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; 10http; 11http). 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 (7http; 8http; 9http; 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.,
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 (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 (Mohammed, 1990; Rashid, 1991; Yang et al., 1995; Owen and Miller, 1996; Bhat and Dhar, 2000; Sakila et al., 2007; Biswas et al, 2007, Yang et al., 1999) show that shoots might be induced from meristematic tissues since actively dividing cells are highly susceptible to hormonal treatments, which alter their normal developmental pathways.

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 betacea*. 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 widespread 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 from 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. (Kaepppler and Philips,
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 Brinthurst, 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) chromosome 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; ¹http).

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<sub>12</sub> B<sub>60</sub> C<sub>12</sub>). This shows the genetic relatedness of in vivo and in vitro plants of D. indica. The slight differences observed in the calli (A<sub>12</sub> B<sub>58</sub> C<sub>14</sub>) 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 (Kaepppler 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 (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,
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; Graham et al., 1996; Harrison et al., 1997; Porebshi and Catling, 1998; Ontivero et al., 2000; Congiu et al., 2000; Becerra et al., 2001; Degani et al., 2001; Kuras et al., 2004; Gambardella et al., 2005; Hokanson et al., 2006; Milella et al., 2006).

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 hexacontanoic 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
rosaceous 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
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 $2n = 12x = 84$. 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 novel micropropagation 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.