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# Isolation of drought influenced micro RNAs from *Hevea brasiliensis*

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## INTRODUCTION

MicroRNAs (miRNAs) are single-stranded non-coding RNAs, ~20 to 24 nucleotides in length that play critical roles in regulating gene expression at the post-transcriptional level by repressing translation or enhancing degradation of specific target mRNAs (Bartel, 2004). They are involved in regulating various developmental and metabolic pathways, signal transduction, response to environmental stress such as oxidative stress, nutrient stress, dehydration and mechanical-stress (Sunkar *et al.*, 2004; Shukla *et al.*, 2008). No studies have been made in *Hevea* to identify the role of miRNA in stress alleviation/ tolerance. Hence, an attempt was made to isolate miRNA from drought stressed *Hevea brasiliensis* leaves with an objective to analyse their role eventually in regulation of gene expression under drought stress conditions. Isolation of miRNA from *Hevea* has been standardized successfully for the first time and the results are discussed in this paper.

## MATERIALS AND METHODS

*Hevea brasiliensis* (clone RRIM 600 which is relatively drought tolerant) grown in polythene bags were exposed to drought in the field of Rubber Research Institute of India (RRII), Kottayam. One set of plants (n=10) were subjected to water stress by withholding water for 14 days and the other set of plants (n=10) was watered on alternate days to maintain field capacity which was the control. Leaf samples were harvested in liquid $N_2$ after assessing the drought status of the plants by measuring the net $CO_2$ assimilation rate (A) and stomatal conductance (gs) using a portable photosynthesis system (LI-6400), LI-COR, USA. miRNA was isolated from frozen leaf samples using mirVana miRNA isolation kit, Ambion (USA). Qualitative and quantitative estimation of the small RNAs isolated was done by using a mini spectrophotometer (Nanodrop ND -1000). About 2 µg of small RNA with an equal volume gel loading buffer were loaded on a 12% denaturing (7M urea) polyacrylamide gel. miRNA marker was loaded as size control for the identification of RNAs in the 20-25 nucleotide (nt) size range. The gel was stained with SYBR gold nucleic acid stain and the small RNAs were visualized on a UV transilluminator. The eluted miRNA fragments (21-25 bp) were ligated with a 3′ and a 5′ linker using miRCat cloning kit (IDT, USA) in two separate reactions followed by cDNA synthesis using SuperScript III Reverse Transcriptase (Invitrogen). The cDNAs were further PCR amplified and purified by phenol: chloroform purification method.

## RESULTS AND CONCLUSIONS

Well watered plants maintained a high rate of A (11.28 µ mol $m^{-2}$ $s^{-1}$ ) compared to those that were

subjected to moisture deficit stress for 14 days (3.24 μ mol $m^{-2}$ $s^{-1}$ ) (Fig. 1a) in which gs was reduced to near zero during this period (Fig. 1b). This clearly implies that the plants are stressed and the leaf samples collected would certainly have drought stress related gene expression and associated miRNA population.

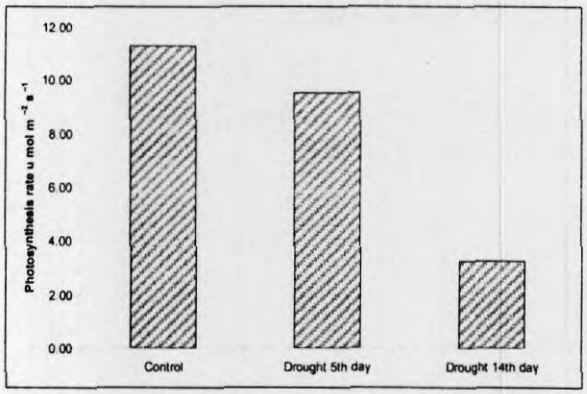


**Fig. 1a.** Photosynthesis Rate (A) of control and drought stressed *Hevea* plants.

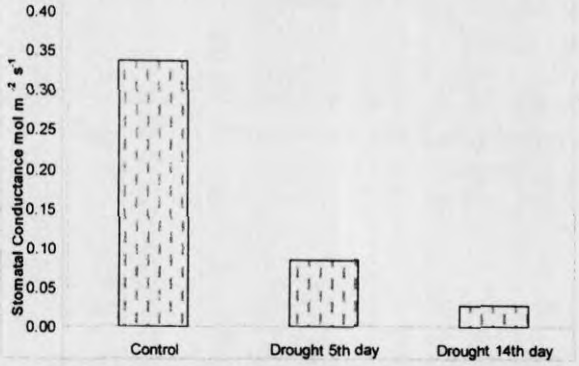


**Fig. 1b.** Stomatal Conductance (gs) of control and drought stressed *Hevea* plants.

Attempts to isolate miRNA was made from leaves of stress treated and irrigated plants of clone RRIM 600 using miRVANA columns. The final elute from these columns containing the miRNA was subjected to electrophoresis on a polyacrylamide gel along with miRNA markers. The results (Fig. 2a) indicate the presence of miRNA bands in the range of 20-24 nucleotide (nt) size. The miRNA visualized on gel have to be eluted for further cloning and identification by DNA sequencing. For this purpose, cut bands of miRNA were eluted and purified to proceed with the next step which involves attachment of linkers to the miRNA which facilitate the reverse transcription of miRNA. Linkers were attached on

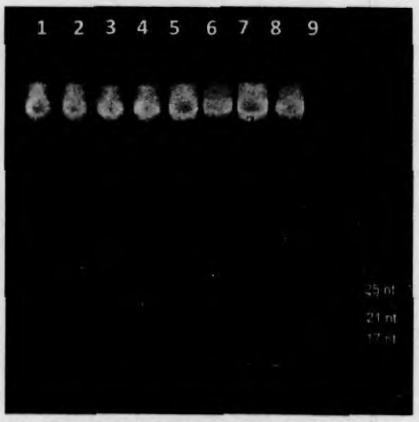


**Fig. 2a.** Profile of miRNA isolated from leaf samples of drought stressed *Hevea brasiliensis* (clone RRIM 600); Lane 1-8 = miRNA from 8 samples; Lane 9 = miRNA marker

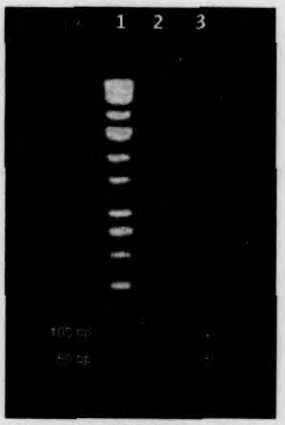


**Fig. 2b.** PCR amplified products of miRNA (with an approximate size of 62 bp which includes 3′ and 5′ linkers); Lane 1= DNA marker; Lane 2-3= linker attached miRNA

both 3′ and 5′ ends of the miRNA fragments followed by reverse transcription using linker specific primers.

PCR amplification using the above mentioned linker specific primers follows then which is possible only if the 3′ and 5′ linkers are ligated properly with the miRNA population eluted at the first instance. The PCR amplified products when visualized on agarose gel (Fig. 2b) indicate the presence of amplicons in the range of 62 bp size. These amplicons were further purified to proceed with cloning and sequencing. The strategies adopted in Isolation of miRNA have proved that good quality miRNA can be isolated from leaf samples of *Hevea*. This attempt involves a standardization of steps like adaptor ligation, cDNA synthesis and PCR amplification which we could successfully carry out for the first time in *Hevea*. The subsequent cloning and sequencing of these miRNA would help identifying them, their target mRNA and their possible role in stress tolerance/alleviation.

## REFERENCES

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