CHAPTER TWO

REVIEW OF LITERATURE

The following review of literature includes Sugarcane (Saccharum officinarum L.) importance, tissue culture, somatic embryogenesis, salinity tolerance and finally molecular markers (RAPD-PCR).

2.1. Background and Importance of Sugarcane

Sugarcane is a perennial, tropical or subtropical crop grown worldwide, within 30° of the equator, for its high sucrose accumulation (**Ming *et al.* 2006**). Commercially, the crop is established by means of seed cane and ratoons, when the bud and root primordia of the stool left after harvesting produce stubble (**James, 2004**).

Sugarcane grows well in medium to heavy, slightly alkaline, soils with good drainage, high organic matter (**Anonymous, 2003**) and an annual water supply of 1200-1500 mm^3 (**Tarimo and Takamura, 1998).**

Sugarcane has one of the most efficient photosynthetic mechanisms, capable of fixing 2-3 % radiant solar energy and achieves a high CO_2 coefficient (**Almazan *et al.* 1998**).

Sugarcane belongs to the genus *Saccharum* L., a part of the *Andropogoneae* tribe of the family *Poaceae* (grasses) (**Selman-Housein *et al.* 2000**). Among the recognized species are *S. officinarum, S. spontaneum, S. sinense, S. edule, S. barberi and S. robustum* (**Tarimo and Takamura, 1998**).

According to **Grivet *et al.* (2004),** sugarcane genetic resources can be divided into three groups: (i) Traditional cultivars: noble cultivars which have brightly coloured stalks and are rich in sugar e.g. *S. officinarum* L. and the North Indian and Chinese cultivars which have thinner stalks, flatter colours and lower sugar content, e.g. *S. barberi;* (ii) Wild relatives: related to the traditional cultivars, grouped into the '*Saccharum* complex', have little or no sugar and have diverse morphological and ecological adaptations, e.g. *S. spontaneum* L.; (iii) Modern cultivars: created by Dutch breeders in Java in the early 1900s (**Burnquist, 2001**); hybrids of traditional cultivars and *S. spontaneum* L. and replaced the traditional cultivars during the 20th century.

The modern sugarcane cultivars are highly polyploid and aneuploid, originating from crosses between *S. officinarum* L. (2n = 80) and *S. spontaneum* L. (2n = 40:128) and backcrossing the interspecific hybrids with

the *S. officinarum* L. parent (**Stevenson, 1965; Sreenivasan *et al.* 1987; Butterfield *et al.* 2001; Lakshmanan *et al.* 2005; Ming *et al.* 2006; Singh *et al.* 2010**). In some of these cultivars, 10% of the chromosomes are inherited entirely from *S. spontaneum*, 80 % entirely from *S. officinarum* and 10 % results from recombination of chromosomes from the two ancestral species (**D'Hont *et al.* 2008).**

Approximately 75% of the world's sugar is obtained from sugarcane and 25 % from sugar beet (*Beta vulgaris* L.) (**Ming *et al.* 2006**). Although over 100 countries cultivate sugarcane, the bulk of the world's production is produced by a few countries, including South Africa (SA) (**Fischer *et al.* 2009**).

All over the world, the increase of sugar production through the genetic improvement of the two most important sugar crops, i.e. sugar cane (*Saccharum officinarum* L.) and sugar beet (*Beta vulgaris* L.), is an ongoing process, since more than 2000 years. Sugarcane is cultivated mainly in Upper Egypt where it is well adapted to the extreme summer heat **(Thomas, 2000).**

Total sugarcane area has been stabled around 115,000 Hectares (HA) for the last three years and is expected to remain almost unchanged for the next few years. About 2000 feddan (840 HA) of new reclaimed dessert land in Aswan governorate have been cultivated with sugarcane in May 1999 **(Thomas, 2000).**

In Egypt sugar cane is considered among the most important industrial converting crops. It is the main source of sugar industry and the sole source for molasses industry. Sugar cane is cultivated in about 270 thousand feddans producing about 1,100,000 tons of sugar annually. In Egypt, although sugar beet crop is a supplement for sugar industry, its byproducts are used for the production of untraditional animal feed, as well as many other secondary industries. It is cultivated in about 370 thousand feddans in the North of the Delta, producing about 910,000 tons of sugar annually **(El-Sheikh, 2013).**

The main byproducts of sugar production are molasses, used as stock feed and in the production of ethanol (**Zuurbier and Van de Vooren, 2008**), and bagasse. The fibrous residue left after the juices are extracted from the cane, are used as fuel and in the production of cardboard, fiber board, furfural and wall board (**Almazan *et al.* 1998). Mohan *et al.* (2005**) also used bagasse as an alternative to agar in apple micropropagation

2.2. Sugarcane and Tissue Culture

Singh, (2003) successful in obtaining plant regeneration from tissue culture of sugarcane and applied to breeding programs for rapid screening of clones for disease resistance, salt tolerance, drought tolerance, herbicide resistance and early maturity and high sugar. The author reported that a newly identified variety with desirable character, e.g pest/disease resistant, high sugar content, stress resistant can be propagated through tissue culture and made available to the farmer for commercial benefit that is otherwise not possible through conventional means that take appox 8-10 years.

Ammirato, (1987) reported that, somatic embryogenesis is considered an efficient and high volume propagation system for the production of large number of plantlets in fewer steps, with a concomitant reduction in lab our, time and cost.

Blanco *et al.* (1997) reported that, plant regeneration from cultured immature inflorescence segments of sugarcane (*Saccharum sp.*) was obtained via somatic embryogenesis. The results obtained that, regeneration on MS medium supplemented with 3mg L^{-1} NAA.

Arencibia *et al.* (2000) reviewed that tissue culture techniques are widely used in sugarcane improvement programs. Somatic embryogenesis in cell and callus cultures has also become the choice for high volume propagation systems would be desirable in multiplying new sugarcane varieties. Production of transgenic plants through any transformation method (Physical, chemical or biological) requires highly efficient and reliable plant regeneration systems.

Somatic embryos offer a great potentiality in crop improvement by cell selection, genetic transformation, somatic hybrid and polyploid plant production (**Marcano *et al.* 2000**). In recent years somatic embryos are used for developing synthetic seeds, shortening the breeding cycle and transformation. Induction of somatic embryogenesis from young leaves of sugarcane.

Embryos were cultured on MS medium containing 0.015 g/l AC, 452 µM 2, 4-D and 2iP by **Gallo-Meagher and Green, (2002)**. These results indicated that, clusters of SEs developed from all immature zygotic embryos 5 weeks after culture initiation. After 12 weeks, explants were transferred to the same medium with the amount of 2, 4-D reduced to 90.4 µM which resulted in SE proliferation. The authors had noted that without AC in the medium, oxidation was high with all zygotic embryos becoming brown and failing to respond.

When AC present in the medium no oxidation or phenolic accumulation was observed and the embryos swelled to approximately four times their original size in 2 weeks. After 5 weeks, all explants began to develop translucent clusters of SEs. Probably AC may absorb PGRs present in the medium and hence high concentration of PGRs was used for induction.

Lal, (2003) reported that, sub-apical slices and leaf-roll explants of sugarcane *(Saccharum* hybrids) cv. B.O. 91 formed callus on Murashige and Skoog's (MS) basal medium supplemented with 5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D). Callus was maintained on 3 mg/l 2,4-D augmented medium, Concentrations of 2,4-D resulted in differences in growth rate and morphogenetic patterns. Rapid regeneration via somatic embryogenesis was obtained on 1 mg/I kinetin. 0.5-2.0 cm tall shoots were obtained within 4 weeks on Kin containing medium, however, the root growth remained suppressed. The resulting shoots were rooted on 1/2 strength basal medium (sucrose @ 20 g/l) supplemented with 0.2 mg/I c~-naphthalene acetic acid (NAA). Within 5 weeks healthy plantlets (12.2 + 1.8 cm) bearing 14.3 _+ 7.3 roots were obtained on rooting medium. These were successfully established in soil following a 3 week hardening phase. The procedure holds promise for genetic manipulation via cellular/molecular breeding in this cultivar.

The plant tissue culture allows the control of stress homogeneity and the characterization of cell behavior under stress conditions, independently of regulatory systems that take place at the whole plant level (**Lutts *et al.* 2004)**.

Nine sugarcane genotypes (CP59-73, CP63-588, CP80-314, SP71-1081, F160, L62-96, CP70-321, CP57- 614 and Clone III) were evaluated for their callus induction capacity, embryogenic callus production and plant regeneration ability by **Gandonou *et al.* (2005)**. Leaf cylinders were used as explants using Murashige and Skoog (MS) based medium supplemented with 3 mgl^{-1} 2,4-dichlorophenoxy acetic acid. Plant regeneration was accomplished on hormone free modified MS medium supplemented with casein hydrolyzate. These results showed that, the genotypes tested showed high callus induction percentage (69 to 95%) and high embryogenic callus percentage (60 to 100%). These genotypes also showed excellent regeneration capacities, with regeneration percentages ranged between 88 and 100%. Significant differences were observed between genotypes for callus induction capacity, embryogenic response and plant regeneration ability indicating that these criteria are genotype dependent. Plant regeneration ability is highly correlated with

embryogenic callus production. The *in vitro* regenerated plants were successfully rooted and well acclimatized in growth cabinet conditions.

Ahmad *et al.* (2007) provided that, the *in vitro* culture technique is a controlled and uniform environment for studying physiological and biological processes in plants of particularly salt induced ionic and osmotic stresses at cellular level.

Badawy *et al.* (2008) present research work has been carried out to study the response of three genotypes of sugarcane (*Saccharum* species hybrids) for callus induction, embryogenic callus production and their *in vitro* salt tolerance. For callus induction and embryogenic callus production, leaf base segments were subjected to *in vitro* culture on Murashige and Skoog (MS) medium supplemented with 3 mg l^{-1} 2,4 Dichlorophenoxyacetic acid for 4 weeks. To evaluate salt tolerance of the varieties, cultured calli were exposed after two subsequent subcultures to different concentrations of NaCl (0, 17, 34, 68 and 102 mM) added to the culture medium for 4 weeks. Comparison of genotypes was based on callus induction percentage, embryogenic callus production percentage and relative fresh weight growth. For salt tolerance, necrosis percentage and relative fresh weight growth of callus were used. The three genotypes responded well to callus induction. The results showed that, the high per cent of embryogenic callus obtained for the three varieties indicated that these genotypes have a high capacity for embryogenic callus production. The effects of NaCl resulted in calli necrosis and a reduction of their growth. Growing calli derived from genotypes GT 54-9 and NCo310 showed less necrosis percentage and less relative fresh weight growth reduction under salt stress. They appeared to be more salt tolerant *in vitro* than Co 413.The finding of superior genotypes GT 54-9 and NCo 310 and inferior one Co 413 for salt tolerance together with their high potential for embryogenic callus induction may be a model varieties to study physiological mechanisms associated with *in vitro* salt tolerance and *in vitro* selection for salt tolerance in sugarcane.

Asad *et al.* (2009) developed an efficient regeneration protocol from sugarcane callus through inclusion of amino acids in regeneration medium. Sugarcane (*Saccharum officinarum* L.) SP-241 callus was induced from meristematic explants cultured on Murashige and Skoog medium supplemented with B5 vitamins containing 13.6 µM 2-4, dichlorophenoxy acetic acid, 0.05% (w/v) casein hydrolysate, 10% (v/v) coconut water and 3% glucose. Five levels (0.1, 0.25, 0.5, 0.75, 1.0 mM) of five different amino acids (glutamine,

asparagine, glycine, cysteine and arginine) were tested on the same medium containing 6.8 µM 2,4 -D to compare their ability to induce somatic embryogenesis and shoot regeneration from six week old callus. Among the tested amino acids, glycine (0.75 mM), arginine (0.5 mM) and cysteine (0.25 mM) showed significant effect on smatic embryogeensis (94%) and shoot production as compared to non- amino acid medium. Of the evaluated amino acids, glycine was most effective to promote somatic embryogenesis and maximum shoot regeneration. Except low callus growth index (G.I) amino acid treatment resulted in high percentages of somatic embryogenesis and shoot regeneration as compared to non-amino acid medium. Regenerated shoots when transferred to same medium supplemented with 19.7 µM IBA, grew normal and developed roots. These results indicate the efficacy of amino acids in stimulating sugarcane plant regeneration from non-embryogenic callus, and may be suitable for future use in genetic transformation studies to enhance regeneration of transgenic sugarcane plants.

Sani and Mustapha, (2010) investigated the effect of genotype and 2, 4-D on callogenesis in sugarcane (*Saccharum spp.*) hybrid cultivars; SP726180, B47419, M1176/77 and M2119/88. To evaluate the response of the genotypes to callus induction and embryogenic callus production, leaf base explants were cultured on Murashige and Skoog (MS) basal medium supplemented with 2.5, 3.0, 3.5, or 4.0mg/L) 2,4-D for 8wks. While, the number of responding explants was found to generally increase with the increase in 2,4-D concentration from 2.5 to 4.0mg/L, embryogenic callus production was highest on media supplemented with 3.0 and 2.5mg/L. the results clearly indicated that, there were no significant differences in the number of responding explants and embryogenic callus production among B47419, M1176/77 and M2119/88, these genotypes however exhibited a significantly higher response when compared to SP726180 indicating their high propensity to *in vitro* callogenesis.

Roy *et al.* (2011) reported that, leaf sheath explants of an indigenous variety Isd-16 of sugarcane (*Saccharum officinarum* L.) produced light yellow friable callus after culturing on to MS with 2,4-D (2 - 4 mg/l) and NAA (3 - 5 mg/l) singly. Callus formation was the maximum on MS + 3 mg/l 2,4-D. Callus underwent embryogenesis producing huge number of somatic embryos when sub cultured on MS with 15 - 30 mg/l L-proline, 3 mg/l 2,4-D + 5 - 10% coconut water (v/v) and 3 mg/l 2,4-D + 10% CW (v/v) + 300 - 500 mg/l CH. L-proline significantly enhanced somatic embryo- genesis and 25 mg/l L-proline in MS was the best culture medium formulation. Most of the somatic

embryos germinated and developed plantlets after 1 - 2 weeks of incubation in proline-supplimented medium. On the other hand, maturation and germination of embryos were achieved on half-strength MS with or without 0.25 - 1.0 mg/l L-proline, and 5% coconut water (v/v). Somatic embryos derived plantlets were then successfully transferred to natural condition through successive phages of acclimation.

Alcantara *et al.* (2014) regenerated plants from somatic embryos and analyze the origin and division pattern of cells during the different embryonic developmental stages of sugarcane cultivars RB855156 and RB72454. For both cultivars, the best results for embryogenic callus induction were obtained with explain cultures on culture medium containing 13.5 μM 2.4-dichlorophenoxyacetic acid (2,4-D) followed by callus culture on solid medium with 4.5 μM 2,4-D. A higher rate of shoot induction was observed in RB855156 with die addition of 8.9 and 17.8 μM benzylaminopurine (BAP); this higher rate was also observed in RB72454 with the addition of 17.8 μM BAP. Murashige and Skoog (1962) (MS) medium containing 2.5 and 5.0 μM indolebutyric acid (IBA) for RB855156 and RB72454, respectively, was suitable for rooting. The plantlets were successfully acclimatized and the plant survival rate was 100% for both cultivars. Histological analysis revealed that the shoots in both cultivars originated from somatic embryogenesis.

2.3. Drought Tolerance in Sugarcane

Tissue culture system is useful for the evaluation of tolerance to environmental stresses because the stress conditions can be easily controlled *in vitro* (**Tal, 1983).** Moreover, *in vitro* culture provides a uniform population of synchronously developing plant cells without involving regulatory mechanisms that naturally repaired at the whole plant level.

Dutta Gupta *et al.* (1995) observed moderate increase of Na^+ and Cl^- within callus tissue might avoid water loss and ensure an economic way to adjust osmotically . However, when the ability of the cells to compartmentalize the ions into the vacuole is exceeded, ions build up in the cytoplasm and lead to severe ion imbalances and to conformational changes in the plasma membrane electrical potential.

Yeo, (1998) reported that, salt and drought stresses are expressed by a series of morphological, physiological, metabolic and molecular changes that adversely affect the plant growth and productivity. Plants have evolved complex mechanisms for adaptation to osmotic and ionic stresses caused by high salt. These mechanisms include osmotic adjustment through an accumulation of compatible solutes, such as glycine betaine and proline and reduction of toxic concentrations of ions in the cytoplasm by restricting Na^+ influx, or its sequestration into the vacuole and/or extrusion . The contributory role of glycine betaine to osmotic adjustment has been unequivocal.

Watanabe *et al.* (2000) reported that, salt treatment reduced growth rate significantly as compared to the control. Also, mannitol induced stress decreased considerably both RGR and WC values. The results showed that the highest RGR and WC decrease were noticed under mannitol-induced osmotic stress, which indicated that RGR inhibition is due to the reduction of water availability and the loss of turgor.

Basu *et al.* (2002) mentioned that, under induced stress, the increase in Na^+ concentration and the subsequent decrease in K^+ concentration. This could be due to the fact that some species were able to substitute K^+ by Na^+ to ensure the osmotic adjustment.

Zhu, (2002) studied different abiotic stress factors such as osmotic stress, oxidative stress and protein denaturation in plants, which lead to similar cellular adaptive responses such as accumulation of compatible solutes, induction of stress proteins, and acceleration of reactive oxygen species scavenging systems.

This is in part due to the complexity of interactions between stress factors and various molecular, biochemical and physiological phenomena affecting plant growth and development.

Sairam and Tygai, (2004) reported that, proline is a stress resistance marker has been widely adopted. As well, they detected that, proline can serve as an organic nitrogen reserve ready to be used after stress relief to sustain both amino acid and protein synthesis**.** Generally, it can be seen that the increase in iso-osmotic stress parallel with the accumulation of proline.

Wahid, (2004) summarized that Sugarcane (*Saccharum* species hybrid) is one of the important agro-industrial sugar crops of the world, cultivated in more than 20 million hectares, a little about 2 % of the total cropped area. With increased salinization of soil and water, abiotic stress is assuming as a major limiting factor for plant growth and will soon become even more severe as desertification covers more and more of the world's terrestrial area. Sugarcane being a glycophyte, high levels of salt in the soil affects growth rate, cane yield as well as the sucrose content in cane.

Gandonou *et al.* (2005) reported that, Sugarcane (*Saccharum sp.*) the source of 65% of sugar production in the world. Unfortunately, the production of this crop is restrained by several diseases and abiotic stresses such as salinity, drought and freezing. The improvement of sugarcane plant resistance to these stresses is of great importance. *In vitro* selection of favorable somaclonal variant strains from callus culture is a supplementary tool to traditional breeding for production of stress-resistant plants. The introduction of a given genotype in *in vitro* selection program depends on its aptitude to *in vitro* culture, essentially to embryogenic callus induction and plant regeneration

Errabii *et al.* (2006) obtained Calli from two sugarcane cultivars (R570 and CP59-73) were exposed to different osmotic stress intensities followed by a period of stress relief. Relative rate growth, callus water content and changes in organic and inorganic solutes were determined at the end of stress and relief periods. After the stress period, calli derived from both cultivars showed a decrease in RGR, but at lesser extent in R570 than CP59-73 cultivar. Same tendency was recorded in the callus water content under mannitol- induced osmotic stress. The inorganic solutes seemed to have no contribution in the osmotic adjustment in mannitol-stressed calli since K^+ and Ca_2^+ concentrations decreased drastically while Na+ and Mg_2^+ concentrations were not affected. The accumulation of proline occurred in both cultivars and was

more marked in CP59-73 than R570 cultivar. At the end of the relief period, we observed that all the considered parameters have recovered completely to reach the control levels. According to these results, we conclude that the drought stress-induced changes are reversible, at the least at the cellular level, in sugarcane cultivars.

Ashraf and Foolad, (2007) showed that Glycine betaine (GB) and proline are two major organic osmolytes that accumulate in a variety of plant species in response to environmental stresses such as drought, salinity, extreme temperatures, UV radiation and heavy metals. Although their actual roles in plant osmotolerance remain controversial, both compounds are thought to have positive effects on enzyme and membrane integrity along with adaptive roles in mediating osmotic adjustment in plants grown under stress conditions. While many studies have indicated a positive relationship between accumulation of GB and proline and plant stress tolerance some have argued that the increase in their concentrations under stress is a product of, and not an adaptive response to stress. In this article, review and discuss the evidence supporting each of these arguments. As not all plant species are capable of natural production or accumulation of these compounds in response to stress, extensive research has been conducted examining various approaches to introduce them into plants.

Errabii *et al.* (2007) investigated the effects of NaCl and mannitol iso-osmotic stresses on calli issued from sugarcane cultivars (cvs.) R570, CP59-73 and NCo310 in relation to callus growth, water content, ion and proline concentrations. The results showed that, callus growth and water content decreased under both stresses with the highest reduction under mannitol-induced osmotic stress. The ion concentration was drastically affected after exposure to NaCl and mannitol. Salt stress induced an increase in Na^+ and Cl^- accumulation and a decrease in K^+ and Ca_2^+ concentrations. Under mannitol-induced osmotic stress, K^+ and Ca_2^+ concentrations decreased significantly while Na^+ and Cl^- concentrations remained unchanged. Free proline accumulation occurred under both stresses and was more marked in stress-sensitive cv. than in stress-resistant one. Our results indicated that the physiological mechanisms operating at the plant cell level in response to salt and osmotic induced stress in sugarcane cvs. are different. Among the cvs., we concluded that the stress resistance is closely related to the maintain of an adequate water status and a high level of K^+ and Ca_2^+ under both stresses and a low level of Na^+ concentration in the presence of NaCl. Thus, sugarcane (*Saccharum* sp.) can be regarded as a Na^+ excluder. We also provided evidence

that proline accumulation is a stress-sensitive trait rather than a stress resistance marker.

Salama *et al.* (2007) studied the responses to osmotic stress in *Sesuvium portulacastrum*, a halophyte potentially usable for saline soil stabilization and covering. Cuttings were multiplicities and cultivated in nutrient solution supplemented with 100 mM NaCl. They were exposed for 12 days to osmotic stress induced by either mannitol or polyethylene glycol (PEG 6000). Growth, tissue water content, relative water content, and contents in inorganic (Na^+, K^+) and organic (proline, soluble sugars) solutes were determined at regular intervals. Both PEG and mannitol reduced growth, decreased leaf number and leaf mean surface area, and led to a significant reduction of leaf water and K^+ contents. However, these effects were significantly less severe in plants submitted to mannitol, as compared to PEG treated ones. The contents in soluble sugars contents and in Na^+ remained unchanged, while that of proline strongly increased, particularly in mannitol-stressed plants. A positive relationship was observed between growth, relative water content, potassium content, and proline accumulation in leaves. However, proline remained a minor component in the pool of solutes.

Nieves *et al.* (2008) reported that, somatic embryogenesis is known as an important pathway for plant regeneration and a useful model system for the investigation of molecular, biochemical and morphological events that take place during the early development of plants.

Badawy *et al.* (2008) reported that, Callus RFWG decreased as the concentration of NaCl increased in the culture medium. This decrease was in cultivar Co 413 in comparison with the two other genotypes. For example, at 17 mM NaCl (the lowest NaCl concentration used), RFWG were about 1.219, 0.966 and 0.578 respectively for NCo 310, GT 54-9 and Co 413, corresponding to 95%, 90% and 63%, respectively, to that of the control. At highest NaCl concentration used (102 mM), callus RFWG for NCo310, G.T.54-9 and Co413 were 0.715, 0.64 and 0.334, which correspond to 56%, 59% and 37% of control, respectively. Analysis of variance for RFWG showed significant differences ($p < 0.001$) among the different NaCl concentrations and among genotypes.

In vitro **Patade *et al.* (2008)** studied the responses of embryogenic sugarcane (*Saccharum officinarum* L.; cv. CoC-671) calli stressed with different levels of NaCl (0.0, 42.8, 85.6, 128.3, 171.1, 213.9 or 256.7 mM). The results showed that a significant decrease in callus growth and cell viability occurred

with C85.6 mM NaCl. Higher amounts of free proline and glycine betaine were accumulated in NaCl stressed calli. Although the leached and retained Na^+ contents increased, the retained K^+ content decreased with increasing levels of NaCl. Such a mechanism implies that sugarcane can be considered as a Na^+ excluder. The accumulation of salt ions and osmolytes could play an important role in osmotic adjustment in sugarcane cells under salt stress.

Suriyan Chaum and Kirdmanee, (2009) investigate the biochemical, physiological and morphological responses of sugarcane to iso-osmotic salt and water-deficit stress. Disease-free sugarcane plantlets derived from meristem cuttings were photo autotrophically grown in MS media and subsequently exposed to -0.23 (control), -0.67 or -1.20 MPa iso-osmotic NaCl (salt stress) or mannitol (water-deficit stress). Maximum quantum yield of PSII (Fv/Fm), stomatal conductance and transpiration rate in the stressed plantlets were significantly reduced when compared to those of plantlets of the control group (without mannitol or NaCl). In addition, physiological changes and growth parameters of plantlets in the salt stress conditions were more sharply reduced than those in water deficit stress conditions. On the other hand, the proline content and non-photochemical quenching (NPQ) in the leaves of stressed plantlets increased significantly, especially in response to iso-osmotic salt stress. The multivariate biochemical, physiological and growth parameters in the present study should be further used to develop salt, or drought, tolerance indices in sugarcane breeding programs.

Begum *et al.* (2011) reported drought continues to be a major setback for the production of crop plants. Drought is an environmental stress which causes important agricultural losses in arid and semiarid areas. Sugarcane requires 10–12 months from planting to harvesting and faces severe drought and other factors. Drought is one of the principal environmental stress which constraint sugarcane production.

Haq *et al.* (2011) reported that, after somatic embryo induction, calli were sub-cultured on plant regeneration medium. Their results indicated that the maximum plant regeneration was observed in control plant regeneration cultures of each cultivar. So regeneration efficiency was decreased with the increase in NaCl stress.

Mantri *et al.* (2011) showed that salt stress affects plants by causing ion imbalance and hyper-osmotic stress, which often led to oxidative damage. It is now well documented that, reactive oxygen species (ROS) production is

increased under saline conditions and their higher concentrations in the absence of any protective mechanism seriously disrupt normal metabolism of plants through oxidation of membrane lipids, proteins and nucleic acids.

Rai *et al.* (2011) reviewed that biotic and abiotic stresses impose a major threat to agriculture. Therefore, the efforts to develop stress- tolerant plants are of immense importance to increase crop productivity. In recent years, tissue culture based *in vitro* selection has emerged as a feasible and cost effective tool for developing stress-tolerant plants. Plants tolerant to both the biotic and the abiotic stresses can be acquired by applying the selecting agents such as NaCl (for salt tolerance), PEG or mannitol (for drought tolerance) and pathogen culture filtrate, phytotoxin or pathogen itself (for disease resistance) in the culture media. Only the explants capable of sustaining such environments survive in the long run and are selected. *In vitro* selection is based on the induction of genetic variation among cells, tissues and/or organs in cultured and regenerated plants. The selection of somaclonal variations appearing in the regenerated plants may be genetically stable and useful in crop improvement. This review focuses on the progress made towards the development of stress-tolerant lines through tissue culture based in vitro selection. Plants have evolved many biochemical and molecular mechanisms to survive under stress conditions.

Rasheed *et al.* (2011) reported that high temperature strongly hampers the plant growth particularly at early growth stages. In this study, changes in some physiological and anatomical characteristics and possibility of mitigating the adversities of heat stress by soaking sugarcane nodal buds in 20 mM proline and glycinebetaine (GB) solutions have been explored. The results indicated that, heat stress reduced the rate of bud sprouting nonetheless soaking the setts in proline followed by GB was beneficial. In addition, heat stress reduced the bud fresh and dry weights, generated H_2O_2, reduced the tissue levels of K^+ and Ca^+, while increased the osmolytes synthesis in a time course manner. Heat stress also delayed the emergence and expansion of new bud leaves, by restricting the number and area of mesophyll cells. It also caused poor and aberrant development and diffused appearance of mesophyll cells and vascular bundles in the bud leaves. However, soaking of buds in proline and GB solutions substantially reduced the H_2O_2 production, improved the accumulation of soluble sugars and protected the developing tissues from heat stress effects; although proline was more effective than GB. Correlations of various attributes indicated that soaking in GB and proline restricted the

H_2O_2 generation, improved K^+ and Ca_2^+ contents, and increased the concentrations of free proline, GB and soluble sugars eventually improving the heat tolerance of buds. Cost benefit analysis showed that, considering increase in sprouting of buds, soaking in 20 mM solution of both osmoprotectants is economical.

Basnayake *et al.* (2012) reported that development of genotypes tolerant to drought stress is one of the main objectives of sugarcane research programs in Brazil; however, achievement of such goal is hampered by the highploidy level of modern sugarcane varieties and by the fact that drought tolerance is mutagenic and is a quantitative trait .

Karpe *et al.* (2012) Assessed salt stress responses of sugarcane (*Saccharum officinarum* L.) var. CoC 671 and Co 86032 using *in vitro* plantlets by subjecting them to increasing concentrations of NaCl (0, 50, 100, 150, 200 and 250 mM) and checking relative growth rate (RGR), membrane damage rate (MDR), soluble proteins, osmolytes (proline, glycine betaine), ions (Na^+ and K^+) and activity antioxidant enzymes (peroxidase, ascorbate peroxidase, guaiacol peroxidase, catalase and superoxide dismutase). As the concentration of NaCl increased, the RGR was found to decrease by 42.1 and 77.7%, the MDA level increased by 32.5 and 55.8% and proline increase of about 43 and 189% was seen in CoC 671 and Co 86032 respectively. CoC 671 was adapted to a higher Na^+ concentration (150 mM) than Co 86032. As for the K^+ accumulation, it displayed similar patterns as in Na^+ accumulation. In general, it was observed that in all cases except catalase, CoC 671 displayed higher tolerance to NaCl (up to 150 mM) than Co 86032 (up to 100 mM). Based on the results, it is suggested that CoC 671 displayed NaCl tolerance up to about 150 mM, while that of Co 86032 was around 100 mM. The study also indicates that *in vitro* plantlets can be used for screening salt tolerance in sugarcane.

Patade *et al.* (2012) cultured sugarcane (*Saccharum officinarum* L. cv. Co 86032) calli were cultured on media containing NaCl or polyethylene glycol (PEG) 8000 that exerted the same osmotic pressure (-0.7 MPa) in order to discriminate between the ionic and osmotic components of salt stress. PEG stress exposure for 15 days led to significant growth reduction and loss in water content than salt stressed and control tissues. Osmotic adjustment (OA) was observed in callus tissues grown on salt, but was not evident in callus grown on PEG. Oxidative damage to membranes, estimated in terms of accumulation of thiobarbituric acid reactive substances TBARS and electrolytic leakage was

significantly higher in both the stressed calli than the control however, the extent of damage was more in the PEG stressed calli. The stressed callus tissues showed inhibition of ascorbate peroxidase activity, while catalase activity was increased. These results indicate sensitivity of cells to PEG mediated stress than salt stress and differences in their OA to these two stress conditions. The sensitivity to the osmotic stress indicates that expression of the stress tolerance response requires the coordinated action of different tissues in a plant and hence was not expressed at the cellular level.

Roa and FTZ, (2013) developed callus lines in sugarcane. Results showed that, high molecular weight PEG was used as selective agent. Selected callus line grew better than non-selected callus when grown on different concentrations of PEG. The activity of antioxidant enzymes like CAT, POX, APX and SOD were high in selected callus than in non-selected callus. Osmolytes like proline and ascorbic acid were at higher levels in selected callus than in nonselected callus, however at higher concentrations (20–30 %) of PEG, levels of proline and ascorbic acid decreased. The frequency of organogenesis and number of plantlets decreased in selected callus than in non-selected callus. The results can be used for *in vitro* screening and manipulations of sugarcane for improvement of drought tolerance.

Patade *et al.* (2014) reported that, antioxidant defense system provides protection against oxidative damage caused by abiotic stresses including salinity. Ameliorative effects of L-proline, L-glutamine, glycine betaine (GB) on growth, proline accumulation and antioxidant enzyme activities were studied using cultured cells of sugarcane against salt (NaCl) stress. NaCl stress reduced growth rate significantly over the control however, proline or glutamine supplementation resulted in growth revival. Proline supplementation to media with or without salt increased accumulation of free proline significantly than the controls and other (proline, GB and glutamine) treatments. Salt stress led to increase in superoxide dismutase (SOD) and glutathione reductase activity whereas guaiacol peroxidase (GPX), catalase and ascorbate peroxidase activities were significantly suppressed. Proline supplementation to the salt medium improved the GPX activity over the salt media supplemented with glutamine or glycine betaine. The activity ratio between SOD and H_2O_2 scavenging enzyme activities, which is considered as a working hypothesis for biochemical marker for salt tolerance, was lower in salt medium supplemented with proline. Thus, the higher growth rate and the lower activity ratio suggest maximum salt stress

ameliorative potential of proline in sugarcane cultured cells. System for *in vitro* selection of drought tolerant

2.4. Molecular Studies

Genetic markers represent genetic differences between individual organisms or species. Generally, they do not represent the target genes themselves but act as 'signs' or 'flags'. Genetic markers that are located in close proximity to genes (i.e. tightly linked) may be referred to as gene 'tags'. Such markers themselves do not affect the phenotype of the trait of interest because they are located only near or 'linked' to genes controlling the trait. All genetic markers occupy specific genomic positions within chromosomes (like genes) called 'loci' (singular 'locus'). There are three major types of genetic markers: (1) morphological (also 'classical' or 'visible') markers which themselves are phenotypic traits or characters; (2) biochemical markers, which include allelic variants of enzymes called isozymes; and (3) DNA (or molecular) markers, which reveal sites of variation in DNA (**Jones *et al.* 1997; Winter and Kahl, 1995**).

Are usually visually characterized phenotypic characters such as flower color, seed shape, growth habits or pigmentation (**Sumarani *et al.* 2004**). Isozyme markers are differences in enzymes that are detected by electrophoresis and specific staining. The major disadvantages of morphological and biochemical markers are that they may be limited in number and are influenced by environmental factors or the developmental stage of the plant **(Winter and Kahl, 1995)**.

However, despite these limitations, morphological and biochemical markers have been extremely useful to 171 plant breeders (**Eagles *et al.* 2001; Weeden *et al.* 1994**).

Paterson, (1996) reported that, DNA markers are the most widely used type of marker predominantly due to their abundance. They arise from different classes of DNA mutations such as substitution mutations (point mutations), rearrangements (insertions or deletions) or errors in replication of tandemly repeated DNA, These markers are selectively neutral because they are usually located in non-coding regions of DNA.

DNA markers are unlimited in number and are not affected by environmental factors and developmental stages of the plant (**Winter and Kahl, 1995**). Apart from the use of DNA markers in the construction of linkage maps, they have numerous applications in plant breeding such as assessing the level of genetic diversity within germplasm and cultivar identity (**Baird *et al.*, 1997;**

Henry, 1997; Jahufer *et al.* 2002; Weising *et al.*, 1995; Winter and Kahl, 1995).

Other researchers such as **Gupta *et al.* 1999, Jones *et al.* 1997; Joshi *et al.* 1999; Winter and Kahl, 1995**, reported that, DNA markers may be broadly divided into three classes based on the method of their detection: (1) hybridization-based; (2) polymerase chain reaction (PCR) based and (3) DNA sequence based.

Essentially, DNA markers may reveal genetic differences that can be visualized by using a technique called gel electrophoresis and staining with ethidium bromide or silver nitrate or detection with radioactive or colorimetric probes. They reveal differences between individuals of the same or different species. These markers are called polymorphic markers, whereas markers that do not discriminate between genotypes are called monomorphic markers. DNA markers are widely accepted as potentially valuable tools for crop improvement in rice **(Mackill *et al.*, 1999; McCouch and Doerge, 1995)**, Wheat **(Eagles *et al.* 2001; Koebner and Summers, 2003; Van Sanford *et al.*, 2001)**, maize **(Stuber *et al.* 1999; Tuberosa *et al.* 2003)**, barley **(Thomas, 2003; Williams, 2003)**, oilseeds **(Snowdon and Friedt, 2004)**, horticultural crop species **(Baird *et al.* 1996, 1997)** and pasture species **(Jahufer *et al.* 2002).**

Many studies suggested that DNA markers will play a vital role in enhancing global food production by improving the efficiency of conventional plant breeding programs (**Kasha, 1999; Ortiz, 1998**). Although there has been some concern that the outcome of DNA marker technology as proposed by initial studies may not be as effective as first thought, many plant breeding institutions have adopted the capacity for marker development **(Eagles *et al.* 2001; Kelly and Miklas, 1998; Lee, 1995)**.

Analysis of the changes that occur at the DNA level resulting from culture-induced somaclonal variation and mutagenic treatments are important to understand the resulting variation **(Hoezel and Green, 1998; Rasheed *et al.* 2005)**. Evaluation of variation based on visible traits is not reliable as they are dependent on the environment and age of plants. Molecular markers (DNA and protein based) are more reliable as they identify internal changes that have a genetic origin **(Kunert *et al.* 2003)**.

Afiah *et al.* (2007) mentioned that, RAPD results in amplification of few random segments of DNA, allowing for variation in length and number of amplified segments when the sequence of the segments is altered. RAPDs have

been used widely for analysis of genetic variation. Also, recommended the use of RAPDs for analysis of somaclonal variation.

DNA marker systems used in analysis of such variation include Amplified Fragment Length Polymorphism (AFLP) **(Chuang *et al.* 2009),** Restriction Fragment Length Polymorphism (RFLP) **(Patzak, 2003)** and Random Amplified Polymorphic DNA (RAPD) **(Rasheed *et al.* 2005).**

RAPD is a simple and time efficient technique compared to RFLP **(Garcia *et al.* 2004)**. It results in amplification of few random segments of DNA, allowing for variation in length and number of amplified segments when the sequence of the segments is altered **(Hoezel and Green, 1998)**. RAPDs have been used widely for analysis of genetic variation **(Afiah *et al.*, 2007; Ehsanpour *et al.* 2007; Ngezahayo *et al.* 2007; Cuesta *et al.* 2010)** recommended the use of RAPDs for analysis of somaclonal variation

Bae *el al.* (2010) reported that, Osmotic stress induces changes in the expression of various genes including those associated with drought tolerance, cell wall metabolism and defense. They isolated 852 cDNA clones, the expression of which is induced by osmotic stress, from cells of a hybrid poplar (Populus alba Populus tremula var. glandulosa) by suppression subtractive hybridization after mannitol treatment. they examined how stress affected their expression using cDNA microarray analysis, which identified 104 genes significantly up-regulated by osmotic stress. These include genes with functions related to transcription, signal transduction, cell wall metabolism and defense. Other gene transcripts encoding cysteine protease and aquaporin are also up regulated during osmotic stress. The function of about one-third of the genes in poplar cells that were significantly up-regulated by stress is not known, suggesting that the cell suspension may offer an opportunity of finding novel genes otherwise never expressed and that we still need more information at the molecular level.

Lata and Prasad, (2011) reviewed that one alternative approach to the development of drought tolerant plants is to genetically engineer plants to introduce stress- tolerant genes, including genes for transcription factors (TFs). TFs recognize specific DNA sequences in the regulatory regions of target genes and lead to activation of downstream genes responsive to abiotic stresses. One relevant class of transcription factors is the DREBs.

Khan *et al.* (2013) reported that, drought tolerance is polygenic and complex trait interplay with the environment makes phenotypic evaluation

difficult. Hence, the use of DNA markers can help breeders in improving the speed as well as reliability of the process. Gene tagging and DNA fingerprinting is particularly suitable for pyramiding of desired traits.

Reis *et al.* (2014) reported that drought is one of the most challenging agricultural issues limiting sustainable sugarcane production and, in some cases; yield losses caused by drought are nearly 50%. DREB proteins play vital regulatory roles in abiotic stress responses in plants. The transcription factor DREB2A interacts with a cisacting DRE sequence to activate the expression of downstream genes that are involved in drought, salt and heat stress response in Arabidopsis thaliana. In the present study, we evaluated the effects of stress-inducible over-expression of At DREB2A CA on gene expression, leaf water potential (L), relative water content (RWC), sucrose content and gas exchanges of sugarcane plants submitted to a four-days water deficit treatment in a rhizotron-grown root system. The plants were also phenotyped by scanning the roots and measuring morphological parameters of the shoot. The stress-inducible expression of AtDREB2A CA in transgenic sugarcane led to the up-regulation of genes involved in plant response to drought stress. The transgenic plants maintained higher RWC and L over 4 days after withholding water and had higher photosynthetic rates until the 3rd day of water-deficit. Induced expression of AtDREB2A CA in sugarcane increased sucrose levels and improved bud sprouting of the transgenic plants. Our results indicate that induced expression of AtDREB2A CA in sugarcane enhanced its drought tolerance without biomass penalty.