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Novel Biotechnological Approaches for the Enhancement of Sugarcane Production

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Abstract

A major challenge observed by sugarcane farmers is to acquire greater biomass production. As a consequence of sugarcane's weak defense against viruses, pathogens, and insect attacks. Growing healthy plant is a difficult task. Glyphosate tolerance and insect borer resistance are significantly important characteristics considering the genetic development of different crops. Transformation of insect borer resistant genes Bt. is an efficient and cost-effective approach used for improving the resistance of several crops against insect borers. Presently, commercially developed GM crops, for example, maize and cotton, consisted of genes associated with insect borer resistance and herbicide tolerance which are helpful qualities in a larger production. Herbicide-resistant transgenic crops signify the importance of genetically modified crops grown throughout the world, considering that 83% of land committed for GM crops was grown with herbicide-resistant species. Transformed lines stacked with various transgenes are generally achieved through cross-hybridization amongst the diverse transformed plants or re-transformation of multiple genes. These different procedures for combining the genes created some considerable restrictions such as cross-hybridization is lengthy and labor exhaustive. Considering this, scientists successfully developed a rapid and efficient procedure for transforming the many genes constructed individually with promoter and terminator through T-DNA in the specific plant genome by a singular transformation step. Sugarcane (*Saccharum officinarum* L.) is an important cash crop. It makes the availability of about 80% of the worldwide sugar and almost 90% in Pakistan. Lepidopteran insects can damage the sugarcane crop, causing more than 30% loss with reference to yield globally. Sugarcane is commonly grown in rainy areas, so it is typically exposed to weed infestation, which causes yield loss as well. Several studies reported the successful introduction of Bt. genes in sugarcane against lepidopteran insects. Some other studies reported the transformation of PAT/bar genes and developed resistant plants for glufosinate-ammonium. Currently, no study is available which introduced the Bt. genes such as *Cry1Ac*, *Cry2A*, and *Glyphosate Tolerance* gene (*GT*-gene) all together in sugarcane for commercial purposes.

Keywords: Sugarcane; *Cry1Ac*; *Cry2A*, *GT gene*; Insecticides; Herbicides

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Introduction

Sugarcane is a useful cash crop due to its ability for greater biomass production. Agriculturally, a suitable environment of the top five sugarcane producing countries including Pakistan attracts its higher production. Brazil is the principal country regarding sugarcane covered area and alone encompasses a 25% share in the world sugar production (FAO 2014). Sugarcane industry is producing a huge number of products, such as biofuel, electricity, pharmaceuticals, enzymes, beverages, alcohol, furfural, dextran, chipboard, paper, confectionery, chemicals, plastics, paints, synthetics, insecticides, detergents etc. [1-4]. The mature sugarcane stem consists of soluble sugar contents ranging from 12% to 16%, fiber, 11% to 16%, water and 2% to 3% constituents other than sugars. Sugarcane juice makes availability of carbohydrates, protein, minerals for example calcium, iron, potassium, sodium, zinc, thiamine, riboflavin, and antioxidants including flavonoids and phenolic compounds which makes a defensive set up of human body to save it from dangerous diseases including cardiovascular problems and cancer. For, achieving maximum benefits from sugarcane, its traits can improve through its genetic modification [5]. The incorporation and constitutive

expression of insect and herbicide tolerant characteristics are now achievable in the required crops [1,6]. Regarding advancements in biotechnology, crops with enhanced insect resistance is possible now through gene insertion methods. According to James [7], 2009 the farming of biotech crops on large levels from 1996 to 2009 was just possible because of their single-handedly insect resistance quality or along with herbicide resistant trait has made them possible to rank 2nd world widely [7].

Morphological Characteristics

Sugarcane considered indigenously C4 crop throughout the year growing grass species of the tropical and the subtropical areas of the world [8], under family Poaceae and genus *Saccharum*. Mature cane plants made up of the lateral shoots at the bottom, which multiplies into stems approximately 10 ft to 13 ft in height and nearly 5 cm in diameter. On maturity, cane stems become seventy five percent of the entire plant. Genus *Saccharum* divides into six species, two of them are considered as wild *Saccharum spontaneum* and *Saccharum robstum* whereas remaining four promoted for cultivation as *Saccharum edule*, *Saccharum officinarum*, *Saccharum barberi* and *Saccharum sinense* [9]. All the species belong to the diverse regions like *Saccharum barberi* belongs to India; *Saccharum edule* and *Saccharum officinarum* belong to New Guinea. Among these, the best reliable Hybrid *Saccharum officinarum* produced from manifold crossings of *S. spontaneum*, *E. asundinaceus* and *M. sinensi* [10].

Sugarcane consists of complicated aneuploid although decidedly heterozygous cultivars [1]. Being polyploidy, the number of chromosomes ranges from 80 to 120 [11]. The highest growing latitude for this crop is mostly at 34°N, at the same time at 37°N is furthermore stated from Spain. Its propagation is continued by taking immature shoots or stem portions popularly named as setts. From the top part, the third segment of the stem aged 8 to12 months is considered most excellent for cane seed (setts). For most favorable growth of sugarcane, cane seed with fresh live buds are accounted the best. Cane seeds vegetate from 10 to 14 days covered with light soil under suitable growing environmental conditions. Pakistan, regarding area wise sugarcane cultivation is on 5th position whereas 15th considering its production and numbered 60th for contributed sugar [12].

Cane and Yield Production: Causes and Challenges

There are several issues creating hindrance for achieving the maximum production as well as yield from sugarcane. The biggest reasons for these are selected varieties with respect to growing area, undesirable growth of weeds and unavailability of strong defense systems against cane borers. Weeds usually create destructive problems for the desired crop plants through competition for soil nutrients, water availability, light plus other essential growth required factors [13]. This unnecessary competition creates negative effects on the desired yield of the crop. The resulting consequences always caused major losses by producing low quality crop production. Neftim [14] stated almost 12% to 72% yield achievement failures are reasoned by weeds. Weeds were considered as an actual reason for k15 to 30% loss of yield in Pakistan [15]. A variety of control systems were adopted to lessen these damages through chemical procedures, mechanical techniques and biological developments [16]. For chemical procedures, herbicides or combinations of different herbicides tried throughout the world. It's a simple approach for avoiding crop competition by weeds. Conventionally, uprooting of weeds is a general practice. It involves massive manual labor which sometimes causes shearing and tearing of the field surface.

Cane-borers are considered the chiefly crop destructive *Lepidopteran*. They include stem and shoot borer, top-borer, root borers and Gurdaspur borer etc. More than an 80% decrease in yield has been recorded [17] through the crop destruction of these most damaging *Lepidopteran* pests including *Diatraea saccharalis* (Stem borer), *Emmalocera depressalis* (root borer), *Chilo terrenellus* (sugarcane top borer), *Sesamia inferens* (pink borer) and Eoreuma loftini (Maxican rice borer) [18]. Among sugarcane varieties, pests' defiant germplasm is unavailable. Deficiency of this quality characteristics making up to 10% decrease in cane yield per annum regarding sugarcane globally. Sugarcane borer, (*Proceras venosatus*) single-handedly made 7% crop damage annually in China [6].

Wide-ranging and bigger quantities of insect killing sprays are in use possibly to make sure crop safety and damage control by insects. *Ceratovacuna lanigera*, Woolly aphid is the main sucking pests regarding Asian countries [19]. Along with these insects, moth and butterfly, *Fulmekiola serrate* as sugarcane thrips, *Melanaphis sacchari* as cane aphid, *Oxya chinensis* as grasshopper, as well as burrowing bug (*Scaptocoris talpa*) included among yield destroying insects. As a root destroying agents cane beetles (*Tomarus subtropicus*), giant termite (*Mastotermes darwiniensis*) and ants affected the cane crop production through the damaged roots. Just because of these borers, insects, and pests up to 10% loss in sugarcane production is recorded all over the world [20,21].

Among these pathogens, causing agents is fungus, bacteria in addition to different viruses etc. The famously known reported diseases caused by fungi are red rot by *Physalospora tucumanesis anamorph*; *Colletotrichum falcatum*, root rot by *Pythium graminicola*, Rhizoctonia sheath and shoot rot by *Rhizoctonia solani*, pineapple diseases by *Thielaviopsis paradoxa*, downy mildew by *Peronosclerospora sacchari*, wilt by *Fusarium sacchari*, rust by *Puccinia melanocephala*, *P. kuehnii*, smut by *Ustilago scitaminea* and seedling blight by *Alternaria alternata*. Smut is observed frequently prevailing in the African and South-East Asian Countries [22].

Tissue Culture Studies

Sugarcane with complicated genome of both polyploid and aneuploid has a narrow gene pool, long-term selection cycle that slow its breeding improvement, making it a genuine candidate for plant molecular breeding. With the use of new genetic transformation techniques, insertion of insecticidal and herbicide resistant transgenes in the *Saccharum* germplasm is practicable for achieving the maximum yield task [23]. Advanced scientific techniques kin seed biotechnology exemplified the exploitation of desired genes in any crop to modify its genome or genetic editing for enhanced characteristics (transformed characters), for example, making these crop plants tolerant for a latest and highly effectual herbicide and resistant against insect pests.

In Vitro Callus Formation and Organogenesis in Monocots

A number of studies have confirmed the callus formation and regeneration from different cereal crops successfully [24]. Embryogenesis is a reliable description of the dictum of plant totipotency, hybrid embryos and scutellar tissue, mesocotyl tissues

and leaf tissues. Apical meristems, Root tips, inflorescence, anthers, pollen and seeds. But anthers and immature embryos have been used frequently as an explant source for initiation of morphogenic culture in cereal tissue culture [25,26].

Commonly higher amounts of auxin and lower quantities of cytokinin are used in the growth media for enhancement of callus formation and cell division [27,28]. Normally, synthetic 2,4-D as an auxin are in use for the callus formation from the explant. In contrast, lower amount auxin plus higher quantities of cytokinin added in the medium for shoot developments [29]. The use of the Dicamba (3,6-dichloro-o-anisic acid) in plant tissue culture as a hormone or growth regulator, was considered similar in structure to well recognized auxin 2,4-D. Though, a higher amount of the Dicamba is suggested for the initiation of callus in comparison to the 2,4-D [30]. Significant roles of Thidiazuron (TDZ) have been studied in the tissue culturing of the dicots especially throughout the *in-vitro* developmental stages.

Somatic Embryogenesis in Monocots

Embryogenic capability, regeneration and through this whole plant development are innovative achievements. Several experiments faced many limitations regarding tissue culture in the cereal crops, presently tissue culture is established in the cereal crops through somatic embryogenic capabilities for example in maize, in rice, sugarcane, sorghum, wheat, barley and tritordeum, and in rye. For the regeneration of complete plantlets *via* somatic embryogenesis specifically in the rye, the genotype dependence was not as much of as regarding the wheat as well as barley. In rice tissue culturing 2,4-D induced the somatic embryogenesis while BAP causes the stimulation of shoot induction. It was found from regeneration procedure by the formation of the callus with leaf in wheat (*Triticum aestivum* L.), within limited time, earlier induction was reported by adding the BAP in the callus inducing media, but BAP did not show any improvement regarding the embryogenic callus and plant developments. Desired concentrations of the 2,4-D, BAP and NAA showed parallel regeneration rate to the control.

Specifically, 2,4-D has proved itself an efficient hormone for inducing the embryos in the numeral monocots such as sugarcane, in wheat, and rye crops. The synthetic coating of growth media on embryo produced synthetic seeds [31].

Tissue Culture Studies in Sugarcane

Until 1960, sugarcane varieties were improved *via* conventional breeding. These techniques depend on locations with required weather for flower formation, crossing to improve seeds. Baday SJ [32] studied effects of various hormones for callus formation and its culturing, and micro-propagation using auxin and cytokinin in basal medium. Other growth hormones like IAA, IBA and myoinositol were also studied.

Comparison between Murashige and Skoog (MS) basal media and Chu-N6 medium for embryogenic callus formation from immature leaves showed a positive increase in embryogenic calli from the *Saccharum* spp. Both media were supplemented with 2,4-D for callus initiation. The N6 media gave good results by sustaining somatic embryogenic calli and totipotent abilities. The addition of growth regulators kinetin along with coconut water in callus inducing media inhibits the conversion of somatic cells into embryos. Use of MS medium with 2,4-D (14 mg/L to 4 mg/L conc.) and this media with BAP (0.54 mg/L to 2 mg/L range) inducted direct formation of somatic embryos in sugarcane cultivar-CO 671 and embryogenesis is completed in 12 to 15 days without callus inter-phase. 2 mg/L of 2,4-D plus 1 mg/L BAP in media showed good results for callus initiation and maintain its embryonic potential [33].

Salokhe [34] reported the somatic embryo formation from sugarcane leaf rolls on the MS medium enhanced with 2,4-D, 3 mg/L, and observed somaclonal varieties developed through regenerated young leave rolls from 2 sugarcane clones *viz.* BL4 and AEC 81-S415 on the growth media supported with 2,4-D, 4 mg/L. Shoot induction was studied on a MS medium with IAA 2 mg/L, IBA 2 mg/L along with Kn 2 mg/L, while rooting was achieved on half strengthened MS media with addition of 1 mg/L IBA. Somatic embryos were studied in sugarcane (CP 84-1198) callus from young leaf segments on media with 13.6 uM 2,4-D and various growth hormones like BAP, Kn, 2Pi, Zeatin (Z), TDZ in presence of 2.5 uM NAA to assess calli regeneration potential. Growth medium with 2.5 uM TDZ considered good for shoot initiation. Effects of organic constituents for *in-vitro* tissue culturing were also studied [35].

Shoot development obtained in the absence of 2,4-D with 12 h light and dark period as well as rooting with the similar environment. Different phases of embryo growth have been presented by histological studies from the sugarcane calli of the cultivar H50-7209. Lesser quantities of auxin produced maximum embryogenesis, though shoot development was observed without auxin. Meristematic cells from the apical regions were understood better for micropropagation required germplasm comparing the callus [36].

The development through *in vitro* micro-propagation concluded lesser plant production through leaves than shoot tip. For the sugarcane, similarly like others monocots different tissues were used as explant for its tissue culture such as on maturity the intermodal parenchymal parts, parenchymal tissue from the shoot apices and the leaf segments of *Saccharum* spp. Comparatively, leaf segments were excellent for the callus formation from all the genotypes in sugarcane, however; the explants have no limitations, considering the greenhouse plantings and seasonal dependence [37].

Transformation Studies

Genome modification procedures made proficient contribution through the development of the two controlled dealings based on *Bacillus thuringiensis* or the *Bt.* genes. One is bio-pesticides through the genetic modification of microbe's strains. Secondly, more effectual scientific way is the insertion of the *Bt.* genes directly as a part of their genome in the desired crop.

Bacillus thuringiensis with insect resistant δ-Endotoxins

From the beginning, the *Bacillus thuringiensis* (*Bt.*) with insect killing toxins received the center of attention with reference to scientific studies [38]. The foremost bio-pesticide, *Bt.* has rod shape structure, gram positive, facultative anaerobic, spore forming bacteria, their isolation was doing from the different regions globally, most likely from the soil, insects, stored-product dust, and deciduous and coniferous leaves.

After the preliminary characterization of *Bt.*, its insecticidal capabilities were documented through their Crystal proteins named as Insecticidal Crystal Proteins (ICPs), such as Crystal (*Cry*) and Cytolytic (*Cyt*) proteins, formed as parasporal inclusions during spore production. It is distinctly demonstrated that *Cry*, and *Cyt*.,

proteins as toxins act together in a specific and synergistic approach in insect's gut to create toxicity. These parasporal excretions consist of comparatively high amounts of glycoprotein recognized as δ-endotoxins or *Cry*-toxins. Variability in the size of these proteins was observed. The δ- helices of the domain-I are commonly inside the cells however their locality is generally exterior to the spore. The production of insect toxic proteins and their interaction with the stationary stage within the *Bt.* are controlled through many processes occurring during the transcriptional, post transcriptional and post translation levels. The δ-endotoxins classification was made considering their insecticidal characters and molecular associations [39].

Mode of Action of the δ-Endotoxins

Considering the toxins groupings, *Cry*-I is the principal one against *lepidopteran*, protein sizes are ranging from 130 KDa to 138 KDa and crystal structure is bi-pyramidal parasporal. These *cry* proteins or toxins were dissolved in the alkaline medium interior of the midgut by forming a free pro-toxin type. Then, trypsin and chymotrypsin like proteases converted these free pro-toxins as active toxins inside the gut [40]. Digestion of the *Cry*-lA pro-toxins begins in a progressive mode from C-terminus then preceding the toxic core (55 kDa to 65 kDa). Following the processing period, the C-terminal is recessively concise in the sections of 10 KDa. The active Cry-proteins allocated two jobs from which one receptor binding and second ion channel activity. Scientific research considering the δ-endotoxin shape showed three domains (Figure 1).

The studied work confirmed the three domains of δ-endotoxin and integration in the cell membrane of the gut by creating a pore for ions movement. Domain-II showed structure similarity with the antigen-binding constituency of the immune-globulins with the confirmation that loops in the domain-II affects irreversible/reversible binding through the hydrophobic communications with the receptor's molecule. Conclusion showed that a toxin may not effectual being receptor's un-recognition against one insect but can be more efficient considering the others. Firstly Domain-III generated stability for the protein, however later on it was thought that Domain-III B-sandwich as well take part in the functions such as receptor binding, particular site recognition to end with ion channel is gating [41].

(a) Ingesting and solubilizing phase of the pro-toxin in the midgut (b) Proteolytic activation of the toxin at N and C-termini (c) Interacting phase of the toxin with binding sites on epithelial membrane (d) conformational change revealing δ 4-5 helical hairpin (e) Oligomerization and incorporation in the membrane of midgut for making ion channel or pores. By conformational change toxins explained binding with epithelial receptors on the midgut. The communication of the toxin with high-affinity receptors is considered greatly important which illustrates the insecticidal uniqueness of these *Bt.* toxins (Figure 2). Finally, this binding of toxins or toxic proteins provides stimulation for the pore development by the epithelial membranes in the insect's midgut. Conclusions in pH can be also a reason to modify the δ-helices/domain-I structural change ultimately influence the pore formation capability in the δ-endotoxin [42].

Transformation and Genetic Modification of Sugarcane

The incorporation of desirable traits in the best cultivars of sugarcane would be of huge importance; however its complicated

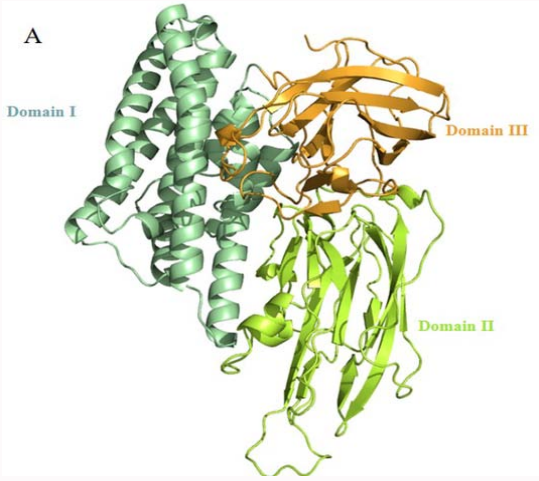

Figure 1: δ-endotoxin with three domains.

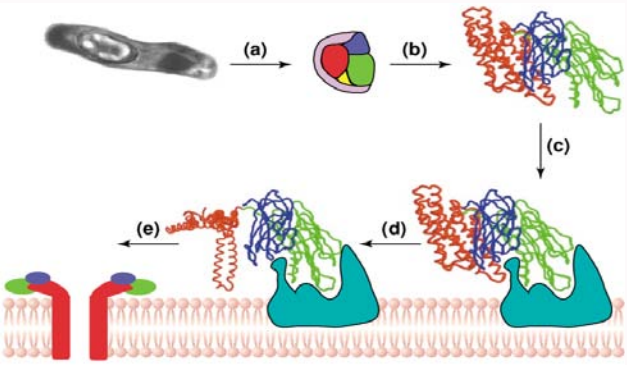

Figure 2: Complete way of action projected for toxin or Cry proteins.

genotypes and highly polyploidy 28 (2n-36-170) made conventional breeding and genome scientific research studies difficult. Genetic engineering procedure advantaged to solve these difficulties. Genetic modification in sugarcane has been possible from the protoplast transformation *via* PEG then through electro-proration. The transformation potential comparison was made among the commercially important 12-cultivars of sugarcane, by transforming pDP687 (Poineer Hi-Bred International Inc.) contained two anthocyanin transcriptional activators, coated on tungsten micro-projectile particles (M17, Bio-Rad Laboratories CA). pDP687 was transformed in the plants, no harm or disadvantage in the form of cells death was observed. Among sugarcane cultivars NCo310 along with NC0367 provided better results considering the anthocyanin protein expression. Being a natural host, dicot plants are considered best for transformation *via A. tumefaciens* in contrast to monocotyledons [43].

Expression vector known as *p*GT GUSBAR, binary construct, transformed in the *Agrobacterium tumefaciens* through direct transformation method. For these four individual methods, each one studied with hundred explants. Maximum transformation efficiency was observed in treatment-A during which co-culturing and post co-culturing was experimented with solid media. Field trials were made to check the herbicide resistant transformed plants through the application of BASTA spray. To confirm the integration of transgenes many molecular techniques were used including polymerase chain reaction, Southern, Northern and Western blot. From

transformation studies with *Saccharum* varieties Ja60-5 and B4362 through *A. tumefaciens* consisted of the binary expression vector pGT GUSBAR evaluation was done considering the effects of three antioxidant compounds, ascorbic acid, cysteine and silver nitrate on the cellular viability of meristematic cells along with *A. tumefaciens*. Conclusion confirmed the improvement in transformation efficiency by antinarcotic pretreatment in the monocotyledons [44].

The callus suspension cells and electroporation of protoplast showed probability of transgenic calli. For these experiments, suspension cells from 3-days older cultures were selected for bombardment of *p*BARGUS expression constructs. Southern hybridization and PAT activity were carried out to confirm integration; however, no transformants were obtained. Transformation efficiency from this study was as low as in wheat on making comparison studies [44].

Several heterogeneous promoters were constructed to strengthen the transgene expression but post-transcriptional genes silencing resulted even in young transformants of monocotyledon. Maize Ubiquitin-I was highly recommended for the sustainable protein expression. The expression vector pMon 19344 contained the two gene sequences *nptII* and *Cry*1Ac. The gene expression was studied with the insect larvae of *Elasmopalpus lignosellus* (zeller), the LCB. Conclusion showed that *Bt.* transgenes hold good potential against the LCB and in selecting the resistant sugarcane varieties [46]. From the maize, anthocyanin regulatory regions R and C were used for study of transient expression and higher helium pressure to bombard the plasmid coated micro particles increased the transient expression [47]. These higher levels of expression adversely shorten the height of the transgenic plants, even not a single plant with this anthocyanin expression showed more than 3 cm in height. From a study on transformation in 12 commercially available sugarcanes (*Saccharum* spp. hybrids) cultivars, used expression construct *p*D*p*687 (Pioneer Hi-Bred International) coated on tungsten micro-projectile (M17, Bio-Rad Laboratories CA). Particle flow gun delivered plasmid construct coated on particles to the embryogenic callus. Sugarcane cultivars NCo310 and NCo376 resulted in more positive response while N20 expressed no transgene protein [48].

The herbicide tolerant sugarcane variety NC0310 through the micro-projectile DNA delivery construct pAHC20 consisted with bar sequences under maize Ubiquitin promoter (Ubi-10) was transformed. A comparison was made between the plasmid coated gold and tungsten particles. No significant difference was observed. Transformation of the construct *p*MON19344 contained the *nptII* and *Cry*lAc genes in three cultivars H62-4671, 1173-6110 and 1178-4153 was done through the gene gun method and transformed lines were obtained. They were subjected for bioassay analysis with insect larvae. Comparisons between calli and leaf bioassay showed low insect mortality percentages on the transgenic leaf. It was also confirmed that 1 to 5 copies of the integrated genes existed in transgenic plants. The ten sugarcane cultivars were used to check the more appropriate one. South African cultivar NCo310 considered as a standard for this experiment. Expression constructs transformed named as P1-contained maize anthocyanin gene, P2 contained the *nptII* and P3 contained the *Eldana succharina*, (*Lepidoptera*: Pyralidae) resistant transgene CERD and P4 contained sequences of *nptII* and ERI. After transformation of Pl-construct, genotype differences were observed among callus of all cultivars. Higher transient expression was in bombardment with P2, P3, and P4. Transgenic callus with P4 construct has a smaller number of plants such as 1 plant in N12, 5 plants in N12 and 12 N19 [49].

Two resistant cultivars SP80-3280 and SP80-1842 of Brazilian commercial sugarcane against the cane borer were developed. Three expression vectors individually constructed with genes and co-transformed through gene gun method. (a) Expression vector pC1B4421 constructed with *Cry*1Ab under maize PEP-C (b) plasmid vector pCIB4426 constructed with *Cry*1Ab under pith promoter (c) vector pHA9 constructed with neo sequences. Evaluation made on the basis of insect mortality through leaf bioassays, phenotypic or agronomic traits and yield characters. Conclusion confirmed effective toxins production and developed resistance against insect borers observed during the field study. Many crops with these improved characters are now commercially available for example soybean, corn and cotton.

Earlier reported research evidently proved that sugarcane modified with *Cry*1Ac is good approach for advancing the traits [21]. Shoot borer *Chilo infuscatellus* commonly damaged the crop plants at earlier phases of their growth in the field and ultimately concluded in higher yield reduction. Mostly shoot borer *Chilo infuscatellus* attacked the crop in dry hot time periods. The crop yielded losses by a decrease in the cane weight 19.63% and 1.72% to 8.02% sugar recovery. Expression plasmid constructed with *Cry*1Ac and bar sequences was transformed by gene gun method in FN15 Variety [50]. The transformed plants were screened first by adding the Phosphinothricin and Basta in the growth medium and then PCR. Fourteen transformed lines were recovered. Results confirmed that transgenic lines with low copy number produced maximum expression for the stem borer. Insect larvae death occurred on stem parts with symptoms of *Cry* protein toxicity. Transgenic lines contained double *Bt.* transgenes *Cry*1Ac plus *Cry*2A were better when compared with the non-transformant. It's concluded that transgenic lines were constantly resisted by insect attacks. During five years of field study, eight generations were studied with the confirmation and production of stably expressed transgenes in the form of insect toxin. Gene sequences of *Cry*1ac and *Cry*2a showed less than 45% homology. Making comparison between the two genes, *Cry*2A protein expression critically performed for the death of diptera and *lepidopteran* borers as well.

The expression of the toxic protein (*Cry*2A) was formed by using an artificial diet and experimenting with *lepidopteran* borers, also, biochemical analysis gave the confirmations that *Cry*2A and *Cry*1Ac did not share the same binding sites in the insect's gut. Many scientific research studies already confirmed that toxins form both transgenes is highly effective against the insects Latif et al. [16]; Bakhsh et al. [51]; Qamar et al. [52]; Bakhsh et al. [53] showed variability in the protein expressions of the two transformed genes *Cry*1Ac and *Cry*2A considering the time intervals and concluded that expressions of these transgenes decrease with the aging of the transformed plants of the similar variety [16,51-53].

Monocot Transformation

Monocotyledons are considered the most difficult genome as genetic exploitation of the monocots in agronomical cereal grains most of the time are not possible *via* Ti-plasmid-mediated transformation procedure. Firstly, that was just because of the particular and restricted host choices of *Agrobacteriurm* to deliver the desired gene. Secondarily, death of the cells on the wounded spot compared with

plants of other crops that were genetically manipulated with this type of infection. Substitute methods were implemented to make monocots genetically modified. At first, gene was delivered by electroporation, next by polyethylene glycol induction, and then *via* micro-injection. Afterward on, for intact tissues techniques i.e. electroporation procedure, direct delivery of the gene in the protoplast and tissues, with *Cry*1Ab into the callus, *via* Silicon Carbide Fiber technique in the suspension culture. Biolistic procedure in the corn plants, also pioneerly studied the genetic modification through the bombardment method. After the establishment of this protocol, numerous DNA labeled particles delivery method in the monocotyledons such as the corn, rice as well as wheat, etc [54].

Generally, resistant sequences or selection markers are developed that hindered the development of the non-transgenics and develop resistance for antibiotics to stop the expansion of the cells other than transformed. Selection marker genes limit the duration of screening from transformed crops. These selection sequences were transformed either separately through an independent vector or constructed already within the expression construct along with the desired specific genes to transform the genome. It was 100% for transformation and 50% for other constructs. Commonly, expression was made through the constitutively promoters e.g. Cauliflower Mosaic Virus (CaMV35S), Ubiquitin, actin etc. Selection marker genes provide resistance against antibiotics e.g. kanamycin, aminoglycosides, geneticin, paromomycin, *SAT3* gene sequences against streptornycin, other examples can be to facilitate agronomic traits or to save crop production e.g., for herbicide tolerance [55].

The screened resistant sorghum plants were reported by expressing the hygromycin (*hph*) plus kanamycin (*nptII*) genes through their concentration optimizations in growth medium. From this experiment though the gene gun method transformed plants were proficiently regenerated. Further, other genes named reporter genes differentiates between the transformed and non-transformed tissues exclusive death of non-transgenics [56].

Innovating ideas were made to control the expression of proteins in the transgenics. Up till now, transformation with numerous stacked sequences in a single crop is a difficult task. Very few commercially available transgenic crops consisted with the stacked/pyramided traits.

Obviously, these reporter genes contribute for screening the transgenics even during tissue culturing period. Among the nominated sequences as reporter genes, uidA gave efficiently transient expression which remained stable in several crop plants. To make rapid screenings and visual expression among transgenic and non-transgenics GUS considered the best marker. Various reporter transgenes expressed in the differentiating proteins which on a few treatments will express in transgenics. For example, the GUS transformed tissues when treated with enzymatic substrate developed the bluish green color. Commonly, uidA used in plant transformations as a reporter transgene with respect to protein expression analysis. GUS visualization is simple, enzymes denaturation is little difficult with stable precipitate [50].

Lentz et al. [57] recommended genetically modified viruses to ensure the GUS was transformed and expressed. Maximum enhancement in the bluish green stains was studied. Several gene sequences for example encoding β- Glucosidase, β-Calaclosidase and nos were reported for the genome transformation considered as the reporter transgenes. Although, gene gun protocol was made that still faced challenges to attain maximum results. One of the reasons is incompetence as well as lower regeneration percentage. Müller et al. [58] reported that maximum protein expression from the transformed genes was observed within the transgenic cells following coated DNA delivery by adding the 0.25 M mannitol in the growth medium before and after transformation with gene gun procedure, use silver thiosulphate instead of calcium chloride and exclude spermidine during the DNA coating on the micro-particles. Various factors made their contributes to sequencing, silencing and optimization are used for selection and regeneration.

Luo et al. [59] through micro-projectile DNA delivery technique successfully achieved stable expression in the *Sorghum vulgare*. Transformants containing the desired genes or expression vector were observed through Gel blot analyses. Rye, through comparisons, showed less positive for the regeneration. Liu et al. [60] studied rye and produced the fertile transgenics through the Biolistic method; molecular analyses gave confirmation for the uidA and bar expressions with Mendelian transmission in the progenies. Likewise, fertile, oat transformants were achieved by the Fatmawati [61] *via* bombardment in embryogenic oat callus with a construct encoding the bar and uidA transgenes. From these three independent transgenics of hygromycin phosphotransferase (*hpt*) were achieved. Makkar et al. [62] studies showed two derivational functional transformed varieties of the rice Basmati 370 and M7. Transformation was made through the Biolistic method on 6-days aged calli resulting from the scutella of matured seeds, with the expression construct pROB5 contained with synthesized *Cry*-2A *Bt.* gene along with *hpt* coded sequence co-transformed with pWRG1515 consisted of GUS (*uid*A) and hygromycin resistant sequences. Both, the genes were individually constructed with CaMV35S promoter plus NOS terminator [63,64]. Transgenics were obtained on the selection media added with hygromycin, plantlets transferred under the house conditions in the soil pots, subsequently transgenes were verified through molecular analyses in transgenics such as Southern Blot for the integrated genes.

Conclusion

Sugarcane (*Saccharum officinarum* L.) is an important cash crop. As a result, *lepidopteran* insect's damaging the sugarcane crop is a significant problem, causing more than 30% loss with reference to yield globally. Sugarcane is commonly grown in rainy areas, so it typically experiences weed infestation and provokes yield lost as well. Although a number of studies reported the successful introduction of *Bt.* genes in sugarcane against *lepidopteran* insects however, using PAT/bar genes and developed resistant plants for glufosinate-ammonium, currently, no study is available which introduced the *Bt.* genes such as *Cry*1Ac, *Cry*2A and *Glyphosate Tolerance* gene (*GT*-gene) altogether in sugarcane for commercial purposes. There is a need to develop sugarcane varieties resistant to insects and Weedicides.

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