Chapter 1

INTRODUCTION

Sugarcane (Saccharum species complex) is a monocotyledonous plant belonging to the grass family Poaceae. It is a fast growing, perennial grass that grows the best under moist, hot, sunny climate in almost every type of soil. Being one of the most important cash crops of India, sugarcane provides livelihood to about 34 lakh farmer families in Uttar Pradesh. It has emerged as a multipurpose crop providing not only sugar as the main product but also a series of value added by-products such as ethanol and other alcohol derived chemicals, paper, particle board, animal feed, antibiotics, wax, bio-fertilizer and raw material for generating electricity.

Sugarcane, being the most valuable commercial crop of India, sustains not only sugar and distillery industries but also holds a key position in the national economy by earning foreign exchange. Besides conventional products like Gur and Khandsari, it also provides raw materials of economic importance such as molasses, bagasse, wax etc to several other industries. Sugarcane based sugar industry is second largest agro-industry of India who is the second largest producer of sugarcane and largest consumer of sugar. Uttar Pradesh occupies first position in the country with regard to area as well as production of
sugarcane however its productivity is lower as compared to the average productivity at national level. Thus, Uttar Pradesh faces a continuing challenge to increase both cane yield and sugar recovery in the state. This can be easily achieved by replacing the old deteriorated varieties with newly released ones. Newly developed varieties possess higher yield and quality potential and better resistance to a number of abiotic and biotic stresses than several varieties presently being cultivated in Uttar Pradesh.

Cytogenetic researches have shown that the *Saccharum officinarum* (noble cane) was domesticated from *S. robustum*. Investigations indicated that *S. officinarum* originated from the area of Papua New Guinea. About 2000 years ago, India first time produced crude form of sugar from sugarcane. For this purpose, the cane was cut into small pieces, crushed by a wooden pestle and mortar and the juice thus obtained was boiled and stirred until it became solid. These solids, being of uneven shapes and sizes, were formerly called ‘Sarkara’ and the modern term 'sugar' was derived from it.

Sugarcane clones, cultivated on commercial scale in several cane producing countries, are believed to have originated in New Guinea and Northern parts of India, the two different geographical centers. The erect, fast growing and elongated tropical species of sugarcane (*viz.*, *S. officinarum*) was considered to be originated in New Guinea from a wild species *viz.*, *S. robustum*. These sugarcanes included some important noble canes like Cheribon, Bourbon, Tonna or Caledonia and were characterized by thick cane stalks with soft rind, low fibre, high cane yield and high sugar contents. Subsequently, the above mentioned clones modified and developed into new species by the process of natural interspecific hybridization. The north Indian canes, like *S. barberi* and *S. sinense* have developed through natural hybridization among the migrating species of *S. officinarum* and *S. spontaneum*.
Stevenson, 1965). This concept obviously presupposes that the presence of *S. officinarum* in India-Burma region antedated the origin of north Indian species.

Sugarcane is classified into the grass family- *Poaceae* (earlier known as ‘*Gramineae*’), sub-family *Panicoideae*, super tribe *Andropogoneae*, sub-tribe *Saccharinaeae* and genus *Saccharum* (Watson et al., 1985). Most of the commercially cultivated varieties of sugarcane have been developed through crosses between different cultivars/species or selected from self population and therefore designated as ‘*Saccharum* species Complex’ or ‘*Saccharaum* species Hybrid’.

The genus *Saccharum* includes two widely accepted wild species (*S. spontaneum* L. and *Saccharum robustum* JESWIET et BRANDES) and four domesticated species. Tropical regions of, Asia, Africa and Oceania is rich of the wild species *S. spontaneum* L. Another wild species of sugarcane *viz.*, *S. robustum* JESWIE et BRANDES is confined to New Guinea and its nearby islands. *S. robustum* is morphologically characterized by bamboo like elongated plants attaining heights of up to about 10 meter. It is generally used by the villagers for making walls and roofs of houses in rural areas and also in making fence posts. This cane is highly resistant to insect-pest and disease infestations.

*S. officinarum, S. barberi, S. sinense* and *S. edule* are the sugarcane species which are domesticated. Out of these, *S. officinarum* L. is categorized as a noble cane bearing the stalks with comparatively higher girth. Longitudinal stripes in various colours are generally present on the stalks. The plants attain heights of 3-4 m and bear few tillers. The stalks possess soft rind and high sucrose content. Noble cane is considered as to be original, sweet in taste and soft chewing cane. These canes are presently cultivated all over the country for commercial production of sugar. Sugarcane is cultivated in larger part of India because of the agro-climatic conditions of tropical (0-10° latitudes) or
subtropical region (10°-30° latitudes) which is suitable for sugarcane cultivations. However, the cultivation of sugarcane round the year is more promising in tropical climate than in sub-tropical one regarding cane yield and sugar recovery.

*S. barberi* Jesw (Indian sugarcane) is characterized by thin stalked and hard canes that grow best in semi-tropical and temperate climates. The canes of this species were first used for the manufacturing of sugar. The canes of species *S. sinense* Roxb are usually tall, vigorous in growth and hardy which were developed from the hybridization between *S. spontaneum* and *S. officinarum*. About 100 years ago, a variety namely ‘Uba’ became much popular when it replaced the 'noble canes' due to disease susceptibility in several sugar industry areas. Cultivation of *S. edule* – HASSK (a mutant of *S. officinarum* and bearing edible inflorescences) was restricted only to Indonesia and Melanesia.

Today’s commercially cultivated sugarcane varieties are highly complex interspecific hybrids having genes and chromosomes derived from *S. officinarum* (2n=80), *S. barberi* (2n = 82-124), *S. sinense* (2n = 116-120), *S. spontaneum* (2n = 40-128) and *S. robustum* (2n = 60-80). The hybrid varieties are more successful to sustain the sugar industries for a longer period of time because of their specific characteristics, such as high sugar contents, high yielding potential and resistance to pests and diseases. The number of chromosomes of complex sugarcane varieties varies from 100-120 or even more (Price, 1963; Rao et al., 1983).

Sugarcane being an important industrial crop occupies an area of about 4.90 million hectares that accounts approximately 3% of the total cultivated area in the country. The annual cane production is about 305 million tonnes (2015-2016). Thus, the crop contributes about 8.0 percent to the total agricultural production in India.
Over 50 million sugarcane cane grower and equivalent population of laborers depend on this crop for income. The sugar mills gives job to approximately 5 lakhs technical and skillful employees. About 520 sugar mills use to crush 240 million tonnes of cane and produce about 25 million tonnes of sugar. The average yield of sugarcane in the country is about 71 tonnes per hectare. Thus, India ranks second next only to Brazil in cane and sugar production in the world. In addition, abundant open pan Khandsari and Jaggery (Gur) units are also supported by this crop.

In India, Uttar Pradesh is the largest sugarcane cultivating and producing state of subtropical part of the country. Sugarcane is cultivated in about 21.69 lakh hectares area with an average yield of about 67.0 t/ha and sugar production of about 145.38 lakh tonnes in 2015-16 (Anonymous, 2016). Uttar Pradesh is lagging behind in the rate of average yield and sugar recovery from the national average. The present challenge of sugarcane production in Uttar Pradesh can be resolved by developing newer varieties with improved potential for cane and sugar yield along with the disease resistance.

For last several decades, the development of new high yielding and high sugar varieties of sugarcane are being done through selection using breeding methods. Various sugarcane research institutes are utilizing conventional breeding methods for developing new varieties with improved potential for cane and sugar productivity. However, this method requires a time of over 8-10 years or even more to develop and release a new variety. Thus, this strategy has not been proved satisfactory up to the desired extent. It is, therefore, needed to use some cost effective, efficient and rapid approaches (e.g. Biotechnology) to evolve newer varieties of sugarcane possessing high yielding potential and improved sugar contents.
New sugarcane varieties are interspecific hybrids and usually complex aneuploids of cultivated species *viz.*, *S. officinarum* and *S. spontaneum* with high number of chromosomes. Due to genetic complexity, the sugarcane is lagging behind the other crops in the use of molecular markers to exploit its genomics and produce transgenics. The major raw material of sugar production is sugarcane and it accounts for about 65% of its cost of production. Research efforts to improve cane yield and sugar content and to reduce its cost of production per unit area and time are, therefore, of critical importance for improving the efficiency of sugar industries and making it competitive in the world market. Sugar recovery for the last 40 years has been stagnating around 10%. The compound growth rate in yield during 1949-1950 to 2010-11 has been only around 1.21% per annum as against 1.58% for all the crops.

U.P. Council of Sugarcane Research, Shahjahanpur has so far released over 212 varieties of sugarcane. Among these, varieties *viz.* CoS 510, CoS 687, CoS 8436, CoS 88230, CoS 95255, CoS 96268, Co 0238, CoS 08272 etc have played an important role in increasing sugarcane and sugar production in Uttar Pradesh. There are however lesser number of early maturing sugarcane varieties under commercial cultivation, therefore, to economize the cost of sugar production, more and more thrust is required for the development of early maturing varieties having high yield and high sugar with adequate level of disease resistance. In respect of improvement, major efforts have been made through inter-varietal hybridization but now possibilities are also being explored through distant hybridization programme. With this strategy, considerable success has been achieved during recent years in identifying and developing high yielding varieties. Even then, there are certain constraints and limitations to evolve the cultivars with desired traits through hybridization as sugarcane varieties are genetically highly heterozygous and complex polyploids.
Modern commercial sugarcane varieties are developed by the process of breeding involving a multi-level selection scheme requiring a time span of over 10 years for identifying a few elite clones from a very large population of hybrid seedlings. The cost of selection and identification per cultivar through conventional breeding programme is also very high. The complex polyploid nature, heterozygosity and the associated problems in flowering and seed set make the application of traditional breeding approaches very difficult in sugarcane. Sugarcane is propagated slowly through vegetative means, hence fast spreading of newly released varieties is another main limitation. Seed cane of newly released varieties is available in a very limited quantity during time of release. Therefore, it requires the time span of 8-10 years to cover the vast area of the state for commercial planting if multiplied through conventional sett planting method of seed multiplication. By the time the variety begins deteriorating in yield and quality traits. Thus, it seems to be difficult to fulfill the increasing requirements of planting material of newly developed and released clones timely through conventional method of seed multiplication. It is the call of time to evolve and use biotechnological methods for quick multiplication of new clones. In recent years, plant biotechnology have several advanced techniques that can be applied for developing new improved varieties through precision breeding for defined objectives and also for rapid production of elite varieties to meet the increasing demand of planting material of new varieties.

During the past more than eight decades, attempts are being made through conventional breeding methods to develop high yielding and high sugared varieties of sugarcane. Consequently, a large number of varieties were developed from time to time which revolutionized the sugar industry to a considerable extent. The conventional breeding methods, however, require a longer period of over eight years for developing an elite clone through selection
from a very large population of seedlings. Moreover, the clones developed through sexual hybridization may suffer from several drawbacks.

It has been opined that the genetic variability in clones gets exhausted as a consequence of continuous selection and exploitation of available genetic resources resulting in the narrowing down of the genetic base of sugarcane crop. Liu (1972) has stated that the genetic variability created by gene segregation resulting from sexual crossing may not adequately allow improvement in a particular desired character. Further, it may not be possible to rectify a specific defect in otherwise well adopted sugarcane varieties through sexual breeding methods because of the higher genetic complexity existing in this crop (Heinz et al., 1977).

To overcome the aforesaid problems, it seems quite essential to exploit the modern biotechnologies in developing new elite cultivars of sugarcane for sustaining the sugar industries for longer period of time. Tissue culture technique is a promising and an important tool of biotechnology in crop improvement and rapid production in different crops.

Haberlandt for the first time utilized in vitro techniques in 1902 to demonstrate the phenomenon of totipotency in plant cells. A totipotent cell is capable of performing all the developmental functions, similar to a zygote, to develop into a whole plant. Earlier efforts to demonstrate the totipotency of cells resulted in the innovation of in vitro techniques for growth and culture of plant cells on artificial nutrient media under controlled environmental conditions. Brilliant contributions made by R.J. Gautheret (France) and P.R. White (U.S.A) in early decades of 20th century, were helpful in achieving this goal. Hannig (1904) first time established the embryo culture from mature embryos of some crucifers which were later successfully grown to maturity.
Subsequently, Guha and Maheshwari in 1964 produced haploid plants from pollen grain culture for the first time which set the milestone of anther and pollen culture for development of haploid plants. Later, the techniques were developed by many other workers who demonstrated that the complete plants of tobacco could be produced \textit{in vitro} using isolated microspores.

After discovery of hormonal regulations in growth and organ formation by Skoog and Miller in 1957, Steward \textit{et al.} (1958) and Reinert (1958) also independently demonstrated the cellular totipotency of plant cells and reported the development of somatic embryos from cultured cells.

Recently, tremendous research work has been done in different national and international institutes to improve the genotype of different crops such as cereals, pulses, fruits, forestry and many other crops applying the biotechnological approaches. By applying the technique, it became possible to develop elite clones with improved characteristics such as development of high yielding potential and disease resistance through somaclonal variations. Somaclonal variation has been well documented in sugarcane by Larkin and Scowcroft (1981) and numerous reports on somaclones with improved traits have been published. Rejuvenation of old cultivars and conservation of genetic resources have also been made possible using \textit{in vitro} approaches.

MS medium is used in micropropagation and different plant growth regulators (hormones) are externally added to it for desired results. Cytokinins and auxins are generally used in various concentrations, alone or in combination, for responses like explant establishment, shoot multiplication and root development. Cytokinins such as BAP, Kinetin, TDZ etc. play important role cell division and auxins like IAA, NAA, IBA etc are also used for rooting of shoots. Different permutation combinations are tried for better results. Plant growth regulators are used in the media for better growth and elongation
however the responses depend on the species/varieties in many crops including sugarcane (Ashraf et al., 2014, Tolera et al., 2014). TDZ was synthesized for defoliation of cotton by German Schering Corporation (Arndt et al., 1976). Originally, TDZ was accepted to have cytokinin like morphogenetic response. Besides playing role in shoot regeneration, shoot multiplication, induction of axillary bud formation, it has been reported to regulate the level of endogenous auxin (Wang et al., 1986, Grabkowaska et al., 2014, Kou et al., 2016). TDZ is also effective in inducing varied morphogenetic responses depending upon exposure time. Pretreatment with TDZ can predispose a tissue to accept other inductive stimuli in some plants (Guo et al., 2011). No reports are available on the use of TDZ in tissue cultures of sugarcane.

The environmental condition for tissue culture is important as it can produce mutagenesis during cell differentiation as reported by Leshem (1990). The light spectrum is known to exert remarkable effect on cells, plant tissue growth and the biosynthesis of metabolites (Ferreira et al., 2017). Similarly, the physiological process of the shoot/plant depends upon various environmental factors like photoperiod, light intensity, temperature and pH of medium hence optimization of these factors is important for successful micro propagation of any crop and maintenance of genetic stability of clone population.

In vitro root development is very important step of micropropagation technique. A healthy root makes the plant more compatible to survive in the green house during acclimatization and thereafter in the field. Various protocols have been suggested for better root development in the regenerated shoots. These protocols emphasizes on media composition of auxin and sucrose (Nadar and Heinz, 1977; Lee, 1986; 1987; Ramanand et al., 2007). TDZ has also been reported a promising hormone for root development in plants (Kou et al., 2016).
Lack of adequate procedures for rapid multiplication of newly released varieties of sugarcane has been experienced by the scientists since a long time (Singh et al., 1995). Traditional methods of vegetative propagation exhibit a slow rate taking ten or more years to build up sufficient stock of seed cane of newly released varieties for general cultivation.

Micropropagation is one of the most important and perhaps the most utilized technique of plant tissue culture. From time to time, several investigators developed various protocols to be used for in vitro micropropagation of sugarcane (Hendre et al., 1975; 1983; Sreenivasan and Sreenivasan, 1992; Shukla et al., 1994; Lal and Singh, 1997; Singh, et al., 2001; Kumari and verma 2001; Pawar et al., 2002 a,b; Ramanand and Lal, 2004; Jalaja et al. 2008; Kaur and Sandhu, 2015). Micropropagation involves establishment of axenic shoot cultures and production of large number of plantlets of a variety using specific plant parts known as explants. The choice of explants for micropropagation depends on the objectives and targets. For producing virus free plants, preferably apical shoot meristems are selected (Quark, 1977) while for seed multiplication, shoot tips, nodal segments and even large vegetative buds can be used as the explants. The rate and mode of propagule multiplication determines the success of micropropagation. Morphogenesis and proliferation rate of cultures depend on various factors influencing the relative incidence of organogenesis or embryogenesis. Sterilization of the explant is very important as in this process integrity of biological property should be maintained and only contaminates to be removed. Mercuric Chloride is most commonly used as sterilizing agent of explants (Mekonnen et al., 2013). Some other choice of sterilizing agent like sodium hypochlorite in place of media autoclaving had been used by Sawant and Tanwar (2011).
Slower multiplication ratio (1:10) in the field through conventional sett planting method has been a major constraint in rapid propagation of newly released varieties. *In vitro* micropropagation technique is more competent for rapid spreading of new sugarcane varieties by reducing the time span between release and large-scale cultivation. Micropropagation using shoot tip (or apical meristem) is very efficient and it saves considerable time of seed multiplication in comparison to conventional method in sugarcane crop (Hendre *et al*., 1983).

However, micropropagation of many species including sugarcane sometimes shows phenotypic instability which can be regarded as a disadvantage in micropropagation system (Bailey and Bechet, 1989; Irvine *et al*., 1991; Taylor *et al*., 1995). Therefore, assessment of genetic fidelity and presence of any of somaclonal variation in tissue culture derived plants seems to quite desirable. Now molecular markers, a kind of genetic markers, are also being used for the assessment of genetic variability in regenerated clones.

DNA sequences having specific location in the genome, are inherited and follow the law of inheritance, are known as molecular markers. Various molecular markers have been used for fidelity analysis of various plants for rapid and reliable assessments (Taylor *et al*., 1995; Rani and Raina, 2000; Devarumath *et al*., 2007). Various molecular marker techniques have been developed and being used such as expressed sequence tags (EST), microsatellite primed PCR (MP-PCR), amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), simple sequence repeats (SSR), Directed amplification of minisatellite-region DNA (DAMD) etc. PCR based molecular markers require small amount of DNA for analysis and no prior information regarding the sequence is needed in RAPD, SPAR, AFLP and ISSR. They are capable of screening many genes in a cost effective manner.
Different PCR based molecular markers like RAPD, SSR and DAMD have been used in sugarcane. DAMD has been found to be more efficient for being a species specific marker and it has been recommended to use in combination with molecular markers to get maximum efficiency (Murthy et al., 2013, Lamare et al., 2015). RAPD analysis is comparatively simple and quick methodology to assess the genetic fidelity of micropropagated clones and it also requires a very small quantity of DNA. RAPD analysis is less expensive in comparison to different other markers viz., RFLP, SSR, AFLP and ISSR (Williams et al., 1990).

The success of the micropropagation depends on the successful transfer of plantlet from in vitro condition to the green house, and from green house to the field soil. The in vitro raised sugarcane plantlets should be successfully acclimatized in green house for optimum survival in the soil. Different approaches had been tried by various researchers for acclimatization of plants. Role of different types of substrate, pots, soil mixture in acclimatization of plant has been reported in different plants (Davis et al., 2009; Mengesha et al., 2013; Resende et al., 2015).

The use and exploitation of modern biotechnologies for crop improvement has been emphasized by several investigators in recent years. Tissue culture techniques have proved to be an effective approach for improvement of many economically important crops. To emphasize the utility of somaclonal variation in crop improvement, sugarcane is often cited as an example along with the potato in all publications dealing with this subject.

Sugarcane is considered to be an ideal plant material for such studies because of the existence of polyploidy, the capacity of the plants to tolerate chromosomal aberrations and the capacity of the deviant cells to differentiate into plants. However, when a somaclone with a desirable trait is identified and
selected, for example disease resistance, it has to pass through all the stages of testing and adaptive trials and thus the time taken for their ultimate release as a cultivar will remain almost the same as that of sexual populations. Sreenivasan and Jalaja (1995) were of the view that selection among somaclonal population would be effective mainly for rectifying one or two specific defect(s) and not for an overall improvement of a cultivar.

Some efforts have also been made to understand the molecular control of cell differentiation (Heinz et al, 1971, Dey et al., 1998). The best approach for understanding the molecular events taking place during morphogenesis is to identify response specific biochemical/ molecular marker(s) which could also be helpful in understanding the differential gene expressions during in vitro morphogenesis.

In addition to successful application of in vitro micropropagation technique in many fruits, aromatic and ornamental plants, it is now being used to multiply newly released varieties of sugarcane also. However, some constraints and limitations associated with micropropagation of sugarcane, such as high production cost, somaclonal variation etc need to be reduced. In the field of agricultural biotechnology, tissue culture technique is a new emerging tool for rapid multiplication of newly developed sugarcane varieties and crop improvement through selection of somaclonal variants. Although several herbaceous crops have been extensively studied, the members of Poaceae family including sugarcane have not received much attention.

**OBJECTIVES OF THE STUDY**

Keeping the above points in view, the present study has been proposed to be carried out with the following objectives:
1. To develop an improved protocol for rapid multiplication of sugarcane through *in vitro* micropropagation technique for seed production.

2. Studies on acclimatization and survival of micropropagated plants under greenhouse conditions.

3. Evaluation of micropropagated plantlets for clonal fidelity through molecular techniques.