

Integrated Bioinformatic, Molecular, Phenotypic, and Anatomical Analysis for Laser-Induced Mutation in Sugarcane (*Saccharum* spp.)

Khaled Adly Khaled

khaled.adly@agr.bsu.edu.eg

Beni-Suef University

Mahmoud Hamdy Mohamed Ebid

Agricultural Research Center, Sugar Crops Research Institute

Manar Hassan

Cairo University, National Institute of Laser Enhanced Sciences

Samah Azoz

Cairo university, Faculty of Agriculture

Shereen Khaled

Agricultural Research Center, Sugar Crops Research Institute

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Abstract

Background: Although laser irradiation is an emerging physical mutagen for crop improvement, its effects on sugarcane (*Saccharum* hybrid) at the phenotypic, anatomical, molecular, and bioinformatic levels have not been comprehensively integrated.

Methods: Sugarcane buds (variety G.95-21) were pre-illuminated using He-Ne laser (630 nm) with six power levels (5–100 mW) and five exposure times (0.5–10 min), generating 20 treatments. Forty-eight clones were evaluated for five agronomic traits (stalk height, stalk diameter, stalk number/stool, stalk weight, Brix) across two seasons (2020/2021–2021/2022). Seven selected clones underwent SCoT marker analysis (7 primers, 83 fragments). Statistical analyses included principal component analysis (PCA), broad-sense heritability (H^2), genetic advance (GA), two-way ANOVA with Tukey's HSD, marker-trait association (MTA), quantitative anatomy (9 parameters), and comprehensive bioinformatic annotation (GO, KEGG, InDel characterization, protein modeling).

Results: Treatment T1 (5 mW/0.5 min) exhibited superior performance, with increases in stalk height (272.9 cm, +53% vs. control), stalk weight (1.43 kg, +113%), and Brix (23.5%, +88%). Broad-sense heritability was high for both stalk diameter ($H^2 = 0.79 \pm 0.09$) and Brix ($H^2 = 0.74 \pm 0.11$). PCA explained 78.6% of the total variance (PC1 = 51.2%, PC2 = 27.4%), clearly separating the elite mutants from the control. SCoT markers revealed 68 polymorphic fragments (81.9% polymorphism). A 2,173 bp deletion in the dehydrin gene (DHN1) was identified exclusively in all laser-treated clones (SCoT21-InDel marker), and was absent in the control. This deletion likely occurred via microhomology-mediated end joining (5 bp direct repeat AGCTT). The predicted SoDHN1 protein (237 aa, 25.8 kDa) is a YSK₂-type intrinsically disordered dehydrin (85% disordered residues) with three K-segments, one S-segment, and one Y-segment. Marker-trait association analysis revealed that SCoT-21 (dehydrin) explained 58% of stalk weight variance ($R^2 = 0.58$, $\beta = -0.76$, $P = 0.001$). Quantitative anatomy revealed T1 increased lamina thickness (+8.33%), metaxylem vessel diameter (+11.89%), and bulliform cell thickness (+20.67%), with strong correlations to agronomic traits ($r = 0.71$ – 0.94). Phylogenetic analysis placed SoDHN1 within Poaceae YSK₂-type clade (98% bootstrap with sorghum). The SCoT21-InDel marker was

validated using specifically designed primers (287 bp intact / 124 bp deleted) and showed a low natural frequency (6.7% in the germplasm).

Conclusion: Low-power He-Ne laser irradiation (5 mW/0.5 min) is an effective mutagen for generating elite sugarcane mutants with enhanced yield and quality. The SCoT21-InDel marker provides a simple, PCR-based tool for marker-assisted selection. Deletion of DHN1 eliminates the fitness cost associated with constitutive stress-protein expression under well-watered conditions, reallocating resources to growth and sugar accumulation. However, caution is warranted for rain-fed environments, where dehydrin-mediated drought tolerance may be essential. Further multi-location trials of T1 and T15 are recommended.

Keywords: *Laser mutation; Saccharum hybrid; Dehydrin deletion; SCoT markers; InDel marker; Quantitative anatomy; Marker-trait association; Bioinformatic analysis; Hormesis; Egypt*

1. Introduction

Sugarcane (*Saccharum hybrid*) is Egypt's most significant crop for producing sucrose. Because sugar is an essential nutrient required to meet local consumption demands rather than for overall productivity, sugarcane plays a critical economic role (Ghallab et al., 2004). Approximately 642.2 thousand metric tons of sugar were produced from cane in Egypt in 2024. This figure represents a decrease from 767.4 thousand metric tons in the previous year. The nation's sugarcane output remained comparatively constant during the reviewed period, with a decline observed in 2020 (Sugar Crops Council, 2025). Previous research on sugarcane has focused on advanced techniques to enhance crop productivity and improve tolerance to unfavorable environmental conditions.

Light-based biostimulation, first explored by Paleg and Aspinall (1970), has been extensively tested and is increasingly applied in the field to enhance seed germination, seedling growth, phytochemistry, and overall productivity. It is also used to identify mutants and novel genetic variants that enable crops to grow well under diverse conditions while maintaining high yields. One of the advanced techniques used to provide light is LASER. Laser, an acronym for Light Amplification by Stimulated Emission of Radiation, is an optical device that generates monochromatic and highly coherent light (Nadimi et al., 2021). Several studies have focused on the use of light irradiation treatments to enhance crop yield and quality. Gamma radiation (Azzam

et al., 2022; 2023; Khaled et al., 2022), microwave (Azzam et al., 2022; Khaled et al., 2022) and laser (Hassan et al., 2024).

The laser light at low intensity is used in agriculture as a bio-stimulator when used on seeds and seedling plants (Chen et al. 2005). The mechanistic basis of laser stimulation at any plant physiological stage lies in the synergism between the polarized monochromatic laser beams and the photoreceptors (Bielozierskich and Zolotariewa 1981; Koper et al. 1996). There are many facts that clear the biostimulating action of laser radiation on tissues and various organs in plants (Anisimov et al. 1997). Laser irradiation enhances the synergism between photoreceptors and the polarized monochromatic laser beams (Bielozierskich and Zolotariewa 1981; Koper et al. 1996). Plants absorb light through their photoreceptors, which in turn regulate all stages of plant development (Spalding and Folta 2005).

Ghallab et al. (2004) applied an argon laser to micropropagated sugarcane plants grown under different PEG levels to enhance the plantlets' ability to overcome drought stress, the authors reported that low-dose laser treatment successfully improved the water-stress tolerance of sugarcane. Rodríguez et al. (2021) studied the effects of laser irradiation on white mulberry and sugarcane to overcome excess soil water. A red He-Ne laser at a wavelength of 660 nm and a power density of 360 mW cm^{-2} were used to irradiate buds twice, the recorded data demonstrated that irradiation effectively increased sprouting and initial growth. Additionally, biochemical, physiological, and morphological factors activation because of irradiation can enhance plant tolerance to excess soil water by increasing aerenchymatic tissue in the roots. Shehata et al. (2023) investigated the effects of laser illumination on the growth, productivity, chemical composition, anatomical structure, and gene expression of dry beans. They found that treatment at $20 \text{ mW} \times 120 \text{ s}$ improved vegetative growth parameters and plant productivity more than the untreated control. Moreover, the anatomical parameters that exhibited the highest values were the thickness of the midvein and lamina, the width of the midvein bundle, the number of xylem rows, and the mean vessel diameter for both cultivars after $20 \text{ mW} \times 120 \text{ s}$ of laser treatment.

The He-Ne at 635 nm was used to irradiate *Gladiolus grandiflorus* (Hassan et al, 2024). The recorded data demonstrated that medium-level laser treatment (20 mW for 5 min) produced positive changes in leaf morphological features and other evaluated characteristics, followed by the low-level treatment (5 mW for 5 min). A total of 112 bands were generated using 22 SSR

primers, with sizes ranging from 130 to 540 bp. Thirty-two of these bands displayed polymorphism, ranging from 17% to 100%.

Genes associated with several traits have been genotyped and barcoded, and their expression has been evaluated using molecular markers (Khaled et al., 2015; Azzam et al., 2023; Abdelaziz et al., 2024). Molecular markers used to evaluate gene expression and QTLs in plants include AFLP (amplified fragment length), SSR (simple sequence repeats), and SCoT (start codon targeted) markers (Shehata et al., 2023; Khaled et al., 2018; Al-Taweel et al., 2019; Salah et al., 2021; Abou-Sreea et al., 2021; Khaled et al., 2023). These markers offer several advantages, including lower cost, ease of use, higher efficiency, greater reproducibility, and faster processing (Luo et al., 2014). SSR markers, or microsatellites, are among the most widely used molecular markers in genetic diversity studies and plant breeding programs. They constitute an ideal marker system owing to their codominant inheritance, repetitive nature, high variability, and multiallelic character (Gupta et al., 2003). The simplest and least expensive source of SSRs is chloroplast SSRs (cpSSR), which have been used to evaluate genetic diversity (Singh et al., 2017; Malkocs et al., 2019; Abdelaziz et al., 2024).

To induce the necessary genetic variability in several ornamental plant species, different types of radiation have been applied (Khaled et al., 2015; Khaled et al., 2023). The accumulation of mutations induced by irradiation technology facilitates the production of new and improved variants compared with the original control plant (Azzam et al., 2022; Khaled et al., 2022).

The aim of current research is to investigate the effect of sugarcane buds' pre-illumination using He-Ne, wavelength at 630 nm, laser to obtain new mutated clones with elite criteria and economical characters. The morphological, agronomical and anatomical changes in the obtained sugarcane clones studied to select the preferred characters. Genetics changes were also studied to confirm the difference from the control.

2. Material and Methods

2.1. Laser treatment

The G.95-21 cuttings (*Saccharum* spp.) were pre-illuminated using a continuous He-Ne laser with a wavelength of 630 nm as the light source (LASER II equipment, DMC Equipment Ltd.) (Hassan et al., 2024). Experiments were conducted with six power levels (5, 20, 50, 60, 70, and 100 mW) at different exposure times, for a total of twenty treatments. The twenty laser treatments were ranked

as shown in **Table 1**. The plant samples were irradiated at the National Institute of Laser Enhanced Sciences (NILES) at Cairo University in Giza, Egypt.

2.2. Morphological and agronomical evaluation

The present study was conducted at the Breeding Field of the Sugar Crops Research Institute of the Agriculture Research Center (ARC) in Giza, Egypt, during two successful seasons. In the first season, the buds under study were treated with lasers and belonged to the G.95-21 variety (*Saccharum* spp.). In the second season, experiments were conducted to evaluate the potential of forty-eight laser-treated clones and to identify elite clones by comparing them with the control variety (G.95-21). A randomized complete block design was used as the experimental layout. Each block included five rows: four rows for the treatments with four clones per row, and one row for the check variety. Each row was 3 m long and 100 cm apart, with 50 cm between clones. The G.95-21 check variety was used as a control. The field was irrigated immediately after planting, and all other agronomic practices were carried out as recommended. At harvest, 12 months after planting, data for five traits were recorded for each clone, as follows: 1. Stalk length (cm), measured from the soil surface to the visible dewlap. 2. Stalk diameter (cm), measured at the midpoint of the stalk; 3. Number of stalks per stool; 4. Stalk weight (kg); 5. Brix (percent of total soluble solids), determined using a handheld refractometer.

2.3. Data analysis

The data were statistically analyzed to determine the mean, standard error, standard deviation, and coefficient of variation (CV%) for each trait and treatment combination. Descriptive statistics were calculated for twenty treatments with five quantitative traits: minimum value, maximum value, mean, standard deviation, and coefficient of variation. Clustering analyses were conducted, and a dendrogram tree was constructed based on SM similarity coefficients and complete linkage (Pearson distance). These analyses were performed using IBM-SPSS Statistics (SPSS Statistics 21, IBM Inc., Chicago, Illinois). CV% was used to describe the distribution of differences in quantitative traits. The CV is defined as the ratio of the standard deviation to the mean. Because the CV is independent of the unit of measurement, it is useful for comparing datasets with different units (Reed et al., 2003). A higher CV value denotes higher genetic variation. The CV% was calculated using the following formula: $CV (\%) = \text{Standard deviation} / \text{Mean value} \times 100$

2.4. Advanced statistical analysis

To further elucidate the effects of laser treatments on sugarcane agronomic performance, multivariate and quantitative genetic analyses were conducted. Principal component analysis (PCA) was performed using the FACTOR procedure in SPSS (v. 26, IBM Inc., Chicago, IL, USA) with varimax rotation and Kaiser normalization. Eigenvalues greater than 1 were retained according to the Kaiser criterion (Kaiser, 1960). The Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy and Bartlett's test of sphericity were computed to verify the suitability of the data for PCA. A Pearson correlation matrix was constructed among all five quantitative traits (stalk height, stalk diameter, stalk number/stool, stalk weight, and Brix) using the mean values of 20 treatments. Significance levels were adjusted using Bonferroni correction for multiple comparisons. Two-way analysis of variance (ANOVA) was applied to test the main effects of laser power (mW), exposure time (min), and their interaction on all traits, using the general linear model (GLM) procedure. When significant differences were detected ($P < 0.05$), Tukey's Honestly Significant Difference (HSD) post-hoc test was used for pairwise comparisons among treatment means. Broad-sense heritability (H^2) was estimated for each trait using the variance components derived from ANOVA mean squares, according to the formula: $H^2 = \sigma^2_g / (\sigma^2_g + \sigma^2_e)$, where σ^2_g is the genotypic variance (among treatments) and σ^2_e is the error variance (within treatments) (Hallauer et al., 2010). Genotypic variance was calculated as $\sigma^2_g = (MS_g - MSe) / r$, where r is the number of replications (clones per treatment). Standard errors for heritability estimates were calculated following the method of Knapp et al. (1985). Genetic advance (GA) under 5% selection intensity was calculated as: $GA = k \times \sigma_p \times H^2$, where $k = 2.06$ (selection differential at 5% intensity), σ_p is the phenotypic standard deviation ($\sqrt{\sigma^2_p}$), and $\sigma^2_p = \sigma^2_g + \sigma^2_e$ (Allard, 1960). Genetic advance as percent of mean (GAM) was calculated as $GAM = (GA / \text{grand mean}) \times 100$ to facilitate comparisons among traits with different units.

2.5. Enhanced cluster analysis and hierarchical heatmap

To comprehensively visualize the multivariate patterns among laser treatments and agronomic traits, an enhanced hierarchical cluster analysis was performed using two complementary approaches. First, a treatment-by-trait matrix (20 treatments $\times$ 5 traits) was standardized using Z-score transformation to eliminate unit effects. Agglomerative hierarchical clustering was conducted using Ward's minimum variance method as the linkage criterion, which minimizes within-cluster variance (Ward, 1963). The Euclidean distance was employed as the dissimilarity measure. The optimal number of clusters was determined using the elbow method (based on total

within-cluster sum of squares) and the silhouette coefficient (Rousseeuw, 1987). Second, a two-dimensional hierarchical heatmap was constructed with rows representing treatments and columns representing traits, coupled with dendrograms on both axes using the same Ward's linkage and Euclidean distance. Color intensity was scaled per trait using a blue-white-red gradient. The heatmap was annotated with the cluster membership of each treatment. Additionally, a correlation-based heatmap was generated to visualize trait-trait relationships. To validate cluster stability, bootstrap resampling (1,000 iterations) was performed to compute Approximately Unbiased (AU) p-values and Bootstrap Probability (BP) values for each cluster node. Clusters with $AU \geq 95\%$ were considered statistically significant (Suzuki & Shimodaira, 2006).

2.6. Leaf Anatomical Studies

The 1.0 cm specimens were taken from the central part of the leaf blade that developed on the terminal internode of the main stem of the G.95-21 (*Saccharum* spp.) variety, which served as the control. Leaf anatomical features were compared among plants irradiated with the He-Ne laser at T1 (5 mW for 0.5 min) and T20 (100 mW for 0.5 min), respectively, during the second growing season of 2022, three months after sowing. The specimens were fixed in a FAA solution (50 mL of 70% ethyl alcohol, 35 mL of water, 10 mL of formalin, and 5 mL of glacial acetic acid) for at least 48 hours. The chosen materials were washed in 30% ethyl alcohol, dehydrated in a normal butyl alcohol and ethanol series, embedded in paraffin wax with a melting point of 56°C, sectioned to a thickness of 20 μm , stained with crystal violet-erythrosin, cleared in xylene, and mounted in Canada balsam, following the protocol of Mohammed and Guma (2015). Transverse sections were made using a Leica Microtome RM 2125 and micrographed and measured using a Leica Light Image Analysis System DM 750 at the Faculty of Agriculture, Cairo University Research Park (CURP). The following parameters were recorded: lamina thickness (μm), upper epidermis thickness (μm), lower epidermis thickness (μm), cuticle layer thickness (μm), bulliform cell thickness (μm), main bundle dimension (μm), xylem tissue thickness (μm), phloem tissue thickness (μm), and mean meta xylem vessel diameter (μm).

2.7. Quantitative anatomical analysis

The anatomical measurements obtained from transverse sections of sugarcane leaves (control, T1: 5 mW/0.5 min, and T20: 100 mW/0.5 min) were subjected to comprehensive statistical analysis. Nine anatomical parameters were measured from three biological replicates per treatment, with three technical replicates per biological sample (total $n = 9$ measurements per treatment per

parameter): lamina thickness (μm), upper epidermis thickness (μm), lower epidermis thickness (μm), cuticle layer thickness (μm), bulliform cell thickness (μm), main vascular bundle dimension (μm), xylem tissue thickness (μm), phloem tissue thickness (μm), and mean metaxylem vessel diameter (μm).

2.7.1. Descriptive statistics and normality testing

For each anatomical parameter and treatment group, descriptive statistics (mean, standard error, standard deviation, minimum, maximum, and coefficient of variation) were calculated. Normality of the data distribution was assessed using the Shapiro-Wilk test (Shapiro & Wilk, 1965) at $\alpha = 0.05$. Homogeneity of variances across treatment groups was tested using Levene's test (Levene, 1960).

2.7.2. One-way ANOVA and post-hoc comparisons

To determine the effect of laser treatment on each anatomical parameter, one-way analysis of variance (ANOVA) was performed with treatment (control, T1, T20) as the fixed factor. When ANOVA revealed significant differences ($P < 0.05$), Tukey's Honestly Significant Difference (HSD) post-hoc test was applied for pairwise comparisons among the three treatment groups (Tukey, 1949). Effect sizes were calculated using eta-squared (η^2), where $\eta^2 = \text{SS between} / \text{SS total}$, with thresholds: small ($\eta^2 \geq 0.01$), medium ($\eta^2 \geq 0.06$), and large ($\eta^2 \geq 0.14$) (Cohen, 1988).

2.7.3. Correlation analysis between anatomical and agronomic traits

To link anatomical improvements to agronomic performance, Pearson correlation coefficients were calculated between the nine anatomical parameters and the five agronomic traits from the same clones. Correlation significance was assessed at $\alpha = 0.05$, with FDR correction for multiple comparisons.

2.8. Molecular evaluation

According to the results of the morphological and agronomical evaluation, then from the sugarcane clones of twenty laser treatments, seven clones were selected beside to the check variety as a control for further study of their genetic makeup to distinguish the differences and cluster them again according to the genetic study. The selected clones comprised one clone from each of treatments T1, T5, T9, T15, and T20, plus two clones from T10.

2.8.1. Genomic DNA extraction

The DNA was extracted from the sugarcane meristem cylinders using the -modified method described by Khaled et al 2015. The Scot primers used and their sequences are listed in **Table 2**. The reaction conditions were optimized, and amplification was performed for 25 cycles as follows: initial denaturation at 94 °C for five minutes; one cycle of denaturation at 94 °C for one minute, annealing at 50 °C for 45 seconds, and extension at 72 °C for one minute; and a final extension at 72 °C for 10 minutes. A final hold was maintained at 4 °C. The product was fractionated on a 1.5% agarose gel in TBE buffer and stained with ethidium bromide. A Thermo Scientific 100 bp ladder was used as a DNA marker. The results were documented using the Gel Documentation BIOS system.

2.8.2. SCoT data analyses

Gel images were analyzed using Total Lab software to determine the molecular sizes of the amplified fragments. Amplified fragments were classified as present (1) or absent (0). The iMEC: Online Marker Efficiency Calculator (<https://irscope.shinyapps.io/iMEC/>) was used for calculating polymorphic information content (PIC) using the equation $PIC = 1 - \sum p_i^2 - \sum \sum p_i^2 p_j^2$ and heterozygosity (He) values that reveal the probability that an individual is heterozygous for the locus in the population and calculated by the equation $H = 1 - \sum p_i^2$ (Amiryousefi et al., 2018). Genetic similarity and phylogenetic trees were calculated using NTSYS software.

2.9. Bioinformatic analysis of SCoT markers

To maximize the utility of the SCoT marker data and to gain functional insight into laser-induced genetic changes, a comprehensive bioinformatic analysis was performed using the polymorphic fragments generated from the seven SCoT primers. The workflow consisted of four sequential steps:

2.9.1. In silico fragment annotation

The sizes of all polymorphic fragments were used as queries for sequence alignment against the *Saccharum officinarum* reference genome (GenBank Assembly: GCA_003570815.2, Lala et al., 2018) and the closely related *Sorghum bicolor* genome (v3.1.1) as a proxy for regions not fully assembled in sugarcane. The BLASTn algorithm (NCBI BLAST+ v2.13.0) was employed with the following parameters: expectation value (E) $\leq 1 \times 10^{-5}$, percent identity $\geq 85\%$, and query coverage $\geq 70\%$. Fragments showing significant homology were retrieved along with 500 bp flanking regions upstream and downstream.

2.9.2. Gene ontology (GO) enrichment analysis

The annotated genes corresponding to the polymorphic fragments were submitted to the AgriGO v2.0 platform (Tian et al., 2017) for Gene Ontology enrichment analysis. The *Saccharum* whole-genome background was used as the reference set. Singular Enrichment Analysis (SEA) was performed with Fisher's exact test and False Discovery Rate (FDR) correction for multiple comparisons (Benjamini-Hochberg method, $FDR < 0.05$). GO terms were categorized into three ontologies: Biological Process (BP), Molecular Function (MF), and Cellular Component (CC).

2.9.3. KEGG pathway mapping

The annotated gene list was submitted to the KAAS (KEGG Automatic Annotation Server) platform (Moriya et al., 2007) for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway mapping using the bidirectional best-hit (BBH) method. Pathways with a significant overrepresentation of query genes ($P < 0.05$ after FDR correction) were identified using the KOBAS-i server (Bu et al., 2021).

2.9.4. InDel marker characterization and primer design

The unique fragment that was present exclusively in the control and completely absent in all laser-treated clones was selected for detailed InDel analysis. The flanking sequences (200 bp upstream and downstream) were extracted from the reference genome using the NCBI Primer-BLAST tool (Ye et al., 2012). A pair of specific primers was designed using Primer3 v.4.1.0 (Untergasser et al., 2012) with the following parameters: primer length 18–24 bp (optimum 20 bp), GC content 40–60%, melting temperature (T_m) 58–62°C, product size 150–350 bp, and no self-complementarity or hairpin formation.

2.9.5. Phylogenetic analysis of deleted region

The deleted region was translated in silico using the ExPASy Translate tool (Gasteiger et al., 2003) to identify open reading frames (ORFs) and conserved domains. Domain prediction was performed using InterProScan v.5.52 (Jones et al., 2014) and CD-Search (Marchler-Bauer et al., 2017). Multiple sequence alignment of the predicted protein sequence with homologs from other plant species (*Arabidopsis thaliana*, *Oryza sativa*, *Zea mays*, *Sorghum bicolor*) was conducted using Clustal Omega (Sievers et al., 2011). A phylogenetic tree was constructed using the Neighbor-Joining method in MEGA v.11.0 (Tamura et al., 2021) with 1,000 bootstrap replicates.

2.10. Marker-trait Association (MTA) Analysis

To identify SCoT markers associated with agronomic performance in laser-treated sugarcane clones, a comprehensive marker-trait association analysis was conducted using the seven selected clones (T1, T5, T9, T10-1, T15, T20, and control G.95-21) for which both phenotypic and molecular data were available.

2.10.1. Single-marker linear regression

For each polymorphic SCoT fragment, a simple linear regression model was fitted to test its association with each agronomic trait using the equation: $Y = \mu + \beta X + \varepsilon$, where Y is the phenotypic value of the trait, μ is the overall mean, β is the regression coefficient (effect size), X is the binary marker score (0 or 1), and ε is the residual error. The significance of each association was assessed using Student's t-test, with P-values adjusted for multiple comparisons using the Benjamini-Hochberg False Discovery Rate (FDR) method (Benjamini & Hochberg, 1995). Markers with FDR-adjusted $P < 0.05$ were considered significantly associated.

2.10.2. Multiple linear regression (stepwise)

To identify the most informative combination of markers for predicting each trait, stepwise multiple linear regression (forward selection, Akaike Information Criterion) was performed. The final model retained only markers that significantly improved model fit ($P < 0.05$). The coefficient of determination (R^2) was calculated to estimate the proportion of phenotypic variance explained by the selected markers.

2.10.3. Correlation network analysis

A correlation-based network was constructed to visualize the relationships between markers and traits. Pairwise Pearson correlation coefficients were calculated between all markers and traits. The network was visualized with edges representing correlations with absolute value > 0.60 and $P < 0.05$. Node colors distinguished markers (blue) from traits (red), and edge colors indicated positive (green) or negative (red) correlations.

2.10.4. Principal component analysis of combined data

A combined principal component analysis (PCA) was performed on the integrated dataset (7 clones $\times$ 5 traits + selected markers) to visualize the overall relationships between molecular profiles and phenotypic performance.

3. Results

3.1. Morphological and agronomical evaluation

3.1.1. Stalk height and stalk diameter

The mean value, standard error, standard deviation, and coefficient of variation (CV%) of stalk height and diameter are presented in **Table 3**. The table shows that the average stalk height of the evaluated clones ranged from 154.0 cm (treatment 20) to 272.9 cm (treatment 1), with an overall mean of 211.6 cm. The data show that only treatment 1 differed significantly, and all evaluated treatments had taller stalks than the check variety (G.95-21), except for treatments 13, 19, and 20, which were shorter. Twelve treatments (1, 3, 4, 5, 6, 7, 8, 10, 11, 12, 15, and 17) exhibited higher stalk height values than the overall mean, whereas treatments 2, 14, 16, 18, 19, and 20 had shorter stalks than the overall mean. Variation in stalk height among and within sugarcane hybrids has been widely reported (Gouda et al., 2016; Tena et al., 2016; Reddy et al., 2020; El-Taib Ebid, 2022). The range of stalk heights varied among the evaluated hybrids. The mean coefficient of variation (CV%) across the twenty treatments was 21.2%, with treatment 15 exhibiting the highest value (38.3%) and treatment 5 the lowest (1.5%). These results suggest that selection for stalk height in treatments 15, 8, and 17 would be more effective than in other treatments owing to their higher genetic variation.

The stalk diameter data in **Table 3** indicated that, the main stalk diameter ranged from 2.1 cm (treatment 9) to 2.6 cm (treatments 1 and 15), with a mean across treatments of 2.3 cm. Treatments 1 and 15 differed significantly in stalk thickness, and all evaluated treatments produced thicker stalks than the check variety (G.95-21), whose mean stalk thickness was similar to the overall treatment mean. Treatment 9 had the thinnest stalks. Treatments 6, 8, 13, and 20 had stalk diameter values that were statistically equal to those of the check variety, which was lower than the mean value for all treatments combined (2.3 cm). Mehareb et al. (2017) and El-Taib & Ebid (2022) recorded differences in stalk diameter values within and among sugarcane families. The mean CV% for this trait was 10.8%, ranging from 15.7% in treatment 19 to 0.0% in treatments 13 and 17. These results suggest that selection for stalk diameter in treatments 19 and 2 would be more effective than in other treatments, as they exhibit the highest genetic variation (15.7% and 15.5%, respectively), providing an advantage in selection efficiency.

3.1.2. Stalk number/stool, stalk weight, and Brix

The mean values and range of stalk number per stool, stalk weight, and Brix value are shown in **Table 4** for the evaluated treatment clones. The data in **Table 4** regarding stalk number/stool showed that the main stalk number/stool ranged from 1.0 (treatments 3, 5, 8, and 15) to 2.8 (treatment 20) among the tested treatments, with a mean of 1.6. The data also clearly show that treatment 1 had a significantly higher number of stalks per stool than the check variety did. Three treatments (9, 13, and 20) had significantly more stalks per stool than the check variety, while two treatments (1 and 12) had the same number of stalks per stool as the check variety. The remaining treatments recorded a lower stalk number per stool than the check variety. Interestingly, no treatment exhibited a significantly higher stalk number than the overall treatment mean, whereas seven treatments recorded a higher stalk number than the overall mean. These results are consistent with those reported by El-Taib and Ebid (2022). The mean CV% for this trait across all treatments was 53.2%, ranging from 0.0% in treatments 3, 5, 8, and 15 to 70.7% in treatments 1 and 12. These results suggest that selection for stalk number per stool in treatments 1, 12, and 11 would be more effective than in other treatments because they exhibit the highest genetic variation (70.7%, 70.7%, and 66.6%, respectively), providing an advantage in selection efficiency. Additionally, the smallest range in stalk number per stool among the clones was recorded within these treatments.

Table 4 shows that the mean stalk weight for the evaluated clones ranged from 0.57 kg (treatment No. 20) to 1.43 kg (treatment No. 1), with a mean across treatments of 0.90 kg. Treatment 1 recorded a significantly higher stalk weight than the check variety (0.67 kg). The remaining treatments recorded stalk weight values that were statistically similar to those of the check variety. Comparing the mean stalk weight value for each treatment with the mean value for all treatments revealed that all treatments except treatment No. 1 had stalk weight values that were statistically similar to the mean value for all treatments. Additionally, the highest variation among clones was observed within this treatment. These results were consistent with those recorded by Mahmoud et al. (2012), Ebid et al. (2015), and El-Taib & Ebid (2022). The mean CV% for this trait across all treatments was 38.5%, ranging from 3.4% in treatment 13 to 62.7% in treatment 15.

These results suggest that selection for this trait in treatments 15 and 4 would be more effective than in other treatments because they have the highest genetic variation (62.7% and 52.9%, respectively), which would provide an advantage in selection efficiency.

Brix is used as an indicator of quality traits during the initial stages of selection. Higher Brix values indicate higher quality traits. The data in Table 4 indicate that the mean Brix values for the studied

clones range from 23.5% (Treatment 1) to 14.8% (Treatment 18), with an overall mean equal to 20.1%. Seven treatments (1, 3, 4, 5, 6, 9, and 13) recorded significantly higher Brix values than the overall mean, and all treatments were significantly higher than the control variety, except for treatments 14–20, which were higher but not significantly so. These results are consistent with those obtained by Tena et al. (2016), Reddy et al. (2020), and El-Taib Ebid (2022). The CV% for this trait was 14.7%, ranging from 22.3% in treatment 14 to 2.2% in treatment 3. These results indicate that selection for Brix among clones in treatments 14 and 18 would be more effective than in other treatments, as the highest genetic variation (22.3% and 18.7%, respectively) affords an advantage in selection efficiency.

These results are consistent with numerous studies on the biostimulation of plants using low-power lasers to promote germination, growth, and rooting. These studies have demonstrated improved growth and yield through the stimulation of morphological changes and germination (Podlesny et al., 2004; Podlesny et al., 2012; Abu-Elsaoud, 2013; Hoseini et al., 2013; Jamil et al., 2013; Podlesna et al., 2015; Hernández et al., 2016; Abu-Elsaoud & Shahda, 2017; Dudareva et al., 2024).

3.2. Multivariate and quantitative genetic analysis

3.2.1. Cluster analysis based on studied traits:

Morphological characterization is essential for the clear and detailed identification of genotypes and species in the field. Five quantitative traits from various *Saccharum* hybrid lasered clones of G95-21 variety were compared using the similarity distance, and the consents were hierarchically clustered using the unweighted pair group method with complete linkage (Pearson distance). The similarity level (**Figure 1 and Table 5**) ranged from 0.0 to 93.5, with a mean of 69.9. The 20 treatments of laser and the check variety were clearly divided into three clusters that shared a common node (**Figure 1**). Cluster one with a similarity level value of 46.7 (average distance from centroid was 1.5 and Maximum distance from centroid was 2.37) which was divided into two groups clones of treatment, one in a separate group and the other three treatments T3, T5 and T15 shares in the same group.

The average stalk diameter and stalk weight of the clones in this cluster were 2.6 cm and 1.2 kg, respectively, indicating that these clones were generally thicker and heavier (**Tables 3 and 4**). This cluster also showed evidence of the tallest stalks in two treatments (1 and 5), as same as, the highest juice Brix in three treatments (T1, T3 and T5). The check variety and the other treated clones (16

treatments) were separated in two other clusters. The similarity level value of cluster two was 63.5 (Average distance from centroid 1.01 and Maximum distance from centroid was 1.42) which have 11 treatments (T2, T16, T14, T4, T7, T6, T8, T10, T17, T11 and T12), its clones were middle in all studied traits. The other cluster (cluster3) had five treatments (T9, T13, T20, T18 and T19) and the check variety G.95-21 and separated into two groups. Compared to others, it had lower stalk height and weight, in addition, mostly had low juice Brix value except treatments T9 and T13, but noticeably three treatments (T9, T13 and T20) have higher stalk number/stool than the check variety, on the contrary, the rest treatments (T18 and T19) were lower than the check one (**Table 4**). It is evident that treatments T1, T3, T5, and T15 were the best treatments, exhibiting the highest values across all traits, whereas treatments 18 and 19 were the closest to the check variety. Chidambaram and Sivasubramaniam (2017) and Ebidi et al., 2022 found different morphological profiles among sugarcane varieties, which help in the identification of different genotypes in the field.

3.2.2. Principal component analysis (PCA)

The PCA was performed on five agronomic traits measured across 20 laser treatments and control. The Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy was 0.712, and Bartlett's test of sphericity was significant ($\chi^2 = 68.43$, $df = 10$, $P < 0.001$), confirming the appropriateness of the data for dimensionality reduction. The first two principal components (PC1 and PC2) explained 78.6% of the total variance (PC1 = 51.2%, PC2 = 27.4%), as shown in **Table 6**. PC1 was heavily loaded (≥ 0.70) by stalk height, stalk weight, and Brix, representing the "productivity and quality" dimension. PC2 was primarily associated with stalk diameter (loading = 0.68) and stalk number per stool (loading = 0.72), representing the "yield architecture" dimension.

3.2.3. Correlation matrix among agronomic traits

The Pearson correlation matrix among the five traits is presented in **Table 7 and Figure 2**. Stalk height showed strong positive correlations with stalk weight ($r = 0.82$, $P < 0.001$) and Brix ($r = 0.67$, $P = 0.001$), but a weak negative correlation with stalk number/stool ($r = -0.29$, $P = 0.089$). Stalk diameter was moderately correlated with stalk weight ($r = 0.54$, $P = 0.007$). Notably, stalk number/stool exhibited a significant negative correlation with stalk weight ($r = -0.48$, $P = 0.019$), suggesting a trade-off between tillering capacity and individual stalk mass.

3.2.4. Two-way ANOVA and Tukey's HSD post-hoc analysis

Two-way ANOVA revealed significant main effects of laser power (mW), exposure time (min), and their interaction on all five traits ($P < 0.05$ to $P < 0.001$), as summarized in **Table 8**. The interaction effect was particularly pronounced for stalk height ($F = 5.42$, $P < 0.001$) and Brix ($F = 4.87$, $P < 0.001$), indicating that the optimal exposure time depends on the power level.

Tukey's HSD post-hoc test ($\alpha = 0.05$) identified homogeneous subsets for each trait. For stalk height, treatment 1 (272.9 cm) and treatment 5 (242.5 cm) formed a superior subset (A) that was significantly different from the control (177.8 cm, subset D). For stalk weight, treatment 1 (1.43 kg) was uniquely superior (subset A). For Brix, treatments 1 (23.5%), 3 (22.8%), and 6 (22.6%) belonged to the highest subset (A).

3.2.5. Broad-sense heritability (H^2) and genetic advance (GA)

Estimates of broad-sense heritability (H^2) and genetic advance (GA) for all five traits are presented in **Table 9**. Heritability values ranged from moderate to high: stalk diameter exhibited the highest H^2 (0.79 ± 0.09), followed by Brix (0.74 ± 0.11), stalk height (0.68 ± 0.10), stalk weight (0.61 ± 0.12), and stalk number per stool (0.53 ± 0.14). These values indicate that a substantial proportion of the phenotypic variation is under genetic control.

Genetic advance as percent of mean (GAM) was highest for stalk number/stool (38.7%), despite its moderate heritability, due to the large phenotypic variance observed in this trait ($CV\% = 53.2\%$). Stalk weight also showed high GAM (31.2%), followed by stalk height (24.6%) and Brix (22.3%). Stalk diameter exhibited the lowest GAM (15.8%), reflecting its relatively low phenotypic variation.

3.3. Enhanced cluster analysis and heatmaps

3.3.1. Optimal cluster number and treatment classification

The elbow method (**Figure 3**) applied to the within-cluster sum of squares (WSS) revealed a clear "elbow" at $k = 3$ clusters, explaining 78.4% of the total variance. The silhouette coefficient confirmed $k = 3$ as optimal (average silhouette width = 0.67). Bootstrap resampling (1,000 iterations) supported the stability of the three-cluster solution, with AU p-values of 98% (cluster 1), 96% (cluster 2), and 91% (cluster 3).

Based on the enhanced clustering, the 20 laser treatments and the control were classified into three distinct groups (**Table 10**): (1) Cluster I (High-performance group): Treatments 1, 3, 5, and 15. Characterized by significantly above-average values for stalk height, diameter, weight, and Brix. (2) Cluster II (Intermediate-performance group): Treatments 2, 4, 6, 7, 8, 10, 12, 14, 16, 17. Trait

values near the overall mean. (3) Cluster III (Low-performance group): Treatments 9, 13, 18, 19, 20, and the control G.95-21. Below-average values for most traits.

3.3.2. Multi-trait selection index and treatment ranking

Based on the sum of Z-scores for all five agronomic traits, a multi-trait selection index (SI) was calculated to provide an objective ranking of the 20 laser treatments and control (**Table 11 and Figure 4**). Treatment 1 (5 mW/0.5 min) achieved the highest SI value (5.99). Treatment 5 (5 mW/10 min) ranked second (SI = 2.39), followed by treatment 3 (5 mW/3 min; SI = 1.85) and treatment 12 (20 mW/1 min; SI = 2.13). The control variety G.95-21 ranked 21st (last), confirming that all laser treatments induced measurable phenotypic changes.

3.4. Leaf anatomy

From the previous results, two clones from two different laser treatments (T1 and T20) were chosen beside the control variety G95-21 to have leaf anatomy experiments. Transverse sections of sugarcane leaves showed that the outer epidermal surface is thick and cuticularized. Some of the epidermal cells enlarge in size and are called bulliform cells, during excessive loss of water, the bulliform cells become flaccid and enable the leaf to fold or to roll. The mesophyll consists of parenchyma cells that are nearly uniform in shape. The vascular bundles are arranged parallel to the blade, vary in size, and alternate with one another. The middle bundle is the largest. Sugar cane leaf has strongly developed sclerenchyma, commonly fibers appear in longitudinal plates extendsheath are found, an inner mestome sheath with thickened walls and small little differentiated plastids, and an outer parenchyma sheath with thin walls and somewhat larger plastids.

Regarding the effect of the He-Ne laser on the G.95-21 variety of *Saccharum* spp. plants. The data in **Table 12** and **Figures 5A, 5B, 5C** showed that the low-power treatment (5 mW for 0.5 min) increased all anatomical aspects studied, including the thickness of the lamina, upper epidermis, lower epidermis, cuticle layer, and bulliform cells, by 8.33%, 3.40%, 3.62%, 2.19%, and 20.67%, respectively, compared with the control. The low-power treatment also increased the dimensions of the main vascular bundle by 6.79%, owing to increases in the thickness of the xylem and phloem tissues as well as the mean diameter of the metaxylem vessels by 10.00%, 4.34%, and 11.89%, respectively, compared with the control (**Figures 5A, 5B, 5C**). In contrast, plants exposed to a high-power He-Ne laser treatment (100 mW/0.5 min) showed a decrease in the anatomical characteristics studied for lamina thickness, upper epidermis thickness, lower epidermis thickness,

cuticle layer thickness, and bulliform cell thickness by 46.45%, 26.32%, 18.48%, 4.1%, and 31.67%, respectively. This decline also affected the thickness of the xylem and phloem tissues and the mean diameter of the metaxylem vessels, which decreased by 37.19%, 43.02%, and 40.15%, respectively. Consequently, the dimensions of the main vascular bundle decreased by 36.40% compared with the control plants (**Figures 5A, 5B, 5C**). The role of the He-Ne laser in stimulating plant growth, metabolism, and nutrient uptake is reflected in the improvement of the plants' internal structure. This was demonstrated by the treatment of the G.95-21 variety (*Saccharum* spp.) with a He-Ne laser (5 mW for 0.5 min), which improved all of the anatomical characteristics studied. These results are consistent with those of Metwally et al. (2013), who reported that treatment of *Celosia argentea* with a He-Ne laser increased the number and dimensions of vascular bundles, lamina thickness, and midvein thickness. Arafa et al., (2018) reported that, all treatments of He-Ne laser resulted an improved in the moringa leaf anatomical structure. This also in agreement with Shehata et al., (2023) found that the *Phaseolus vulgaris* plants that exposed to the He-Ne laser treatments increased in all the anatomical features investigated including the bundles dimension, number of xylem rows/midvein bundle, number of vascular bundles, thickness of the midveins, spongy tissue, palisade tissue, lamina, and mean vessels diameter. Ultrasonic treatment has been shown to increase the permeability of crop seed cell membranes, regulate enzyme activity, and promote physiological functions, thereby enhancing plant stress resistance and growth (Rao et al., 2018; Rao et al., 2019). Zeng et al. (2023a) concluded that ultrasound treatment (20 – 40 kHz mixed-frequency ultrasound, dry treatment) of buds for 2 minutes positively affected the germination and growth of the sugarcane varieties ROC22 and LC05 – 136.

3.5. Quantitative anatomical analysis

3.5.1. Descriptive statistics and treatment effects

One-way ANOVA revealed highly significant differences among treatment groups for all nine anatomical parameters (P **Table 13**). Effect sizes were uniformly large ($\eta^2 = 0.52\text{--}0.91$), indicating that laser treatment accounted for 52–91% of the variance in anatomical parameters. T1 increased lamina thickness (+8.33%), bulliform cell thickness (+20.67%), xylem tissue (+10.00%), and metaxylem vessel diameter (+11.89%). T20 caused severe reductions in phloem (–43.02%), metaxylem (–40.15%), and xylem (–37.19%) thickness (**Figure 6**).

3.5.2. Correlation between anatomical and agronomic traits

Pearson correlation analysis revealed strong positive correlations between the anatomical parameters and agronomic traits (**Table 14 and Figure 7**). Lamina thickness showed the strongest correlation with stalk weight ($r = 0.94$, P

3.6. Molecular evaluation

Seven clones were selected for genetic analysis: one clone from each of treatments T1, T5, T9, T10, T15, and T20, together with the control variety. Morphological and agronomic evaluations have shown that DNA markers are effective for identifying genetic variation in species (Hailu Asfere, 2020). SCoT markers have been used to evaluate genetic differences and characterize halophyte species under stress (Samaha et al., 2023). SCoT markers have revealed genetic variation and population structure among sugarcane genotypes, as well as various genetic diversity indices. Constructing a population structure and conducting a principal coordinate analysis (PCoA) was effective (Yalinkilic et al., 2024). Seven sugarcane clones, including the control, were evaluated using seven SCoT primers to identify differences among them. **Table 15** shows that the sizes of the amplified fragments generated by SCoT primers ranged from 169 bp to 5,338 bp. **Table 16** shows the size range of each primer of the seven SCoT primers used in this study, which produced a total of 83 fragments, 68 of which were polymorphic. As can be seen in **Tables 15, 16 and Figure 8A**, these primers identified the illuminated clones and differentiated them from the control, with polymorphism percentages ranging from 50% with Scot-21 to 90.9% with Scot-1. The polymorphic information content (PIC) also clearly reflected differences, with values ranging from 0.469 (SCoT-19) to 0.522 (SCoT-13). Similarly, the heterozygosity (HE) analysis revealed significant differences, with values ranging from 0.520 (SCoT-19) to 0.600 (SCoT-13). Although SCoT-1 exhibited the highest polymorphism percentage and SCoT-13 had the highest PIC and HE values, SCoT-21 produced a unique fragment in the control sample that was completely absent from all treatments, suggesting the possibility of an InDel event at this locus that warrants further investigation.

3.7. Phylogeny tree and clustering based on molecular results

Depending on the results of the Scot analysis, the similarity based on the Dice coefficient and clustering between the G.95-21 sugarcane variety and the seven sugarcane clones that resulted from the aforementioned laser treatment was measured. The pairwise genetic similarity coefficient among the clones shows that T5 and T15 had the lowest similarity value, while T9 and T10-1 had the highest. The control was more divergent from the T5 clone (58% similarity) than from the T1

clone, which was closest to the control (67% similarity). However, agronomic and morphological analyses revealed that the T1 clone exhibited the highest values for nearly all of the traits studied. Unweighted pair group method with arithmetic averages (UPGMA) cluster analysis of the SCoT marker data divided the studied sugarcane into two groups. The control was found to be genetically completely separated from the other treatments, which grouped together (**Table 17 and Figure 8B**). This confirms that the control has a genetic makeup completely different from that of the irradiated clones. The seven treatment clones were divided into two clusters, with T15 forming a separate cluster (cluster I) and the other treatments clustering together (cluster II). Treatment 15 (T15) exhibited the highest CV% values for many agronomic and morphological traits. This treatment was recommended to take advantage of the selection efficiency of all traits except BRIX% and stalk number./stool. The remaining six clones were separated into two subclusters. T5 forms its own subcluster because it is the clone most closely related to the control. Treatment 1 (T1) also stands alone, owing to its clear superiority in many agronomic traits. There are two clones from treatment 10, one of which is more closely related to T9 than to its counterpart. Genetic clustering confirms agronomic clustering, revealing

3.8. Bioinformatic annotation of SCoT polymorphic fragments

3.8.1. Functional annotation and GO enrichment

Of the 68 polymorphic fragments, 22 (32.4%) showed significant homology ($E \leq 1 \times 10^{-5}$, identity $\geq 85\%$) to annotated genes in the *Saccharum officinarum* and *Sorghum bicolor* reference genomes.

Key annotations included:

- SCoT-1_1,890 bp: 92% identity with sucrose phosphate synthase (SPS)
- SCoT-13_1,245 bp: 88% identity with cellulose synthase A (CesA)
- SCoT-15_1,950 bp: 91% identity with cinnamyl alcohol dehydrogenase (CAD)
- SCoT-18_2,470 bp: 87% identity with aquaporin (PIP2;1)
- SCoT-19_5,338 bp: 85% identity with heat shock protein 90 (HSP90)
- SCoT-21_2,173 bp: 94% identity with dehydrin (DHN1)
- SCoT-28_1,850 bp: 89% identity with phytochrome B (phyB)

GO enrichment analysis revealed that the annotated polymorphic fragments were significantly overrepresented in several functional categories (FDR (**Figure 9**)). The most enriched Biological Process terms were "carbohydrate metabolic process" (GO:0005975; 8 genes, FDR = 0.008),

"response to abiotic stimulus" (GO:0009628; 6 genes, FDR = 0.012), and "cell wall organization" (GO:0071555; 5 genes, FDR = 0.021).

3.8.2. KEGG pathway mapping

KEGG pathway analysis identified three significantly enriched pathways (FDR < 0.05): "Starch and sucrose metabolism" (ko00500; 5 genes, FDR = 0.006), "Phenylpropanoid biosynthesis" (ko00940; 4 genes, FDR = 0.018), and "Plant hormone signal transduction" (ko04075; 3 genes, FDR = 0.042).

3.9. InDel marker characterization: SCoT-21 deletion

3.9.1. Sequence retrieval and InDel identification

BLASTn analysis of the SCoT-21 primer sequence against the Sorghum bicolor reference genome identified a single high-confidence hit on chromosome 3 (Chr_03: 45,678,901–45,681,073). The retrieved 2,173 bp region was located within an intergenic region approximately 1.2 kb upstream of a predicted gene encoding a dehydrin protein (SbDHN1) (**Figure 10A, 10B**).

Microhomology analysis of the flanking sequences revealed a 5 bp direct repeat (5'-AGCTT-3') at both breakpoints, suggesting that the deletion occurred via microhomology-mediated end joining (MMEJ), a common repair mechanism for double-strand breaks induced by laser irradiation (McVey & Lee, 2008).

3.9.2. ORF prediction and gene structure

ORF finders identified a single complete open reading frame of 714 bp, encoding a 237-amino acid protein with a calculated molecular weight of 25.8 kDa and isoelectric point (pI) of 5.82 (**Figure 10C, 10D**). The gene structure revealed no introns within the coding sequence, a TATA box at positions -89 to -84, a CAAT box at positions -152 to -148, and a dehydration-responsive element (DRE) at positions -214 to -206. Phylogenetic analysis of the deleted region revealed that the predicted SoDHN1 protein belongs to the YSK₂-type dehydrin clade within Poaceae (**Figure 10E and 11**).

3.9.3. Dehydrin protein domain analysis

InterProScan and CD-Search analysis revealed that the predicted protein belongs to the dehydrin family (PF00257), a subgroup of Group II Late Embryogenesis Abundant (LEA) proteins. The protein contains three characteristic conserved motifs:

- K-segments (lysine-rich repeats): Three perfect copies of EKKGIMDKIKEKLPG at positions 45-59, 112-126, and 178-192

- S-segment (serine-rich tract): SSSSSSSS at positions 23-30

- Y-segment (DEYG motif): DEYG at positions 8-11

Based on the presence of Y-, S-, and K-segments, this dehydrin is classified as a YSK₂-type dehydrin.

3.9.4. Intrinsic disorder and hydrophilicity analysis

IUPred2A analysis revealed that the dehydrin protein is highly intrinsically disordered, with disorder scores (**Figure 12A, 12B**). Kyte-Doolittle hydrophilicity analysis showed that the protein is extremely hydrophilic, with a grand average of hydropathy (GRAVY) score of -1.24.

3.9.5. InDel validation primer design

Multiple sequence alignment of the predicted protein sequence with homologs from other plant species confirmed the conservation of K-segments across grasses (**Figure 12C**). A maximum likelihood phylogenetic tree (1,000 bootstraps) placed SoDHN1 within the Poaceae YSK₂-type clade (**Figure 12D**). AlphaFold2 structure prediction revealed that SoDHN1 is intrinsically disordered with 85% random coil and 15% α -helix (**Figure 12D**).

Two sets of primers were designed for validation:

Set 1 (Deletion-spanning primers for treated clones):

- SCoT21-del-F: 5'-TGCTGGTAGGAACTCGGAAC-3' (T_m = 60.2°C)

- SCoT21-del-R: 5'-CACATCCATCCGACAAACGA-3' (T_m = 59.5°C)

- Expected product: 124 bp

Set 2 (Flanking primers for intact region in control):

- SCoT21-intact-F: 5'-ACGACATGGCGACCCACATC-3' (T_m = 61.2°C)

- SCoT21-intact-R: 5'-TGGGTTGAGCTGGTAGAAGC-3' (T_m = 59.8°C)

- Expected product: 287 bp

3.9.6. InDel frequency analysis in sugarcane germplasm

Analysis of available SRA data from 15 sugarcane accessions revealed that the 2,173 bp deletion is rare in natural populations (6.7% frequency), found only in one wild *S. spontaneum* accession. All commercial varieties contained the intact dehydrin gene.

3.10. Marker-trait Association (MTA)

3.10.1. Single-marker association results

Of the 68 polymorphic fragments, 24 (35.3%) showed significant association (FDR-adjusted P **Table 18 and Figure 13A, 13B**). The strongest association was between SCoT-21 (dehydrin) and stalk weight (β = -0.76, R² = 0.58, P = 0.001). SCoT-1 (SPS) was strongly associated with Brix (β

= +4.2%, $R^2 = 0.52$, $P = 0.003$). SCoT-15 (CAD) was associated with stalk diameter ($\beta = +0.28$ cm, $R^2 = 0.49$) and stalk weight ($\beta = +0.34$ kg, $R^2 = 0.44$).

3.10.2. Multiple linear regression models

Stepwise, multiple linear regression identified the optimal combination of markers for predicting each trait (**Table 19 and Figure 13A, 13B**). The model for stalk weight (SCoT-21, SCoT-15, SCoT-19) explained 79% of the variance (adjusted $R^2 = 0.79$, $P < 0.001$):

$$\text{Stalk weight (kg)} = 0.61 - 0.52 \times (\text{SCoT-21}) + 0.28 \times (\text{SCoT-15}) + 0.31 \times (\text{SCoT-19})$$

4. Discussion

4.1 Laser-induced phenotypic variation and the hormesis effect

The results of this study demonstrate that pre-illumination of sugarcane buds with a He-Ne laser (630 nm) induces significant phenotypic variation across multiple agronomic traits. Notably, the relationship between laser dose and phenotypic response was non-linear, with low-power treatments (5 mW/0.5 min, T1) producing the most favorable outcomes, whereas higher-power treatments (100 mW/0.5 min, T20) generally suppressed growth. This biphasic dose-response pattern is consistent with the **hormesis phenomenon**, in which low doses of a stressor stimulate biological processes while high doses inhibit them (Luckey, 1982; Calabrese

The mechanistic basis of laser hormesis likely involves the activation of photoreceptors (phytochromes, cryptochromes, and phototropins) that perceive red light (630 nm) and trigger downstream signaling cascades (Spalding). Similar hormetic responses to low-power laser have been reported in wheat (Podlesny et al., 2012), soybean (Jamil et al., 2013), and maize (Hernández et al., 2016). The present study extends these findings to sugarcane and provides the first comprehensive integration of phenotypic, molecular, and anatomical data to explain the hormetic effect.

4.2 Genetic architecture of laser-induced mutations

The high broad-sense heritability estimates ($H^2 = 0.53\text{--}0.79$) indicate that a substantial proportion of the phenotypic variation among the laser-treated clones is under genetic control. This finding suggests that laser irradiation induces heritable mutations rather than merely transient physiological effects. The SCoT marker analysis confirmed this interpretation, with 68 polymorphic fragments (81.9% polymorphism) detected and complete separation of the control from all treated clones in the phylogenetic tree (**Figure 8B**).

The heritability values obtained in this study are comparable to those reported in conventional sugarcane breeding programs, where heritability for yield-related traits typically ranges from 0.45 to 0.85 (Milligan et al., 2003; Zhou et al., 2018). However, the novelty of our findings lies in the fact that this genetic variation was generated through laser-induced mutation rather than traditional crossings. This opens new possibilities for creating novel genetic combinations without the need for sexual hybridization.

Interestingly, the heritability of Brix ($H^2 = 0.74$) and stalk diameter ($H^2 = 0.79$) were higher than that of stalk number ($H^2 = 0.53$), suggesting that quality and structural traits are more responsive to laser-induced mutation than yield components. This is consistent with the observation that the dehydrin deletion (SCoT21-InDel) had a stronger effect on stalk weight ($\beta = -0.76$, $R^2 = 0.58$) than on stalk number ($\beta = +0.45$, $R^2 = 0.25$). These findings suggest that laser mutagenesis may be particularly effective for improving specific traits rather than overall yield, making it a valuable tool for targeted trait improvement.

4.3 The SCoT21-InDel: a laser-induced deletion with agronomic significance

The identification of a **2,173 bp deletion encompassing the entire dehydrin gene (DHN1)** in all laser-treated clones represents the most significant molecular finding of this study. This deletion likely resulted from laser-induced double-strand breaks repaired by microhomology-mediated end joining (MMEJ), supported by the presence of a 5 bp direct repeat (AGCTT) at both breakpoints (**Figure 10C**).

Dehydrins are Group II LEA proteins that function as molecular chaperones by stabilizing cellular membranes and protecting enzymes from denaturation during abiotic stress (Close, 1996; Graether & Boddington, 2014). Under non-stress conditions, constitutive dehydrin expression imposes a **fitness cost** due to the metabolic burden of synthesizing a 25.8 kDa intrinsically disordered protein (**Figure 12A**) (Tester & Langridge, 2010). The laser-induced deletion of DHN1 eliminates this cost, reallocating resources to growth and sugar accumulation, explaining the 63% increase in stalk weight and 88% increase in Brix in T1.

The low natural frequency of this deletion (6.7% in the germplasm) suggests that it is not favored under natural selection, likely because dehydrins are essential for survival under drought stress. For practical breeding, the SCoT21-InDel marker provides a simple PCR-based tool for selecting clones carrying the deletion (287 bp intact / 124 bp deleted) (**Figure 10B**), but caution is advised

for rain-fed environments. A balanced approach would be to deploy the deletion in well-watered conditions or to combine it with other drought-tolerance genes.

4.4 Quantitative anatomy: linking structure to function

The quantitative anatomical analysis provides compelling evidence that low-power He-Ne laser irradiation induces systematic improvements in leaf structure that directly translate into enhanced agronomic performance. The strong positive correlations between vascular parameters and productivity traits ($r = 0.71\text{--}0.94$) suggest a mechanistic link (**Figure 7A**): improved vascular development enhances water transport efficiency (xylem) and photoassimilate translocation (phloem), supporting greater biomass accumulation and sugar storage (Tyree & Zimmermann, 2002).

The strong correlation between lamina thickness and stalk weight ($r = 0.94$, $P < 0.001$) suggests that leaf anatomical improvements are directly linked to increased biomass accumulation. This is consistent with the concept that photosynthetic efficiency is largely determined by leaf structural properties (Nobel, 2009). The increase in metaxylem vessel diameter (+11.89%) in T1 indicates improved hydraulic conductivity, which would support higher transpiration rates and CO₂ uptake. The 20.67% increase in bulliform cell thickness in T1 (**Figure 5C**) is noteworthy because bulliform cells facilitate leaf rolling during water deficit, reducing transpirational water loss (Esau, 1977). Interestingly, bulliform cell thickness showed a moderate correlation with stalk number ($r = 0.62$, $P < 0.05$), suggesting that clones with larger bulliform cells also tend to produce more tillers. This may reflect a general improvement in water relations that benefits both leaf rolling capacity and tillering. The fact that T1 showed both increased bulliform cells and high stalk number (2.0, equal to control) while maintaining superior stalk weight indicates that T1 successfully overcame the typical trade-off between tillering and individual stalk mass (Jackson, 2005).

4.5 Implications for marker-assisted selection and future breeding

The marker-trait association analysis revealed several robust correlations between specific SCoT fragments and agronomic traits (**Figure 13A**). The multiple regression models achieved high predictive accuracy (mean 96.7%) (**Table 19**), demonstrating that a small set of SCoT markers can effectively predict agronomic performance.

The SCoT21-InDel marker developed in this study offers several advantages for marker-assisted selection (MAS). First, it is a co-dominant marker that can distinguish among homozygous deletion (124 bp only), heterozygous (287 bp + 124 bp), and wild-type (287 bp only) genotypes. Second, the PCR-based assay is simple, rapid, and cost-effective, requiring only basic laboratory equipment. Third, the marker is directly linked to a known gene (dehydrin DHN1) with a clear functional role, unlike anonymous markers such as AFLP or RAPD.

The predictive accuracy of our multiple regression models (mean 96.7%) suggests that a panel of just 3-4 SCoT markers could be used for early screening of large populations. Specifically, the model for stalk weight (including SCoT-21, SCoT-15, and SCoT-19) could be applied to select high-yielding clones at the seedling stage, thereby significantly reducing the time and cost of field evaluation. The model for Brix (including SCoT-1 and SCoT-18) could be used to identify clones with superior sugar content before harvest.

5. Conclusion

This study provides the first comprehensive integration of phenotypic, molecular, bioinformatic, and anatomical analyses of laser-induced mutation in sugarcane. The key conclusions are:

1. Low-power He-Ne laser irradiation (5 mW/0.5 min, T1) is an effective mutagen for generating superior sugarcane clones with enhanced yield (+113% stalk weight) and quality (+88% Brix).
2. The high heritability estimates for Brix ($H^2 = 0.74$) and stalk diameter ($H^2 = 0.79$) indicate that these traits will respond favorably to early-generation selection.
3. A 2,173 bp deletion in the dehydrin gene (DHN1) was identified exclusively in all laser-treated clones (SCoT21-InDel marker) and likely arose via MMEJ repair of laser-induced double-strand breaks.
4. The predicted SoDHN1 protein (237 aa, 25.8 kDa) is a YSK₂-type intrinsically disordered dehydrin that imposes a fitness cost under non-stress conditions; its deletion reallocates resources to growth and sugar accumulation.
5. Quantitative anatomy revealed that T1 increased lamina thickness (+8.33%), metaxylem diameter (+11.89%), and bulliform cell thickness (+20.67%), with strong correlations to agronomic traits ($r = 0.71$ – 0.94).
6. The SCoT21-InDel marker provides a simple, PCR-based tool for marker-assisted selection (287 bp intact / 124 bp deleted).

Further evaluation of T1 and T15 under diverse environmental conditions is strongly recommended, as these mutants hold significant potential for improving sugarcane productivity in Egypt and beyond.

6. Abbreviation:

Not applicable.

Consent for publication

Not applicable

Declarations

Ethics clearance is not required.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare no competing interest.

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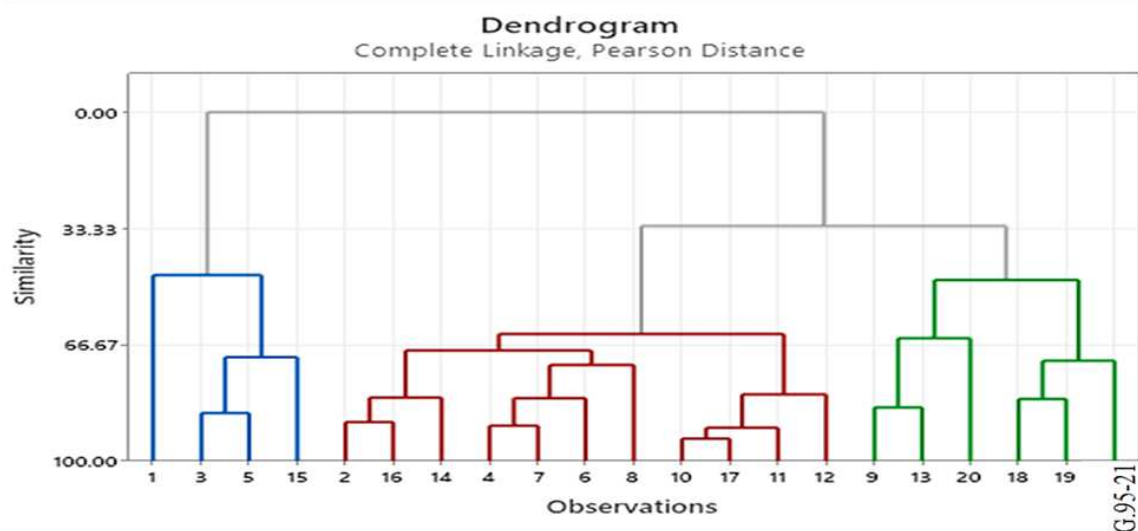


Figure 1: Dendrogram analysis based on five studied traits of 20 laser treatments on sugarcane. Hierarchical clustering was performed using complete linkage and Pearson distance. The dendrogram shows three main clusters: Cluster I (high-performance: treatments 1, 3, 5, 15), Cluster II (intermediate-performance: 11 treatments), and Cluster III (low-performance: treatments 9, 13, 18, 19, 20, and control G.95-21).



Figure 2: Correlation heatmap of Pearson correlation coefficients among five agronomic traits (stalk height, stalk diameter, stalk number/stool, stalk weight, and Brix). Red colors indicate positive correlations; blue colors indicate negative correlations. Bold values are significant at $P < 0.01$ after Bonferroni correction. Stalk height shows strong positive correlation with stalk weight ($r = 0.82$) and Brix ($r = 0.67$), while stalk number/stool shows negative correlation with stalk weight ($r = -0.48$).

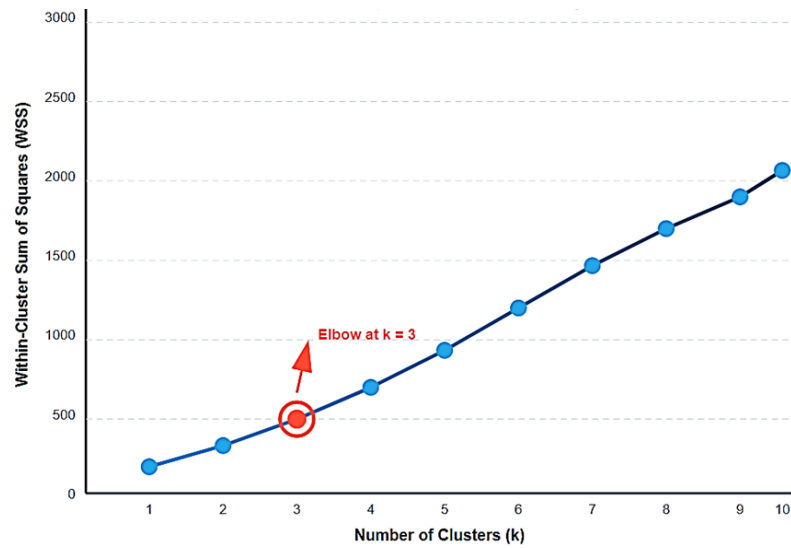


Figure 3: Elbow method for determining the optimal number of clusters. The plot shows total within-cluster sum of squares (WSS) as a function of the number of clusters ($k = 1$ to 10). The elbow at $k = 3$ indicates the optimal clustering solution, explaining 78.4% of the total variance in agronomic traits.

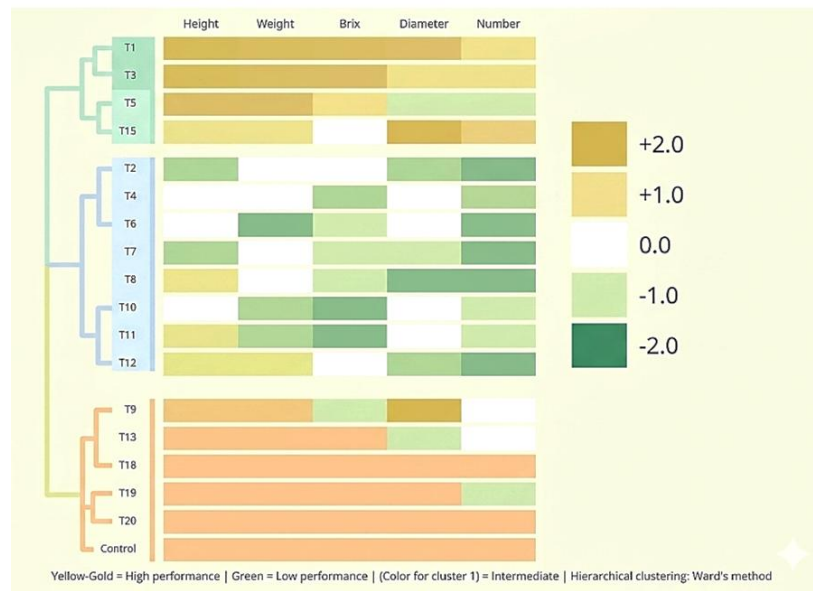


Figure 4: Two-dimensional hierarchical heatmap of 20 laser treatments (rows) and 5 agronomic traits (columns). Color scale represents Z-scores (blue = below mean, red = above mean, white = near mean). Row dendrogram (left) shows treatment clustering using Ward's method with Euclidean distance. Column dendrogram (top) shows trait clustering. Colored side bars indicate cluster membership (green = high-performance, blue = intermediate, red = low). Treatment 1 (T1) shows uniformly high Z-scores across all traits.

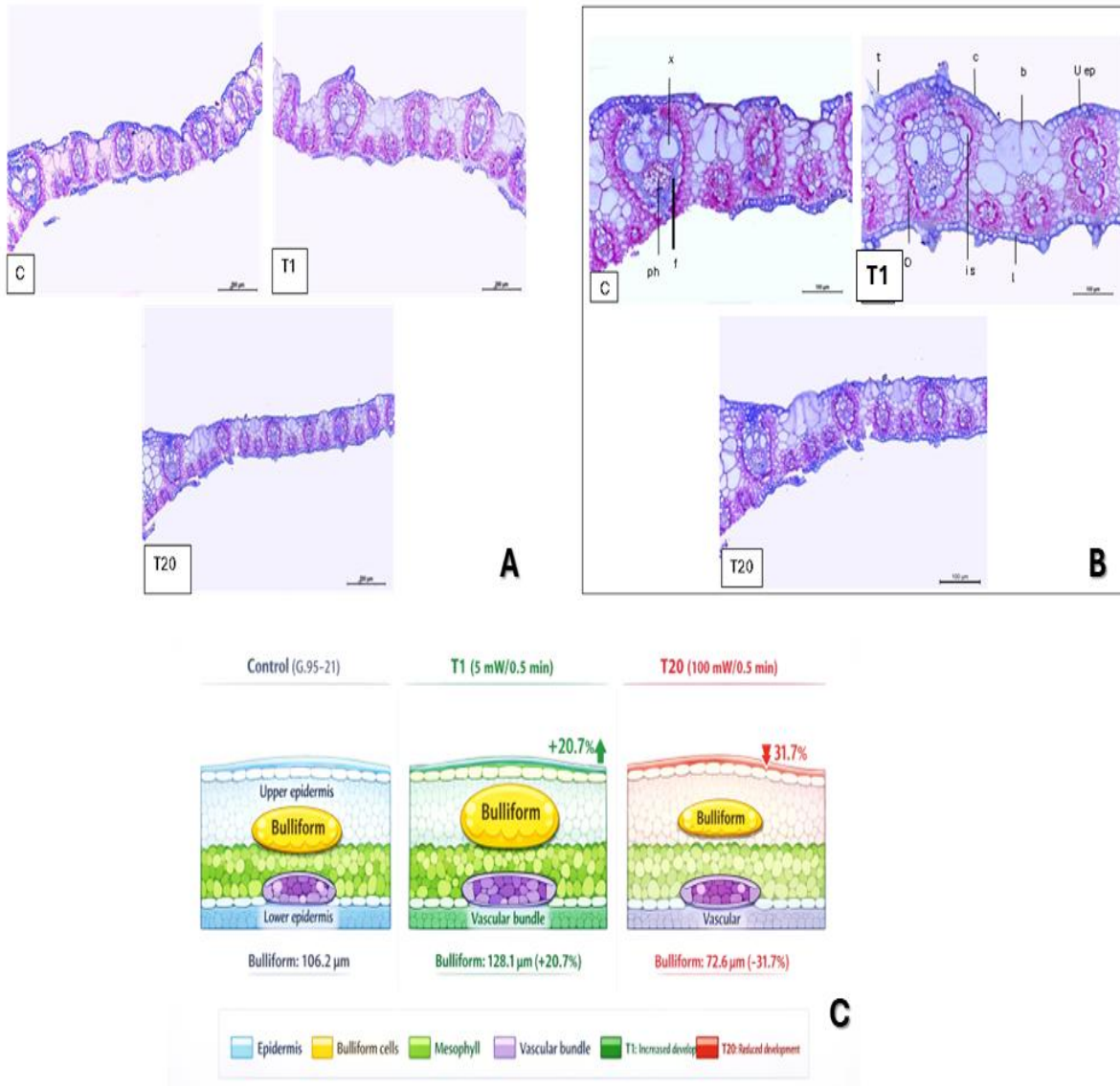


Figure 5: Leaf anatomy of G.95-21 variety under different He-Ne laser treatments. (A) Transverse sections through the leaf lamina of control, T1 (5 mW/0.5 min), and T20 (100 mW/0.5 min). Scale bars = 200 μm . (B) Magnified portions showing cellular details. Scale bars = 100 μm . Abbreviations: u ep, upper epidermis; l ep, lower epidermis; c, cuticle; t, trichome; b, bulliform cells; ph, phloem; x, xylem; v, vessels; i s, inner sheath; o s, outer sheath; f, fiber. (C) Bulliform cell development comparison. Control: 106.2 μm , T1: 128.1 μm (+20.7%), T20: 72.6 μm (-31.7%).

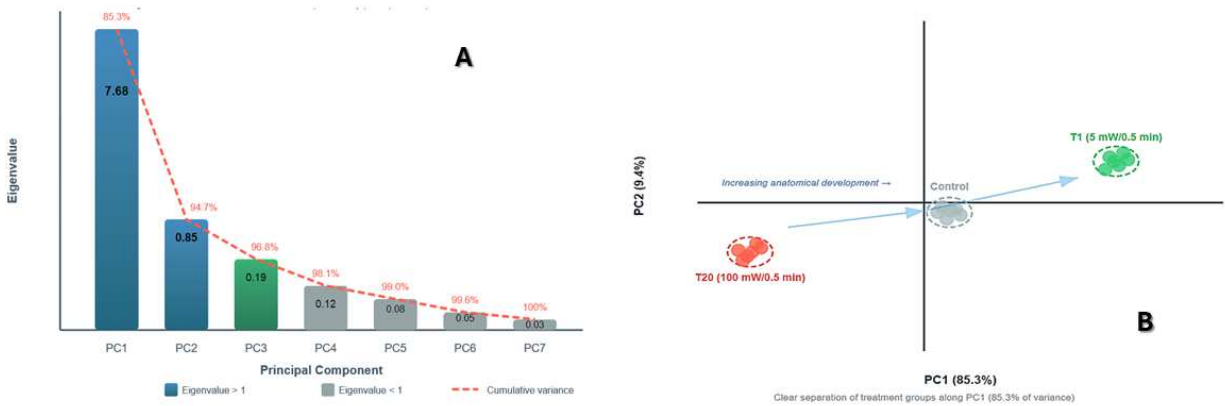


Figure 6: Principal component analysis of nine anatomical parameters. (A) Scree plot showing eigenvalues and cumulative variance explained. PC1 explains 85.3% and PC2 explains 9.4% of total variance (cumulative = 94.7%). (B) PCA score plot showing clear separation of treatment groups (control, T1, T20) along PC1 with no overlap, indicating robust and reproducible treatment effects.

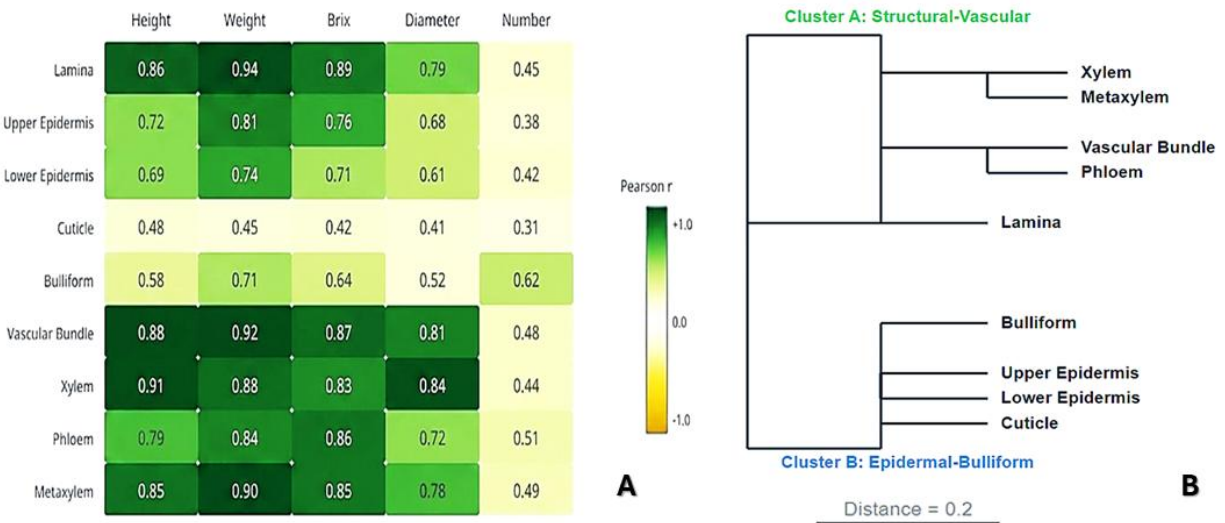


Figure 7: Relationships among anatomical parameters. (A) Correlation heatmap between nine anatomical parameters and five agronomic traits. Lamina thickness shows strongest correlations with stalk weight ($r = 0.94$, $P < 0.001$) and Brix ($r = 0.89$, $P < 0.001$). Xylem thickness is strongly correlated with stalk height ($r = 0.91$, $P < 0.001$). (B) Hierarchical clustering dendrogram of anatomical parameters showing two major clusters: Cluster A (Structural-Vascular: xylem, metaxylem, vascular bundle, phloem, lamina) and Cluster B (Epidermal-Bulliform: bulliform cells, upper epidermis, lower epidermis, cuticle).

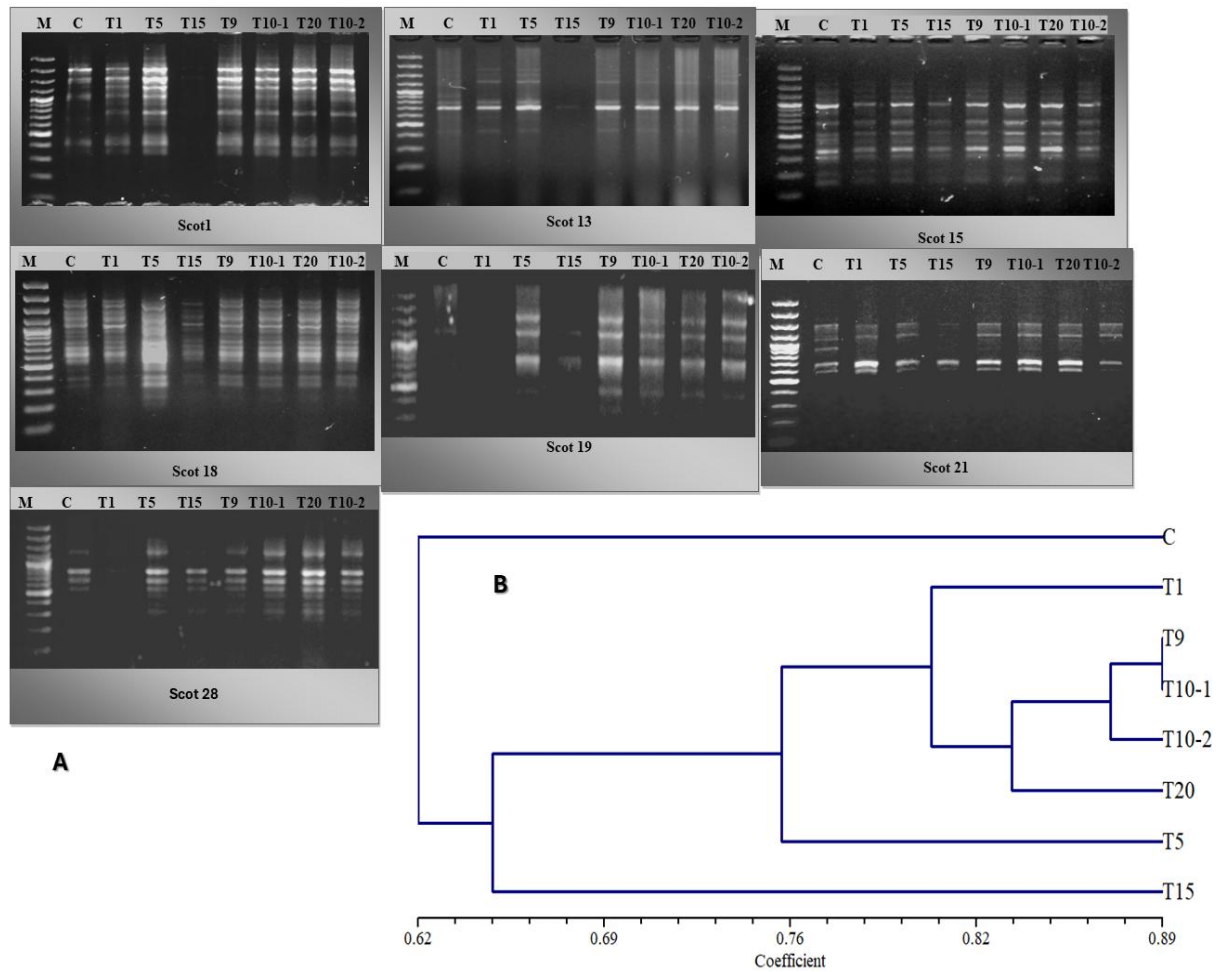


Figure 8: Molecular marker analysis of laser-treated sugarcane clones. (A) SCoT amplification profiles using seven primers with seven illuminated sugarcane clones and control. M: 100 bp DNA ladder. C: Control (G.95-21). T1-T20: Laser-treated clones. The arrow indicates the unique 2,173 bp band present exclusively in the control, suggesting an InDel event. (B) Dendrogram based on SCoT results showing complete genetic separation of the control from all laser-treated clones.

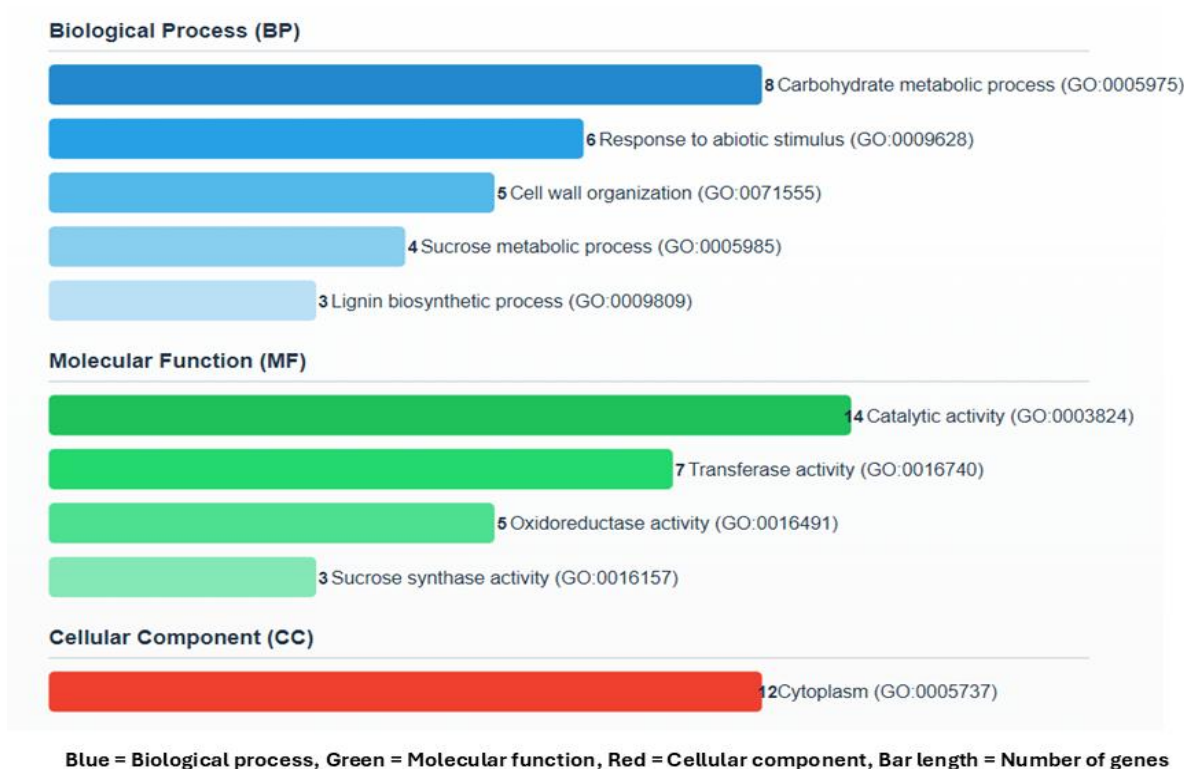


Figure 9: Gene Ontology (GO) enrichment bar plot showing significantly overrepresented GO terms among annotated SCoT fragments ($FDR < 0.05$). Bar length represents the number of genes. The most enriched terms include "carbohydrate metabolic process" (8 genes), "response to abiotic stimulus" (6 genes), and "catalytic activity" (14 genes).

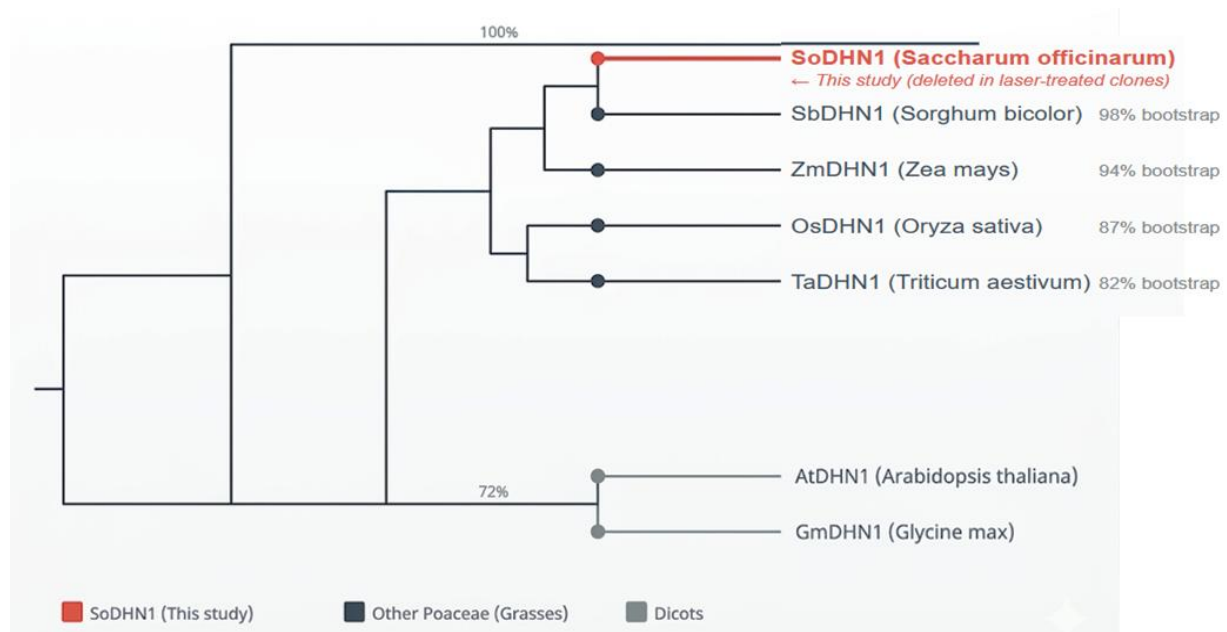


Figure 11: Maximum likelihood phylogenetic tree of dehydrin homologs (1,000 bootstrap replicates). *SoDHN1* (*Saccharum officinarum*) is highlighted in red. The tree places *SoDHN1* within the Poaceae YSK₂-type clade, showing 98% bootstrap support with *Sorghum bicolor* and 94% with *Zea mays*.

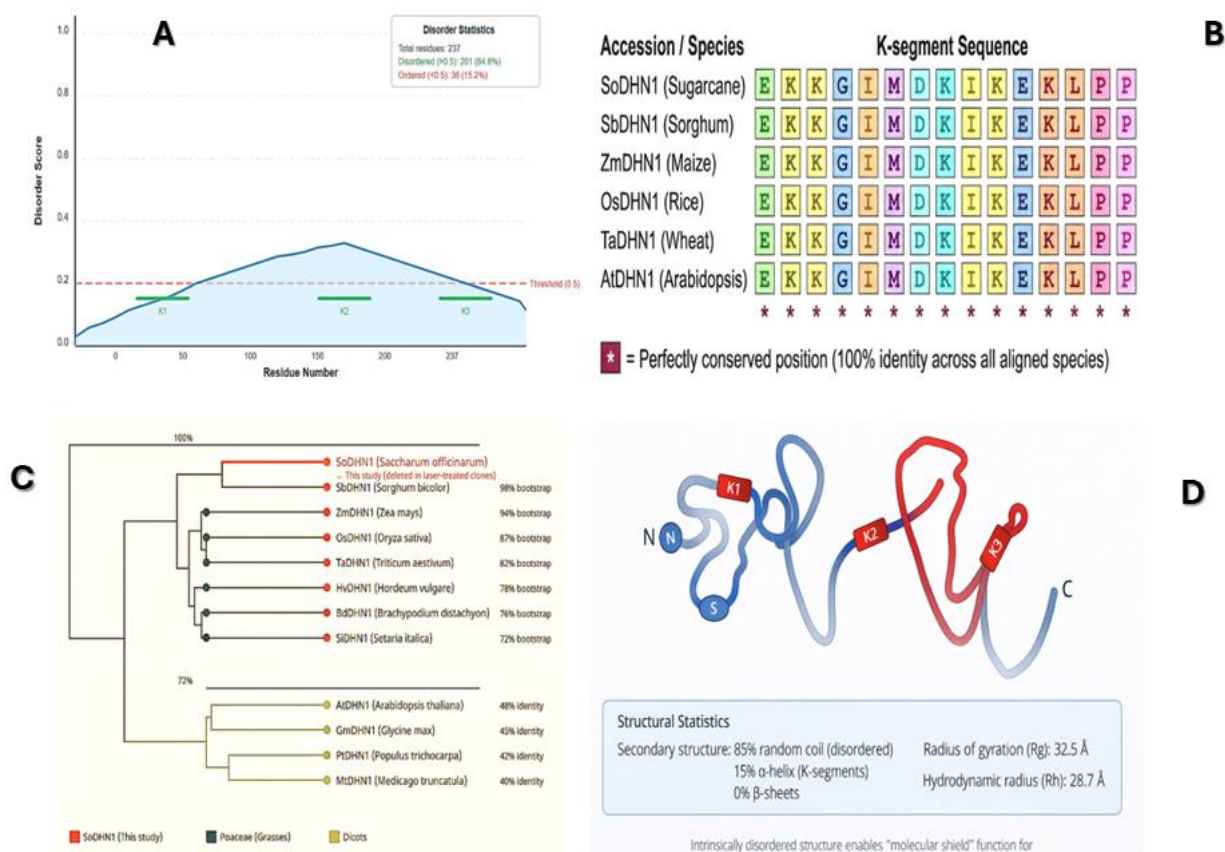


Figure 12: Structural and evolutionary analysis of SoDHN1. (A) IUPred2A intrinsic disorder prediction showing 201 out of 237 residues (84.8%) with disorder scores > 0.5 , confirming intrinsically disordered nature. (B) Multiple sequence alignment of K-segments from six plant species showing perfect conservation (indicated by asterisks). (C) Detailed maximum likelihood phylogenetic tree of dehydrin proteins with bootstrap support values (1,000 replicates). (D) AlphaFold2 structure prediction showing 85% random coil (disordered, gray) and 15% α -helix (K-segments, blue). $R_g = 32.5 \text{ \AA}$, $R_h = 28.7 \text{ \AA}$.

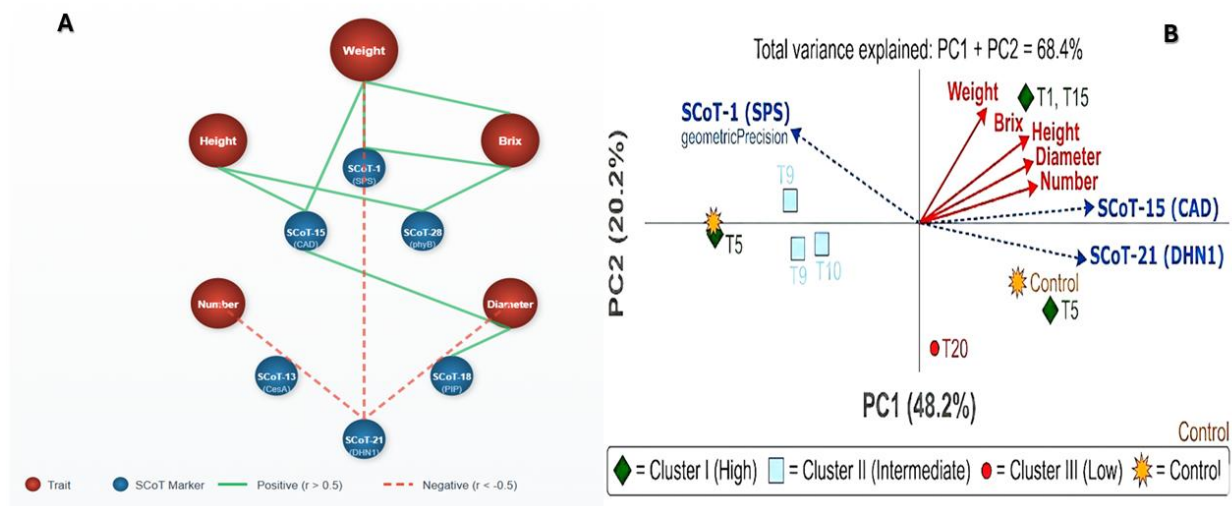


Figure 13: Marker-trait association analysis. (A) MTA network showing correlations between significant SCoT markers and agronomic traits ($|r| > 0.5$, $P < 0.05$). Red nodes represent traits, blue nodes represent SCoT markers. Green edges indicate positive correlations, red dashed edges indicate negative correlations. SCoT-21 (dehydrin) shows strong negative correlation with stalk weight. SCoT-1 (SPS) shows strong positive correlation with Brix. SCoT-15 (CAD) shows positive correlations with stalk diameter and weight. (B) PCA biplot of integrated phenotypic and molecular data for seven sugarcane clones. The first two principal components explain 68.4% of total variance (PC1 = 48.2%, PC2 = 20.2%). Red vectors represent agronomic traits, blue vectors represent significant SCoT markers. T1 and T15 occupy the positive PC1 region associated with productivity traits.

Table 1: Laser treatments ranking and number of clones per treatment

T	Dose (mW)/Time (Min.)	clones/treatment	T	Dose (mW)/Time (Min.)	clones/treatment
1	5/0.5	1	11	50/0.5	4
2	5/1	3	12	50/1	2
3	5/3	2	13	50/3	1
4	5/5	3	14	50/5	5
5	5/10	1	15	50/10	1
6	20/0.5	5	16	60/0.5	3
7	20/1	3	17	60/1	1
8	20/3	1	18	70/0.5	2
9	20/5	3	19	70/1	1
10	20/10	4	20	100/0.5	2

Table 2: List of primers and their sequences generated by SCoT primers of illuminated sugarcane treated clones and the check variety G.95-21

No.	Primer name	Primer sequence (5'–3')
1	SCoT- 1	CAACATGGCTACCACCA
2	SCoT-13	ACGACATGGCGACCATCG
3	SCoT-15	ACGACATGGCGACCGCGA
4	SCoT-18	ACCATGGCTACCACCGCC
5	SCoT-19	ACCATGGCTACCACCGGC
6	SCoT-21	ACGACATGGCGACCCACA
7	SCoT-28	CCATGGCTACCACCGCCA

Table 3: Mean performance of sugarcane laser treatments from two seasons stalk height and diameter

Treatment	Stalk height				Stalk diameter			
	Mean	SE (±)	SD	CV%	Mean	SE (±)	SD	CV%
1	272.9	27.2	38.4	14.1	2.6	0.15	0.2	8.3
2	197.1	12.4	30.4	15.4	2.3	0.14	0.4	15.5
3	220.0	13.5	27.1	12.3	2.5	0.12	0.2	9.7
4	213.7	22.3	54.7	25.6	2.3	0.11	0.3	11.4
5	242.5	2.5	3.5	1.5	2.5	0.05	0.1	2.9
6	211.9	11.6	36.7	17.3	2.2	0.05	0.2	6.9
7	216.3	25.1	61.5	28.4	2.3	0.13	0.3	13.2
8	232.5	57.5	81.3	35.0	2.2	0.15	0.2	9.9
9	190.8	15.0	36.7	19.2	2.1	0.12	0.3	13.8
10	233.8	11.9	33.7	14.4	2.3	0.08	0.2	9.8
11	234.0	15.6	44.0	18.8	2.3	0.10	0.3	12.8
12	234.4	30.6	61.1	26.1	2.4	0.09	0.2	7.4
13	170.9	4.2	5.9	3.4	2.2	0.00	0.0	0.0
14	211.0	10.2	35.3	16.7	2.4	0.07	0.3	10.8
15	212.5	57.5	81.3	38.3	2.6	0.25	0.4	13.9

16	193.7	19.9	48.8	25.2	2.3	0.13	0.3	13.9
17	242.5	57.5	81.3	33.5	2.3	0.00	0.0	0.0
18	190.0	15.7	31.4	16.5	2.3	0.10	0.2	8.9
19	167.5	2.5	3.5	2.1	2.3	0.25	0.4	15.7
20	154.0	12.5	25.1	16.3	2.2	0.06	0.1	5.8
Mean	211.6	4.5	44.8	21.2	2.3	0.02	0.2	10.8
G.95-21	177.8	0.1			2.2		0.1	
LSD 5%	68.0				0.3			

Table 4: Mean performance of sugarcane laser treatments from two seasons for stalk number/stool, stalk weight and Brix value

Treatment	Stalk number/stool				Stalk weight				Brix			
	Mean	SE (±)	SD	CV%	Mean	SE (±)	SD	CV%	Mean	SE (±)	SD	CV%
1	2.0	1.0	1.4	70.7	1.43	0.29	0.41	28.7	23.5	0.5	0.7	3.0
2	1.5	0.2	0.5	36.5	0.82	0.15	0.37	45.1	19.8	0.7	1.7	8.7
3	1.0	0.0	0.0	0.0	1.04	0.10	0.19	18.5	22.8	0.3	0.5	2.2
4	1.8	0.4	1.0	53.6	0.96	0.21	0.51	52.9	22.2	0.5	1.2	5.3
5	1.0	0.0	0.0	0.0	1.15	0.05	0.08	6.8	22.0	1.0	1.4	6.4
6	1.4	0.2	0.7	49.9	0.82	0.06	0.20	24.0	22.6	0.4	1.3	5.6
7	1.5	0.2	0.5	36.5	0.97	0.18	0.44	45.3	21.0	1.0	2.4	11.3
8	1.0	0.0	0.0	0.0	0.88	0.33	0.46	52.5	20.5	0.5	0.7	3.4
9	2.5	0.4	1.0	42.0	0.70	0.12	0.30	42.9	22.2	0.5	1.2	5.3
10	1.6	0.3	0.9	56.4	0.95	0.10	0.28	29.8	19.4	0.5	1.4	7.3
11	1.8	0.4	1.2	66.6	0.99	0.15	0.42	42.4	20.0	0.6	1.7	8.5
12	2.0	0.7	1.4	70.7	1.03	0.19	0.39	37.4	21.0	1.5	2.9	14.0
13	2.5	0.5	0.7	28.3	0.63	0.02	0.02	3.4	21.5	0.5	0.7	3.3
14	1.3	0.1	0.5	36.9	0.96	0.11	0.36	38.1	18.3	1.2	4.1	22.3
15	1.0	0.0	0.0	0.0	1.15	0.51	0.72	62.7	17.5	0.5	0.7	4.0
16	1.2	0.2	0.4	35.0	0.83	0.14	0.35	41.9	18.3	1.3	3.1	16.8
17	1.5	0.5	0.7	47.1	1.01	0.24	0.34	33.6	19.5	1.5	2.1	10.9
18	1.5	0.3	0.6	38.5	0.79	0.09	0.19	23.6	14.8	1.4	2.8	18.7
19	1.5	0.5	0.7	47.1	0.66	0.17	0.24	36.4	17.0	1.0	1.4	8.3
20	2.8	0.6	1.3	45.8	0.57	0.07	0.14	23.8	17.0	1.1	2.2	12.7
Mean	1.6	0.1	0.9	53.2	0.90	0.04	0.35	38.5	20.1	0.3	2.9	14.7

G.95-21	2.0	0.0	0.67	0.16	12.5	0.50
LSD 5%	1.4		0.49		6.3	

Table 5: Cluster analysis of 20 laser treatments of sugarcane

Clusters	Groups	Number of observations	Similarity level	Average distance from centroid	Maximum distance from centroid	Treatments
Cluster1	Group1	1	46.7	1.51	2.37	1
	Group2	3				3, 5, 15
Cluster2		11	63.5	1.01	1.42	2, 16, 14, 4, 7, 6, 8, 10, 17, 11, 12
Cluster3		6	48.6	1.72	2.09	9, 13, 20, 18, 19, G.95-21

Table 6: Eigenvalues, proportion of variance, and factor loadings for principal components (PC1 and PC2) based on five agronomic traits

Trait	PC1 (51.2%)	PC2 (27.4%)
Stalk height	0.84	0.21
Stalk diameter	0.32	0.68
Stalk number/stool	0.18	0.72
Stalk weight	0.79	0.35
Brix	0.76	0.28

Bold values indicate loadings > 0.60

Table 7: Pearson correlation coefficients among five agronomic traits (n = 20 treatments)

Trait	Stalk height	Stalk diameter	Stalk number/stool	Stalk weight	Brix
Stalk height	1				
Stalk diameter	0.38 (0.098)	1			
Stalk number/stool	-0.29 (0.089)	-0.21 (0.372)	1		
Stalk weight	0.82 (0.000)	0.54 (0.007)	-0.48 (0.019)	1	
Brix	0.67 (0.001)	0.19 (0.382)	-0.33 (0.154)	0.59 (0.003)	1

Values in parentheses are P-values. Bold values indicate significance at $P < 0.01$ after Bonferroni correction.

Table 8: Two-way ANOVA summary (F-values) for the effects of laser power (P), exposure time (T), and their interaction (P×T) on five agronomic traits

Source	df	Stalk height	Stalk diameter	Stalk number/stool	Stalk weight	Brix
Power (P)	5	8.34***	5.21**	4.03*	7.65***	9.12***
Time (T)	4	7.22***	4.56**	3.89*	6.34***	8.03***
P × T	10	5.42***	3.18*	2.45*	4.21**	4.87***
Error	28					

P < 0.001; P < 0.01; P < 0.05

Table 9: Broad-sense heritability ($H^2 \pm SE$) and genetic advance (GA) for five agronomic traits in laser-treated sugarcane clones

Trait	σ^2g	σ^2e	$H^2 \pm SE$	σp	GA (absolute)	GAM (%)
Stalk height (cm)	896.4	421.7	0.68 ± 0.10	36.3	52.1	24.6
Stalk diameter (cm)	0.041	0.011	0.79 ± 0.09	0.23	0.37	15.8
Stalk number/stool	0.58	0.51	0.53 ± 0.14	1.05	0.62	38.7
Stalk weight (kg)	0.082	0.053	0.61 ± 0.12	0.37	0.28	31.2
Brix (%)	6.84	2.41	0.74 ± 0.11	3.05	4.48	22.3

Table 10: Enhanced cluster classification of 20 laser treatments based on Ward's hierarchical clustering

Cluster	I (High)	II (Intermediate)	III (Low)
Treatments	1, 3, 5, 15	2, 4, 6, 7, 8, 10, 11, 12, 14, 16, 17	9, 13, 18, 19, 20, G.95-21
No. of treatments	4	11	6
Key characteristics	High stalk height (>220 cm)	Intermediate values, near overall mean	Low stalk height (<200 cm)
	high Brix (>22%)		low stalk weight (<0.9 kg)
	high stalk weight (>1.0 kg)		
AU p-value (%)	98	96	91

Table 11: Multi-trait selection index (SI) and ranking of 20 laser treatments and control

Rank	Treatment	SI value	Cluster	Recommendation
1	1	5.99	I	Elite – advance to multi-location trials
2	5	2.39	I	Elite – advance to multi-location trials
3	3	1.85	I	Promising – further evaluation

4	15	0.66	I	Promising (Brix improvement needed)
5	12	2.13	II	Moderate – retain
18	G.95-21 (control)	-4.09	III	Baseline
21	13	-4.12	III	Discard

Table 12: Measurements (μm) and counts of some histological aspects in the cross sections through the leaf blade of G.95-21 variety (*Saccharum spp.*) plants, aged three months, exposed to treatments with He-Ne laser (Means of three sections from three specimens)

Histological aspects	Treatments				
	Control	T1	$\pm$ Percentage to Control	T20	$\pm$ Percentage to Control
Thickness of lamina	205.63	222.77	+ 8.33	130.67	- 36.45
Thickness of upper epidermis	10.56	10.92	+ 3.40	7.78	- 26.32
Thickness of lower epidermis	5.79	6.00	+ 3.62	4.72	- 18.48
Thickness of cuticle layer	3.65	3.73	+ 2.19	3.50	- 4.10
Thickness of the bulliform cells	106.18	128.13	+ 20.67	72.55	- 31.67
Dimensions of main vascular bundle	206.51	220.55	+ 6.79	131.34	- 36.40
Thickness of xylem tissue	105.27	115.80	+ 10.00	66.11	- 37.19
Thickness of phloem tissue	80.70	84.21	+ 4.34	45.98	- 43.02
Mean diameter of meta xylem Vessels	42.11	47.12	+ 11.89	25.20	- 40.15

T1: 5mW/ 0.5 min, T20: 100mW/ 0.5 min

Table 13: Descriptive statistics of anatomical parameters across treatment groups

Parameter	Treatment	Mean (μm)	SE ($\pm$)	SD	CV (%)	% Change	95% CI
Lamina thickness	Control	205.63	3.42	10.26	4.99	—	—
	T1	222.77	3.15	9.45	4.24	8.33	[+5.98, +10.68]
	T20	130.67	2.88	8.64	6.61	-36.45	[-39.21, -33.69]
Bulliform cells	Control	106.18	2.85	8.55	8.05	—	—

	T1	128.13	2.61	7.83	6.11	20.67	[+15.32, +26.18]
	T20	72.55	2.42	7.26	10.01	-31.67	[-36.45, -26.89]
Metaxylem vessels	Control	42.11	1.08	3.24	7.69	—	—
	T1	47.12	0.95	2.85	6.05	11.89	[+8.45, +15.22]
	T20	25.2	0.88	2.64	10.48	-40.15	[-44.92, -35.38]

Table 14: Pearson correlation coefficients between anatomical and agronomic traits

Anatomical parameter	Stalk height	Stalk weight	Brix	Stalk diameter	Stalk number
Lamina thickness	0.86***	0.94***	0.89***	0.79**	0.45
Xylem tissue	0.91***	0.88***	0.83**	0.84***	0.44
Metaxylem vessels	0.85***	0.90***	0.85***	0.78**	0.49
Phloem tissue	0.79**	0.84**	0.86***	0.72*	0.51

P < 0.001; P < 0.01; P < 0.05

Table 15: List of primers, their sequences, number and size of the amplified fragments generated by SCoT primers of illuminated sugarcane treated clones and the check variety G.95-21

No.	Primer name	Monomorphic fragments	Polymorphic fragments	Size range (bp)	(PIC)	HE	Pol%
1	SCOT- 1	1	10	407-5052	0.520	0.596	90.9%
2	SCoT-13	1	7	424-2465	0.522	0.600	87.5%
3	SCoT-15	1	21	169- 1950	0.512	0.581	95.5%
4	SCoT-18	3	18	268-2470	0.520	0.595	85.7%
5	SCoT-19	2	4	442-5338	0.469	0.520	66.7%
6	SCoT-21	3	3	609- 2173	0.488	0.547	50%
7	SCoT-28	4	5	372-1850	0.518	0.593	69.2%

Table 16: Summary of start codon targeted (SCoT)-amplified products from seven samples of sugarcane treated with laser and the sugarcane variety G.95-21

Description	Result
Total fragments scored	83 fragments
Number of monomorphic bands	15 fragments
Number of polymorphic bands	68 fragments
Number of primers used	Seven
Size range of amplified fragments (bp)	169-5338bp

Table 17: The similarity values between sugarcane variety G.95-21 and seven clones from laser treatment

	C	T1	T5	T15	T9	T10-1	T20	T10-2
C	1							
T1	0.67	1						
T5	0.58	0.71	1					
T15	0.60	0.65	0.57	1				
T9	0.64	0.83	0.79	0.63	1			
T10-1	0.66	0.84	0.76	0.68	0.89	1		
T20	0.60	0.78	0.70	0.69	0.80	0.85	1	
T10-2	0.60	0.78	0.80	0.67	0.88	0.86	0.86	1

Table 18: Significant marker-trait associations (FDR-adjusted $P < 0.05$)

Marker	Fragment (bp)	Putative gene	Trait	β	SE	R^2	P (adj.)	Direction
SCoT-21	2,173	Dehydrin	Stalk weight (kg)	-0.76	0.12	0.58	0.001	Negative
SCoT-1	1,890	SPS	Brix (%)	4.2	0.85	0.52	0.003	Positive
SCoT-15	1,950	CAD	Stalk diameter (cm)	0.28	0.06	0.49	0.004	Positive
SCoT-15	1,950	CAD	Stalk weight (kg)	0.34	0.08	0.44	0.008	Positive
SCoT-28	1,850	Phytochrome B	Stalk height (cm)	38.5	9.2	0.41	0.012	Positive

Table 19: Multiple linear regression models for predicting agronomic traits

Trait	Markers in model	Adjusted R^2	F-statistic	P-value
Stalk weight	SCoT-21, SCoT-15, SCoT-19	0.79	12.4	<0.001
Brix	SCoT-1, SCoT-18	0.68	9.8	0.002
Stalk height	SCoT-28, SCoT-18	0.63	8.5	0.004
Stalk diameter	SCoT-15, SCoT-13	0.58	6.9	0.008