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Mechanistic Insights into Fluoride Stress Mitigation in Vigna mungo: The Interplay Between Host Genotype and Bradyrhizobium Symbiosis

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ABSTRACT
Fluoride (F) toxicity in soil is a growing agricultural challenge that severely limits the growth and nitrogen-fixing potential of pulses like black gram. This study provides mechanistic insights into how Bradyrhizobium symbiosis alleviates stress and investigates the role of host genotype in this protective relationship. By evaluating tolerant and sensitive blackgram genotypes under gradients, we observed that fluoride exposure triggers oxidative damage and disrupts nodulation. However, Bradyrhizobium inoculation significantly mitigated these effects by enhancing the antioxidant defense system (SOD, CAT, and APX). The tolerant genotype exhibited a more robust synergy with the microsymbiont, suggesting that the effectiveness of the symbiosis is governed by the host's genetic architecture. These results highlight the biological buffering capacity of Bradyrhizobium and suggest that integrating specific plant-microbe combinations is a viable strategy for sustainable pulse production in fluoride-contaminated soils.

KEYWORDS: qRT-PCR, Statistics, Fluoride, Ct value

INTRODUCTION
The most electronegative and smallest halogen found in nature is F. It is a common and persistent environmental pollutant which is difficult to degrade, is 13th in abundance in the crust of the earth and is a threat level 12 in the biosphere. Its limited range of safety options has earned it the nickname of the 'two-edged sword (Banerjee *et al.,* 2020; Sruti *et al.,* 2023). In many countries, the main problem is the contamination of soil and groundwater with fluoride (F) at levels above the permitted levels. According to the WHO recommendation that the recommended upper limit of F in drinking water is 1.5 ppm (mg/L) (Yadu *et al.,* 2016). Nowadays, Agricultural soils have high levels of F are typically because of long-term accumulation of F from a number of sources, such as industrial waste and extensive use of phosphate fertilisers and insecticides containing F (Zouari *et al.,* 2017).

Reactive oxygen species (ROS), such as superoxide anion radicals (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-), peroxyl radicals (HOO^-), and singlet oxygen ($^1O_2$), are undoubtedly produced in conjunction with these stresses. These ROS cause peroxidation and degradation of macromolecules, damage to cell membranes, and ultimately cell death. Superoxide dismutase is an antioxidant enzyme that may effectively scavenge reactive oxygen species (ROS) by catalyzing the breakdown of superoxide anion radicals (O_2^-) into hydrogen peroxide (H_2O_2), which is then transformed into harmless water and oxygen (Zhou *et al.,* 2017). Superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) are examples of enzymatic antioxidant defense mechanisms that tightly regulate ROS generation in plant cells. CAT and APX both scavenge H_2O_2 buildup in plant cells; however, APX is mostly found in the cytosol, cell walls, and vacuoles, whereas CAT is found in peroxisomes. By using ascorbic acid (AsA) as the deoxidizer in the cytosol and chloroplast, APX primarily reduces H_2O_2 to H_2O while concurrently generating two molecules of monodehydroascorbate (MDHA) and a short-lived radical (Zhang *et al.,* 2015).

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Both global and specific gene expression profiles are becoming indispensable in advanced molecular biology and molecular breeding studies. The expression of each gene is dynamic and spatiotemporal, influenced by a variety of internal and external variables. Quantification of mRNA is required to understand the amount of gene expression in biological materials. Real-time PCR, a straightforward, sensitive, and adaptable method that can be used to numerous samples (Nicot *et al.*, 2005) and numerous genes in a single experiment, is the conventional approach for gene expression analysis. There are two methods for determining the amounts of gene expression in real-time PCR experiments: absolute quantification and relative quantification. Delta C_t techniques were created for the relative measurement of gene expression levels. The basic idea is to determine differences in means of gene expression levels between groups of samples (or technical duplicates of a single sample), normalize gene expression values using an endogenous control gene, and convert gene expression values into linear form using the $2^{-(value)}$ algorithm.

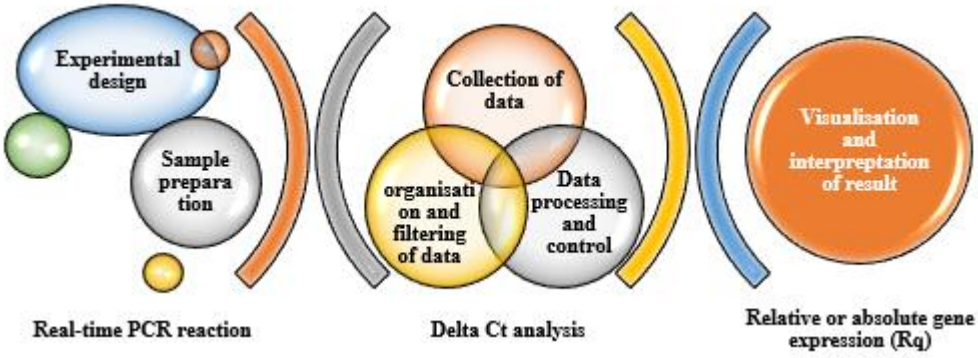

Figure 1. Core steps quantitative Real time – Polymerase Chain Reaction

The 2^{-ddCt} method also involves normalizing C_t values by endogenous control gene; however, the obtained delta C_t (dC_t) values are summarized by means in the compared study groups instead of being exponentially transformed. The ddC_t value is obtained by subtracting the mean dC_t in a control group from the mean dC_t in a study group (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008).

MATERIAL AND METHODS

2.1 Selection criteria and characterization of *Vigna mungo* genotypes

Seeds of contrasting *Vigna mungo* (Blackgram) genotypes Mukundra urd-2 and Pratap urd 1 were precured from agricultural research station, Ummedganj, Kota, Rajasthan. Varieties were selected based on preliminary screening of fluoride tolerance indices and their phenotypic response to abiotic stress. Healthy seeds were surface sterilized using 0.1% (w/v) $HgCl_2$ for 3 minutes, followed by 5 successive washing with deionized water to remove surface contaminant.

2.2 Symbiotic inoculation and stress regimes

The plants were cultivated in a controlled pot culture condition. Symbiotic treatment was established by inoculating seeds with a compatible *Bradyrhizobium* strain (MTCC2380) which was precured from Microbial type Culture collection and gene bank, Chandigarh, India. The strain was cultured on yeast extract mannitol (YEM) broth at 28±2°C for 24-48 h until reaches logarithmic phase. To evaluate the molecular response to fluoride toxicity, the experimental sets were subjected to two discrete concentrations of sodium fluoride (NaF): 30 and 40ppm. For control set, seeds without fluoride stress and bacterial treatment were considered as it serves a baseline for comparative physiological and transcriptional analysis. The leaf tissue from each tested set was harvested after 14 days of sowing, flesh frozen in liquid nitrogen and stored at -80°C for downstream molecular analysis.

2.3 Transcriptional profiling via qRT-PCR: assay designing and gene selection

To elucidate the molecular mechanism governing fluoride tolerance, we investigated the transcriptional dynamics of suite of core antioxidant enzymes: Superoxide dismutase (SOD), Catalase (CAT) and Ascorbate

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peroxidase (APX). These targets were strategically selected due to their pivotal role in scavenging reactive oxygen species and maintain redox homeostasis under environmental perturbations.

2.3.1 Quantitative transcriptional profiling: RNA integrity and cDNA synthesis

High-quality RNA was successfully extracted from 100 mg of leaf samples using the Trizol technique, which produced a distinct RNA pellet after precipitation and a clear phase separation. This technique offered a dependable and effective RNA isolation strategy that could be used in a number of downstream procedures, including real-time PCR and cDNA synthesis. This method was optimized to remove polyphenols common in legume plants. RNA quantity and quality was assessed using nanodrop spectrophotometer (A_{260}/A_{280} ration~2.0).

First stand cDNA was synthesized from1 µg RNA of total RNA using the instruction given by manufacturer on reverse transcription kit, FIREScript RT cDNA synthesis kit (Solis BioDyne). Nanodrop was used to quantify the cDNA, and a 1.5% agarose gel was used to assess its quality. 25s rRNA (housekeeping gene) was used to normalize the cDNA. PCR amplification using gene-specific primers was performed using normalized cDNA.

2.3.2 Stress-Responsive Gene Primer and PCR Amplification

NCBI blastn was used to create the primers needed to amplify the chosen genes. 150–250 bp amplicon sizes were chosen. Barcode BioSciences (Bengaluru, Karnataka) produced the primers. After the primers were reconstituted, gradient PCR with temperatures between 55 and 65 ℃ was used to normalize the annealing temperature (Tm). The resulting cDNA was made less concentrated to reach a level of 10 ng/ µl. The total cDNA was used for measurement with SYBR green Universal PCR Master Mix (2X). The reactions were carried out using a StepOnePlus Real-time PCR system. Three technical copies were run in a 96-well plate setup. At the same time, a no-template control (NTC) was included for each set of primers to check for any possible contamination, non-specific DNA fragments, or primer dimer formation. The thermal cycling process for the qPCR involved an initial step of 2 minutes at 95 ℃ to activate the enzyme, followed by 10 seconds at 95 ℃ for DNA denaturation, 30 seconds at 60 ℃ for annealing and extending the DNA strands, and this cycle was repeated for 40 to 45 times, during which data was collected. The RNA concentration across different samples was adjusted based on the amount of the housekeeping gene Actin (HKG) present in each RNA sample.

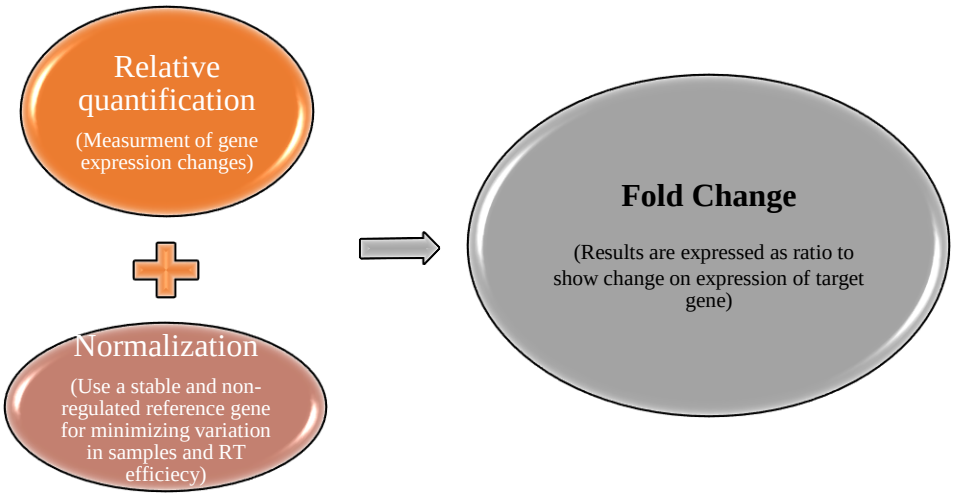

Figure 2: Core concept for quantification of Relative gene expression

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2.4 Statistical analysis and Bioinformatic integration

Relative fold-changes in gene expression was calculated using the comparative $C_T(^{-2\ \Delta\Delta Ct})$ method, with actine serving as internal reference gene for normalization. To ensure the technical and biological reproducibility, all qRT-PCR assays were performed in triplicates (n=3) for each biological sample

Calculation of Relative gene expression

Step-1: Calculation of Δ C_t (C_t stands for Threshold cycle)

Each target gene is normalized to reference gene using formula mentioned below:

$\Delta C_t = C_t$ (Target)-C_t (Reference)

Step-2: Calculation of ΔΔ C_t

Each ΔC_t value to normalized to control employing formula:

$\Delta\Delta C_t = \Delta C_t$ (Sample)- ΔC_t (Control)

Step-3: Calculation of fold change

Relative expression is calculated by $2^{-\Delta\Delta Ct}$.

RESULT AND DISCUSSION

Quality assessment of RNA and validation of reference gene stability

RNA integrity was validated using A_{260}/A_{280} ratio ensuring high quality cDNA template synthesis. Our samples exhibit A260 and A280 ratios of 1.8-2.0 and 2.0-2.2, respectively. These measures ensure the excellent quality of the RNA for use in subsequent processes such as RT-qPCR. However, lower values were also found in a small number of samples. These samples were processed independently for the cDNA synthesis.

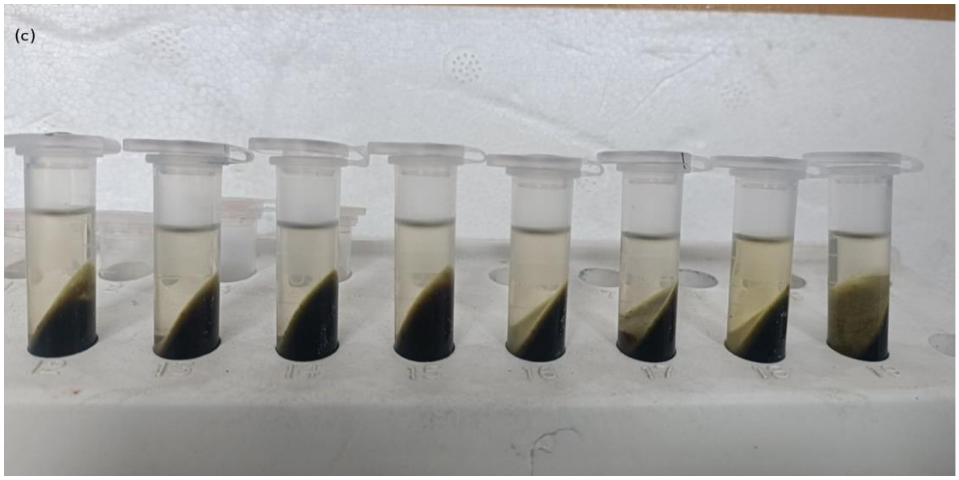

Figure 2. RNA extraction via Trizol method (Phase separated after adding chloroform (RNA in top colorless aqueous phase, DNA in interphase and at the bottom organic phase

Table 1. Primer sequence of SOD, CAT and APX of both the varieties

Sr. No.	Antioxidant enzymes	Primers	Sequence 5'-3'	Length
1	SOD	Forward primer	GTGAATCCTCTTCCATCTGACG	22
2		Reverse primer	TTT CTCCCTCTCCACAATTCG	21
3.	APX	Forward primer	GTTCCTCACTCCTTCGTCAC	20
4		Reverse primer	CGTTCCGTATCCTTCACTCG	20
5	CAT	Forward primer	AACCCTAAGAACCACATCCAG	21
6		Reverse primer	CCTCCAATAGACACTTCACACC	22

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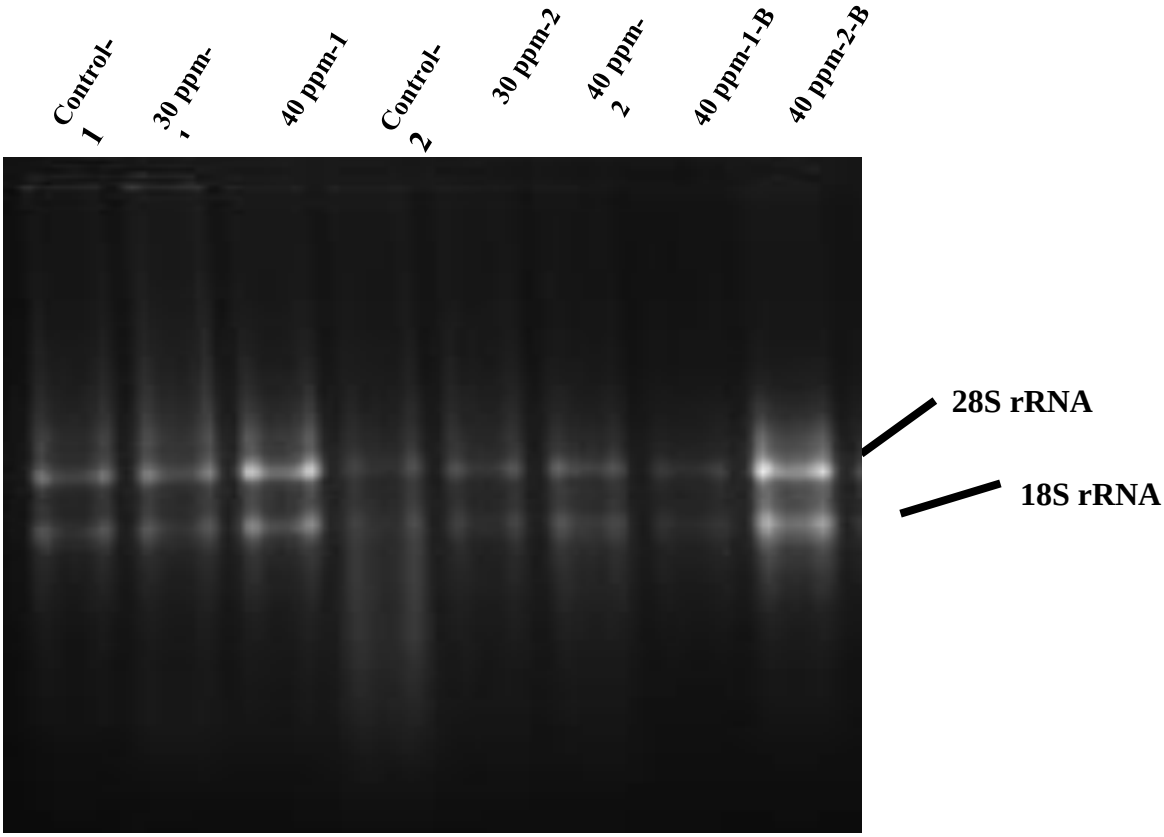

Figure 3. RNA has been isolated from shoots of two *Vigna mungo* cultivars, PU 1 and MU 2, and was checked on a 1.2% MOPS-containing denaturing agarose gel. The RNA samples that looked good quality were then used for cDNA synthesis and to estimate the relative transcription levels.

Each gene produced an expected size amplified APX, CAT, SOD, and actin, which showed precise and targeted amplification. To determine whether genomic DNA contamination was present, a non-reverse transcriptase sample of RNA was used as a template. The fact that the Actin gene was not amplified in this assay indicates that the amplicons are produced exclusively from cDNA synthesised and that the RNA products are not contaminated with DNA.

The extracted RNA was processed by DNase to remove any remaining DNA, and then the actin-specific primers were used to screen for DNA contamination. Not all samples analysed had an amplicon. The RNA content of each sample was corrected to 1.0/1.10 µl by a nanodrop test using water treated with DEPLC to avoid nucleases. The presence of Actin transcript after conversion of this RNA to cDNA was examined.

Impact of Fluoride Intensity on Antioxidant Transcript Abundance

Quantitative analysis revealed that Fluoride stress induces a drastic transcriptional collapse at higher concentration (40ppm). In Pratap Urd 1 transition from 30ppm to 40ppm caused SOD C_t values to plummet from an active 18.40 to a suppressed 32.55. Our findings were supported by previous study conducted by Singh *et al.*, 2021 who found that high concentrations of fluoride act as metabolic inhibitors, repressing the gene expression of CAT by up to 51% in sensitive *Vigna* species, while moderate fluoride stress can stimulate antioxidant activity. Similarly, in Mukunda 2 the 40ppm F treatment result in significant downregulation of SOD (C_t 32.24) and CAT (C_t 28.87), indicating that high fluoride levels severely impair the basal expression of the enzymatic defence machinery. The data clearly show that 40-ppm fluoride cat as a critical threshold for *Vigna mungo* the sharp increase in C_t values indicate a state of *metabolic exhaustion* where plant can no longer synthesise protective mRNA which reflects the dose-dependent molecular behaviour. The *transcriptional silencing* is a key indicative of fluoride toxicity, likely due to direct inhibition of transcriptional factors or

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increased oxidative degradation of RNA template.

***Bradyrhizobium* inoculation Induced Recovery and Transcriptional Priming**

The addition of *Bradyrhizobium* serves as a powerful rescue agent particularly at 40ppm stress levels. In Pratap Urd 1 bacterial treatment at 40ppm showed a massive restoration of CAT transcript (17.93) compare to fluoride only treatment (29.34). A similar trend was observed in Mukundra urd 2 where bacterial treatment reduces the SOD C_t from 32.24 to 26.72, effectively increasing the relative transcript abundance to combat oxidative damage. Our findings were conclusive of ability of *Bradyrhizobium* to reverse fluoride induced suppression.

Table 2. C_t values of gene analyzed in control and treated plant samples of Pratap urd 1 and Mukundra urd 2

Enzyme	C_t value (30ppm)	C_t value (30ppm+Brady)	C_t value (40ppm)	C_t value (40ppm+Brady)
Mukundra urd-2				
SOD	23.80579185	33.13447571	32.24349594	22.22059631
CAT	23.66988564	30.88954163	28.87065125	17.9374485
ACTIN	22.84574127	21.10664749	24.0377636	22.48548889
APX	22.37465858	24.20924568	28.685709	25.81879616
Pratap urd 1				
SOD	19.44008636	18.40818024	32.55200195	22.22059631
CAT	29.34453773	15.53603649	29.34453773	17.9374485
ACTIN	21.9481411	21.66622353	25.36908913	22.48548889
APX	20.99539566	17.23552513	23.4042263	25.81879616

The dramatically lowering of C_t values at 40ppm with bacterial inoculation suggests that symbiont primes the host's antioxidant pathways. The microbial mediated enhancement of CAT and SOD transcripts likely provides the necessary backbone to neutralize fluoride induce H_2O_2 by protecting the photosynthetic and nitrogen fixing apparatus. Mott *et al.,* (2022) and recent microbial adaptation studies suggest that *Bradyrhizobium* inoculation does not merely fix nitrogen but actively primes the host's antioxidant genome. Our finding that *Bradyrhizobium* restores *APX* and *SOD* expression in fluoride-stressed plants extends the "Bio-Shield" hypothesis proposed in recent heavy metal research, confirming that the symbiont protects the nodule's redox state by sustaining the transcription of host defense enzymes even when physiological thresholds are exceeded.

Genotypic divergence in molecular defence

The differential resilience between Mukundra urd-2 and Pratap urd 1 aligns with the tolerant vs sensitive paradigms established in comparative legume studies. Our data shows Mukundra urd-2 maintains a stable basal gene expression (consistent *ACTIN* and moderate *APX* C_t values) under stress, a characteristic of tolerant genotypes identified by Singh *et al.,* 2021. In contrast, the erratic stress response of Pratap urd 1 (extreme *CAT* upregulation to C_t 15.53) parallels the behaviour of fluoride sensitive cultivars described in recent transcriptomics profiling, where hypersensitivity leads to rapid but unsustainable gene induction followed by systemic failure (Fikret *et al.,* 2013)

Bioinformatic Correlation: Linking Gene Expression to Symbiotic Performance

Calculating log base 2 values using 2^{-DDCt}, we found that the levels of SOD, CAT and APX genes differed between samples of treated and untreated of the bacteria of both varieties. Our results were in line with Livak and Schmittgen, 2001.

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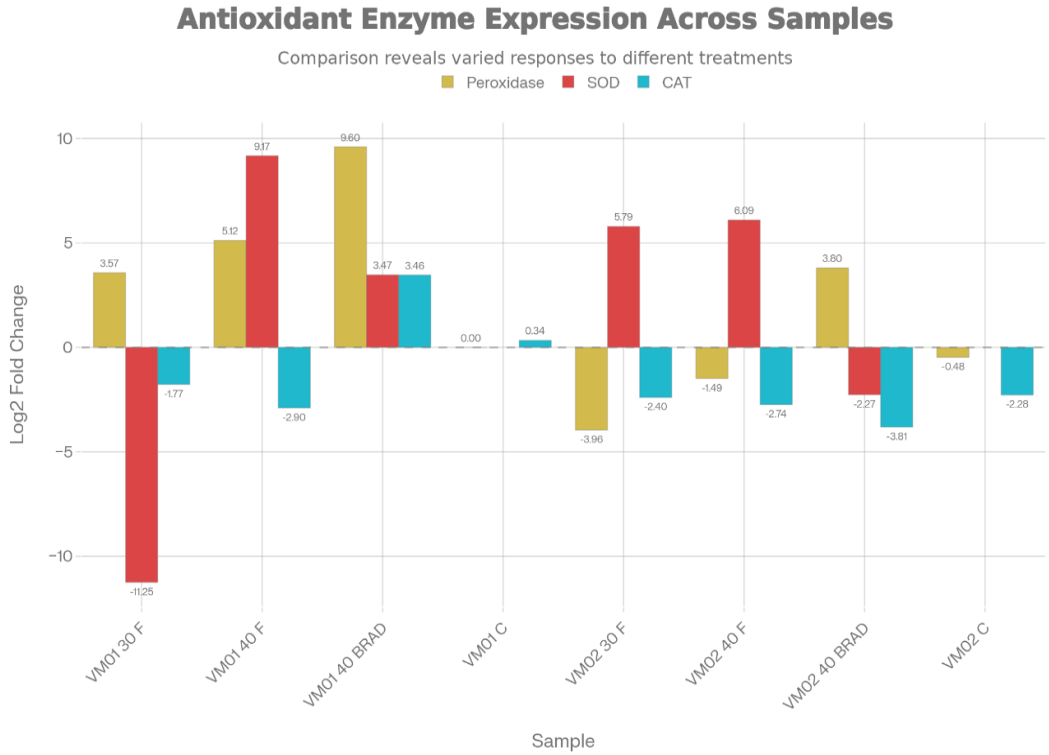

Figure 4. Using the $2^{\wedge(-\Delta\Delta CT)}$ method (Livak and Schmittgen 2001) to calculate the log base 2 of the relative expression levels, we found that the SOD, CAT, and APX gene expression levels varied in the *Vigna mungo* samples VM01 (Mukundra urd -2) and VM02 (Pratap urd 1) at different concentrations in comparison to the control.

Mukundra urd-2 (40 ppm + *Bradyhizobium*) shows the highest peroxidase up-regulation at 9.60 log_2, suggesting that bacterial and fluoride stress are optimal factors for expression of peroxidase. Mukundra urd-2 activates peroxidase under all stress conditions. Conversely, Pratap urd exhibits genotypic downregulation by 30 ppm (3.96), suggesting that there is different regulation of peroxidase response by both genotypes. The SOD expression showed a large difference of 20.4-fold between 30 and 40 ppm for Mukundra urd 2 (11.25 and 9.17) log_2 FC compared to Pratap urd 1 in Fluoride. SOD maintains its status as a highly fluorine sensitive gene. The high variability between 11.25 (Mukundra urd 2, 30 ppm) and 9.17 (Mukundra urd-40 ppm) indicates that fluoride is primarily of importance as an adaptive response to stress.

All three antioxidant genes are co-upregulated by *Bradyrhizobium* 40 ppm (3.46), with primary upregulation occurring in *Bradyrhizobium* in mukundra urd -2. Although *Bradyrhizobium* is an antagonist of Pratap urd 1 40 ppm + *Bradyrhizobium* with CAT, which is the lowest expression of the antioxidant defence (3.81), treatment with *Bradyrhizobium* appears to increase the overall mobilisation of antioxidant protection in Mukundra urd-2.

M Control SOD CAT APX ACT

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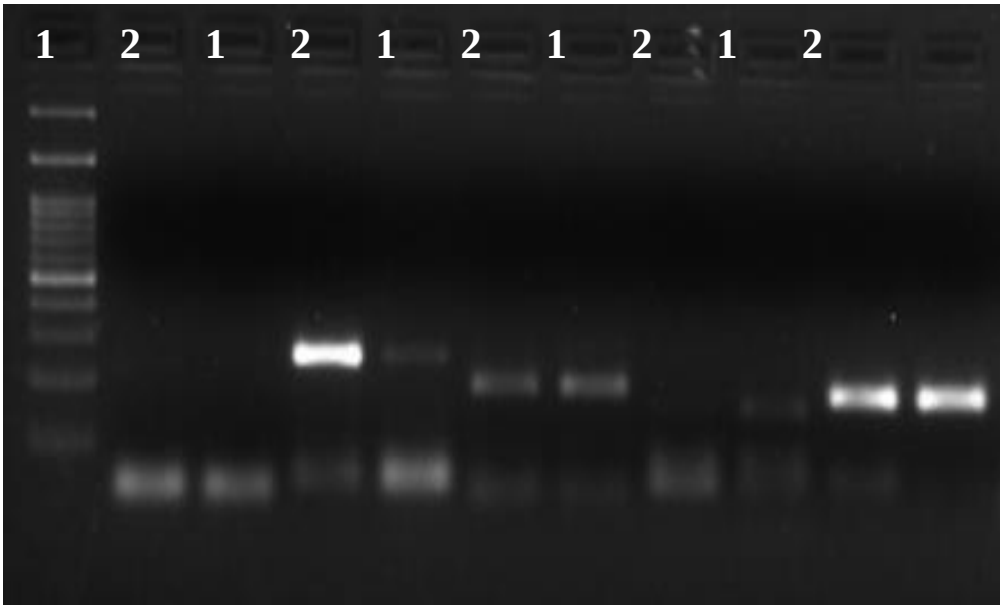

Figure 5. Gel photograph showing presence of amplicons for various transcripts in 1 (Pratap Urd 1) and 2 (Mukunda Urd 2) samples. M indicate 100bp Ladder

Fluoride acts as an inducing factor for SOD in Pratap urd 1 (5.79 at 30ppm and 6.09 at 40ppm), but it gets suppressed by *Bradyrhizobium* inoculation. CAT is still largely downregulated. Fluoride acts as an inducer of SOD in Pratap urd 1 (5.79 at 30 ppm and 6.09 at 40 ppm), but is suppressed by inoculation with *Bradyrhizobium.* CAT is still to a large extent unregulated.

CONCLUSION

This study provides a definitive molecular blueprint for fluoride (F^-) stress mitigation in *Vigna mungo* by elucidating the transcriptional synergy between the host genotype and *Bradyrhizobium* symbiosis. Our qRT-PCR analysis demonstrates that while stress suppresses the genetic machinery responsible for ROS scavenging, inoculation with *Bradyrhizobium* triggers a robust "transcriptional rescue," significantly upregulating the expression of Superoxide Dismutase (SOD), Catalase (CAT), and Ascorbate Peroxidase (APX). This coordinated induction of the Foyer-Halliwell-Asada pathway at the mRNA level suggests that the microsymbiont actively reprograms the host's antioxidant defense to maintain cellular redox homeostasis. Notably, the superior resilience observed in specific genotypes is directly linked to a more efficient and rapid upregulation of these transcripts, identifying these genes as critical molecular markers for fluoride tolerance. By proving that *Bradyrhizobium* serves as a genetic primer for stress resilience, this research offers a sophisticated biotechnological strategy for securing legume productivity in F^- effected landscapes through the targeted use of symbiotic bio-inoculants.

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