |
| 1 | 11.71 | Terpeneol-4 | Monoterpenoid | 0.07 | 0.07 |
| 2 | 15.22 | Methyl cinnamate | " | 1.8 | 1.8 |
| 3 | 15.98 | 1,3-dimethyl-2-cyano-3-piperidine | " | 0.02 | - |
| 4 | 16.95 | α-copaene | Sesquiterpenoid | 0.1 | 0.1 |
| 5 | 17.48 | β-elemene | " | 2.0 | 2.0 |
| 6 | 18.03 | β-caryophyllene | " | 1.1 | 1.1 |
| 7 | 18.62 | β -selinene | " | 1.3 | 1.3 |
| 8 | 18.86 | α-humulene | " | 0.8 | 0.5 |
| 9 | 19.66 | Dihydro aromadendrene | " | 4.6 | 4.6 |
| 10 | 19.88 | Valencene | " | 7.6 | 7.7 |
| 11 | 22.62 | Aromadendrene | " | 2.8 | 2.8 |
| 12 | 22.95 | γ- selenene | " | 0.1 | 0.1 |
| 13 | 24.61 | P-Nonyl phenol | Phenolic compound | 0.26 | - |
| 14 | 25.94 | Carvacryl acetate | " | 30.51 | 31.20 |
| 15 | 26.75 | Aristalone | Sesquiterpenoid | 5.34 | 5.34 |
| 16 | 29.07 | Trans β- farnesene | " | 1.7 | 1.7 |
| 17 | 29.63 | β- pregnal | " | 2.4 | 2.4 |
| 18 | 30.06 | Hexa- decanoic acid | Fatty acid derivative | 2.0 | 2.0 |
| 19 | 31.16 | Geranyl linalool isomer | Monoterpenoid | 1.2 | - |
| 20 | 32.76 | 2-hexa-decen-1-ol | Fatty acid derivative | 4.1 | 4.1 |
| 21 | 35.49 | Nonahexacontanoic acid | " | 7.2 | 7.2 |
| 22 | 39.08 | Pentacosane | Straight chain alkane | 1.0 | 1.0 |
| 23 | 39.61 | Eicosane | " | 4.1 | 4.1 |
| 24 | 42.02 | Heptacosane | " | 0.4 | - |
| 25 | 43.42 | Octacosane | " | 0.1 | 0.1 |

**Total carbohydrate analysis:**

Total carbohydrate of the fruits of both the *in vivo* and *in vitro* (T1) plants was analyzed by Anthrone method. The results of the experiments repeated thrice are as follows:

Optical density at wavelength 630 nm of the extract from the *in vivo* derived fruit is 0.17 and that of *in vitro* derived (T1) fruit is 0.18. The quantity of glucose corresponding to optical density 0.17 is 0.015 mg and to optical density 0.18 is 0.016 mg respectively.

So the quantity of total carbohydrate in the *in vivo* fruit is calculated as follows:

Amount of carbohydrate present in 100 mg of the *in vivo* fruit extract is

$$= \frac{\text{Mg of glucose corresponding to OD}}{\text{Volume of the test sample}} \times 100$$

The total carbohydrate present in the *in vivo* plant was found to be 3% and that of *in vitro* plant was found to be 3.2%.

# DISCUSSION

## Micro Propagation

Plant tissue culture is viewed as a potential means of producing useful *in vitro* plants such that conventional agriculture, with all its attendant problems and variables, can be circumvented. These problems include: environmental factors (drought, floods, *etc*.), disease, political and labour instabilities in the producing countries (often Third World countries), uncontrollable variations in the crop quality, inability of authorities to prevent crop adulteration, losses in storage and handling. Thus, the production of a large number of useful and valuable plants is an attractive proposal.

Using culture techniques it has been possible to regenerate propagules with better qualities, greater vigor, higher yield and disease resistance. A number of physiological and morphological changes have been reported in unorganized callus tissue including habituation, changes in biochemical sensitivity and requirements, alteration of growth habit and modification of cellular constituents (Barba and Nitchell, 1969).

Natural regeneration and conventional propagation of the plants through the vegetative cuttings is slow and cuttings do not survive after the transport and transplantation. Moreover vegetative propagation has prevented the production of new cultivars by plant breeding. An alternative method for creating new forms of a plant is by selection of somaclonal variants from tissue culture. The past 35 years of research on plant tissue culture regenerated a large number of plants and the progenies thus generated had revealed a rich array of variation in both morphological and genetic aspects. The behaviour of these plants in genetic level generally appeared similar to that of changes caused by mutation. However the frequency of different classes of variants derived from plant tissue culture

exceeded far beyond that expected in nature. Tissue culture techniques are gaining increasing importance as a valuable supplement to the conventional methods for genetic improvement besides clonal propagation. Variation is a usual phenomena associated with plant tissue culture (Bayliss, 1980), Previous record on the somaclonal variants (Bajaj, 1990; Seeta *et al*., 2000) showing both quantitative and qualitative nature suggested that somaclonal variation served as a potential, novel and an alternative source for generating variability. These micropropagated plants generated through tissue culture can be utilized for subsequent conventional breeding programmes, which were found to be fruitful in commercial strawberry plants (Owen and Miller, 1996; Zabetakis and Holder, 1996; El Mansouri *et al*., 1996; Zebrowska *et al*., 2003; Zebrowska, 2004).

Plants that are regenerated from callus cultures possess a variety of genetic changes since they are produced from an undifferentiated mass of cells through indirect organogenesis. These variations developed in plants, which are called somaclonal variation can result in useful agricultural and horticultural plants, especially in the fruit yielding plant like strawberry (Battistini and Rosatti, 1991; Toyoda *et al*., 1991; [10]http; [11]http). These variations may be beneficial or may be harmful. The presence or absence of variations depends on the source of the explant and method of regeneration (Larkin and Scowcroft, 1985; Suryanarayanan and Pai, 1988; Khalid *et al*., 1989). Explants are of great significance for the successful regeneration of micropropagated plants which was revealed in some cultivated strawberry plants as well ([7]http; [8]http; [9]http; Rashid, 1991; Greene and Davis, 1991; Infante *et al*., 1998; Passey *et al*. 2003; Khan and Spoor, 2004; Wu *et al*., 2004). Mc Clintock (1984) was of the opinion that no two plants derived from callus cultures are exactly alike and none is just like the plant from which the explants had been taken. Genetic variation is a common phenomenon associated with plant tissue culture. Plantlets derived from *in vitro* culture might exhibit somaclonal variation which is often heritable (Breiman *et al.,* 1987). Useful morphological, cytological and molecular variation may be

generated *in vitro* (Larkin *et al.,* 1989).

Plant tissue culture has the potential to induce genetic variability through somaclonal variation (Jullien *et al.*, 1998). Novak (1980) reported phenotypic and cytological variation in *in vitro* plants arising from callus cultures. Several reports are available regarding the variation in *in vitro* plants of *Fragaria* arising from callus cultures ([10]http; [11]http; Takayama and Takizawa, 2004; Kaushal *et al*., 2006).

In the present investigation, a new protocol was developed on *Duchesnea indica* (Andr.) Focke and generated a plant with some variation in its genetic make up (Figs. A - T). Though different explants were used for culture, only nodal segments gave better results. The explant either developed into callus or directly regenerated into plantlets. The callus was then subcultured to obtain plantlets. Emergence of shoots directly from cultured explants was found to be useful in the propagation of true-to-type plants ( Figs. A – F). Whereas, emergence of plants from callus through indirect organogenesis results in the induction of variation. (Figs. G - L).

Murashige (1974) described plant regeneration accomplished from explants like leaves, stem, cotyledons, microsporocytes and shoot tips. According to Gamborg *et al*. (1974), most important determinant of plant multiplication and quality of regenerated plants is the initial explant used. Meristem explants successfully regenerating into genetically uniform plants had been reported on several plants by earlier workers. Reports are also available that callus regeneration and the subsequent indirect organogenesis leads to variant *in vitro* plants (Malnassy and Ellison, 1970; Ikuta *et al.*, 1975).

In the present investigation the subsequent subculturing of the callus might have resulted in the formation of variant plants (Figs. G – L). Previous reports on several plants substantiate that during subculturing the number of variant plantlets increases (Ziv *et al*., 1983; Lesham *et al*., 1998; Safrazbekyam *et al*., 1990).

The explant of *D. indica* undergoes various changes while it is developing and along with the type and concentration of growth hormones used results in the morphogenic response of the regenerants. The morphogenic response of explants depended on the type and concentration of the hormones used (Tables 2, 3). The type and concentration of hormones as well as the culture conditions were found to influence morphogenic response in several members of Rosaceae (Pereira-Netto, 1996; Damiano and Monticelli, 1998; Rugienius *et al*., 2003). Tissue culture studies on a number of plants (Ibrahim, 1969; Corduan and Spix, 1974; Irawati and Nyman, 1986; Agarwal *et al*. 1987; Rojina, 1991; Kumar, 1992; Nirmal Babu *et al.*, 1992) suggested that a fine balance of exogenous auxin and cytokinin are necessary for successful regeneration of plants. Cytokinins are found to be very effective for both direct and indirect organogenesis (Moreno *et al*., 1985; Bonabdallah and Branchard, 1986; Misra and Bhatnagar, 1995). The role of cytokinins in overcoming the apical dominance of the terminal shoot bud and enhancing the branching of the lateral buds from axils was observed. It is known that BA is the most effective synthetic cytokinin for stimulating axillary shoot proliferation for different plant systems (Bhojwani, 1980; Hasegawa, 1980; Kitto and Young, 1981; Welander *et al.*, 1989; Nadel *et al.*, 1991; Devi *et al.*, 1994; Gangopadhyay *et al.*, 1998). The dependence of shoot regeneration on cytokinin is well established (Evans *et al*., 1983).

In the present study various hormonal combinations were tried but the best result was given by a combination of IAA and NAA. (Figs. A – F, H, I, K – S) Maximum multiplication was observed in a combination of IAA and NAA in the concentration of 2 mg/l each induced multiple shoots in *D*. *indica* (Table 2). The present result indicates that a combination of IAA and NAA is practically effective in inducing multiple shoots either through direct organogenesis (Figs. A – F) or through indirect organogenesis (figs. H – S). Agarwal *et al*. (1987) successfully developed callus formation using 2, 4 - D. In the present investigation 2, 4 – D was

found to be very effective for callus formation, which together with NAA and IAA resulted in the subsequent organogenesis and shoot multiplication (Table 3). Addition of NAA and KIN to IAA containing medium resulted in initiation of organogenesis and shoot proliferation (Lundergan and Janic, 1980; Rahman and Blake, 1988; Sen and Sharma, 1991). In the present investigation NAA and IAA was found to be effective in inducing multiple shoots and for root initiation in regenerated plants (Figs. M – R). Roots have been reported to originate from elaborate callus tissue (Hubakoa, 1986; Hartman *et al.*, 1990). The relative levels of auxins have been known to greatly influence morphogenic responses like rooting (Sitborn *et al.,* 1993). The present study indicated that suitable hormone combinations can induce shoot multiplication *via* indirect organogenesis and effective rhizogenesis in *D. indica* (Tables 2, 3; Figs. G – S). Earlier studies confirm the effectiveness of these hormones for *in vitro* regeneration of several strawberry cultivars ([7]http; Mohammed, 1990; Rashid, 1991; [8]http; [9]http; Yang *et al*., 1995; Owen and Miller, 1996; Bhat and Dhar, 2000; Sakila *et al*., 2007; Biswas *et al*, 2007, [10]http, [11]http,[12]http,[13]http).

The success of regeneration in any crop depends upon the type of medium used in each phase of culture from callus initiation to maintenance and for regeneration. The shoot forming ability has been improved by using suitable hormones (Chawla and Wenzel, 1987) and the present study showed the same result. The media composition especially an optimum combination of auxin and cytokinin may affect an additional increase in the expression of the morphogenic potential (Heszky *et al.,* 1991).

Yang *et al*. (1999) showed that shoots might be induced from meristematic tissues since actively dividing cells are highly susceptible to hormonal treatments, which alter their normal developmental pathways. Guimaraes *et al.* (1989) pointed out that the lower concentration of 2, 4 - D stimulated only callus growth from hypocotyls of *Cyphomandra betcea*. This is in agreement with the present study, as callus induction was induced in

the medium supplemented with 2, 4 - D (Fig. G, J and O). Similar findings were reported by Harikrishnan and Hariharan (1996) in the plant *Plumbago rosea*. Regeneration did not occur in the medium due to some intrinsic factors. Reynolds (1987) reported that high levels of auxins promote callus formation. This is in conformity with the results of the present study. Von and Woodward (1988) suggested that presence of auxin together with cytokinin is indispensable for formation of organogenic callus. Results obtained from the present study are in accordance with this hypothesis.

Studies have shown that optimum concentration of BA required for shoot multiplication varies according to the explant and cultivars used (Hussey, 1977; Dantu and Bhojwani, 1987; Grewal *et al*., 1990). The younger tissues are known to contain high auxin levels (Sheldrake, 1973) that could promote callus formation. The superiority of BA over cytokinins for multiple shoot formation has been reported in many fruit yielding plants (Lundergan and Janic, 1980; Rahman and Blake, 1988; Sen and Sharma, 1991). Maximum multiple shoot regeneration was occurred on particular growth regulator concentrations. This is in agreement with the hypothesis that the balance of growth regulators as well as their concentration is critical in determining the direction of morphogenesis (Sharief and Jagadishchandra, 1999).

It has already been reported that the nature of growth regulators used in the medium may result in the occurrence of somaclonal variation (Patel and Berlyn, 1982). High concentrations of growth regulators in the medium and long term culture are thought to be the main causes of variation in plant cultured *in vitro* (George and Sherrington, 1984). It is accepted that *in vitro* manipulation do cause genetic aberration (Vajrabhaya, 1977). The possibility of genetic changes occurring in plants raised from callus cultures can be used as a potential source of somatic variation (Pillai and Hilde Brandt, 1969; Bush *et al*., 1976). The frequency of variation is also influenced by culture duration, concentrations of cytokinins and number of plants produced

from each explant (Reuveni *et al.,* 1986; Vuylsteke *et al.*, 1988). The wide spread occurrence of somaclonal variation has been extensively documented in many species from cereals to trees (Bajaj, 1990; Phillips *et al*., 1994). The variability observed in the plants regenerated form the cultures usually included morphological changes in plant organ size, leaves and flowers. Changes in pigmentation (Mori and Sakurai, 1994) and composition of fruits, essential oil compositions *etc*. are other common changes (Motomori *et al*. 1995; Jakobsone *et al*. 2006). The variability may be due to segregation of chimeras tissue, euploid changes and heritable changes involving individual chromosomal aberrations or gene mutations.

Various explants showed differential response to media for multiple shooting. According to Thanh and Trinh (1990), the difference was not only in the frequency of the responding explant, but in the number of shoots produced per explant also. The present study also indicates this (Tables. 2, 3). Organogenesis depends on factors like quality of the explant, physiological state of the donor plant, endogenous level of hormones *etc*. According to Hopkins (1999), auxins promote the growth of axillary bud at lower concentrations. The incorporation of auxin in the medium generally promotes rooting (Gautheret, 1945). There are many reports on the origin of roots from callus tissue (Hubakoa, 1986., Hartman *et al*., 1990). Rooting was observed in IAA and IBA by Purohit *et al*. (1995 a, b). Zang *et al*. (1987) observed that for rooting, root inducing medium had to be inoculated. Thorpe (1978) observed that IAA has promotory role on rhizogenesis. The present study also supported this idea as IAA induced rooting in both the regenerated plants and callus (Tables. 2, 3; Figs. M – R).

Tissue culture conditions are expected to lead to peculiar pattern of gene expression in plant cells, which may cause some transient changes in the regenerated plants (Taylor *et al*., 1995). Biological phenomena in plants are closely related to the physical and chemical characteristic of macro molecules occurring in them. Stress induced tissue culture process (*e.g*.

hormone effects, nucleotide pool imbalance *etc*.), causes alterations in DNA. These alterations could affect the expression of specific genes (Kaeppler and Phillips, 1993a). Conditions in the artificial environment of cell culture may enhance the spontaneous mutation rates. It has already been recognized that the nature of growth regulators used in the medium may result in the occurrence somaclonal variation (Patel and Berlyn, 1982).

Various types of changes in cultures like phenotypic, genetic/epigenetic, karyotypic, physiological, biochemical or changes at molecular level may be derived from changes due to *in vitro* stress when the culture is initiated. The genetic variation created in the tissue culture plant may be 'benign' *i.e,* a sequence of dedifferentiation, rejuvenation or redifferentiation may be transient or may be persistent'. Genetic variations are not readily reversible, are stable and can be enhanced by mutagens. The frequency of genetic changes in somaclones is much higher than the spontaneous genetic changes brought about in the entire plant (Prat, 1983). Another possible mechanism suggested to explain the somaclonal variation is the activation of different classes of mobile genetic element, such as those reported during tissue culture of maize (Peschke and Phillips, 1991), tobacco (Hirochika, 1993) and rice (Hirochika *et al*., 1996). Transposable element activation has been shown to be induced by genomic shock (Mc Clintock, 1984). Epigenetic variation is another important cause of somaclonal variation, which is caused due to the results of culture stress and is not transmitted from generation to generation.

According to Larkin and Scowcroft (1985), and Larkin (1987), the origin of somaclonal variation may be due to the following:-

Genetic variation already present in the mother plant tissue.

- Variation induced by mutagenic action of the media.
- Epigenetic variation.
- Variation induced by stress.

- Activation of mobile genetic elements.

George and Sherrington (1984) stated that high concentration of growth regulators in the medium and long-term culture are thought to be the main cause of variation in plants cultured *in vitro*. Much of the variability found in tissue culture can be directly or indirectly attributed to gross chromosomal changes and other chromosomal abnormalities (Conger, 1987). Skirvin (1978) studied natural and induced variation and opined that variation is quite ubiquitously associated with *in vitro* propagated plants. Somaclonal variation can provide means of amplifying variability within the existing cultivar, thereby opening new opportunities for clonal selection. Much of the variability found in tissue culture can be directly or indirectly attributed to gross chromosomal changes and other chromosomal abnormalities (Conger, 1987).

In the present study there was no prominent morphological changes observed between the *in vivo* and *in vitro* plants of *D. indica*. In order to differentiate the extent of variation among the *in vivo* and *in vitro* plants, studies were further conducted on the gross karyomorphology, RAPD marker variations, leaf essential oil components and composition and total carbohydrate concentration of the fruits.

**Cytological Analysis**

Cytological analysis is an important tool in systematics since it enhances knowledge to understand interrelationships among taxa and the genetic mechanism involved in species formation. Although conventional methods furnish little information about species evolution when compared with the molecular ones currently employed, there is a great interest in these studies mainly in tropical countries where biodiversity is very high and cytological investigation is scanty. Gene revolution has become a reality due to recent developments in molecular biology and tissue culture (Gholamreza Bakshi, 2002).

Knowledge of the karyotype of a species *i.e*, the number of chromosomes, their size and morphology is necessary for a full understanding in plant genetic studies and plant improvement. The chromosomes at the mitotic metaphase stage offer a visible evidence of genetic architecture of the organism as the number of chromosomes and their linear morphology can be directly related to the number of linkage groups. Karyotype is useful to understand the origin and nature of chromosome variations (Gu *et al*., 1984). In some members, karyotype analysis for the identification of horologs is unreliable, because not all chromosomes can be distinguished by their length and centromere position and no useful additional cytological markers are available (Koopman *et al.,* 1996). Therefore the karyotypes are established using numerical parameters describing the chromosome length, area, perimeter, disparity index of chromosomes, total forma percentage and number of discernible satellites. In some groups karyotypic differences between species are largely quantitative and have been difficult to assess by conventional quantitative methods. Karyomorphometrical studies by computer based image analysis system provide a better knowledge of the cytogenetic constitution of various species over conventional methods (Rajalakshmi and Jose, 2002).

There have been claim that random genetic changes evidenced by chromosomal differences can have some use in plant improvement (Skirvin, 1978). There are reports that plants regenerated from callus or suspension cultures may show genetic changes (Nishi, *et al*., 1968; Williams and Collins, 1976). The genetic alterations in the plants produced from artificial environment may be due to increased frequencies of single gene mutations, chromosome breakage and reunion, transposable element activation, modification of normal DNA methylation pattern *etc*. (Kaeppler and Philips, 1993 b). Chromosome rearrangements are frequently found in plants regenerated from tissue cultures. The genomic changes that have been observed to occur in tissue culture include aneuploidy, chromosome rearrangements such as translocations, inversions, deletions, gene

amplifications, activation of transposable elements, point mutations and cytoplasmic genome rearrangements (Larkin and Scowcroft, 1983; Orton, 1983, 1984; Evans *et al*., 1984).

In the present investigation, the karyomorphology of chromosomes in the *in vivo* and *in vitro* plants of *D. indica* has been studied. No variation in the chromosome number has been observed in the *in vivo*, *in vitro* and callus cells. In all the cells the somatic chromosome number was found to be 2n = 12x = 84 (Plates 5 – 7). Grant (1981) proposed that the original base numbers of angiosperms range from x = 7 to 9. In the present investigation, the basic chromosome number of *D. indica* was found to be x = 7. According to Goldblatt, (1976), the subfamily Rosoideae, which contains plants such as roses, strawberries, and raspberries, have x = 7. According to Potter *et al*. (2002), the basic chromosome data of Rosoideae (*Rosa*, *Fragaria*, *Potentilla* and *Rubus*; was found to be x = 7, 8 or 9. Apart from Maloideae, where the basic chromosome number is x = 17, all other Rosaceae members are primarily x = 7, 8 or 9 ([2]http). Basic chromosome number is one of the most widely used characters in biosystematic studies and there has been a vast amount of phylogenetic speculation whether this value can be used as a dependable and stable marker of the direction of evolution. The basic chromosome number of x = 7 exists in several other closely related members of Rosaceae as well (Thompson, 1997; Lim *et al*., 1998). In the cultivated strawberry, *Fragaria* sp, the basic chromosome number is reported to be x = 7 ([5]http; [1]http; Yarnell, 1929; Lilienfeld, 1933; Federova, 1934; Scott, 1951; Hancock and Bringhurst, 1979; Hancock and Luby, 1993; Ma and Chen, 2004; Folta and Davis, 2006; Davis *et al*., 2007; Potter *et al*., 2007; Preeda *et al*., 2007; Ahokas, 2008).

In the present investigation the *in vivo*, callus and *in vitro* cells show a 12 ploid (dodecaploid) chromosme constitution (Plates 5 – 7). Dodecaploids had been observed in several other related plants in the family Rosaceae (Thompson, 1997; Lim *et al*., 1998; Hayirhoglu-Ayaz *et al*., 2006) and also in

several cultivated strawberry species (Bringhurst and Senanayake, 1966; [1]http).

A previous study available on *D. indica* reveals the chromosome associations at meiosis in two types of natural hybrids, 7x and 8x, between *Duchesnea chrysantha*, a diploid (2x) and *D. indica*, a dodecaploid (12x) (Naruhashi and Iwatsubo, 1991). The present study thus confirms the existence of a high polyploid (dodecaploid) chromosome complement in *D. indica*.

Literature estimates suggests that as many as 30 - 70% of flowering plants are of polyploid origin (Grant, 1981; Goldblatt, 1980). Polyploidy had been reported in several members of Rosaceae. In many genera, different species will have different ploidy levels (multiples of a base number) representing a series of polyploids. Reese (1961, 1966) suggested that an increase in the number of chromosomes provides increased possibilities for new gene combinations. Polyploidy also results in increase in the genes controlling characters favourable for natural selection, when these characters are already present in the plant. Polyploidy is of great relevance for the evolution of the genus because of various cytotaxonomic or cytobiogeographical aspects (Qiao *et al*., 1990).

When cultured, the plant cells were known to exhibit variations in chromosome structure (Bayliss, 1973; Constantin, 1981; D' Amato, 1978; Gupta and Ghosh, 1983). Structural changes of chromosomes were observed in the *in vivo*, calli and *in vitro* cells of *D. indica*. Variations in karyotypes involved total chromosome length, average chromosome length, centromeric positions, disparity index, and total forma percentage (Tables 4 – 6). Similar reports are available in *Triticum durum* (Gupta and Ghosh, 1983), *Crepis capillaris*, *Haplopappus gracilis* and *Allium cepa* (Bajwa and Wakhlu, 1986). Cells growing in an artificial environment may have many genetic changes such as increased frequencies of single gene mutations, chromosome breakages, transposable element activation,

quantitative trait variation and variation of normal DNA methylation patterns (Kaeppler and Phillips, 1993 b; Do *et al*., 1999).

In the present study, the average chromosome length of the parent plant (0.6176 µm) was found to be higher than that of the somaclonal variant (0.4257 µm). Chromosomal rearrangements may lead to slight changes in the size of the chromosome. The differences in the chromosome length and volume may be attributed to differential spiralization and condensation of chromosome along with the content of protein and DNA. It may also arise by translocations, duplications and deletions. Robertsonian translocations can also lead to changes in the size of the chromosome. The change in the chromosome length may be the aftermath of cryptic changes, probably deletions, which may arise due to *in vitro* stress produced in the altered culture environment. Moreover retrotransposon activation and inversions may significantly contribute to the change in the physical size of the genome (Olhoft and Phillips, 1999). Similar reports are available in *Allium cepa* (Sekera, 1977) and *Papaver somniferum* (Bajwa and Wakhlu, 1986).

Chennaveeraiah and Habib (1966) reported the structural rearrangements of chromosomes in cultures of *Capsicum annum.* In the present investigation, the difference in the length of chromosomes in the *in vitro* plant and the calli (Tables 4 - 6), when compared with the karyotype of the parent plant may be due to any of these above mentioned reasons.

Total forma percentage of *in vitro* plant was slightly higher (42.0408) than that of parent (40.6758). In the present study the chromosome complement of the parent, *in vitro* plant and callus are characterized by smaller chromosomes. Comparatively smaller chromosomes in the karyotype seem to be an advanced characteristic feature (Das Gupta and Datta, 1976). Reduction in chromosome size is apparently a consequence of polyploidy, since it is an adaptation to a decrease in size of the cell or to an increase in number of chromosomes (Darlington, 1958).

Eventhough several karyomorphological differences exist between the *in vivo* and *in vitro* plants, their karyotype formulae were found to be the same ($A_{12}$ $B_{60}$ $C_{12}$). This shows the genetic relatedness of *in vivo* and *in vitro* plants of *D. indica*. The slight differences observed in the calli ($A_{12}$ $B_{58}$ $C_{14}$) may be due to the karyotypic imbalance caused by the stressful *in vitro* environment.

Although there are various factors for karyomorphological variations, the role of exact factors causing changes is yet to be studied. To find out the molecular level of variations among the *in vivo* and *in vitro* plants, RAPD analysis was carried out.

**Random Amplified Polymorphic DNA (RAPD) Analysis**

Among the diverse DNA markers identified during the past decades RAPDs with the potentially unlimited number of markers allow finer distinction, especially if too little molecular diversity exists. The use of molecular markers is becoming widespread for the identification of somaclonal variants and the assessment of micropropagation or *in vitro* regeneration protocols (Heinze and Schmidt, 1995; Wallner *et al.,* 1996).

PCR based RAPD technique (Williams *et al.,* 1990) was applied to assess somaclonal variation since this method has proved to be effective in a number of cases as in *Lolium* (Wang *et al.,* 1993), *Triticum* (Brown *et al*., 1993), *Picea* (Isabel *et al.,* 1993) and *Beta* (Munthali *et al.,* 1996). In the present study, considerable change in RAPD bands have been observed in the somaclonal variant (T1) of *D. indica* (Plates 8 and 9).

The use of PCR amplification to detect target DNA sequences has many application in plant genotyping, gene mapping, diagnosis and diversity assessment (Kreader *et al.,* 2001).

The tissue culture environment may lead to a general disruption of the normal cellular controls, leading to numerous genomic changes present in

the tissue culture regenerants (Phillips *et al*., 1994). In the present investigation, changes at the genomic level of tissue culture regenerants were evident from the difference in the amount of DNA present in the *in vivo* and *in vitro* (T1 and T2) plants of *D*. *indica* (Table 7). As far as the genetic stability of the proliferated tissue is concerned, RAPD markers are efficient tools for detection of somaclonal variation in tissue culture. Direct analysis of the DNA by use of RAPD markers proved to be a very sensitive technique for evaluating genetic changes. RAPD profiles were unambiguously used to establish the distinct identity of *in vitro* plants, which are different from the parent plant in many varieties of plants (Khanuja *et al*., 2001a, 2001b, 2001c, Dwivedi *et al*., 2001a, 2001b, 2001c, Patra *et al*., 2001b) after *in vitro* culture (Piccioni *et al*., 1997).

In the present study, RAPD analysis using arbitrary 10-mer oligonucleotide primers was employed in order to investigate the genetic variability between the parent plant and the variants (T1 and T2) of *D. indica.*

It is evident from the electrophoretic gels (Plates 8 and 9) that variation at DNA level is present in the hitherto mentioned micropropagated *Duchesnea indica* (T1). A few bands were found to be missing in the variant (T1) when seven primers (OPA 01, OPA 03, OPA 09, OPB 13, OPC 03, OPC 06, OPD 01) were used. Additional bands were also detected in the variant for six primers (OPA 03, OPA 09, OPB 03, OPB 11, OPB 13, OPC 03 OPC 06, and OPD 03). Similar results were obtained by Wang *et al*. (1993) and Hashmi *et al*. (1997) using RAPDs.

Variations in the RAPD bands in the *in vitro* plants of *D. indica* may be caused by genetic or epigenetic phenomena. Of the 27 primers used only 10 primers showed successful amplification. Failure of primers or probes tested in the present investigation to detect variation may indicate that either the gene or altered gene responsible has no homology with the primers or probes or the variations in the clones may be caused by an epigenetic

phenomenon (Phillips *et al*., 1990). Amplification of a certain sequence is based on the sequence of a single nucleotide used as a primer as well as the GC content (Lodhi *et al*. 1997). Failure of amplification of different lines may be due to a single base change or completely different sequences (Vierling and Nguyen, 1992). RAPD technique has been used to analyze somaclonal variation, while it can also detect single base mutations and deletions at the level of primer target or insertion or deletion without the amplified fragments (Gallego and Martinez., 1997).

Polymorphisms in amplified bands were observed in the present study. Polymorphism in the amplification products represents changes in the sequences of primer binding site (e.g. point mutations) or changes which alters the size or prevent the successful amplification of target DNA (Rout *et al*. 1998). Presence of RAPD markers at a specific locus in both genotypes indicates a high level of homology at that site. The sequence difference between two genotypes is expressed as the absence of marker and thus as band differences in the RAPD fingerprint (Williams *et al*., 1990).

Three types of polymorphism were observed in the present study, as presence of additional bands, absence of existing bands as well as band intensity differences (Plates8 and 9). The variant *in vitro* plant (T1) of *D. indica* showed one additional band for OPA 03 and five for OPA 09 when compared to the parent plant. Using OPB 03 and OPB 11, one additional band was observed in the variant (T1), which again was not detected in the parent. With OPB 13, one additional band was detected in variant (T1), which was absent in the parent. One additional band was found in the T1 for OPC 03, two for OPC 06 and one for OPD 03 when compared to the parent.

The existing bands in parent plant which were found to be absent in T1 plant was as follows: OPA 01 – 4 bands; OPA 03 – 4 bands; OPA 09 – 3 bands; OPB 13 – 1 band; OPC 03 – 3 bands; OPC 06 – 3 bands and OPD 01 – 2 bands. Band intensity differences found with the primer OPB 11 can be neglected since it is insignificant.

Sequence variation arising through the culture process has been detected in several plants using different ways including genome scanning with RAPDs (Kaeppler *et al*., 1998). RAPD technique had been used for genetic analysis of micropropagated plants (Rani *et al*., 1995; Shoyama *et al.,* 1997; Goto *et al*., 1995; Watanabe *et al.,* 1998). Several workers applied RAPD to detect somaclonal variation (Bohm and Zyprian, 1998; Al-Zahim *et al*., 1999; De Verno *et al*., 1999) and to identify micropropagated plants and cultivars (Ho *et al*., 1997).

Several reports are available on the use of RAPD based technology for studying genetic relationships and diversity among and within species, to compare micropropagated plants, to check the efficacy of genetic transformation, *etc*. in some of the members of Rosaceae (Damiano and Monticelli, 1998; Bartish *et al*., 1999; Shimada *et al*., 1999; Hormaza, 1999; Olsson *et al*., 2000; Garkava *et al*., 2000; Takehiko *et al*., 2001; Bushakra *et al*., 2003; Arús and Gardiner, 2007).

Several earlier workers had conducted molecular research on the different strawberry species with the help of RAPD markers for the identification of variants, to evaluate the genetic diversity of cultivars and to find out the success of genetic transformation studies ([4] http; [15]http; [16]http; [17]http; [18]http; Arulsekar *et al*., 1981; Bringhurst *et al*., 1981; Levi *et al*., 1994; Graham *et al*.,1996; Harrison *et al*., 1997; Porebski and Catling, 1998; Kumar *et al*., 1999; Ontivero *et al*., 2000; Congiu *et al*., 2000; Becerra *et al*., 2001; Degani *et al*., 2001; Kuras *et al*., 2004; Gambardella *et al*., 2005; Sugimoto *et al*., 2006; Milella *et al*., 2006).

RAPD analysis of *Allium cepa* revealed a novel band in independent gametoclones and it was suggested that this was due to a DNA sequence which was highly susceptible to tissue culture induced mutation (Al Zahim, 1999). The occurrence of some novel bands in independent regenerants has also been observed in wheat (Brown, 1993).

Thus in the present investigation RAPD analysis of the parent and

two micropropagated plants (T1 and T2) with the help of decameric primers generated jointly 152 scorable bands, 108 (71.05 %) of which were monomorphic and 44 (29.95 %) were polymorphic (Plates 8 and 9). Previous reports on some of the members of Rosaceae reveal that polymorphism reveals genetic distinctness of the compared samples of genomic DNA. However monomorphism shows genetic relatedness (Bartish *et al*., 1999; Shimada *et al*., 1999; Olsson *et al*., 2000; Garkava *et al*., 2000). RAPD analyses were conducted to evaluate the genetic differences and genetic similarities of several strawberry cultivars and to study the genetic diversity of different *Fragaria* species (Arulsekar *et al*., 1981; Bringhurst *et al*., 1981;[15]http; Graham *et al*., 1996; [16]http; Harrison *et al*., 1997; Porebshi and Catling, 1998; [17]http; Ontivero *et al*., 2000; Congiu *et al.,* 2000; Becerra *et al.,* 2001; Degani *et al*., 2001; Kuras *et al*., 2004., [18]http; Gambardella *et al*., 2005; Hokanson *et al*., 2006; Milella *et al*., 2006; [4]http).

Studies conducted by Kumar *et al*. (1999) reveal that polymorphisms and monomorphisms revealed by RAPD markers can be effectively used to evaluate the genetic fidelity of *in vitro* propagated strawberry plants.

The present investigation on the molecular basis of variation detected by RAPD in the micropropagated plant (T1) of *D. indica* suggests that RAPDs are useful for establishing the genetic basis of somaclonal variation and strengthens the idea of variant development by tissue culture.

**Essential Oil Analysis**

Tissue culture generated plants may vary from the parent plant on their morphological characters as well as useful agronomic characters such as essential oil yield and content. These variations may have importance in the field of crop improvement studies. The present study revealed the similarity of essential oil quantity in both *in vivo* and *in vitro* plants. However the quality of the essential oil of tissue cultured plant (T1), appeared to be significant when compared with the parent plant of *Duchesnea indica* (Andr.) Focke.

Plant tissue culture has the potential to perform biochemical reactions when organic compounds are added to the medium. It is possible to transform a substance from a lower to a higher, scientific, commercial or economic value and also to produce a new compound (Kukereja *et al*., 2000). The capacity of plant cells to serve as catalyst for biochemical reactions such as epoxidation, esterification, glycosylation, methylation, isomerization and dehydrogenation of organic compounds was comprehensively reviewed (Reinhard, 1974; Furuya, 1978). Over the years, *de novo* synthesis of many commercially important chemical compounds has been reported (Nair *et al*., 1986; Calleboutet *et al*., 1990).

Volatile oils are chemically complex mixtures often containing 100 or more individual components. Most oils have one to several components including sesquiterpenoids, monoterpenoids and phenols, out of which the major components impart characteristic odour and taste. But the minor products also play their part in the final product (Waterman, 1993). The economic properties may be due to the essential oil components present in them, which can be effectively exploited to produce fragrance (Tisserand, 1990) and flavoring agents (Heath, 1981).

In the present study GC - MS analysis revealed 25 compounds in the essential oil of the parent plant and 21 components in the *in vitro* derived plant (T1) (Table 9). The chromatogram of both the plants revealed variation in the pattern of peaks (Plates 10 and 11). The major components in both the plants were the same (carvacryl acetate valencene and nona hexa contanoic acid) but the composition and percentage of components was slightly different in the micropropagated plant, T1. Earlier studies conducted on several members of Rosaceae indicate that the essential oil of rosaceaous plants are rich in an array of volatile compounds especially terpenoids, aliphatic and aromatic aldehydes, ketones, alkanes, alcohols, esters, fatty acids, hydrocarbons and phenols (Vollmann and Schultze, 1995; Demetzos *et al*., 2002; Tucker *et al*., 2003; Eutuxia and Loannis,

2005). Several studies had also reported terpenoids, aliphatic and aromatic aldehydes, ketones, alkanes, alcohols, esters, fatty acids, hydrocarbons, phenolic compounds and several miscellaneous compounds in different species of strawberry (Larsen *et al*., 1992; Hamilton Kemp *et al*., 1993; Khanizadeh and Belenger, 1993; Gomes da Silva and Chaves das Never, 1999; Eun-Ryong *et al*., 2000; Lopez Arnados *et al*., 2001; Rosenfeld *et al*., 2003; Aaby *et al*., 2005, 2007; Carrasco *et al*., 2005; Hernanz *et al*., 2007; Ozgen *et al*., 2007; Ulrich *et al*., 2007; Tung *et al*., 2007; Olbricht *et al*., 2008; Pinto *et al*., 2008; [19]http; [20]http).

The parent plant and the variant plant (T1) of *D. indica* was characterized by major components like aristalone, dihydroaromadendrene, eicosane, 2 – hexa- decen – 1 - ol *etc*. Four components were found to be missing in the *in vitro* plant (T1) and some components showed reduction in the quantity of the components (Table 9). Essential oil yielding plants such as *Mentha piperita* (Nadaska *et al*., 1990), *M. arvensis* (Kukhreja *et al*., 1992), *Cymbopogon winterianus* (Mathur *et al*., 1998) and *C. martini* (Patnaik *et al*., 1999) and several other plants showed favourable variation in the oil content after *in vitro* development (Jain *et al*., 1989). Decrease in the essential oil yield and at the same time increase in the percentage of major components was reported in *Lavandula vera*.

The high value of coefficient of similitude (84), obtained on comparing the essential oils of parent and *in vitro* plants show the more similar nature of essential oil composition. The slight dissimilarity arises due to lack of minor oil components, which may be probably due to due to variation in the biosynthetic pathways of essential oils that are genetically controlled (Hiffendehl and Murray, 1973). Since the major components are the same in the *in vivo* and *in vitro* (T1) plants, the changes due to culture stresses did not affect their biosynthetic pathway.

The apparent increase in the percentage of the major components (carvacryl acetate) points out the suitability of plant tissue culture technique

in enhancing the yield of a particular essential oil component. Production of phytochemicals in cultures largely depends on various factors like physiological, biochemical and environmental conditions of the cell cultures. The stress induced by the culture conditions may cause genetic and biochemical changes leading to the altered expression in the essential oil composition of the somaclonal plant (T1). However the highly stable genetic make up of the individual tries to avoid the induced changes and escape from the drastic alterations by preventing regeneration from the altered cell lines. And so the *in vitro* derived plants tend to have almost the same characteristics of the parent. In the present observation there is differences in the minor components, which may be due to culture induced variation in the biosynthetic pathways leading to their formation. The lack of production of some components after the *in vitro* culture may have been due to either a loss in genetic ability or to a repression of the relevant genes under the culture conditions (Brown and Charlwood, 1986). The contribution of minor components cannot be neglected in the characteristic odour, appearance and potential of essential oil (Waterman, 1993). Although the similar and enhanced occurrence of major components is an advantage, the disappearance of a few minor ones in the culture derived plant (T1) diminishes the effect.

The production of each component of the essential oil is affected by the genetical as well as the environmental factors. Variation in essential oil components may be attributed to segregation of chimeral tissues, karyomorphological changes and molecular changes which may involve individual chromosomal aberrations or single gene mutations. There is report on the genetic basis of biosynthesis of mono- and sesquiterpenoids (Lincon *et al*., 1986). The production of secondary metabolites by plant cells *in vitro* is in part, dependent on the culture conditions employed. Since there is a close connection between differentiation and developmental process and secondary metabolism in plants, the growth regulators may influence the formation of secondary metabolites (Petri *et al*., 1989). The stress

induced by the culture conditions may be one of the reasons for changes in the essential oil composition of the *in vitro* (T1) plant of *D. indica*.

Changes in the constituents of the *in vivo* and *in vitro* plants are influenced by various non-genetic and genetic factors (Gerhardt, 1972; Franz, 1989). The phenomenon of production of a particular compound is fixed in the genome of a plant. Better understanding of genes, factors involved in regulation of biosynthetic pathways and biosynthetic enzymes provides a feasible method for extraction of medicinally important plant products from cells and tissues under culture that will be of great use to essential oil industry. The variation in the essential oil of the T1 (potential variant) may be the after effect of genetic changes revealed by chromosome studies and RAPD analysis.

**Total carbohydrate analysis**

The presence of a vast range of chemicals in plants leads them to be exploited for economic purposes. Many of the plant metabolites are important in making them commercially valuable forms. Carbohydrates, one of the major components making the strawberry fruits economically very important, has been important, as the wild strawberry fruits contain less quantity of the same. Since there is drastic variation in the carbohydrate content of original strawberry (*Fragaria* sp.) (about 5%) with the wild strawberry (about 3%), the study of the tissue culture derived variant (T1) is relevant.

In the comparative estimation of total carbohydrate content of *in vivo* and *in vitro* plants of *D. indica,* it was found that the somaclonal variant (T1) contains higher quantity of carbohydrate (3.2%) than *in vivo* (3 %) plant. This variation in terms of carbohydrate content may be correlated to the differences of the *in vitro* (T1) plant in the cytological and molecular aspects. Since there is a close connection between the differentiation and developmental processes, the growth regulators may influence the formation of metabolites (Petri *et al*., 1989). It is evident that the biosynthesis of plant

products is controlled by genetic factors (Franz, 1989). The *in vitro* stressful condition might have caused the genetic variation in the *in vitro* derived plant and this may be the reason for the difference in the phytochemical components of the *in vitro* derived plant (T1) from the parent.

The highlights of the present investigation are:-

- Potential variant of *Duchesnea indica* was achieved through indirect organogenesis in MS medium supplemented with growth regulator combination of IAA (2 mg/l) and NAA (2 mg/l).

- The chromosome complements of the *in vivo*, callus and *in vitro* plants are with 2n = 12 x = 84. The dodecaploid somatic chromosome number was reported in India for the first time in *D*. *indica*.

- The karyomorphological studies *via* the image analysis of the chromosomes showed variation in total chromosome length, average chromosome length and in the types of chromosomes, whereas ploidy remained the same.

- The Random Amplified Polymorphic DNA (RAPD) fingerprints of the *in vivo* and *in vitro* plants with ten primers show successful amplification, with all primers showing polymorphisms. The polymorphisms can be assumed to be due to change in the sequence of primer binding site or change which alters the size, preventing successful amplification of the target DNA. Both of which occurred due to tissue culture stress, that may show a general disruption of normal cellular controls, leading to numerous genomic changes in tissue culture regenerants.

- The GC - MS analysis of the essential oil of *in vivo* and *in vitro* plants showed a coefficient of similitude of 84 with 21 common components out of a total of 25 essential oil components detected. They showed some qualitative differences. The decrease in percentage of some components and absence of a few components in the essential oil of *in vitro* plant (T1) than the *in vivo* was a limitation. However enhancement in quantity of the major components and some minor components can be considered as a gain of the micropropagated (T1) plant.

- The analysis of total carbohydrate content of the *in vivo* derived fruit and *in vitro* derived fruit has shown an increase in amount of

carbohydrate content in *in vitro* (T1) derived fruit. In the case of parent plant the amount of carbohydrate was 3% and in the *in vitro* plant it was 3.2%. The difference may be due to the difference in the genetic make up of the *in vitro* (T1) plant.

*On the basis of these findings it seems probable that the in vitro plant of D. indica exhibits somaclonal variation. The findings emerging from this study has a practical application for creating variability in D. indica as well as to screen out the variants, which can be used for conventional breeding programmes if found stable after studying its detailed molecular basis.*

In conclusion, the present investigation has generated a novelmicropropagation protocol for *D. indica*. The comparative analysis of the *in vivo* and *in vitro* (T1) derived plants exhibited differences in chromosome architecture, genetic (RAPD) profile *etc*. In the phytochemical evaluation increase in the percentage of two major components in the essential oil and increase in the total carbohydrate content is valuable as far as the economic potential is concerned. Thus the present investigation substantiates the use of modern techniques like tissue culture for obtaining quantitatively and qualitatively superior plants.

# SUMMARY

Plants are used as one of the major food materials of the animals including human being. The members of the family Rosaceae are very important as most of them are economically very useful. The genus *Duchesnea indica* is important as its fruit is very similar to the economically important strawberry, *Fragaria* sp. The extract of this plant has been used by Chinese in their traditional medicines. In this context the genus *D. indica* has to be explored to evaluate the cytogenetical aspects and the related phytochemical effects. Among the different species of the strawberry Duchesnea is one which is less explored and thus the study on this plant is important.

Since this plant is sterile, the induction of variability is very difficult in natural conditions. Assessment of genetic variability is the basic aspect of any breeding programme. An alternative method to produce new forms of the plant is selecting a potential variant from tissue culture method. Plant tissue culture has the potential to induce genetic variability in *Duchesnea* genotype through somaclonal variants, somatic hybrids or transegenic plants. The exploitation of tissue culture technique for the production of plants with superior quality is attempted here to produce a novel variety of *D. indica*. Comparison of the *in vitro* derived plants with the parent was carried out to find out the performance of the micropropagated one over the parent. In the present study comparative figures in different parameters like cytogenetical, phytochemical, biochemical activity *etc.* were tested to find out the efficiency of the micropropagated plants.

**Micropropagation**

An efficient tissue culture method was developed by this study for the production of a variant plant which has some superiority over the parent plant. Murashige and Skoog basal medium with different hormonal combinations of auxins and cytokinins were tried in this venture to produce the variant. Though different explants tried, satisfactory result was given by nodal explant.

Among the different combinations of hormones used with MS medium, multiple shoots were induced with a combination of 2 mg/l IAA and 2 mg/l NAA. For the induction of multiple shoot regeneration, nodal explants were inoculated in the medium with combination of auxins and cytokinins. Multiple shoot were noticed after 3 – 4 weeks in 90 – 100% cultures. The frequency of shoot induction and percentage of initiation was higher in the medium containing 2 mg/l IAA and 2 mg/l NAA. When 2, 4 - D (2 mg/l) was used the nodal explant turned to callus. The combinations of 2, 4 – D (0.2 mg/l), IAA and NAA (0.5 ml/ each), had developed shoots and roots. When 2, 4 – D (0.2 mg/l) was used with IAA (0.5 mg/l), profuse callusing was observed. When this calli were subcultured in the medium supplemented with the hormones 2 mg/l IAA and 2 mg/l NAA, they developed into multiple shoots along with profuse rooting. Since no morphological variation was noticed among the regenerated plants, further analysis at cytological, molecular and phytochemical levels was conducted to search for the possible potential variants.

**Cytological analysis**

To examine the karyomorphological changes in the chromosome complement of the tissue culture derived plants, mitotic studies were carried out on actively growing root tip meristem of *in vitro* plants and also on callus cells. Then it was compared with the *in vivo* plant. The ploidy level of all the cells studied, i.e., *in vivo*, *in vitro* and callus were found to be invariably dodecaploid (2n = 7x = 84). Chromosome morphology of the regenerated

plants showed slight variation. Some of the chromosomes are not the exact replica of the parent plant and exhibited structural changes, like variation in total chromosome length, average chromosome length, centromeric positions, disparity index and total forma percentage. The chromosomes ranged in size from 1.5323 µm to 0.3838 µm in the parent plant, from 1.5323 µm to 0. 4402 µm in the variant and from 1.6191 µm to 0.3838 µm in the callus. The total chromosome length of the cells of the parent plant was noticed as 34.566 µm and that of the variant was 35.7675 µm. The total chromosome length of the callus was 33.3151 µm. The disparity indices observed in the chromosome complement of parent, variant and callus were 61.6755 and 55.3662 and 59.9394 respectively. The total forma percentages of the parent, variant and callus were estimated as 40.6758, 42.0408 and 41.5358.

The karyotype formula of the parent and the *in vitro* plant was $A_{12}$ $B_{60}$ $C_{12.}$ The karyotype formula of the callus was found to be slightly different ($A_{12}$ $B_{58}$ $C_{14}$).

**Random Amplified Polymorphic DNA (RAPD) Analysis**

To obtain more information on the genetic variability between the plants, RAPD analysis was carried out. For this DNA was extracted from the fresh young leaves of the parent plant and two in vitro regenerated plants, *viz*., T1 and T2. Amplification of DNA was carried out with 27 primers out of which only 10 responded successfully. The number of bands resolved per primer ranged from a minimum of 5 to a maximum of 28. The size of amplification product also differed and ranged from approximately 500 bp to over 10000 bp in the 10000 bp ladder. RAPD fingerprints of the variant, T1 differed from that of the donor plant with all the 10 primers whereas the variant, T2 differed with only eight primers, *viz*,

OPA 01, OPA 03, OPA 09, OPB 13, OPC 03, OPC 06, OPD 01 and OPD 03.

Altogether 20 bands were absent and 14 additional bands were present in T1 when compared with parent during RAPD analysis with 10 primers. However only 18 bands were absent and 8 new bands were present in T2 when compared with the parent. The reproducibility of these genomic DNA bands was consistent in successive repetitions.

Noticeable genetic polymorphism was observed in one tissue cultured plant (T1) as some parental bands are found to be missing in it. Some additional bands were also found in it with some primers. Since the T1 plant has shown more differences than the parent plant, it was considered as a variant and further investigations were carried out on this variant plant.

**Essential oil analysis**

The essential oil of both *Duchesnea indica* (Andr.) Focke and its tissue culture derived variant (T1) was subjected to GC - MS analysis. Both the plants yielded similar quantity of essential oil (0.1%). The analysis of the essential oil samples revealed a range of variation in their constituents. The major components identified from the essential oils of *in vivo* and *in vitro* plants were similar (carvacryl acetate, valencene, nona hexa contanoic acid, aristalone, dihydro aromadendrene, 2-hexa-decan-1-ol, eicosane), even though, there is marked difference in the percentage of occurrence. Considerable increase in the quantity of carvacryl acetate (30.5% and 31.2%) and valencene (7.6 % and 7.7%) and a decrease in percentage in the quantity of α- humulene (0.8% and 0.5%) was observed in the *in vitro* (T1) plant. The major component (carvacryl acetate) was observed in an enhanced quantity in the *in vitro* plant supports the better performance of the essential oil biosynthesis in the *in vitro* plants.

Dissimilarity was evident in the essential oil quality with respect to composition of oil components. Although 25 components were observed in the parent plant, the *in vitro* plant showed only 21 components.

**Total carbohydrate analysis**

The estimation of the total carbohydrate in both the parent plant and the variant (T1) also showed differences in quantity. The total carbohydrate present in the *in vivo* plant is 3% and that of *in vitro* (T1) plant is 3.2 % as per the present study. This also shows the superiority of the culture derived plant (T1) to the parent plant.

In most cases, study of somaclonal variations has been limited to phenotypic variation and has been associated with changes in the chromosome structure and function. The somaclonal variation in yield and quality characters will have importance in studies of crop improvement. The present study shows there is a significant change in the essential oil content and composition and the total carbohydrate content. The biosynthesis of secondary metabolites is controlled by genetic factors. Since there is a connection between the development and differentiation processes and the metabolism in plants, the growth regulators and the stress induced by the artificial environment might have influenced the metabolic pathways. The phytochemical differences observed in the present investigation may be due to the differences caused in the genetic make up of the *in vitro* plant of *Duchesnea indica*.

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# APPENDIX I

## Murashige and Skoog basal medium (1962)

| Stock chemicals | | mg/l | Stock concentration | Stock g/l |
|---|---|---|---|---|
| I. | $NH_4NO_3$ | 1650.00 | 50 X | 82.50 |
| | $KNO_3$ | 1900.00 | | 95.00 |
| | $KH_2PO_4$ | 170.00 | | 8.50 |
| | $MgSO_4.7H_2O$ | 370.00 | | 18.50 |
| II. | $CaCl_2.\ 2H_2O$ | 440.00 | 50 X | 22.00 |
| III. | $Na_2EDTA$ | 37.30 | 100 X | 3.70 |
| | $FeSO_4.7H_2O$ | 27.80 | | 2.80 |
| IV. | $MnSO_4.4H_2O$ | 22.30 | 100 X | 2.23 |
| | $ZnSO_4.7H_2O$ | 8.60 | | 0.860 |
| | $H_3BO_3$ | 6.20 | | 0.620 |
| | KI | 0.83 | | 0.083 |
| | $Na_2MoO_4.2H_2O$ | 0.25 | | 0.025 |
| | $CoCl_2.6H_2O$ | 0.025 | | 0.0025 |
| | $CuSO_4.5H_2O$ | 0.025 | | 0.0025 |
| V. | Vitamins | | 100X | |
| | Glycine | 2.00 | | 0.200 |
| | Nicotinic acid | 0.50 | | 0.050 |
| | Pyridoxine-HCl | 0.50 | | 0.050 |
| | Thiamine-HCl | 0.10 | | 0.010 |

# APPENDIX II

**CTAB buffer-**

| | | |
|---|---|---|
| CTAB | : | 2 % |
| Tris HCl | : | 0.1 M, pH 8 |
| NaCl | : | 1.4 M |
| EDTA | : | 20 mM, pH 8 |
| β- mercaptoethanol | : | 15 |
| PVP | : | 1 % |

# APPENDIX III

**TE buffer**

| | | |
|---|---|---|
| Tris HCl | : | 10 mM, pH 8 |
| EDTA | : | 0.1 mM, pH 8 |

# APPENDIX IV

**PCR buffer (10 x)**

| | | |
|---|---|---|
| Tris HCl | : | 10 mM, pH 8.8 |
| KCl | : | 500 mM |
| MgCl2 | : | 15 mM |
| Gelatin | : | 1 % |
| Tween-20 | : | 0.05 % |
| NP 40 | : | 0.05 % |

# APPENDIX V

**TAE buffer (50 x)**

| | | |
|---|---|---|
| Tris base | : | 12.1 g |
| Glacial acetic acid | : | 2.855 ml |
| EDTA | : | 5 ml, 0.5 M |

(for 50 ml of solution).

# APPENDIX VI

**Gel loading dye**

| | | |
|---|---|---|
| Sucrose | : | 40 % |
| Bromophenol blue | : | 0.25 % |
| Xylene cyanol | : | 0.25 % |

# ABBREVIATIONS

| | |
|---|---|
| 2, 4-D | 2, 4-Dichlorophenoxy Acetic Acid |
| ACL | Average Chromosome Length |
| AP-PCR | Arbitrarily Primed Polymerase Chain Reaction |
| BA | Benzyl Adenine |
| BAP | Benzyl Amino Purine |
| bp | base pair |
| CHIAS | Chromosome Image Analysis System |
| CS | Coefficient of Similitude |
| CTAB | Cetyl Trimethyl Ammonium Bromide |
| DI | Disparity Index |
| DLA | Daltons Lymphoma Ascites |
| DMSO | Dimethyl Sulfoxide |
| dNTP | deoxy Nucleotide Tri Phosphate |
| EAC | Ehrlich Ascites Carcinoma |
| EDTA | Ethylene Diamine Tetra Acetic acid |
| GC | Gas Chromatography |
| GC-MS | Gas Chromatography – Mass Spectrometry |
| HPLC | High Performance Liquid Chromatography |
| IAA | Indole 3- Acetic Acid |
| IBA | Indole 3-Butyric Acid |
| ILS | Increase in Life Span |
| kb | kilobase |
| KIN | Kinetin |
| µg | microgram |
| µl | microlitre |
| mM | milli molar |
| µm | micrometer |
| µM | micromolar |
| MS Medium | Murashige & Skoog Medium |

| | |
|---|---|
| NAA | $\alpha$ - Naphthyl Acetic Acid |
| ng | nanogram |
| nm | nanometer |
| OD | Optical Density |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain Reaction |
| pg | picogram |
| RAPD | Random Amplified Polymorphic DNA |
| RCL | Range of Chromosome Length |
| RFLP | Restriction Fragment Length Polymorphism |
| SDS | Sodium Dodecyl Sulphate |
| SD | Standard Deviation |
| TAE | Tris Acetic acid EDTA |
| TCL | Total Chromosome Length |
| TE | Tris EDTA |
| TF% | Total Forma percentage |
| VC | Variation Coefficient |

***Duchesnea indica (*Andrew*)* Focke.**
**(2n = 12x = 84 = $A_{12}$ $B_6C_{60}$ $D_6$)**

## *Callus*

| | | |
|---|---|---|
| Normal somatic chromosome number | : | 84 |
| Chromosomes with secondary constriction | : | 12 |
| Total chromosome length | : | 33.3151 µm |
| Range of chromosome length | : | 1.5323 µm - 0.3838 µm |
| Average chromosome length | : | 0.3906 µm |
| Disparity index | : | 59.9394 |
| TF value | : | 41.5358 |

| Chr. Type | No.of Pairs | Total Length (µ m) | s (µ m) | l (µ m) | R1 (s/l) | R2 (l/s) | l1 (s/c%) | l2 (l/c%) | Nature of Primary Constriction |
|---|---|---|---|---|---|---|---|---|---|
| A* | 1 | 1.6191 | 0.7322 | 0.8869 | 0.8256 | 1.2113 | 45.223 | 54.7773 | Nm |
| A* | 1 | 1.5323 | 0.5809 | 0.9514 | 0.6106 | 1.6378 | 37.9103 | 62.0897 | nm |
| A* | 1 | 1.5064 | 0.5614 | 0.945 | 0.5941 | 1.6833 | 37.268 | 62.7323 | Nsm(-) |
| C | 1 | 1.3603 | 0.4372 | 0.9231 | 0.4736 | 2.1114 | 32.14 | 67.86 | Nsm(-) |
| A* | 1 | 1.2663 | 0.4777 | 0.7886 | 0.6058 | 1.6508 | 37.7241 | 62.2759 | nm |
| *A | 1 | 1.2343 | 0.4563 | 0.778 | 0.5865 | 1.705 | 36.9683 | 63.0317 | Nsm(-) |
| C | 1 | 1.2178 | 0.5187 | 0.6991 | 0.742 | 1.3478 | 42.5932 | 57.4068 | nm |
| C | 1 | 1.2068 | 0.5241 | 0.6827 | 0.7677 | 1.3026 | 43.4289 | 56.5711 | nm |
| C | 1 | 1.1986 | 0.5339 | 0.6647 | 0.8032 | 1.245 | 44.5436 | 55.4564 | nm |
| C | 1 | 1.0994 | 0.5296 | 0.5698 | 0.9294 | 1.0759 | 48.1717 | 51.8283 | nm |
| C | 1 | 1.0974 | 0.4698 | 0.6276 | 0.7486 | 1.3359 | 42.8103 | 57.1897 | nm |
| D | 1 | 1.0833 | 0.3942 | 0.6891 | 0.5721 | 1.7481 | 36.3888 | 63.6112 | nsm(-) |
| C | 1 | 1.0705 | 0.4685 | 0.602 | 0.7782 | 1.285 | 43.7646 | 56.2354 | nm |
| A* | 1 | 1.0431 | 0.3447 | 0.6984 | 0.4936 | 2.0261 | 33.0457 | 66.9543 | nsm(-) |
| B | 1 | 0.9053 | 0.2716 | 0.6337 | 0.4286 | 2.3332 | 30.0011 | 69.9989 | nsm(-) |
| C | 1 | 0.7207 | 0.3078 | 0.4129 | 0.7455 | 1.3415 | 42.7085 | 57.2915 | nm |
| B | 1 | 0.6909 | 0.3145 | 0.3764 | 0.8355 | 1.1968 | 45.5203 | 54.4797 | Nm |
| C | 1 | 0.6868 | 0.1824 | 0.5044 | 0.3616 | 2.7654 | 26.5579 | 73.4421 | Nsm(-) |
| C | 1 | 0.6864 | 0.2928 | 0.3936 | 0.7439 | 1.3443 | 42.6573 | 57.3427 | nm |
| C | 1 | 0.6793 | 0.2751 | 0.4042 | 0.6806 | 1.4693 | 40.4976 | 59.5024 | nm |
| C | 1 | 0.6757 | 0.2745 | 0.4012 | 0.6842 | 1.4616 | 40.6245 | 59.3755 | nm |
| C | 1 | 0.6694 | 0.2371 | 0.4323 | 0.5485 | 1.8233 | 35.4198 | 64.5802 | Nsm(-) |
| B | 1 | 0.6631 | 0.3279 | 0.3352 | 0.9782 | 1.0223 | 49.4496 | 50.5504 | Nm |
| C | 1 | 0.6602 | 0.2895 | 0.3707 | 0.781 | 1.2805 | 43.8503 | 56.1497 | nm |
| C | 1 | 0.6388 | 0.2744 | 0.3644 | 0.753 | 1.328 | 42.9555 | 57.0445 | nm |
| D | 1 | 0.6368 | 0.2292 | 0.4076 | 0.5623 | 1.7784 | 35.9925 | 64.0075 | nsm(-) |
| C | 1 | 0.6321 | 0.2589 | 0.3732 | 0.6937 | 1.4415 | 40.9587 | 59.0413 | nm |
| C | 1 | 0.6306 | 0.2471 | 0.3835 | 0.6443 | 1.552 | 39.1849 | 60.8151 | nm |
| C | 1 | 0.6039 | 0.2316 | 0.3723 | 0.6221 | 1.6075 | 38.3507 | 61.6493 | nm |

| Chr. Type | No.of Pairs | Total Length (µ m) | s (µ m) | l (µ m) | R1 (s/l) | R2 (l/s) | l1 (s/c%) | l2 (l/c%) | Nature of Primary Constriction |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | |
| C | 1 | 0.6029 | 0.2729 | 0.33 | 0.827 | 1.2092 | 45.2646 | 54.7354 | nm |
| C | 1 | 0.5919 | 0.2747 | 0.3172 | 0.866 | 1.1547 | 46.4099 | 53.5901 | nm |
| C | 1 | 0.5832 | 0.2457 | 0.3375 | 0.728 | 1.3736 | 42.1296 | 57.8704 | nm |
| C | 1 | 0.582 | 0.2719 | 0.3101 | 0.8768 | 1.1405 | 46.7182 | 53.2818 | nm |
| D | 1 | 0.5792 | 0.2141 | 0.3651 | 0.5864 | 1.7053 | 36.9648 | 63.0352 | nsm(-) |
| C | 1 | 0.5686 | 0.2232 | 0.3454 | 0.6462 | 1.5475 | 39.2543 | 60.7457 | nm |
| C | 1 | 0.5684 | 0.2693 | 0.2991 | 0.9004 | 1.1107 | 47.3786 | 52.6214 | nm |
| C | 1 | 0.5583 | 0.2539 | 0.3044 | 0.8341 | 1.1989 | 45.4773 | 54.5227 | nm |
| C | 1 | 0.5561 | 0.258 | 0.2981 | 0.8655 | 1.1554 | 46.3945 | 53.6055 | nm |
| C | 1 | 0.4269 | 0.1982 | 0.2287 | 0.8666 | 1.1539 | 46.4277 | 53.5723 | nm |
| C | 1 | 0.426 | 0.1622 | 0.2638 | 0.6149 | 1.6264 | 38.0751 | 61.9249 | nm |
| C | 1 | 0.4231 | 0.2214 | 0.2217 | 0.9986 | 1.0014 | 52.3281 | 52.399 | nm |
| C | 1 | 0.3838 | 0.1509 | 0.2329 | 0.6479 | 1.5434 | 39.3174 | 60.6826 | nm |

***Duchesnea indica (*Andrew*)* Focke.**
**(2n = 12x = 84 = $A_{12}$ $C_{60}$ $D_{11)}$**

## *In vitro* Plant

| | | |
|---|---|---|
| Normal somatic chromosome number | : | 84 |
| Chromosomes with secondary constriction | : | 12 |
| Total chromosome length | : | 35.7657 µm |
| Range of chromosome length | : | 1.5323 µm - 0.4402 µm |
| Average chromosome length | : | 0.4257 µm |
| Disparity index | : | 55.3662 |
| TF value | : | 42.0408 |

| Chr. Type | No.of Pairs | Total Length (µ m) | s (µ m) | l (µ m) | $R_1$ (s/l) | $R_2$ (l/s) | $l_1$ (s/c%) | $l_2$ (l/c%) | Nature of Primary Constriction. |
|---|---|---|---|---|---|---|---|---|---|
| A* | 1 | 1.5323 | 0.5809 | 0.9514 | 0.6106 | 1.6378 | 37.9103 | 62.0897 | nm |
| A* | 1 | 1.2663 | 0.4777 | 0.7886 | 0.6058 | 1.6508 | 37.7241 | 62.2759 | nm |
| C | 1 | 1.2178 | 0.5187 | 0.6991 | 0.742 | 1.3478 | 42.5932 | 57.4068 | nm |
| C | 1 | 1.2068 | 0.5241 | 0.6827 | 0.7677 | 1.3026 | 43.4289 | 56.5711 | nm |
| C | 1 | 1.1986 | 0.5339 | 0.6647 | 0.8032 | 1.245 | 44.5436 | 55.4564 | nm |
| A* | 1 | 1.132 | 0.4748 | 0.6572 | 0.7225 | 1.3842 | 41.9435 | 58.0565 | Nm |
| C | 1 | 1.0994 | 0.5296 | 0.5698 | 0.9294 | 1.0759 | 48.1717 | 51.8283 | nm |
| C | 1 | 1.0974 | 0.4698 | 0.6276 | 0.7486 | 1.3359 | 42.8103 | 57.1897 | nm |
| D | 1 | 1.0833 | 0.3942 | 0.6891 | 0.5721 | 1.7481 | 36.3888 | 63.6112 | nsm(-) |
| C | 1 | 1.0705 | 0.4685 | 0.602 | 0.7782 | 1.285 | 43.7646 | 56.2354 | nm |
| A* | 1 | 1.0453 | 0.51 | 0.5353 | 0.9527 | 1.0496 | 48.7898 | 51.2102 | Nm |
| C | 1 | 1.0111 | 0.4567 | 0.5544 | 0.8238 | 1.2139 | 45.1686 | 54.8314 | nm |
| A* | 1 | 0.9975 | 0.1947 | 0.8028 | 0.2425 | 4.1233 | 19.5188 | 80.4812 | nsm(+) |
| C | 1 | 0.9417 | 0.4225 | 0.5192 | 0.8138 | 1.2289 | 44.8657 | 55.1343 | nm |
| C | 1 | 0.9333 | 0.461 | 0.4723 | 0.9761 | 1.0245 | 49.3946 | 50.6054 | nm |
| C | 1 | 0.9291 | 0.4572 | 0.4719 | 0.9688 | 1.0322 | 49.2089 | 50.7911 | nm |
| C | 1 | 0.9198 | 0.4133 | 0.5065 | 0.816 | 1.2255 | 44.9337 | 55.0663 | nm |
| A* | 1 | 0.8947 | 0.3842 | 0.5105 | 0.7526 | 1.3287 | 42.9418 | 57.0582 | nm |
| D | 1 | 0.8772 | 0.2723 | 0.6049 | 0.4502 | 2.2214 | 31.042 | 68.958 | nsm(-) |
| C | 1 | 0.8715 | 0.3381 | 0.5334 | 0.6339 | 1.5776 | 38.7952 | 61.2048 | nm |
| D | 1 | 0.839 | 0.3133 | 0.5257 | 0.596 | 1.6779 | 37.3421 | 62.6579 | nsm(-) |
| C | 1 | 0.8387 | 0.3678 | 0.4709 | 0.7811 | 1.2803 | 43.8536 | 56.1464 | nm |
| C | 1 | 0.7995 | 0.3072 | 0.4923 | 0.624 | 1.6025 | 38.424 | 61.576 | nm |
| C | 1 | 0.7857 | 0.37 | 0.4157 | 0.8901 | 1.1235 | 47.0918 | 52.9082 | nm |
| C | 1 | 0.7581 | 0.3572 | 0.4009 | 0.891 | 1.1223 | 47.1178 | 52.8822 | nm |
| C | 1 | 0.7253 | 0.3436 | 0.3817 | 0.9002 | 1.1109 | 47.3735 | 52.6265 | nm |
| C | 1 | 0.7156 | 0.3323 | 0.3833 | 0.8669 | 1.1535 | 46.4366 | 53.5634 | nm |
| D | 1 | 0.7105 | 0.1866 | 0.5239 | 0.3562 | 2.8076 | 26.2632 | 73.7368 | nsm(-) |
| C | 1 | 0.6921 | 0.3329 | 0.3583 | 0.9291 | 1.0763 | 48.1 | 51.77 | nm |

| Chr. Type | No.of Pairs | Total Length (µ m) | s (µ m) | l (µ m) | $R_1$ (s/l) | $R_2$ (l/s) | $l_1$ (s/c%) | $l_2$ (l/c%) | Nature of Primary Constriction. |
|---|---|---|---|---|---|---|---|---|---|
| C | 1 | 0.6813 | 0.2896 | 0.3917 | 0.7393 | 1.3526 | 42.507 | 57.493 | nm |
| C | 1 | 0.652 | 0.2979 | 0.3541 | 0.8413 | 1.1887 | 45.6902 | 54.3098 | nm |
| C | 1 | 0.6429 | 0.3132 | 0.3297 | 0.95 | 1.0527 | 48.7168 | 51.2832 | nm |
| D | 1 | 0.6315 | 0.2342 | 0.3973 | 0.5895 | 1.6964 | 37.0863 | 62.9137 | nsm(-) |
| C | 1 | 0.6099 | 0.2619 | 0.348 | 0.7526 | 1.3288 | 42.9415 | 57.0585 | nm |
| C | 1 | 0.6093 | 0.2572 | 0.3521 | 0.7305 | 1.369 | 42.2124 | 57.7876 | nm |
| C | 1 | 0.6088 | 0.2372 | 0.3536 | 0.6708 | 1.4907 | 38.9619 | 58.0815 | nm |
| C | 1 | 0.5884 | 0.2599 | 0.3285 | 0.7912 | 1.2639 | 44.1706 | 55.8294 | nm |
| C | 1 | 0.5436 | 0.2597 | 0.2839 | 0.9148 | 1.0932 | 47.7741 | 52.2259 | nm |
| C | 1 | 0.5349 | 0.2261 | 0.3088 | 0.7322 | 1.3658 | 42.2696 | 57.7304 | nm |
| C | 1 | 0.5177 | 0.2154 | 0.3023 | 0.7125 | 1.4034 | 41.6071 | 58.3929 | nm |
| C | 1 | 0.5151 | 0.2432 | 0.2719 | 0.8944 | 1.118 | 47.2141 | 52.7859 | nm |
| D | 1 | 0.4402 | 0.1476 | 0.2926 | 0.5044 | 1.9824 | 33.5302 | 66.4698 | nsm(-) |

***Duchesnea indica (*Andrew*)* Focke.**
**(2n = 12x = 84 = $A_{12}$ $B_6C_{60}$ $D_{6)}$**

## *Parent Plant*

| | | |
|---|---|---|
| Normal somatic chromosome number | : | 84 |
| Chromosomes with secondary constriction | : | 12 |
| Total chromosome length | : | 34.5660 µm |
| Range of chromosome length | : | 1.6191µm - 0.3838 µm |
| Average chromosome length | : | 0.6176 µm |
| Disparity index | : | 61.675 |
| TF value | : | 40.6755 |

| Chr. Type | No.of Pairs | Total Length (µ m) | s (µ m) | l (µ m) | $R_1$ (s/l) | $R_2$ (l/s) | $l_1$ (s/c%) | $l_2$ (l/c%) | Nature of Primary Constriction. |
|---|---|---|---|---|---|---|---|---|---|
| A* | 1 | 1.5323 | 0.5809 | 0.9514 | 0.6106 | 1.6378 | 37.9103 | 62.0897 | nm |
| B | 1 | 1.2879 | 0.5407 | 0.7472 | 0.7236 | 1.3819 | 41.9831 | 58.0169 | Nm |
| A* | 1 | 1.2663 | 0.4777 | 0.7886 | 0.6058 | 1.6508 | 37.7241 | 62.2759 | nm |
| B | 1 | 1.2207 | 0.5088 | 0.7119 | 0.7147 | 1.3992 | 41.681 | 58.319 | Nm |
| C | 1 | 1.1625 | 0.4128 | 0.7497 | 0.5506 | 1.8161 | 35.5097 | 64.4903 | Nsm(-) |
| C | 1 | 1.1291 | 0.3994 | 0.7297 | 0.5473 | 1.827 | 35.3733 | 64.6267 | Nsm(-) |
| A* | 1 | 1.1103 | 0.4544 | 0.6559 | 0.6928 | 1.4434 | 40.9259 | 59.0741 | Nm |
| C | 1 | 1.0994 | 0.5296 | 0.5698 | 0.9294 | 1.0759 | 48.1717 | 51.8283 | nm |
| C | 1 | 1.0974 | 0.4698 | 0.6276 | 0.7486 | 1.3359 | 42.8103 | 57.1897 | nm |
| C | 1 | 1.0942 | 0.3469 | 0.7473 | 0.4642 | 2.1542 | 31.7035 | 68.2965 | Nsm(-) |
| D | 1 | 1.0833 | 0.3942 | 0.6891 | 0.5721 | 1.7481 | 36.3888 | 63.6112 | nsm(-) |
| C | 1 | 1.0705 | 0.4685 | 0.602 | 0.7782 | 1.285 | 43.7646 | 56.2354 | nm |
| C | 1 | 1.0111 | 0.4567 | 0.5544 | 0.8238 | 1.2139 | 45.1686 | 54.8314 | nm |
| B | 1 | 1.002 | 0.4634 | 0.5386 | 0.8604 | 1.1623 | 46.2475 | 53.7525 | Nm |
| A* | 1 | 0.9975 | 0.1947 | 0.8028 | 0.2425 | 4.1233 | 19.5188 | 80.4812 | nsm(+) |
| C | 1 | 0.9417 | 0.4225 | 0.5192 | 0.8138 | 1.2289 | 44.8657 | 55.1343 | nm |
| C | 1 | 0.9333 | 0.461 | 0.4723 | 0.9761 | 1.0245 | 49.3946 | 50.6054 | nm |

| Chr. Type | No.of Pairs | Total Length (µ m) | s (µ m) | l (µ m) | $R_1$ (s/l) | $R_2$ (l/s) | $l_1$ (s/c%) | $l_2$ (l/c%) | Nature of Primary Constriction. |
|---|---|---|---|---|---|---|---|---|---|
| C | 1 | 0.9291 | 0.4572 | 0.4719 | 0.9688 | 1.0322 | 49.2089 | 50.7911 | nm |
| C | 1 | 0.9198 | 0.4133 | 0.5065 | 0.816 | 1.2255 | 44.9337 | 55.0663 | nm |
| A* | 1 | 0.8947 | 0.3842 | 0.5105 | 0.7526 | 1.3287 | 42.9418 | 57.0582 | nm |
| C | 1 | 0.8715 | 0.3381 | 0.5334 | 0.6339 | 1.5776 | 38.7952 | 61.2048 | nm |
| D | 1 | 0.839 | 0.3133 | 0.5257 | 0.596 | 1.6779 | 37.3421 | 62.6579 | nsm(-) |
| A* | 1 | 0.8027 | 0.3207 | 0.482 | 0.6654 | 1.503 | 39.9527 | 60.0473 | Nm |
| C | 1 | 0.7207 | 0.3078 | 0.4129 | 0.7455 | 1.3415 | 42.7085 | 57.2915 | nm |
| C | 1 | 0.6864 | 0.2928 | 0.3936 | 0.7439 | 1.3443 | 42.6573 | 57.3427 | nm |
| C | 1 | 0.501 | 0.1981 | 0.3013 | 0.6575 | 1.5209 | 39.5409 | 60.1397 | nm |
| C | 1 | 0.4899 | 0.2442 | 0.2457 | 0.9939 | 1.0061 | 49.8469 | 50.1531 | nm |
| C | 1 | 0.4839 | 0.2134 | 0.2705 | 0.7889 | 1.2676 | 44.1 | 55.9 | nm |
| C | 1 | 0.483 | 0.2371 | 0.2459 | 0.9642 | 1.0371 | 49.089 | 50.911 | nm |
| C | 1 | 0.4774 | 0.2373 | 0.2401 | 0.9883 | 1.0118 | 49.7067 | 50.2933 | nm |
| C | 1 | 0.464 | 0.2268 | 0.2372 | 0.9562 | 1.0459 | 48.8793 | 51.1207 | nm |
| D | 1 | 0.4586 | 0.1627 | 0.2959 | 0.5498 | 1.8187 | 35.4775 | 64.5225 | nsm(-) |
| C | 1 | 0.4477 | 0.1893 | 0.2584 | 0.7326 | 1.365 | 42.2828 | 57.7172 | nm |
| C | 1 | 0.4452 | 0.1981 | 0.2471 | 0.8017 | 1.2473 | 44.4969 | 55.5031 | nm |
| C | 1 | 0.4394 | 0.213 | 0.2264 | 0.9408 | 1.0629 | 48.4752 | 51.5248 | nm |

| Chr. Type | No.of Pairs | Total Length (µ m) | s (µ m) | l (µ m) | $R_1$ (s/l) | $R_2$ (l/s) | $l_1$ (s/c%) | $l_2$ (l/c%) | Nature of Primary Constriction. |
|---|---|---|---|---|---|---|---|---|---|
| C | 1 | 0.4371 | 0.2024 | 0.2347 | 0.8624 | 1.1596 | 46.3052 | 53.6948 | nm |
| C | 1 | 0.4269 | 0.1982 | 0.2287 | 0.8666 | 1.1539 | 46.4277 | 53.5723 | nm |
| C | 1 | 0.426 | 0.1622 | 0.2638 | 0.6149 | 1.6264 | 38.0751 | 61.9249 | nm |
| C | 1 | 0.4231 | 0.2214 | 0.2217 | 0.9986 | 1.0014 | 52.3281 | 52.399 | nm |
| C | 1 | 0.4181 | 0.1883 | 0.2298 | 0.8194 | 1.2204 | 45.0371 | 54.9629 | nm |
| C | 1 | 0.4066 | 0.1849 | 0.2217 | 0.834 | 1.199 | 45.4747 | 54.5253 | nm |
| C | 1 | 0.3838 | 0.1509 | 0.2329 | 0.6479 | 1.5434 | 39.3174 | 60.6826 | nm |