**SHORT COMMUNICATION**

# INTEGRATED MOLECULAR RESPONSES: UNVEILING THE SYNERGIES BETWEEN COCOA POD BORER INFESTATION AND ALUMINUM STRESS IN ACIDIC SOIL

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**ABTRACT -** *This study explores the intricate interplay between molecular responses in cocoa trees facing challenges from Cocoa Pod Borer (CPB) infestation and aluminum stress in acidic soil. Transcriptome analysis of CPB-infested and uninfested cocoa tissues reveals a shared upregulation of critical genes, including chitinase and osmotin. Notably, a subset of these upregulated genes involved in aluminum responses, such as alcohol dehydrogenase, pectin methylesterase, aluminum transporter protein, and heavy metal transport protein, demonstrates a unique dual role in the face of CPB infestation and aluminum-induced plant stress. Commonly planted in slightly acidic soil (ph 5.5-6.5), cocoa trees exhibit a complex but coordinated molecular response to CPB and aluminum stress. The identified aluminum-responsive genes, showing exclusive upregulation in CPB-infested plants, emerge as potential markers for specific CPB clones. The integration of transcriptome analysis and qPCR validates these findings, offering a comprehensive understanding of the shared molecular pathways activated in response to these environmental challenges. This study enhances our knowledge of cocoa tree defense mechanisms and lays the foundation for targeted strategies in breeding programs to fortify cocoa trees against CPB while adapting to aluminum-rich acidic soils.*

***Keywords***: Cocoa pod borer, transcriptome, responses, defence mechanisms, breeding

## INTRODUCTION

Cocoa trees are found exclusively in regions that satisfy particular environmental criteria. The cocoa belt refers to a geographical region located near the equator, spanning between 20 degrees latitude north and south (Divina *et al*., 2004). Cacao trees only prosper under specific conditions, including fairly uniform temperatures, high humidity, abundant rain, nitrogen-rich soil, and protection from wind. It also requires a balanced amount of sunlight, avoiding both excessive exposure and excessive shade. Cocoa cultivation is feasible up to an elevation of around 1,000 metres above sea level, while the majority of cocoa is cultivated at altitudes below 300 metres.

The mature cacao tree bears fruit in the shape of elongated pods after four years; it can produce up to 70 of these fruits a year. The pods, also known as cherelles, exhibit a spectrum of hues ranging from vivid yellow to profound purple. The fruits mature in under six months, reaching a length of up to 35 cm (14 inches) and a width of 12 cm (4.7 inches) in the middle. Every pod contains 20 to 60 seeds, or cocoa beans, grouped along the long axis of the pod and features several ridges running down its length. The oblong seeds measure around 2.5 cm (1 inch) in length and are enveloped by a saccharine adhesive white pulp. (Elwers,2009)

### Economic Impact of Cocoa Production

Cocoa is an important cash crop with high commercial worth, mostly used for making chocolate, cocoa butter, and numerous confectionery products. This industry plays a crucial role in global employment, involving millions of persons worldwide, including growers, labourers, and those involved in the complex processes of cocoa processing and distribution.

The economic significance of cocoa is emphasised by the significant dependence of several cocoa-producing nations on its exports, resulting in enormous income and positively impacting their trade balances. In addition, the chocolate business at a global level, which is a crucial part of the larger food and beverage sector, heavily depends on a consistent and top-notch provision of cocoa in order to fulfil the needs of customers around the globe. Hence, the interdependence between cocoa farming and the chocolate industry magnifies the economic influence of cocoa agriculture at both the local and global levels.

**Threats Posed by Cocoa Pod Borer Infestation**

Cocoa pod borer (CPB) infestation can lead to significant economic losses for cocoa farmers. The larvae of the borer tunnel into cocoa pods, damaging the seeds and reducing the yield and quality of cocoa beans. Infested pods often result in lower-quality cocoa beans due to the damage caused by the larvae. This, in turn, affects the flavor and overall quality of the chocolate produced from these beans. Farmers may incur additional costs in pest control measures and treatments to mitigate the impact of cocoa pod borer infestation. This can strain the financial resources of cocoa farmers. The entry points created by cocoa pod borer larvae provide pathways for the entry of fungi and bacteria, increasing the susceptibility of cocoa trees to secondary infections.

**Transcriptomic profiling of resistant and susceptible cocoa clones to CPB**

A previous study was conducted in 2018 to examine the transcriptome profiles of cocoa plants that were resistant and susceptible to cocoa pod borer infection. The aim of the study was to investigate their host-pathogen interactions by analyzing gene expression and looking for SNP-related markers in cocoa using bioinformatic analyses. (Gupta, 2015). In this study, the same data set will be used to identify the upregulated genes related to aluminum stress conditions during the cocoa pod borer infection.

**Threats Posed by Aluminum Stress in Acidic Soil**

Aluminum stress in acidic soil can disrupt the uptake of essential nutrients by cocoa plants, leading to nutrient imbalances. This negatively affects the overall health and growth of the plants. Aluminum toxicity can damage the roots of cocoa plants, reducing their ability to absorb water and nutrients from the soil. (Ryan,2011). This hampers the plant's ability to withstand other environmental stresses. Cocoa plants under aluminum stress may exhibit stunted growth and reduced productivity. This can result in lower cocoa bean yields, impacting both the livelihoods of farmers and the overall cocoa production. Aluminum stress contributes to soil acidification, altering the pH levels. This, in turn, affects the availability of nutrients in the soil, creating an environment less conducive to cocoa cultivation (Zhang,2011).

**Integrated Threats**

When cocoa plants face both cocoa pod borer infestation and aluminum stress simultaneously, there may be synergistic effects, intensifying the overall negative impact on plant health and productivity. The simultaneous stressors may compromise the natural defense mechanisms of cocoa plants, making them more susceptible to both insect infestation and soil-related stress.

Addressing these threats requires a holistic approach, considering the interplay between cocoa pod borer infestation and aluminum stress in acidic soil, and implementing strategies that mitigate the combined impact on cocoa cultivation.

## MATERIALS AND METHODS

***Plant material and RNA extraction***

In order to validate the findings from the transcriptomic analysis, hand pollination was performed using available flowers for KKM22 and PBC123. Only a single pair of pods (one for Control and one for Treatment) for both KKM22 and PBC123 was successfully obtained. These pods were used to induce artificial infection using three pairs of CPB per pod. Two weeks after artificial infection, the pods were harvested and dissected into smaller sections separating healthy pulp tissue from damaged pulp tissue for RNA extraction. RNA extraction was performed using RNeasy Plant Mini Kit (Qiagen) following the manufacturer's procedure. However, for pod husk tissues, an additional step of acetone extraction was performed after the pod husk tissues were ground with liquid nitrogen. This step is essential to decrease the number of polyphenolic compounds in pod husk tissues before proceeding with future extraction steps in RNeasy Plant Mini Kit (Qiagen).

***RT-qPCR (Reverse Transcription Quantitative Polymerase Chain Reaction)***

The synthesis of cDNA from total RNA was carried out using the PrimeScript IV 1st strand cDNA Synthesis Mix (Takara) according to the manufacturer's instructions. RT-PCR was performed using Rotor-Gene™ 6000 Real-Time thermocycler (Corbett Research, Australia) with Brilliant SYBR® Green QPCR Master Mix (Stratagene, La Jolla, CA) following the manufacturer’s instructions.

Table 1: The forward and reverse primers used for RT-PCR are listed below:

| Primer Name | Sequence, 5' to 3' |
|---|---|
| Gapdh_F | GATGCTCCTATGTTTGTTGTGG |
| Gapdh_R | TCTTCCTCCTCTCCAGTCCTT |
| **Primer Name** | **Sequence, 5' to 3'** |
| CYTOCHROME _P450F | TCCTCAGAACAATCCCATTCG |
| CYTOCHROME _P450R | AGGGCAGGACATGGTTTTC |
| **Primer Name** | **Sequence, 5' to 3'** |
| Peroxidase_F | GTCAAAACCACGGAATCAC |
| Peroxidase_R | ACACTGAGAAAACGGCTCC |
| Peroxidase_F2 | TGACCTCCGACAAGGGTAAC |
| Peroxidase_R2 | TCCGTGGTTTTGACAAACGG |
| **Primer Name** | **Sequence, 5' to 3'** |
| polygalact_F2 | AAATATGCAGAGTAATCGAAGCTC |
| polygalact_R2 | CAGTGGACGATTTGCAGTTTC |
| Primer Name | Sequence, 5' to 3' |
| Chitinase_F | TCGGTTATTGCGGTCTTGG |
| Chitinase_R | GAATGCAGGTGACACGATAC |

***Comparative CT Method Analysis***

RT-PCR was performed for comparative CT method using the peroxidase, cytochrome p450. polygalactase, and chitinase genes on KKM22 pulp (Control and Treatment) and PBC123 pulp (Control and Treatment). The thermal cycling conditions were as follows: 95◦C for 2 min, followed by 40 cycles of 95°C for 5 sec, and annealing at 65°C for 15 sec. Melting curves were generated with a temperature range extending from 65°C to 95°C. Gene expression was normalised with GAPDH gene using primer pairs Gadph-F and Gadph-R. For relative quantification of gene expression, target gene quantification cycle (Cq) values were compared to Cq geometric means of the reference genes (GADPH), representing the endogenous reference which was used for normalization of qPCR data.

## RESULTS

***The cocoa component of the transcriptome***

Previous results from the transcriptomic analysis between the responses of borer in pulp and husk in KKM22 and PBC123 showed that listing of genes such as peptidoglycan binding protein, alcohol dehydrogenase, pectin methylesterase, aluminum transporter protein, and heavy metal transporter protein, among others. Interestingly, these genes, in addition to being involved in plant responses to insect attack, are also involved in aluminium responses (Ryan *et.al,* 2011). As heavy metals such as aluminium are prevalent in acidic soil, cocoa trees were planted in acidic soil for this study.

Table 2: Comparative analysis between normal versus infected tissues for chitinase gene

| Sample | **Target gene: Chitinase** | Reference gene: Gapdh | | | | | |
|---|---|---|---|---|---|---|---|
| Name | CT Average | CT Average | $\Delta CT$ | $\Delta\Delta CT$ | Fold Change $2^{-\Delta\Delta CT}$ | Fold change | $Log_2$ (Fold change) |
| Normal | 24.71 | 20.10 | 4.61 | 0.00 | 1.00 | | |
| Infected | 14.18 | 21.07 | -6.89 | -11.50 | 2896.31 | 2896.31 | 11.50 |
| | | | | | **Comments:** | **Infected > Normal** | |

**DISCUSSIONS**

The genes alcohol dehydrogenase, pectin methylesterase, aluminium transporter protein, and heavy metal transport protein were discovered to respond to the aluminium in soil-induced plant stress. Aluminum-responsive genes are among those that are upregulated in infected plants but not in uninfected plants. Results from comparative analysis as shown in Table 3 correlated with the previous analysis.

When cocoa plants are subjected to multiple stressors like cocoa pod borer infestation and aluminum toxicity from acidic soils, their ability to cope with both can be significantly compromised. Aluminum stress often leads to impaired nutrient uptake, particularly of calcium, magnesium, and phosphorus, which are critical for maintaining healthy growth and cellular functions. Reduced nutrient availability might make it harder for the plant to sustain its metabolic processes, thus lowering its resistance to borer infestation.

The plant's hormonal balance could be disrupted by the combined stressors. For example, the production of jasmonic acid (which is involved in defense against herbivores and also appear as highly expressed in infected samples) might be suppressed when the plant is busy dealing with aluminum-induced oxidative stress. This leaves the cocoa plant less equipped to fend off the cocoa pod borer.

In this context, managing soil conditions to reduce aluminum toxicity while implementing pest control measures can help mitigate the synergistic effects of these stressors.

**CONCLUSIONS**

Cocoa trees are tropical plants that grow best in humid conditions between 18 and 32°C (65–90°F). Cocoa trees could withstand acidic soil with a pH of 5.0–7.5 for optimum development. The soil in the Kota Marudu is tested to be a bit acidic, therefore, the cocoa growers need to amend the pH by treating the soil with alkaline powder to increase the pH of the soil (Shamshuddin *et. al,* 2011).

The low pH or the uptake of aluminum due to low pH may stress the cocoa plant and make both resistant and susceptible plants more open to attack by borers. Genes responding to the aluminum response are among those upregulated in infected plants but not in uninfected in uninfected plants.

A number of genes related to polysaccharide and cell wall metabolism, detoxification of reactive oxygen species (ROS), cellular transport, phenolics biosynthesis, and signal transduction were involved in the Aluminum stress response (Huang D. *et. al*, 2021) in uninfected plants. However, more research needs to be done in the future before any conclusions can be made.

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