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Quantitative Gene Expression Analysis of Selected Genes to Screen Drought Tolerance of Selected *Hevea* Clones

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ABSTRACT

Expansion of rubber (*Hevea brasiliensis*) cultivation to non-traditional areas in Sri Lanka is necessary due to the increase of global demand of natural rubber and limitation of available land in traditional rubber growing areas. To overcome the negative impact of drought stress, which experience in non-traditional rubber growing areas, plants alter their gene expression as an inherent respond strategy. In traditional rubber growing areas, during the period of February to March is considered as wintering period where recorded higher temperature, low rainfall and lower relative humidity (RH%) comparing to the cropping months of October to December. Therefore, wintering period is more over similar to an environmental condition prevailing in non-traditional areas. Stable clones during wintering period can be expected that having more ability to survive in dry areas too. Therefore, this experiment was conducted to select more stress tolerant clones for non-traditional areas and to evaluate the level of drought responsible genes (Catalase (CAT), ascorbate peroxidase (APX) and Glutathione peroxidase (GPX)) expression of selected *Hevea* clones under wintering stress compared to the cropping months. Quantitative gene expression during wintering period compared to the wintering period was carried out using Bio-Rad thermal cycler. The Livack method was used to calculate fold difference between control and treatment plants. Then paired t-test was done using minitab 17 software. According to the results, all the genes were significantly up regulated in the clone RRISL Centennial 3 during the wintering period and second highest yield dropped percentage (55%) was recorded in this clone. However, *Hb*APX and *Hb*CAT gene expressions were significantly upregulated while *Hb*GPX gene expression was significantly downregulated in the clone RRISL 203 and highest yield dropped percentage (56%) during wintering period was recorded in this clone. In contrast the clone RRISL 2006, *Hb*APX and *Hb*CAT gene expressions were significantly down regulated and upregulation of *Hb*GPX gene expression was not significantly at the P≤0.05 level. This results were different from the expression pattern of other two clones and the clone RRISL 2006 showed lowest yield dropped as around 23% during the wintering period. Leaf falling of this clone also delayed compared to the other clones and might be having more drought tolerant ability than the clones RRISL 203 and RRISL Centennial 3. Therefore, this may be some other genes responsible for the situation and should be studied to confirm the results. According to those results, the clones RRISL 203 and RRISL Centennial 3 can be considered as stress susceptible clones compared to RRISL 2006, which can be considered as stress tolerance clone.

Keywords- *Hevea*, stress tolerance, gene expression, Catalase (CAT), Glutathione Peroxidase (GPX), Ascorbate Peroxidase (APX), Real Time PCR

I. INTRODUCTION

The Para rubber tree (*Hevea brasiliensis*) is a tropical tree which belongs to family Euphabaceae and it is native to the amazon rain forest. (Krishan, 2017) Rubber tree prefers a hot humid climate with annual rainfall varying from 2000 mm to 4000 mm, which is well distributed throughout the year and warm and sunny days with a temperature range of 21°C to 35°C and atmospheric relative humidity of 80 % or above. (Verheye, 2010) The traditional rubber growing areas of Sri Lanka are mainly located in wet zone including Colombo, Gampaha, Kalutara, Kandy, Matale, Kurunegala, Rathnapura and Kegalle districts. (Sri Lanka export development board, 2015). But in the dry zone of Sri Lanka the available land for agriculture is higher than the rubber growing traditional areas in wet zone. (Somarathne and Dhanapala, 1996) Most of the dry zone areas in Sri Lanka are underdeveloped and mainly depend on agriculture. This is a big opportunity to expand rubber cultivation to the nontraditional areas.

So, the need of successful productive cultivation of rubber for non-traditional areas under hot and dry climate was emerged. The selection of drought tolerant clones has become a major challenge for rubber breeding programs to ensure yield and minimize additional cost for water management in rubber production. Rubber research institute Sri Lanka (RRISL) has decided to develop suitable clones for smallholders in non-traditional rubber growing areas to accelerate new planting and expand the cultivation as one of the actions from their master plan for 2025. (Master plan-2017/RRISL)

During drought stress rubber plant undergo many changes at the biochemical, physiological as well as molecular level. (Chaves et al, 2003) To control the effect of drought stress, plant alter their gene expression as an inherent respond strategy. (Shinozaki et al, 2003; Bartels and Sunkar, 2005) Multiple genes, interactions among genes and environmental cues are influenced to develop the complex drought tolerance traits (Tardieu and Tuberosa, 2010)

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To overcome the negative impact of drought stress which experience in nontraditional rubber growing areas, plants alter their gene expression as an inherent respond strategy. In traditional rubber growing areas, February to March considered as wintering period where recorded higher temperature, low rainfall and lower relative humidity (RH%) comparing to the other cropping months. Therefore, wintering period is more over similar to an environmental condition prevailing in non-traditional areas. Wintering tolerant/sustainable clones may be having more ability to survive in dry areas too. Therefore, this study was carried out with several objectives;

1. To select stress tolerance clones for non-traditional areas
2. To evaluate the level of stress responsible genes (Catalase (CAT), ascorbate peroxidase (APX) and Glutathione peroxidase (GPX)) expression of selected *Hevea* clones under wintering stress

II. MATERIAL AND METHODOLOGY

Samples were taken from ECT (Estate Collaborative Trials) field at Eladuwa estate which belongs to Genetics and Plant breeding Department of Rubber Research Institute of Sri Lanka (RRISL) and this is situated at 06°50′N 80°05′E coming under climatic zone of low country wet zone (WL1a). Then, laboratory experiment was carried out at Genetics and Plant breeding Department of Rubber Research Institute of Sri Lanka (RRISL), located in Nivithigalakale, Kaluthara district, Sri Lanka.

*Heveabrasiliensis*clones RRISL 203, RRISL 2006 and RRISL Centennial 3 whichwere planted in Eladuwa estate on 22th may 2009 used as experimental materials.

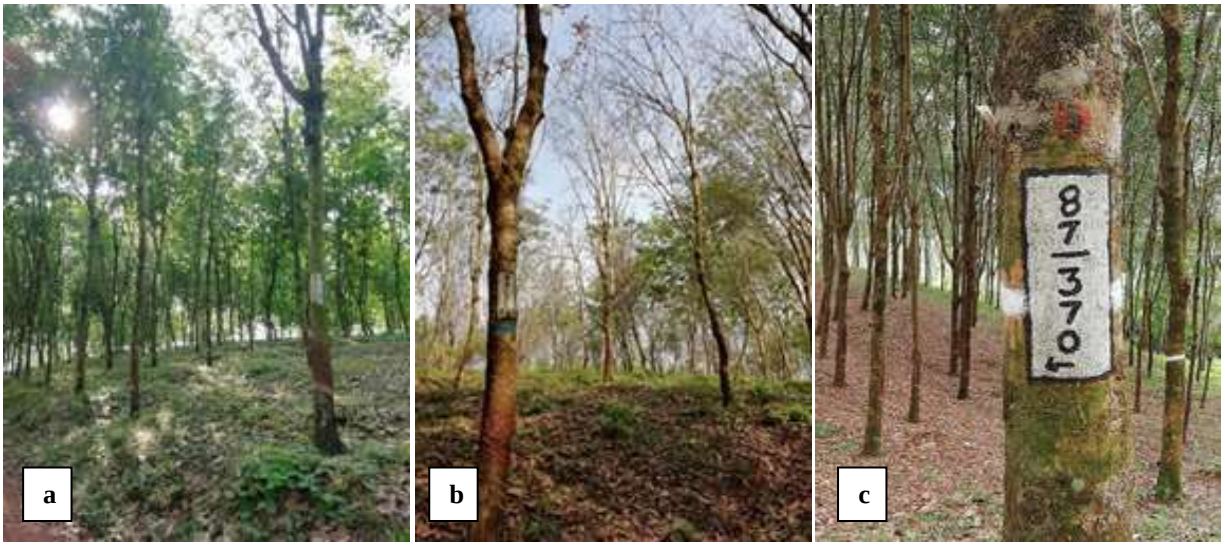
Table 1: clones and their parentages

Clone	Parentage	HP number
RRISL 203	RRIC 100 × RRIC101	74-194
RRISL 2006	IAN 45/710 × PB 28/59	82-14
RRISL Centennial 3	BPM 24 ×RRIC 121	87-370

(PB: PranBesar, BPM: BalaiPenelitian Perkebunan Medan, IAN: Institute Agronomic do Norte, RRIC: Rubber Research Institute of Ceylon, RRISL; Rubber Research Institute of Sri Lanka, HP; Hand Pollination)

The experiment was conducted before wintering (cropping months) and during winteringin period of October 2019 toMarch 2020. For the gene expression analysis, latex samples from each clone were taken during cropping months(January)and wintering months (February)Weather parameters such as temperature (°C), relative humidity (RH %) and wind speed (Km/h) were measured during sample collecting days using portable weather meter and depicted in graphs.

Preliminary trials were conducted to find the best RNA extraction protocol for *Heveabrasileinsis*and best annealing temperature for drought responsible genes (CAT, APX, GPXand GAPDH) by doing gradient PCR (Polymerase Chain Reaction)



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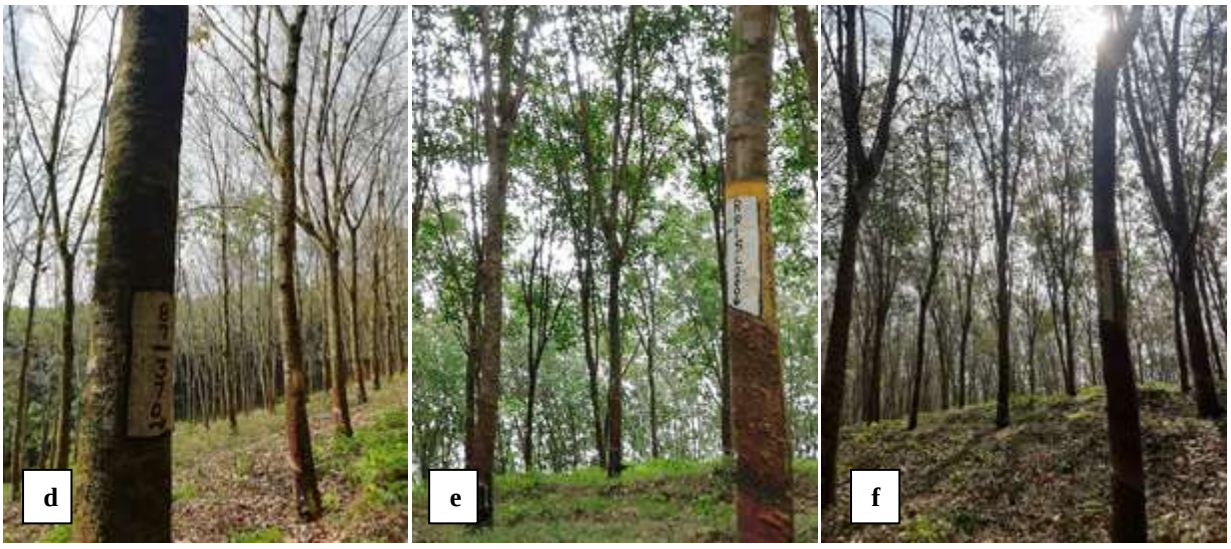

Plate 1: Rubber fields, before wintering (cropping months) and during wintering months

a- During cropping months, the RRISL 203 field((control)
b- *During wintering months, the RRISL 203 field(treatment)*
c- During cropping months, the RRISL Centennial 3 field(control)
d- *During wintering months, the RRISL Centennial 3 field(treatment)*
e- *During cropping months, the RRISL 2006 field(control)*
f- During wintering months, the RRISL 2006 field (Treatment)

Total RNA extraction

Latex samples were collected in early morning and total RNA extraction was done using TRIzol reagent which was described by the Nagahawatta (Nagahawatta et.al.,2017) with few modifications (Gunasekara et.al, 2019)

500µl of TRIzol reagent was added to the Eppendorf which covered by the aluminum foil because TRIzol reagent sensitive to the sunlight. Then 250µl was added to the Eppendorf and mixed well to homogenized the latex and TRIzol reagent. This solution was crushed using autoclaved pipette tips. Those eppendorf was kept in -20˚C freezer for 20 minutes. Eppendorf were thawed and centrifuged at 14500rpm at 4˚C for 20 minutes. Then supernatant was collected to the new eppendorf and kept at room temperature for 10 minutes. Next, 250µl of chloroform was added to the eppendorf and kept at room temperature for 2 minutes. Then centrifuged at 14500rpm, 4˚C for 20 minutes. Supernatant was transferred to the new eppendorf and 400µl of isopropanol was added. Those eppendorf were incubated at 4˚C for 10 minutes. Then supernatant was discarded and 200µl of 90% ethanol. Centrifuged at 6500rpm for 5 minutes. After centrifugation, supernatant was discarded and 200µl of 75% ethanol was added. Again centrifuged at 6500rpm for 5 minutes. Then supernatant was discarded and RNA pellet was air dried. Finally, 25µl of DEPC treated water was added to dissolve the RNA.

cDNA synthesis

First strand cDNA was synthesized by using TaKaRa Prime Script™ RT Reagent Kit, According to the manufacture's instruction. 5µl of total RNA and 1.5µl of RNase free water were mixed with 5µl of reverse transcription mixture (2µl of 5X Prime Script buffer, 0.5µl of Prime Script RT enzyme mixture 1, 0.5µl of OligodT and 0.5µl of Random 6 mers). It was annealed 40˚C for 15 minutes then it was incubated at 85˚C for 0.05 minutes. Finally, cDNA was stored at -20˚C for future uses.

Primer validation

Table 2: List of genes and corresponding primers used for PCR

GENE CODE	ENZYME	FORWARD PRIMER (5'-3')	REVERSE PRIMER(3'-5')
*Hb*CAT	Catalase	TCT CCT TTT CAA TGA CGC ACT	GCT GAG AGA ACC CCA CTT CCT
*Hb*APX	Ascorbate peroxidase	GTA GAG GAT GGT GCC GAC AAC	CCA AAC TTC CCC AGT TAG AAG

			AAA
*Hb*GPX	Glutathione peroxidase	GGG ACA ACA TCA AGT GGA A	CTT CAC ATC CTT CTC GAT GC
*Hb*GAPDH	Glyceraldehyde-3-Phosphate	GCC TGT GAT AGT CTT CGG TGT TAG	GAC GCC TTA TCC TTG TCA GTG AAC

Gene expression analysis was carried out by C 1000 Touch™ Thermal Cycler system (BIO- RAD-USA). PCR was performed in a 20 µl reaction mixture containing 2 µl of first-strand c DNA reaction, 0.5µl of 10 nM of each primer, 12.3 µl Nuclease free water, 2.5 µl Green buffer, 2mM dNTPS and 0.2 µl of DreamTaq polymerase.

For *Hb*CAT gene, PCR was performed by incubating the mixture at 95˚C for 10 minutes, followed by 35 cycles of 95˚C for 10 seconds, 54.4˚C for 30 seconds and 72˚C for 10 minutes.

For *Hb*APX and *Hb*GPX genes, PCR was performed by incubating the mixture at 96˚C for 5 minutes, followed by 45 cycles of 96˚C for 45 seconds, 60.6˚C for 45 seconds and 72˚C for 1 minutes.

Quantitative real time PCR

Quantitative real time PCR was performed in a 10µl reaction mixture containing 2µl of first strand cDNA reaction, 0.5µl of 10 nM of each primer and 5µl of iTaq SYBR Green I.

Master mix (as the fluorescence dye) and 2µl of nuclease free water by using BIO – RAD CFX96 TM Real time system.

Data analysis

The $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) was used to analyze the relative changes in gene expression for quantitative PCR experiments.

(CT – Cycle Threshold value)

(ΔControl = CT Control $_{\text{Target gene}}$ - CT control $_{\text{Housekeeping gene}}$
Δ Treatment = CT Treatment $_{\text{Target gene}}$ - CT Treatment $_{\text{Housekeeping gene}}$
ΔΔCT = Δ Treatment - Δ Control
Fold difference = $2^{-\Delta\Delta CT}$)

Paired t-test was carried out using Minitab 17 software version to find these fold difference values significant or not, between control plants and wintering plants.

III. RESULTS AND DISCUSSION

Preliminary trials were carried out to find best annealing temperature for drought sensitive genes which are Catalase (CAT), Ascorbate peroxidase(APX) and Glutathione peroxidase(GPX) by doing gradient PCR for each gene.

PCR with temperature gradients from 60˚C to 65˚C with 30 thermal cycles and temperature gradients from 45˚C to 55˚C with 40 thermal cycles were carried out for the *Hb*CAT gene. The combination of 54.4˚Ctemperature with 40 thermal cycles was found asbest annealing temperature for *Hb*CAT gene

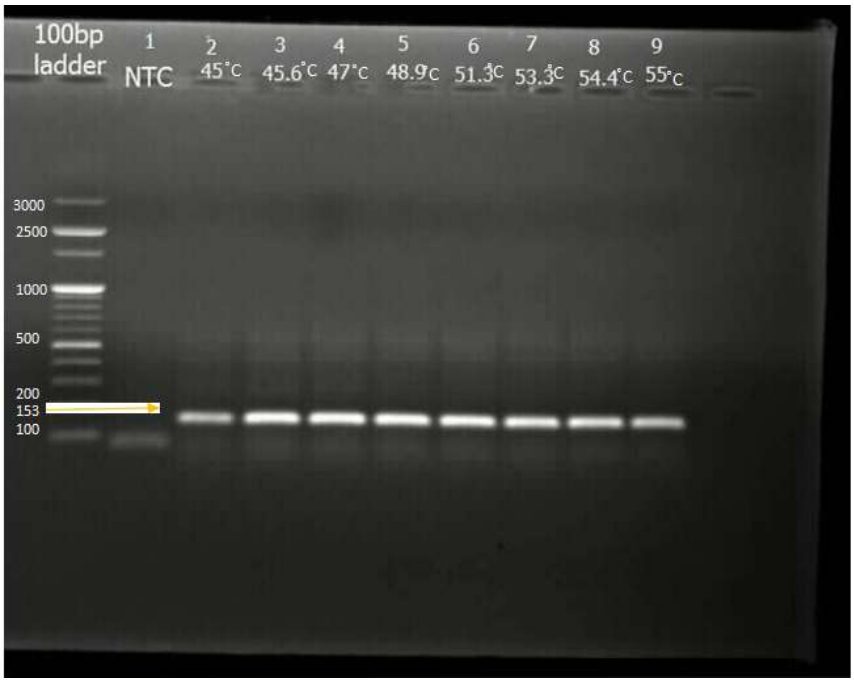

Figure 1: gel run image show the amplicons for PCR products of *HbCAT* gene with genomic DNA

The gradient PCR products from 45˚C to 55˚C temperature, 100 base pair ladder used as marker. No Template Control (NTC) was showed in 1st line. Arrow showed the amplicon size of the *Hb*CAT gene is 153bp. Second line to ninth line showed PCR products which were annealed at 45˚C, 45.6 ˚C, 47˚C, 48.9 ˚C, 51.3 ˚C, 53.3 ˚C, 54.4 ˚C and 55 ˚C respectively.

Temperature gradients from 60˚C to 68˚C with 45 thermal cycles was carried out for the *Hb*APX gene and *Hb*GPX gene using genomic DNA. The best PCRproducts were showed at 60.6˚C annealing temperature for both *Hb*APX gene and *Hb*GPX gene.

For the real time PCR, GAPDH was used as housekeeping gene. So their annealing temperature was checked at temperature gradient from 60˚C to 70˚C.

Weather conditions in Eladuwa estate during cropping and wintering months

During the cropping months (from October of 2019 to January of 2020), total rainfall was recorded as 1,255mm with 68 rainy days and temperature recorded between 28˚C to 31˚C. And also, relative humidity (RH%) was recorded between 72% to 79% in the Eladuwa estate. But during the wintering months (February of 2020) rainfall was recorded as 85mm with 6 rainy days and temperature recorded between 30 ˚C to 36 ˚C. In this period relative humidity was recorded between 68% to 75% in Eladuwa estate. Weather conditions in dry areas of Sri Lanka, where drought spells prevail includes, higher average temperature ranges between 33.3 0C to 34.7 0C in North and North Eastern regions, relative humidity around 60% in driest areas of the dry zone (Anon, 2014). According to these records weather conditions in wintering months showed similar conditions experience in dry areas of Sri Lanka.

Yield and yield dropped percentages in clones

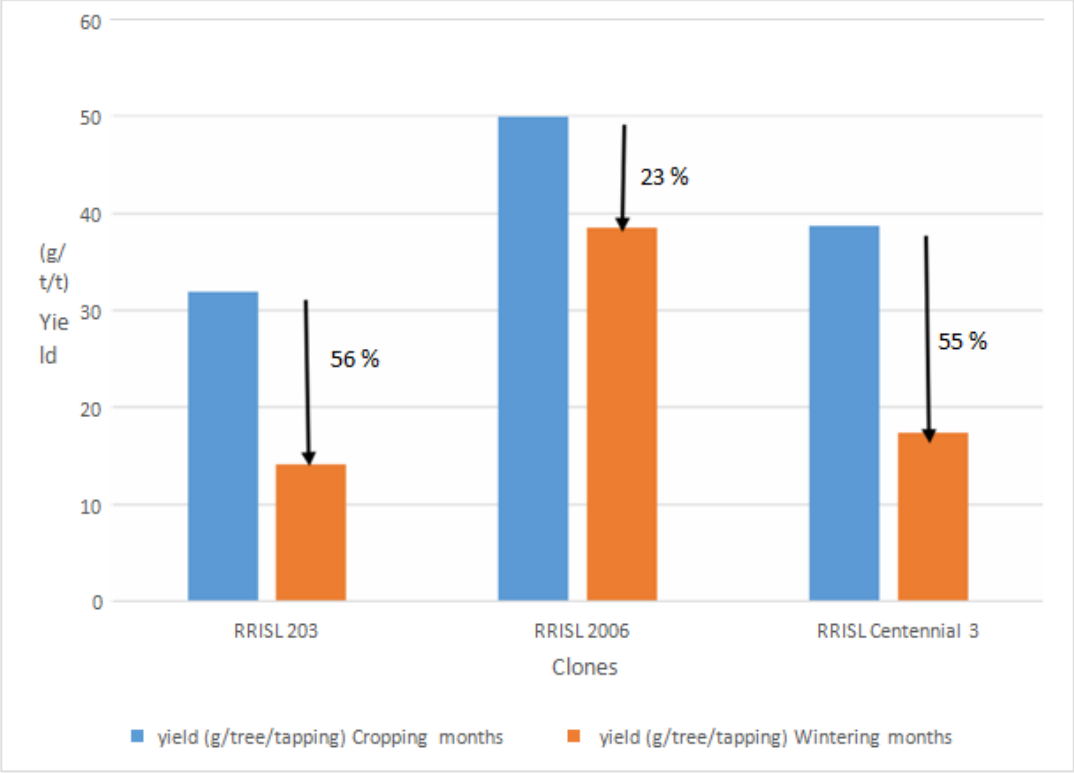

Figure 2: Yield and Yield drop in 10 years old plants of the clones RRISL 203, RRISL 2006 and RRISL

During the cropping months (October, November, December, January) of first three tapping years, the monthly average yields were 31.8 g/t/t (gram/tree/tapping), 50g/t/t and 38.6g/t/t in 10 years old the clones RRISL 203, RRISL 2006 and RRISL Centennial 3 respectively. However, during the wintering months (February and March) when plants were under stress conditions their average yields in the clones RRISL 203, RRISL 2006 and RRISL Centennial 3 were respectively 14g/t/t, 38.4g/t/t and 17.3g/t/t. If we consider percentage of yield drop of these clones, RRISL 203 and RRISL Centennial 3 showed somewhat similar yield drop percentages with 56% and 55% respectively in these clones. But highest yield drop percentages showed in the clone RRISL 203. least yield drop was 23% showed in the RRISL 2006 compared with other two clones. (Figure 2)

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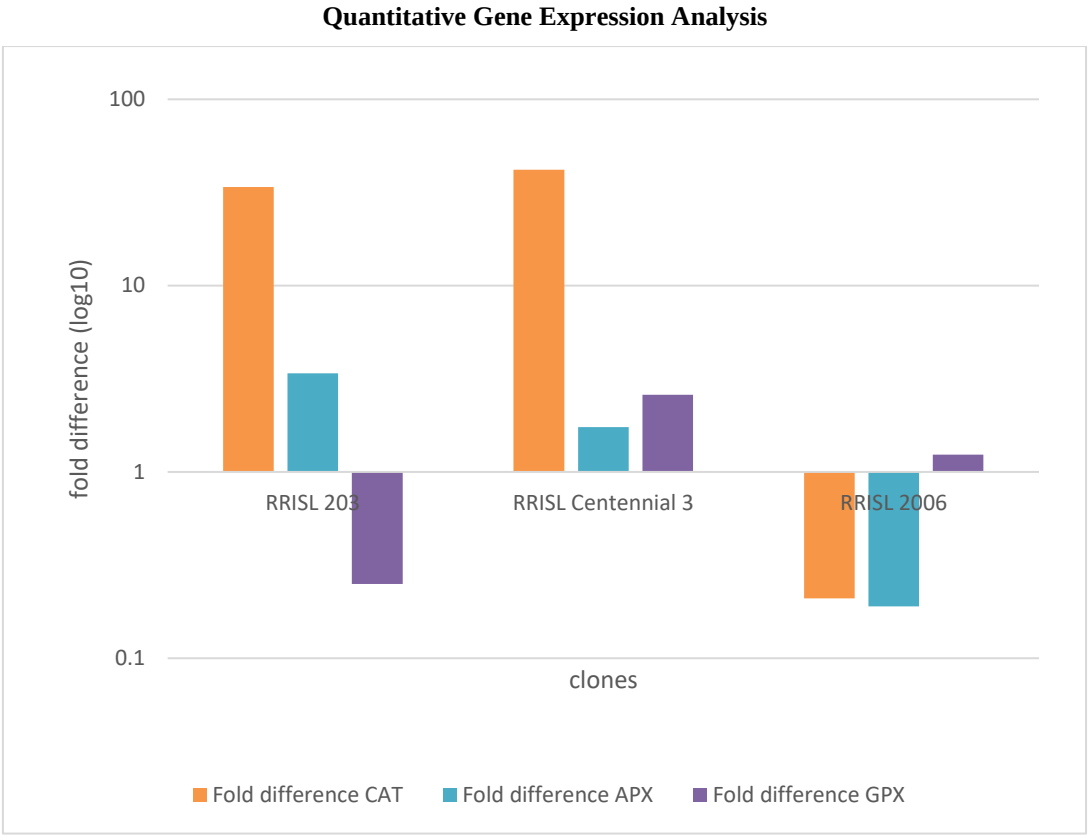

Figure 3: Fold differences of stress sensitive genes in clones in log10 scale

'*' mark shows the value which is not significant at the P≤0.05 level in paired t-test

The *Hb*CAT gene was up regulated in the clones RRISL 203 with 33.88 fold difference and RRISL Centennial 3 with 41.9 fold difference. But *Hb*CAT gene down regulated in the clone RRISL 2006 with 0.21 fold difference in wintered plants.

According to results (Figure 3), the gene *Hb*CAT was significantly up regulated with 33.88 fold difference in clone RRISL 203 and RRISL Centennial 3 during the wintering period and the clone RRISL Centennial 3was showed higher level of fold deference in expression over other clones as 41.9 fold difference. (Figure 3) But *Hb*CAT gene expression was significantly down regulated in the clone RRISL 2006 during the wintered plants compared to during cropping months. it was showed only 0.21 fold difference in the clone RRISL 2006. Previous studies also showed down regulation of CAT gene in *Hevea* clones RRIM 600, RRII 105, RRII 414 and RRII 208 under drought conditions (Luke et al., 2015). Because CAT, GPX and SOD are antioxidant defense enzymes which responsible to break down the harmful end products like Hydrogen Peroxide) H_2O_2 produced in the peroxisomes as a result of oxidative modification of the cell. Under crucial conditions like water stress they remove the high H_2O_2 concentrations and recently cause the down regulations. And also according to previous studies *Hb*CAT gene expression was up regulated in TPD (Tapping Panel Dryness) affected plants (Bandara P. et al. 2018) The clones RRII 208 and RRIM 600 are already identified as more drought stress tolerance clones (Singh et al., 2012) According to this results the clone RRISL 2006 acts more same to the Clone RRIM 600 at stress condition. Drought-sensitive species activate their antioxidative system as their defense system. (Laxa et al., 2019) Catalase has high specificity forH_2O_2 and is unique which it is not required cellular reducing equipment. CAT has a very fast turnover rate but less affinity for H_2O_2than APX. (Luke et al., 2015)

Ascorbate peroxidase (APXs) is a one of crucial enzymes for reducing reactive oxygen species (ROS) in plant cell. According to the results (Figure 3) In case of *Hb*APX gene expression in these clones, *Hb*APX gene was significantly up regulated in the clones RRISL 203 with 3.38 fold difference. And RRISL Centennial 3 with 1.74 fold difference in the wintered plants.

The clone RRISL 203 showed higher level of *Hb*APX gene expression over other clones. (Figure 3) But *Hb*APX gene expression was significantly down regulated in the clone RRISL 2006 with 0.19 fold difference during the wintered plants compared to the cropping months. *Hb*APX is the crucial enzyme, which detoxifies the hydrogen peroxide in plant cytosol, mitochondria, chloroplast and peroxisomes. *Hb*APX

activity was recorded to shoot up with other ROS scavenging enzymes such as glutathione reductase (GR), catalase (CAT) and super oxide dismutase(SOD) under stress condition.(Luke et al., 2015) According to previous studies in *Hevea* at drought stress, *Hb*APX was found significantly up regulated in the relatively tolerant clone RRIM 600 and slightly higher in RRII 105 whereas it was found down regulated in RRII 414 and RRII 208.(Luke et al., 2015) However according to my observations the clone RRISL 2006 showed downregulation of the *Hb*APX gene expression and according to field records this is a late wintering clone. So, it may be has some stress tolerant characters according to these molecular findings, field records and yield records.

When considering the Glutathione peroxidase (GPX) gene expression (Figure 3), *Hb*GPX was showed significantly 0.25 fold difference in wintered the clone RRISL 203. Therefore, *Hb*GPX gene expression was significantly downregulated in this clone. But 2.6 fold difference was recorded in the clone RRISL Centennial 3. So, *Hb*GPX gene was up regulated in this clone. However, 1.24 fold difference was recorded in the clone RRISL 2006. But this value was not significant at the $P \leq 0.05$ level in paired t-test. RRISL Centennial 3 was the only one clone which showed up regulated *Hb*GPX gene expression this experiment. Therefore, further investigation required for the *Hb*GPX gene expression in the clone RRISL 2006.

As the major scavenging APX and CAT activations are stronger in the stress sensitive species than tolerant species. Obviously, stress sensitive plants mainly activate the glutathione- depend scavenging system. On the other hand, tolerant species strongly activate the ascorbate- depend scavenging system compared to the glutathione scavenging- depend system. (Laxa *et al.*, 2019) according to previous results, GPX gene expression was upregulated in TPD (Tapping Panel Dryness) affected plants. (Bandara P, 2018)

IV. CONCLUSION

*Hb*APX, *Hb*CAT and *Hb*GPX gene expressions were upregulated in the clone RRISL Centennial 3 and second highest yield drop percentage was showed in this clone. So, this may be stress susceptible clone.

*Hb*APX and*Hb*CAT gene expressions were upregulated while*Hb*GPX wasdownregulated in the clone RRISL 203 and highest yield drop percentage was recorded in this clone. The clone RRISL 203 may be stress susceptible clone.

In contrast the clone RRISL 2006, *Hb*APX and *Hb*CAT gene expressions were significantly downregulated alter *Hb*GPX gene expression was upregulated in this clone not significantly at the $P \leq 0.05$ level. These results were different from the expression pattern of other two clones and further the clone RRISL 2006 showed around only 23% yield dropped during the wintering period and leaf falling of this clone is delayed compared to the other clones and showed more drought tolerant ability than the clones RRISL 203 and RRISL Centennial 3. Therefore, this may be some other genes responsible for this situation should be further studied to confirm the results. According to those results the clones RRISL 203 and RRISL Centennial 3 can beconsidered as stress susceptible clones and RRISL 2006 can be considered as stress tolerance clone.

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