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UNRAVELING THE MOLECULAR BASIS OF SALT STRESS RESPONSE IN *CYMBOPOGON CITRATUS* (D.C.) STAPF

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SUMMARY

Cymbopogon citratus (lemongrass), a perennial herb from the Poaceae family, is a widely valued plant for its essential oil and medicinal properties. Plants frequently encounter abiotic stresses, such as drought, salinity, extreme temperatures, and heavy metals, which severely limit agricultural productivity. Transcription factors, including MYB (myeloblastosis), WRKY (pronounced worky), and bHLH (basic Helix-Loop-Helix), play pivotal roles in regulating plant responses to such stressors by mediating hormonal and developmental pathways. In this study, three salt concentrations (0, 75, and 150 mM) applied to *C. citratus* had their morphological parameters assessed after 30 days. The results revealed significant reductions in shoot length (73.8%–63.2%), root length (80%–65%), leaf number (72%–66%), tillers (88.4%–76.9%), and both fresh weight (85.5%–73.9%) and dry biomass (80.6%–68.6%) under increasing salt stress. Molecular analysis via PCR confirmed the expression of *CcMYB*, *CcWRKY*, and *CcbHLH*—homologs of *Zea mays* transcription factors—in *C. citratus*. These findings demonstrate a salt-responsive genetic mechanism in *C. citratus* that suggests the identified genes are promising candidates for developing salt-tolerant genotypes. This could potentially pave the way for cultivating *C. citratus* in saline soils and contribute to enhancing stress resilience in related herbaceous crop species.

Keywords: Lemongrass (*C. citratus*), salt stress, transcriptional factors, MYB, WRKY, bHLH

Key findings: Identification of salt stress responsive genes in lemongrass (*C. citratus*) species and their characterization under control and salt stress conditions was successful. This molecular study can benefit the genetic improvement of the *C. citratus* species.

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INTRODUCTION

Soil salinity significantly limits agricultural output, particularly in dry and semi-arid regions where sodium chloride (NaCl) accumulates. This issue adversely affects parts of Asia, Africa, Australia, and the Middle East. Globally, salinity influences over 800 million hectares, representing more than 6% of the Earth's land area. In irrigated regions, salinity alters around 20% of soil (Balasubramaniam *et al.,* 2023). Salt stress in plants manifests through osmotic stress, where high salinity reduces soil water potential, hindering water uptake. This leads to dehydration and physiological responses, such as stomatal closure, which conserves water but restricts CO_2 availability for photosynthesis, ultimately stunting growth due to decreased energy production (Waheed *et al.,* 2024).

Cymbopogon citratus (lemongrass) is a massive, thick grass with a lifespan of four years, which can grow up to 120 cm tall and produce 50–120 kg of oil per hectare. New grass yields 0.2%–0.4% oil (Ranjah *et al.,* 2019). Approximately eight species of *Cymbopogon* are prevalent in Pakistan, with the two main species, *C. flexuosus* and *C. citratus*, renowned for their strong lemon flavor (Hassan *et al.,* 2007).

C. citratus is native to Sri Lanka and India (Tahira *et al.,* 2018). It has been beneficial in traditional and Ayurvedic medicine for over a century (Tarkang *et al.,* 2012). Its essential oil, produced after crushing the leaves, has antifungal, anti-inflammatory, and antioxidant properties. It is also effective in treating diabetes, illnesses, the flu, and pneumonia (Ullah *et al.,* 2020). *Cymbopogon* essential oils contain monoterpenes, such as citronellol, citral, citronellal, 1,8-cineole, elemol, linalool, b-carophyllene, limonene, geraniol, geranylformate, methyl heptenone, and geranyl acetate. Citral, a major component of *Cymbopogon* oil, has commercial applications in fragrance and vitamin A production (Zakir *et al.,* 2015).

Stress-responsive transcription factors (TFs) are crucial in regulating various abiotic stress-related pathways, such as salt-sensory pathways. TFs bind to cis-regulatory regions in the genome, acting as molecular switches for linked genes under specific biological conditions. TFs can interrelate with multiple proteins in transcriptional synthesis and modulate the expression of numerous genes. About 10% of genes in plants can encode TFs, classified based on their DNA-binding domains (Shah *et al.,* 2021).

The basic Helix-Loop-Helix (bHLH) family of genes has been notable in various plant species (Niu *et al.,* 2017), and some bHLH TFs have had hypotheses to improve plant stress tolerance, water deficiency, cold, and salinity (Sun *et al.,* 2018). The bHLH genes, totaling 18 that respond to salinity stress, have occurred in poplar trees (Zhao *et al.,* 2018). The WRKY gene family is another significant family of TFs that plays essential roles in expression control and signaling during biotic and abiotic stresses. WRKY detection and functional studies have progressed on various plant species, including *Populus trichocarpa*, *Pyrus bretschneideri*, *Citrus*, *Glycine max,* and *Daucus carota*, highlighting their importance in plant health (Zhang *et al.,* 2019).

Myeloblastosis oncogene (MYB) and myelocytomatosis oncogene (MYC) are conserved proteins with DNA-binding domains. Salt stress transcription regulates *OsMYB3R*-2, *AtMYC2*, *AtMYB41*, *AtMYB44*, *AtMYB73*, *AtMYB77,* and *AtMYB102,* promoting salinity tolerance in transgenic crops (Shah *et al.,* 2021).

C. citratus is an economically valuable plant due to its medicinal values. An urgent investigation into the salt stress-related properties of *C. citratus* for its commercial cultivation on saline soils in Pakistan is necessary. Enhancing the salt tolerance of *C. citratus* could improve its survival in saline soil conditions.

MATERIALS AND METHODS

Experimental area and plant material

The initial experimentation on *C. citratus* (lemongrass) commenced at the Department of Biological Sciences, University of Veterinary and Animal Sciences (UVAS), Ravi Campus,

Pattoki, Pakistan. Indigenous lemongrass plants collected from the National Agricultural Research Center (NARC), Islamabad, had the germplasm maintained in the nursery at the Department of Biological Sciences, Faculty of Fisheries and Wildlife, UVAS, Ravi Campus, Lahore, Pakistan. The experimental layout was in a completely randomized block design. One lemongrass plant grown per pot had each pot considered as a single replicate. The plantlets sustained growth in triplicate in a potted soil mixture under controlled conditions, with a 16/8 h photoperiod. Plants received daily watering with 200 mL of distilled water, with no pesticide application. The average temperature and humidity had estimates of 29.3 °C and 65%, respectively, with no observed fungal, bacterial, or viral infection during the experiment.

Salinity stress treatment

The salt treatment regime determination for lemongrass was according to the US Salinity Laboratory categories (Richard, 1954). Three salt concentrations (0, 75, and 150 mM) were treatments established (Table 1). Control plants only received distilled water, while treatment plants acquired 75 mM or 150 mM NaCl solution on alternate days. The administration of all treatments was simultaneous on the same days. The study involved adding salt to lemongrass plantlets to maintain consistent salinity levels. The salt's delivery in an aqueous solution prevented osmotic shock. After four weeks, the whole leaf tissues from untreated plants served as a control, while leaf tissues from plants exposed to salt stress (75 and 150 mM NaCl) underwent separate collection in triplicate. The experimental schedule illustration, from growth to harvesting, is available in Figure 1.

Morphological characters

The morphological traits of the *C. citratus* species received assessment before molecular analysis. Evaluations included length of shoot (cm), root (cm), tiller and leaf numbers, and fresh and dry weights of the plant (g). Measurements took place on grown plants from 30 days after the final salt application. The data recording was in triplicate to study variations under salt stress conditions.

Statistical analysis

Applying SPSS Version 20.0 helped compare mean values of different morphological parameters under study by using one-way ANOVA and Duncan's multiple range test (DMRT).

Table 1. Various salt regimes created throughout the current experiment to examine salinity tolerance in lemongrass.

Salt regime	NaCl concentrations
Non-saline	0 mM
Mildly saline	75 mM
Extremely saline	150 mM

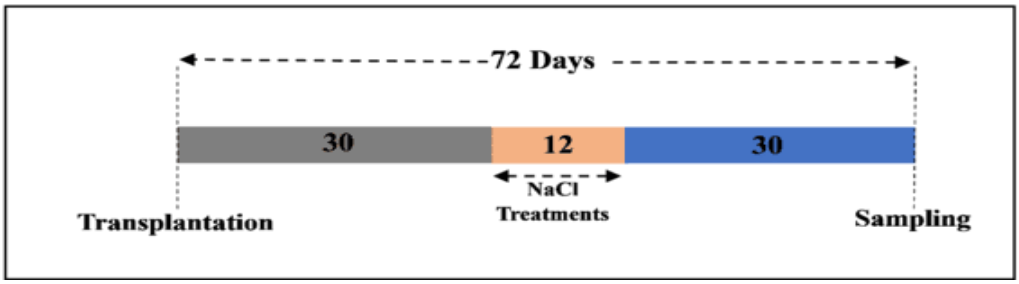

Figure 1. Experimental schedule for salinity stress.

DNA extraction

The use of the CTAB method with minor modifications (Ashwini and Tiwari, 2015) extracted the genome DNA from fresh leaves of 72-day-old *C. citratus* plants. The DNA extraction proceeded by collecting 2 g of leaf samples, washing them with distilled water, and grinding them in liquid nitrogen. The leaf powder weighing 150 mg underwent transferring into preheated extraction buffer before mixing thoroughly. The mixture sustained incubation in a water bath at 65 °C for 30 min, followed by three washings of chloroform isoamyl alcohol. The supernatant, as collected, entailed mixing with chilled isopropanol. The mixture's storage at 20°C took overnight. Then, the sample's centrifugation continued at 15,000 rpm for 20 min to form a hard pellet. The pellet received washing with 70% ethanol and then air-drying. The DNA pellet underwent resuspension in sterile water before storing in a refrigerator.

Agarose gel electrophoresis

The purified genomic DNA's examination used 0.8% agarose gel electrophoresis. The gel preparation utilized 1× TAE buffer, adding 0.5-1 µg/ml of ethidium bromide. The DNA samples' loading onto the gel involved running at 80–100 V for 60 min. The use of the GrabIT 2.5, a gel-documentation system program, took pictures of the gel.

Quantification of genomic DNA

The concentrations of the DNA samples' ready measurements used the Bioanalyzer (3500). Using 1 µL of DEPC-treated deionized water helped obtain blank readings. The measurement of 1 µL of DNA used A260/280 and A260/230 to record the results. A ratio of ~2.0 was the generally accepted amount as "pure" for a DNA sample.

Total concentration of DNA = ng/µL

Salt stress-responsive genes with primer designing

The detection of a subset of salt stress-related genes employed gene-specific primers, with the sequence similarity method used to identify genes that respond to salt stress. Potential candidate genes entailed selection based on their role in salinity tolerance mechanisms in other plant species. The use of the NCBI EST database (dbEST, http://www.ncbi.nlm.nih.gov/dbEST/) for abiotic stresses served for contig assembly, removing duplication with CAP3 software (Huang and Madan, 1999). Sequence contigs underwent comparison to salt-stress tolerance candidate genes in a similarity search. The Primer3 software (version 0.4.0) application for primer designing included *MYB101*, *WRKY74,* and *ZmbHLH* genes. The finalized primers attained designs according to the reference gene sequence for PCR analyses, as shown in Table 2.

PCR amplification of salt-responsive genes

The PCR amplification of selected genes was optimal. PCR amplification of selected *C. citratus* species ensued in a thermal cycler using the standard PCR protocol. Amplified PCR products preceded their placement on agarose gels, stained with ethidium bromide, before

Table 2. PCR primer for salt- responsive transcripts.

Gene ID	Primer ID	Primer sequence	Tm
MYB101	MYB101-F	5′-CATATTGAGGACCAATACTGGC-3′	56.2
	MYB101-R	5′-GCAAGCTCGAAGCATCATG-3′	58.0
WRKY74	WRKY74-F	5′-AAACTCAGGAAGACGTCGC-3′	58.0
	WRKY74-R	5′-TGAGGGGGGGAACATCC-3′	56.0
ZmbHLH	ZmbHLH-F	5′-TCAGGCAGTCCAGCAGGT-3′	58.0
	ZmbHLH-R	5′-GCATTGCTCATCTTCCATTCT-3′	53.8

observation under UV light in a gel documentation system (Jabeen *et al.,* 2012). With 500 ng of template DNA, setting the PCR reaction in 25 µL volume had 1 µL of forward and reverse primers (20 pmol), 2.5 microliters of 10X PCR buffer ($MgCl_2$, 2.5 mM), 1 microliter of 2.5 mM dNTPs, and 1 µL of 2U/µL Taq DNA polymerase. The final volume consisted of deionized**/**nuclease-free water. The PCR running in the thermocycler at different temperatures had a specified time. By using the ZmbHLH, MYB101, and WRKY74 primers, the thermocycler succeeded in programming for denaturation at 95 °C for 4 min, 95 °C for 30 s, followed by annealing at 56 °C for the ZmbHLH primer, 50 °C for the MYB101 primer, and 53 °C for the WRKY74 primer for 30 s. An extension occurred at 72 °C for 30 s before repeating to annealing temperature for an additional 35 cycles. The final extension transpired at 72 °C for 7 min.

Gel electrophoresis

The obtained resolution of final PCR products resulted from running samples on a 1% agarose gel at 80 V for 30 min. A 1% agarose gel prepared in 1X TAE buffer comprised 0.5–1 µg/ml of ethidium bromide. The PCR products of volume 10 µL involved running on the gel, with PCR amplicons visualized under UV light using the GrabIT 2.5 gel-documentation system program.

Gel elution and sequencing of PCR amplicons

PCR amplicons' extraction used the gel extraction kit (Qiagen, Cat. No. 69204) following the manufacturer's protocol. The rechecking of the quality and quantity of the eluted samples continued on 1% agarose gel before sequencing of the eluted samples. PCR-amplified bands that consistently appeared in both control and salt-stressed samples of *C. citratus* underwent separate excision and elution, with the DNA eluted into sterile water. Sequencing of these eluted samples used the automated Sanger sequencing method. The PCR products proceeded for Sanger sequencing to Advance Bioscience International.

Bioinformatical analysis of salt-responsive genes

Sequence data processing utilized the base-calling software (Chromas software version v1.45), and assemblage used BioEdit (7.2) software. High-quality nucleotide sequences or the inferred amino acid sequence of each amplicon underwent comparison with the DNA, EST, and protein sequences from different databases using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.,* 1990) to find homologous sequences. BLAST results confirmed the differential presence of the selected potential salt-responsive genes.

Sequence alignment and phylogenetic analysis

The study used CLUSTALW with default parameters through EMBnet (http://www.ch.embnet.org/software/ClustalW.html) to perform multiple sequence alignment (MSA) on salt stress-related genes from *C. citratus* species (Thompson *et al.,* 1994). Meanwhile, carrying out phylogenetic analysis continued with all protein sequences publicly available for plants and bacteria. For analyzing the evolutionary perspective, the sequence results helped to form a phylogenetic tree using rooted neighbor-joining methods utilizing publicly available online software, MEGA11 (Tamura *et al.,* 2021), from the previously aligned amino acid sequences. Gaps in sequences appeared as missing data. Bootstrap values generated from 1050 replicates determined the relative level of support for the tree topology.

RESULTS AND DISCUSSION

Growth and morphological characters

The morphological characteristics of *C. citratus* underwent assessment after 30 days of final salt stress treatments. Plants examined for different morphological parameters included the length of shoot and root (cm), tiller and leaf numbers, and the fresh and dry weights of the plant (g). The measurements recorded

Table 3. Effect of salinity stress (NaCl) on morphological traits of *Cymbopogon citratus.*

No.	Parameters	Control plants (0 mM NaCl)	Treatment-1 (75 mM NaCl)	Treatment-2 (150 mM NaCl)
1	Shoot Length	22.6000^{a}±3.63731	16.7000^{a}±1.85203	14.3000^{a}±1.55349
2	Root Length	6.0333^{b}±0.81104	4.8000^{ab}±0.40415	3.9333^{a}±0.21858
3	No. of leaves	5.0000^{a}±2.51661	3.6667^{a}±0.88192	4.3333^{a}±1.33333
4	No. of tillers	2.6667^{a}±1.20185	2.3333^{a}±0.66667	3.3333^{a}±0.88192
5	Fresh weight	13.9100^{b}±1.10693	11.8733^{ab}±0.3481	10.2700^{a}±0.28160
6	Dry weight	6.7400^{b}±0.68549	5.4533^{ab}±0.27871	4.6667^{a}±0.44096

According to Duncan's test, means followed by different letters indicate significant differences ($P \leq 0.05$) among the three treatments. Values are means ± SE.

included the control and the two stress-treated groups. Overall, a reduction in all morphological parameters resulted in the treated plants compared with the control plants, with statistically significant differences found between the two stress treatments (Table 3). Similarly, Rehman *et al.* (2022) showed the biochemical and morphological features of lemongrass reduced at 100 mM NaCl concentration, indicating it could not survive a higher salt content. A lower salt threshold level may reduce salt toxicity and ROS damage by enhancing CAT, SOD, and POD enzyme activities. Likewise, Mukarram *et al.* (2022) studied lemongrass growth using parameters like height, dry weight, and leaf area. All salt treatments decreased height, dry weight, and leaf area in a dose-dependent manner, with the highest concentration causing the most harm. Although some harm was evident at low and moderate salinity, the decrease was less than 20%. Lemongrass has moderate resistance to salt stress, as shown by consistent biomass production (Ullah *et al.,* 2020). High and severe salinity can reduce height and dry weight by up to 50%.

DNA extraction and quantification

Genomic DNA from *C. citratus* extraction used the CTAB method and reached resuspension in DEPC-treated water. The purified DNA sustained analysis by agarose gel electrophoresis with ethidium bromide staining, with its concentration quantified using a Bioanalyzer (3500). A single strong peak at 260 nm was evident, indicating good DNA quality and sufficient quantity (Table 4).

PCR amplification of salt-responsive genes

The amplification of all selected potential genes was optimal using a standard polymerase chain reaction (PCR) protocol. With a total of three arbitrary primers (*MYB101*-F, *WRKY7*4-F, and *ZmbHLH*-F) in combination with three anchored primers (*MYB101*-R, *WRKY74*-R, and *ZmbHLH*-R), a total of six differentially expressed gene fragments succeeded in their isolation from leaf samples of treated versus control *C. citratus* plants. The use of a 1% agarose gel separated PCR amplicons from the control and treated samples to compare gene transcript variations in response to salt stress. Upregulated transcripts gained confirmation as final expressed transcripts through reamplification trials, with reamplified PCR products photographed using the GrabIT 2.5 software (Figure 2).

Sequencing of gel-purified PCR amplicons

The study used gene-specific forward and reverse primers to sequence gel-eluted PCR amplicons. The sequence chromatograms of all the identified upregulated transcripts underwent scrutiny using Chromas (V.1.45), generating three sequences: 338 bp for *CcbHLH* (GenBank accession: PX626027), 469 bp for *CcWRKY* (GenBank accession: PX619677), and 487 bp for *CcMYB* (GenBank accession: PX576119). In the control plant sequences, the GC content of the *CcbHLH* gene locus was the highest at 60.22%, followed by *CcWRKY* (57.89%) and *CcMYB* (56.94%). In treated plant sequences, the GC content was highest in the *CcMYB* gene locus at 57.08%,

Table 4. DNA quantification using Bioanalyzer.

Sample identity	Quantity of DNA ng/ µl	Ratio A 260/280
Cymbopogon citratus C_1	679	1.18
Cymbopogon citratus C_2	645	1.31
Cymbopogon citratus T_1	364.5	1.39
Cymbopogon citratus T_2	246.4	1.92

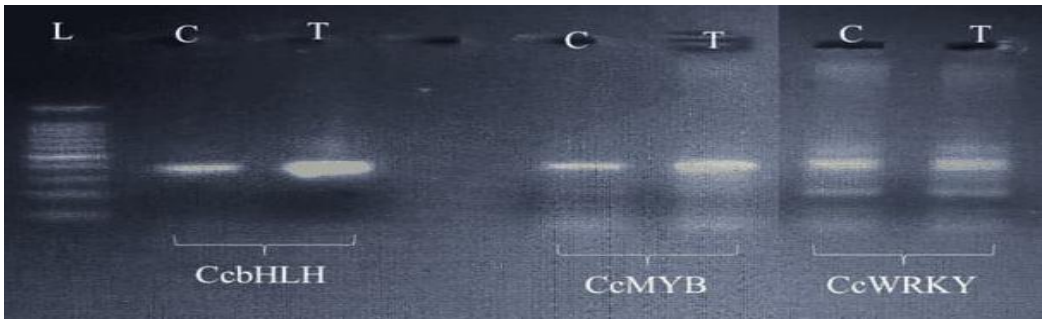

Figure 2. PCR profiling of *Cymbopogon citratus* depicting *CcMYB* (487 bp + Ladder 100bp), *CcWRKY* (469 bp + Ladder 100bp), and *CcbHLH* (338 bp + Ladder 100bp) gene-specific amplicons in control and treated samples, respectively.

Table 5. Statistics derived from the sequencing, alignment, and BLAST analysis.

Sample ID	Sequence length (bp)			GC ratio (%)		
	CcMYB Gene	*CcWRKY* Gene	*CcbHLH* Gene	*CcMYB* Gene	*CcWRKY* Gene	*CcbHLH* Gene
Control Plant	353	304	181	56.94	57.89	60.22
Treated plant	487	469	338	57.08	56.92	54.77

followed by *CcbHLH* (56.92%) and *CcWRKY* (54.77%) (Table 5).

Bioinformatics analysis of the candidate salt-responsive genes

The NCBI BLAST's execution helped identify the gene homologies against land plants (taxid: 3193) with the known genes and proteins using BLASTN and BLASTX. Only 10 significant hits attained consideration by establishing the best matches within a query range, in accordance with the E-value cut-off score of -10.0. The study found high similarity between the *CcMYB* gene and other predicted plant species (*Zea mays*, *Sorghum bicolor*), ranging from 99% to 87%. The *CcWRKY* gene showed similarity with other predicted plant species (*Z. mays*, *Panicum virgatum*), between 88% and 85%, while the *CcbHLH* gene displayed similarity with other predicted plant species (*Z. mays*, *Setaria viridis*) between 96% and 69%. The BLAST results confirmed the presence of the selected salt-responsive genes, *CcMYB*, *CcWRKY*, and *CcbHLH*, with the highest similarity within the same taxon. Similarly, *OsMYB6* is a stress-response gene that, when overexpressed in rice, does not influence transgenic rice growth and development but enhances resistance to salt and drought stress (Tang *et al.,* 2019). The bHLH family is an important defense-related gene family in plants in stress conditions (Li *et al.,* 2021). The WRKY transcriptional factors have emerged to play critical roles in various abiotic stresses like salt, drought, and cold stress (Viana *et al.,* 2021).

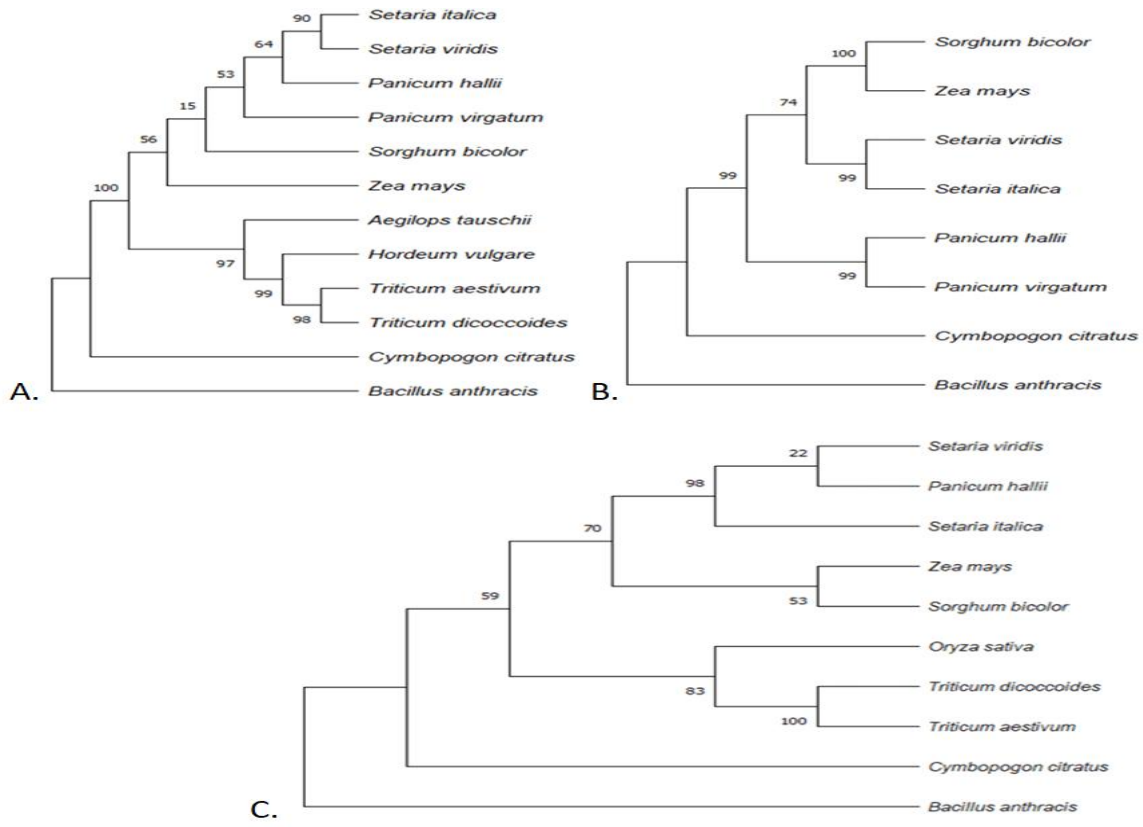

Figure 3. Distance-based neighbor-joining phylogenetic trees of potential gene candidates in *Cymbopogon citratus,* constructed using MEGA11 software with 1050 bootstrap replicates, employing *Bacillus anthracis* as an outgroup: (A) phylogenetic tree of the *CcMYB* gene, (B) *CcWRKY* gene, and (C) *CcbHLH* gene, respectively.

Multiple sequence alignment and phylogenetic analysis

Multiple sequence alignment (MSA) ensued using the known protein sequences of selected salt stress-related genes from *C. citratus* species. Using BLASTX, a sequence similarity search succeeded in determining the top 10 proteins from various plant species whose inferred amino acid sequences show stronger alliance with that of *C. citratus*. The results revealed the *CcMYB* gene provided high similarity with other plant species (*Z. mays*, *S. bicolor*), ranging from 100% to 57%, and the *CcWRKY* gene indicated similarity with other plant species (*Z. mays*, *P. virgatum*), ranging from 100% to 93%. The *CcbHLH* gene expressed high similarity with other plant species (*Z. mays*, *S. bicolor*, *S. italica*), ranging from 96% to 49%. BLAST results confirmed the presence of potential salt-responsive genes—*CcMYB*, *CcWRKY,* and *CcbHLH*—in *Cymbopogon* species. These genes exhibited the maximum similarity within the same taxon. Conserved regions between selected genes and related proteins were notable through multiple sequence alignments.

For phylogenetic analysis, MEGA11 software and bacteria as an out-group served to construct neighbor-joining phylogenetic trees for salt-responsive genes, revealing that *CcMYB* could have a distant relation to *S. italica* proteins and a closer relation to the *Triticum dicoccoides* protein. Based on the phylogenetic tree (Figure 3A), *CcWRKY* proved closely related to the same protein found in *P. virgatum* and distantly related to the protein found in *S. bicolor* (Figure 3B). Moreover, *CcbHLH* initially appeared to have a close relationship with the same protein found in *T.*

aestivum and a distant relationship with proteins found in *S. viridis* (Figure 3C). Each of the 10 related proteins may have shared a common ancestor and diverged relatively late in evolution, placing them all in the same subgroup. The research revealed the expression of the allied variant of the *MYB101* gene of *Z. mays* in *C. citratus*. This contributes to hormone response, drought response, cold stress tolerance, SA-mediated pathway, light response, ABA-mediated pathway, heat stress tolerance, and salt tolerance (Ambawat *et al.,* 2013). The allied variant of the *WRKY74* gene of *Z. mays* also manifested in *C. citratus*, which participates in drought and salt stress, causing cellular dehydration, reactive oxygen species (ROS), and abscisic acid (ABA) accumulation in other species (Chen *et al.,* 2017). The *ZmbHLH* gene variant from *Z. mays* was notable to materialize in *C. citratus*, with 12 *DgbHLH* genes found to improve orchard grass resistance to heat, drought, and salt stress (Lu *et al.,* 2022).

CONCLUSIONS

Lemongrass (*C. citratus*) exhibited high sensitivity to salt stress, affecting its morphology and gene activity. Salt treatments reduced growth in shoots, leaves, and other visible traits. PCR profiling identified three potential salt-responsive gene transcripts, i.e., *CcMYB*, *CcWRKY*, and *CcbHLH*. These gene transcripts showed significant expression in stress conditions and potential for genetic improvement. The findings suggest promoting *C. citratus* cultivation and resilience in saline soils via gene integration. The identified salt stress-related genes have potential application in improving crop resilience for enhancing crop production for industries and pharmaceuticals.

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