



qRT-PCR Analysis of Silicon-induced Defense Genes in Okra Varieties under Root-knot Nematode Stress

Saleha Diwan¹, Mohammedtarik M. Saiyad¹, Harshit Chavda¹, Apexa Rathod¹, Vikram Parmar¹

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ABSTRACT

Background: Silicon (Si) is recognized as a multifunctional element that enhances plant tolerance and improves resistance against insect pests and diseases. Okra (*Abelmoschus esculentus* L.), commonly known as lady's finger or gumbo, is highly susceptible to several pests, particularly the root-knot nematode (*Meloidogyne incognita*), a major pathogen affecting crops of the Malvaceae family. Understanding the molecular defense mechanisms involved in silicon-mediated resistance is essential for improving crop protection strategies.

Methods: Quantitative real-time PCR (qRT-PCR) was employed to analyze gene expression patterns in okra under nematode stress and silicon treatment. Reference genes were used for endogenous normalization to ensure accurate relative quantification of target genes. The expression levels of defense-related genes-PAL (Phenylalanine ammonia-lyase), CAD (Cinnamyl alcohol dehydrogenase) and CHS (Chalcone synthase)-as well as silicon transporter genes Lsi1 and Lsi2, were evaluated under disease and treatment conditions.

Result: Gene expression analysis revealed significant upregulation of PAL, CAD and CHS under nematode-infected conditions, indicating activation of plant defense responses. In contrast, Lsi1 and Lsi2 showed increased expression following silicic acid treatment. The results demonstrate that silicon amendment induces a primed defense response in okra roots, enhancing resistance against root-knot nematodes. Silicon-induced resistance was strongly associated with the activation of key defense-related genes during nematode infection.

Key words: Okra, qRT-PCR, Root-knot nematode, Silicon.

INTRODUCTION

Abelmoschus esculentus L. Moench, commonly known as okra, is a nutritionally rich crop thriving in Mediterranean, tropical and subtropical climates (Ranga *et al.*, 2019). Despite its nutritional value, okra cultivation faces impediments, notably from insect infestations, such as root-knot nematodes (*Meloidogyne* spp.) and fungal pathogens (Maruthi *et al.*, 2025). The deleterious impact of these pests on crop yields not only translates into economic losses for farmers but also contributes to food shortages in affected communities (Ali *et al.*, 2023).

Plant-parasitic nematodes pose substantial threats to vegetable crops, causing considerable annual agricultural production losses globally. Root-knot nematodes, predominant and economically impactful, exhibit a 25-day life cycle at 27°C. Nematode and fungal infestations stand as pivotal biotic factors leading to diminished crop production, with nematodes additionally predisposing plants to heightened susceptibility to fungal diseases. This predisposition is attributed to the modification of plant roots, exacerbating disease severity (Meena *et al.*, 2016).

Silicon (Si) is the second most abundant element in the earth's crust and its importance in agriculture has increased multifold (Bhat *et al.*, 2021). Silicon (Si) is acknowledged for promoting growth and triggering plant-induced resistance against nematodes. This resistance represents an elevated defensive state activated by

¹College of Agriculture, Parul University, Vadodara-391 760, Gujarat, India.

Corresponding Author: Saleha Diwan, College of Agriculture, Parul University, Vadodara-391 760, Gujarat, India.
Email: salehadiwan@gmail.com

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environmental cues, encompassing fungi, bacteria, viruses, nematodes and insect herbivores (Kessmann *et al.*, 1994). Si's role extends beyond growth stimulation, contributing to a robust defense mechanism in plants. Induced resistance manifests as systemic acquired resistance (SAR) and induced systemic resistance (ISR), with distinct elicitor natures and regulatory pathways. Silicon application proves advantageous for plant growth and production, yet variations in silicon accumulation among plant species persist due to variances in root uptake capacity (Takahashi *et al.*, 1990). Silicon accumulation has proven beneficial for various crops such as rice, sugarcane and corn, enhancing their mechanical and physiological traits (Saiyad *et al.*, 2025). Silicon aids plants in overcoming diverse abiotic and biotic stresses, as evidenced by

numerous studies. (Richmond and Sussman, 2003) (Ma *et al.*, 2006) (Zellner *et al.*, 2021).

Quantitative PCR (qPCR), also known as real-time PCR, stands as a widely utilized method for gene expression analysis. Renowned for its high sensitivity, specificity and reproducibility, qPCR enables precise quantification of nucleic acid levels. The real-time monitoring aspect distinguishes it by providing continuous data during the amplification process, making it a fundamental tool in molecular biology for discerning and quantifying gene expression patterns (Brunner *et al.*, 2004). While silicon-mediated resistance against nematodes has been reported in several crop species, there is a lack of comprehensive information on its role in modulating defense-related gene expression in okra under root-knot nematode stress.

The present study was undertaken to evaluate the effect of silicon application on okra growth and defense responses against *Meloidogyne* spp., with particular emphasis on the expression of key defense-related genes using qPCR analysis. It was hypothesized that silicon supplementation enhances okra resistance to root-knot nematodes by activating molecular defense pathways, thereby reducing nematode-induced damage.

Review of literature

Zhan *et al.* (2018) observed that silicon (Si) amendment in rice roots enhances a priming defense response to combat the root knot nematode *M. graminicola*. The Si-induced resistance is linked to increased accumulation of reactive oxygen species (H_2O_2) and phenolic compounds upon nematode attack, illustrating Si's role in fortifying the plant's defense mechanisms.

In a study by Liang *et al.* (2005), the root application of silicon (Si) substantially enhanced the activity of phenylalanine ammonia-lyase in cucumber plants. This increase in enzymatic activity correlated with a notable reduction in the powdery mildew disease index. The findings highlight the potential of silicon-mediated modulation of phenylpropanoid pathway enzymes as a mechanism for enhancing resistance against powdery mildew in cucumber plants.

In a study by Dutra *et al.* (2004), the application of calcium silicate to plants resulted in a significant reduction in the number of root galls and eggs caused by various *Meloidogyne* species in bean, tomato and coffee. This suggests the potential of calcium silicate amendment as an effective strategy for managing nematode infestations and improving plant health in diverse crop species.

Ma *et al.* (2007) explored the genotypic variance in silicon (Si) uptake between japonica var. Nipponbare and indica var. Kasalath. Utilizing real-time reverse transcription polymerase chain reaction, they observed higher expression of Si transporter genes (*Lsi1* and *Lsi2*) in Nipponbare than Kasalath. Immuno staining indicated a consistent subcellular localization pattern of *Lsi1* and *Lsi2* in both varieties, suggesting that Si accumulation

differences arise from varying Si transporter abundance in rice roots.

In Radhakrishna *et al.* (2011) study on miniature roses, the application of 3.6 mM silicon (Si+) enhanced antimicrobial phenolic acids and flavonoids in response to rose powdery mildew. Concurrently, key phenylpropanoid pathway genes (*PAL*, *CAD*, *CHS*) showed upregulation, leading to a 46% reduction in disease severity. The highest concentration of chlorogenic acid, a phenolic compound, increased by over 80% in Si+ inoculated plants, indicating silicon's active role in reducing disease by promoting antifungal phenolic metabolites in response to powdery mildew infection.

Secondary metabolites, including phenolic acids and flavonoids, derived from the phenylpropanoid pathway, are recognized as plant-produced compounds serving as phytoanticipins or phytoalexins. These molecules play a crucial role in plants' defense mechanisms against invading microorganisms, as established by studies such as those by Dixon and Paiva (1995) and Dixon *et al.* (2002).

Si-treated roses after *P. pannosa* inoculation (Shetty *et al.*, 2011). Only a few secondary metabolites have been implicated in Si-induced resistance against fungal diseases (*i.e.* flavonoid phytoalexins in cucumber as well as diterpenoid phytoalexins in rice (Fawe *et al.*, 1998; Rodrigues *et al.*, 2004).

MATERIALS AND METHODS

Experimental design, plant growth conditions and treatments

Seeds of okra (*Abelmoschus esculentus* L. Moench) were obtained from the Main Vegetable Research Station, Anand Agricultural University (AAU), Anand, Gujarat, India. Seeds were surface sterilized using 1% (v/v) sodium hypochlorite solution for 10 min, followed by three rinses with sterile distilled water over a total period of 1 h. Sterilized seeds were sown in plastic pots containing steam-sterilized sandy loam soil and maintained under controlled conditions. Plants were grown until the three-true-leaf stage, at which point nematode inoculation was performed.

Silicon treatment was applied as a seed priming treatment by soaking seeds in 0.1% (w/v) silicic acid solution for 24 h at room temperature, followed by air drying before sowing. Untreated seeds soaked in sterile distilled water served as controls. Nematode inoculation was carried out by introducing 3000 second-stage juveniles (J2) of *Meloidogyne incognita* per plant into small holes made around the root zone. Plants were carefully irrigated after inoculation. Plants were harvested 30 days after nematode inoculation and root samples were collected for gene expression analysis.

The experiment was laid out in a completely randomized design (CRD) with four treatments and three biological replicates per treatment, each replicate consisting of one plant. The treatments were as follows: T1: Control (no silicon, no nematode inoculation).

T2: Silicon treatment (0.1% silicic acid, no nematode inoculation).

T3: Nematode inoculation (*M. incognita* only).

T4: Nematode inoculation + silicon treatment.

Maintenance of pure culture of nematodes

Pure cultures of *M. incognita* were perpetuated in $2.0 \times 1.0 \times 0.5$ m micro plots at the Department of Nematology, B. A. College of Agriculture, AAU, Anand. Continuous multiplication occurred using the root-knot nematode susceptible brinjal cultivar Doli 5, ensuring a year-round supply of nematode cultures for research experiments on okra.

Soil sterilization

Sandy loam soil, collected near the Department of Nematology, was sieved and steam sterilized in aluminum trays ($45 \times 35 \times 15$ cm) at 14 kg/cm^2 pressure for 3 hours. This sterilized soil was employed for pot-based experiments.

Inoculum preparation for root knot nematode

To acquire second-stage juveniles (J_2) of *M. incognita*, galled brinjal roots from dedicated micro-plots were uprooted, thoroughly washed and egg sacs carefully extracted. Fully developed egg sacs were placed on tissue paper supported by a 25-mesh wire gauge over a petri dish with water (Petri-dish Assembly Method by Chawla and Prasad, 1974). After 48 hours, the nematode suspension was collected and J_2 count/ml was determined by averaging five counts under a stereoscopic binocular zoom microscope, readying the nematodes for inoculation.

RNA extraction

Total RNA was isolated from okra root tissues collected at 30 days post-inoculation using the TRIzol reagent method with minor modifications. Approximately 250 mg of root tissue was ground to a fine powder in liquid nitrogen using a pre-chilled mortar and pestle. TRIzol reagent (1 mL) was added and samples were processed according to standard protocols (Sambrook *et al.*, 1989). Chloroform extraction, isopropanol precipitation and ethanol washing steps were performed under RNase-free conditions. The RNA pellet was dissolved in 30-50 μL nuclease-free water and stored at -40°C until further analysis. The present investigation was conducted during the 2023-24 academic year in the Department of Biochemistry, B. A. College of Agriculture, Anand Agricultural University, Anand, Gujarat, India.

Qualitative and quantitative assessment of total RNA

RNA concentration and purity were assessed using a NanoDrop spectrophotometer (Thermo Scientific, USA). Absorbance ratios at A260/A280 and A260/A230 were recorded. Only RNA samples with A260/A280 ratios between 1.8-2.2 and A260/A230 ratios between 2.0-2.2 were used for downstream analysis. RNA integrity was further confirmed by 1.5% agarose gel electrophoresis using $1\times$ TBE buffer. The presence of distinct 28S and 18S rRNA bands indicated good RNA quality.

cDNA synthesis

First-strand cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA). RNA samples ($>100 \text{ ng } \mu\text{L}^{-1}$) were reverse transcribed using random primers following the manufacturer's instructions. The thermal cycling conditions were: 25°C for 10 min, 37°C for 120 min and 85°C for 5 min. Synthesized cDNA was stored at -80°C until use.

Selection and validation of reference and target genes

Housekeeping genes (GAPDH, Actin and UBQ) were evaluated for expression stability across treatments and used as internal controls. Primer sequences for reference and target genes were selected from previously published studies (Ma *et al.*, 2007; Radhakrishna *et al.*, 2011).

Primer specificity was confirmed by:

Primer-BLAST analysis.

Single amplicon detection in melt-curve analysis.

Single band of expected size on 3% agarose gel electrophoresis.

Amplification efficiency for each primer pair was determined using standard curve analysis generated from serial dilutions of pooled cDNA. Primer efficiencies ranged between 90-110%, with correlation coefficients (R^2) ≥ 0.99 , meeting qRT-PCR quality requirements (Table 1).

Quantitative real-time PCR (RT-qPCR)

RT-qPCR reactions were performed using a CFX-96 Real-Time PCR System (Bio-Rad, USA). Each reaction (10 μL final volume) contained 0.3 μM of each primer, 2 μL of diluted cDNA, 6.25 μL of $2\times$ Maxima SYBR Green qPCR Master Mix (Thermo Scientific, USA) and nuclease-free water.

Thermal cycling conditions were:

Initial denaturation at 95°C for 10 min.

40 cycles of 95°C for 15 s and 60°C for 1 min.

Table 1: List of primers.

Target gene		Sequence (5' to 3')
<i>Lsi1</i>	F	CGGTGGATGTGATCGGAACCA
	R	CGTCGAACCTTGTGCTCGCCA
<i>Lsi2</i>	F	ATCTGGGACTTCATGGCCC
	R	ACGTTTGATGCGAGGTTGG
<i>PAL</i>	F	TCCTGACTGGCGAAAAGTTC
	R	GAAGAGGTTACCGTTCCAA
<i>CHS</i>	F	ACAGCAACTCCTCCCAACTG
	R	CGCTGGAATTTCTCCTTGAG
<i>CAD</i>	F	AGGACGGAGGAGGCTAGGTTA
	R	ATGGCATGGGTTACTTCAGC
<i>GAPDH</i>	F	CAACGAGAATGAATACAAGCCA
	R	GAGACCCTCAACAATGCCAA
<i>Actin</i>	F	GACTCTGGTGATGGTGTGAGC
	R	GGCTGGAAGAGGACCTCAGG
<i>UBQ</i>	F	GTGTAATGTTGGATGTGTTCCC
	R	ACACAATTGAGTTCAACACAAACCG

Dissociation curve analysis (55-95°C) was performed to verify amplification specificity. Three biological replicates, each with three technical replicates, were analyzed for every treatment. Non-template controls (NTCs) were included for each primer set.

Statistical analysis of gene expression data

Cycle threshold (Ct) values were obtained using CFX Manager™ software (Bio-Rad). Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Vandesompele *et al.*, 2002), with normalization against the geometric mean of selected reference genes.

Statistical analysis was performed using SPSS version XX/R software (specify if needed). Differences among treatments were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test. Results were considered statistically significant at $P \leq 0.05$.

RESULTS AND DISCUSSION

Real time PCR for endogenous genes study

In qRT-PCR with SYBR Green, synthesized c-DNA amplifies any double-stranded DNA. Observations were recorded in terms of Ct values (Table 2).

Variations of endogenous genes Ct value

Actin demonstrated robust and high expression, evidenced by its low Ct value of 17.01, indicating a strong positive reaction. In contrast, GAPDH exhibited comparatively lower expression, with a higher Ct value of 29.89, suggesting a less intense reaction. Ct levels inversely correlate with target nucleic acid amount; $Ct < 29$ signifies strong positive reactions with abundant target, while Ct values of 30-37 indicate positive reactions with moderate target amounts. Weak reactions with minimal target nucleic acid are associated with Ct values of 38-40.

Selection of stable endogenous gene through ΔCt approach

In Table 3, the stability assessment reveals Actin as a stable reference gene with a low M value of 0.53, while GAPDH (0.78) and UBQ (1.02) exhibit lower stability. Actin, with the lowest M value, is consequently chosen as the stable endogenous reference gene for subsequent qRT-PCR analysis of various target genes.

Expression of defense-related genes under nematode and silicon treatments phenyl ammonia lyase (PAL)

PAL expression was strongly induced under *M. incognita* infection (T3), particularly in AOOL 10-22 and Pusa Sawani, indicating activation of the phenylpropanoid pathway in response to nematode stress. Silicon treatment alone (T2) and combined silicon + nematode treatment (T4) also enhanced PAL expression across all varieties (Fig 1).

Interestingly, PAL expression under T4 was lower than T3, suggesting that silicon-mediated resistance reduced nematode stress severity, thereby lowering the requirement for extreme defense activation. This moderated expression

reflects a primed defense state, where plants respond more efficiently rather than excessively. PAL is a key enzyme linking primary metabolism to secondary metabolite synthesis, including phenolics and flavonoids involved in plant defense (Dixon and Paiva, 1995).

Cinnamyl-alcohol dehydrogenase (CAD)

CAD transcripts were significantly upregulated under nematode infection, with Arka Anamika showing the highest induction, indicating varietal differences in lignification-related defense responses (Fig 2). Silicon supplementation (T2 and T4) further enhanced CAD expression, supporting its role in strengthening cell walls through lignin biosynthesis.

The relatively lower CAD expression in T4 compared to T3 suggests reduced tissue damage under silicon-mediated resistance, consistent with earlier reports linking silicon application to reduced disease severity and controlled activation of phenylpropanoid pathway enzymes (Christian *et al.*, 2012).

Chalcone synthase (CHS)

CHS expression increased markedly under nematode stress (T3), particularly in Arka Anamika and Pusa Sawani, highlighting enhanced flavonoid biosynthesis under biotic stress (Fig 3). Silicon treatment alone and in combination

Table 2: Ct values of endogenous primers obtained through qRT-PCR in root tissue of okra.

Varieties	Treatments	Threshold cycle (Ct) value		
		<i>Actin</i>	<i>GAPDH</i>	<i>UBQ</i>
GAO 5	T1	18.15	27.51	24.43
	T2	17.09	26.44	24.21
	T3	17.41	28.37	24.20
	T4	18.18	27.45	23.98
Pusa sawani	T1	16.74	27.62	25.34
	T2	17.95	26.35	25.67
	T3	18.39	26.64	25.89
	T4	18.12	26.87	23.34
AOL 10-22	T1	18.89	27.19	25.56
	T2	17.58	26.89	24.78
	T3	18.23	26.43	24.87
	T4	18.88	27.23	23.21
Arka anamika	T1	17.56	28.34	25.23
	T2	18.32	28.23	24.89
	T3	17.01	29.89	24.67
	T4	18.13	29.21	25.88

T1: Control, T2: Silicic acid, T3: Inoculated with *M. incognita*, T4: Silicic acid + Inoculated with *M. incognita*.

Table 3: Stability value (M value) of endogenous genes.

Reference genes	Melting temperature (°C)
Actin	0.53
GAPDH	0.78
UBQ	1.02

with nematode infection also upregulated CHS expression, though to a lesser extent than T3.

This pattern supports the role of silicon in priming flavonoid-based defenses, enabling efficient pathogen

resistance while avoiding excessive metabolic cost. Phenylpropanoid-derived flavonoids are known to function as antimicrobial and signaling compounds during pathogen attack (Dixon and Paiva, 1995).

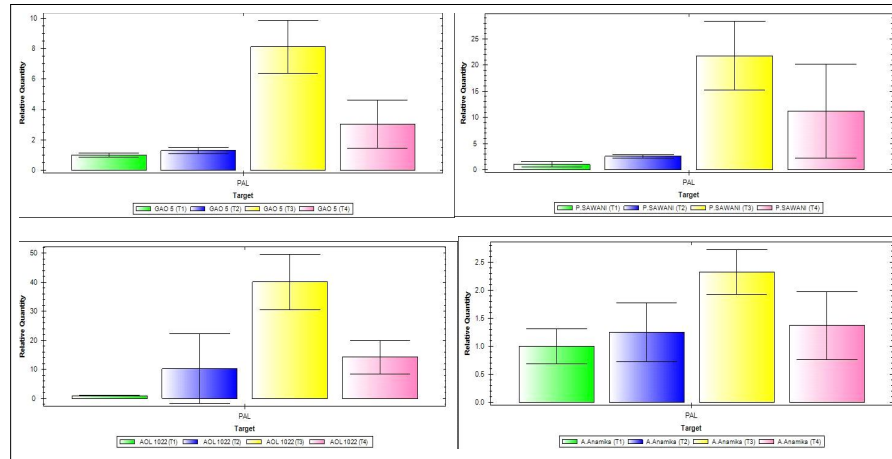


Fig 1: Relative quantity (RQ) plot for target gene *PAL* in okra root.

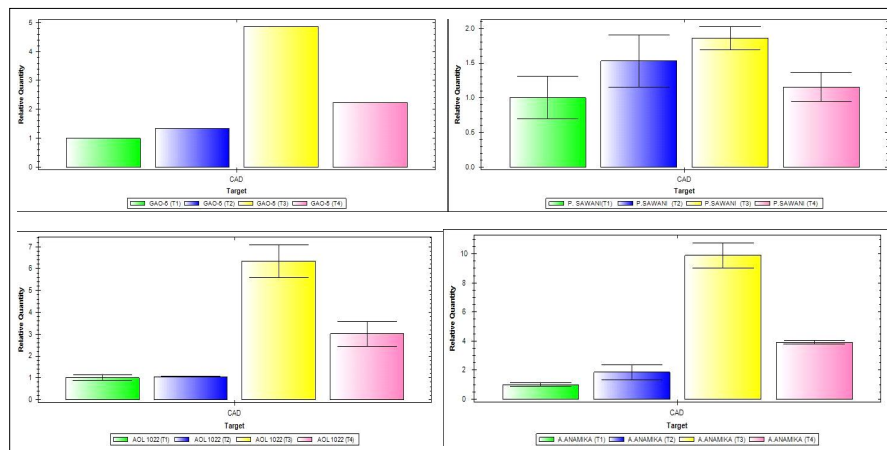


Fig 2: Relative quantity (RQ) plot for target gene *CAD* in okra root.

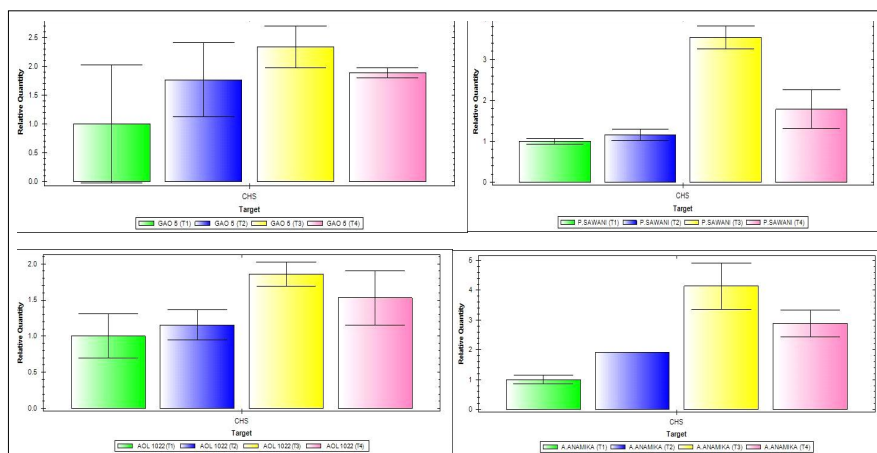


Fig 3: Relative quantity (RQ) plot for target gene *CHS* in okra root.

Expression of silicon transporter genes

Lsi₁

Lsi1 expression was significantly upregulated under silicon treatment (T2) and combined silicon + nematode treatment (T4) across all okra varieties (Fig 4). This confirms the activation of silicon influx mechanisms in response to external silicon availability. Varietal differences were evident, with Arka Anamika and Pusa Sawani exhibiting higher induction, suggesting superior silicon uptake efficiency.

Lsi1 functions as an influx transporter facilitating silicon entry into root cortical cells, thereby enhancing silicon-mediated defense responses (Ma *et al.*, 2007).

Lsi₂

Similarly, *Lsi2* expression increased significantly under silicon-supplemented treatments, with consistent upregulation under T2 and T4 across varieties (Fig 5). The coordinated induction of *Lsi1* and *Lsi2* indicates an active silicon transport system enabling silicon translocation from roots to aerial tissues.

Lsi2 acts as an efflux transporter responsible for silicon movement toward the stele, contributing to systemic stress tolerance (Ma *et al.*, 2007).

Comparative varietal response

Among the okra varieties, Arka Anamika and AOOL 10-22 consistently exhibited stronger induction of defense-related and silicon transporter genes, suggesting higher inherent or silicon-enhanced resistance to *M. incognita*. GAO 5 and Pusa Sawani showed comparatively moderate responses, indicating varietal variability in silicon uptake and defense signaling efficiency.

Biological significance of reduced expression under T4

The observed reduction in defense gene expression under combined silicon + nematode treatment (T4) compared to nematode-only treatment (T3) likely reflects effective silicon-mediated mitigation of nematode stress. Rather than indicating weakened defense, this suggests reduced pathogen pressure due to strengthened physical barriers, enhanced phenolic deposition and improved root integrity.

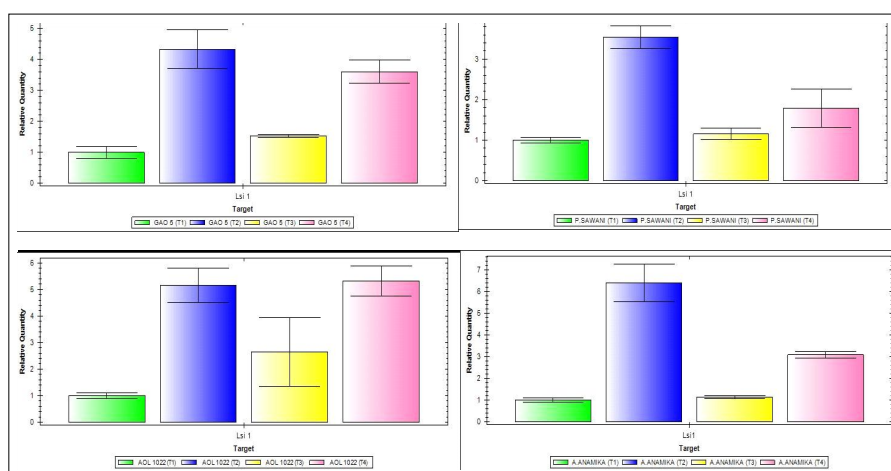


Fig 4: Relative quantity (RQ) plot for target gene *Lsi₁* in okra root.

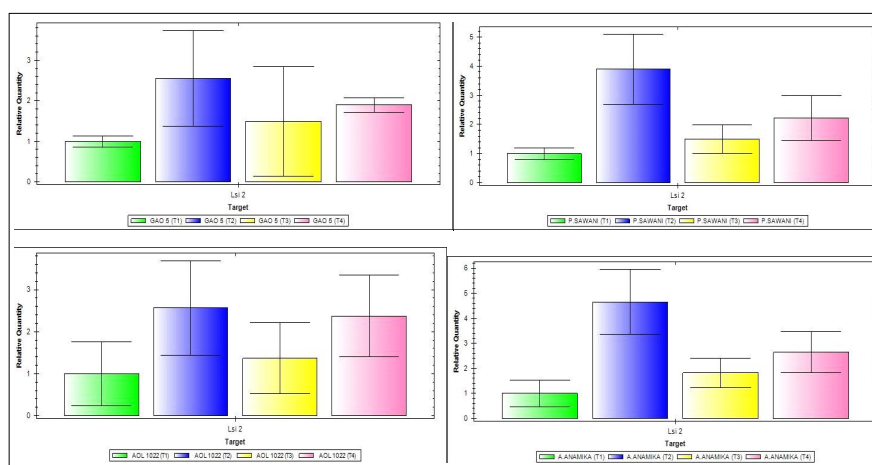


Fig 5: Relative quantity (RQ) plot for target gene *Lsi₂* in okra root.

Radhakrishna *et al.* (2011) investigated silicon-induced changes in roses, finding that 3.6 mM silicon (Si+) application increased antimicrobial phenolic acids and flavonoids in response to rose powdery mildew. Simultaneously, key phenylpropanoid pathway genes were upregulated, correlating with a 46% reduction in disease severity compared to SiO₂-treated leaves. The study concludes that silicon actively reduces disease in roses by inducing the production of antifungal phenolic metabolites in response to powdery mildew infection.

Ma *et al.* (2007) explored genotypic differences in silicon uptake between japonica var. Nipponbare and indica var. Kasalath, finding higher expression of Si transporter genes (*Lsi₁* and *Lsi₂*) in Nipponbare compared to Kasalath, indicating distinct physiological and molecular mechanisms in silicon absorption.

Limitations and future perspectives

Although this study provides molecular evidence of silicon-induced resistance in okra, it is limited to transcript-level analysis under controlled conditions. Future studies should integrate enzyme activity assays, metabolite profiling and field-based evaluations to validate the functional relevance of gene expression changes. Additionally, exploring cross-talk between silicon signaling and hormone-mediated defense pathways would further elucidate the mechanisms underlying silicon-induced resistance.

CONCLUSION

AOL 10-22 exhibited the highest *PAL* upregulation (40.06 fold) in disease conditions, while Arka Anamika showed maximum upregulation for *CAD* (9.89 fold) and *CHS* (4.13 fold). *Lsi1* and *Lsi2* displayed upregulation in treated conditions (T₂ and T₄), with Arka Anamika demonstrating the highest upregulation patterns (*Lsi1*: 6.38 fold, *Lsi2*: 4.65 fold) among all okra varieties. The gene expression study indicates consistent upregulation of *PAL*, *CAD* and *CHS* genes in all okra varieties under disease conditions. The synergistic effect of silicic acid and *M. incognita* (T₄) not only reduced disease severity but also led to downregulation of gene expression compared to T₃, potentially enhancing resistance against biotic stress. Additional research is essential to comprehensively explore the silicon-plant-nematode interaction system, aiming for an enhanced understanding of silicon-induced defence mechanisms in okra against root knot nematodes and various disease conditions, contributing to the development of effective management strategies.

Conflict of interest

The authors declare that they have no conflict of interest regarding the publication of this research. There are no financial or personal relationships with other people or organizations that could inappropriately influence or bias the content of this study.

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