BIOSYNTHESIS OF ALEURITIC ACID IN INDIAN LAC INSECT, *KERRIA LACCA* AND ITS *IN VITRO* PRODUCTION

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by

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Dedicated to

Rafu, Anjumi and the lady who gifted them to me

Certificate

This is to certify that the thesis entitled **"Biosynthesis of aleuritic acid in Indian lac insect, *Kerria lacca* and its *in vitro* production."** being submitted by **Mr. Anees Kaprakkaden** to the **Indian Institute of Technology Delhi**, for the award of the degree of **'Doctor of Philosophy'**, is a record of the bonafide research work carried out by him, which has been prepared under our supervision and guidance in conformity with the rules and regulations of the 'Indian Institute of Technology Delhi'. The research reports and the results presented in this thesis have not been submitted for any degree or diploma in any other University or Institute.

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Date: **(Anees Kaprakkaden)**

Abstract

Aleuritic acid (9,10,16-trihydroxyhexadecanoic acid) is the major acid constituent of the lac resin secreted by Indian lac insect *Kerria lacca*. Use of aleuritic acid as the starting material in perfumery industry as well as in pharmaceutical industries, makes it a high value compound. The current method of alkaline hydrolysis of the lac resin for purification of aleuritic acid to the desired level is very time- consuming. The present study has attempted to decipher the biosynthetic route of aleuritic acid in the lac insect and to reconstitute its pathway in vitro by using heterologous genes, with a view to enable its enzymatic production.

The route of aleuritic acid biosynthesis in Indian lac insect *Kerria lacca* has been proposed based on the evidences from five different approaches. (a) fatty acid methy ester (FAME) analysis of the resin secreting (adult) and resin non-secreting life stages (crawler) of the lac insect using GC-MS, (b) use of broad spectrum inhibitors for epoxide hydrolase (EH) and epoxygenase (EPOX) coupled with the trans-supply of probable intermediates, (c) enzymatic activity of fatty acid desaturase (FAD), epoxide hydrolase and monooxygenase(MONO) in extracts of the adult lac insect compared to that in the crawler (d) Western blotting for epoxygenase (EPOX), fatty acid desaturase and epoxide hydrolase for checking the presence of these enzymes in adult lac insect, and (e) quantitative PCR for FAD, EH, EPOX and MONO genes for the transcript detection in the adult lac insect.

To isolate the genes responsible for the biosynthesis of aleuritic acid in Indian lac insect, cDNA library of the adult lac insect was constructed using pGEMT vector system, wherein 8,057 clones were isolated, and was used for screening of the four categories of genes viz. EH, FAD, EPOX and MONO. Three distinct categories of probes viz. degenerate probes, full-length heterologous genes, and the probes designed based on transcriptome data were

used for cDNA library screening. Also, the use of degenerate primers for direct amplification from cDNA of the adult lac insect was attempted. Approaches like colony blotting and dot blotting followed by secondary and tertiary screening using typical southern blotting procedures were applied for gene isolation. A full-length gene sequence of EH was isolated which showed novel sequence characteristics and similarity to EH sequences of existing database.

To provide a proof of concept for the deciphered pathway of aleuritic acid, in vitro reconstitution of the aleuritic acid pathway was explored using five heterologous genes viz. fatty acid desaturase from *Saccharomyces cerevisiae*, CYP81B1 from *Helianthus tuberosus*, CYP86A4 from *Arabidopsis thaliana*, epoxide hydrolase from *Caenorhabditis elegance* and epoxygenase gene from *Stokasia laevis*. These five genes were cloned, expressed and induced for their use in cell conversion of the probable intermediates of aleuritic acid biosynthesis, viz. 9-hexadecenoic acid and 16-hydroxyhexadecanoic acid. The induced cell lysates of these five genes were used singly, sequentially and in combination along with the trans-supply of intermediates. GC-MS profiling of the products formed was carried out. Synthesis of 9,10-dihydroxyhexadecanoic acid was achieved when the combination of induced cell lysates were incubated with 9-hexadecenoic acid.

This is the first in-depth investigation on the probable route for aleuritic acid biosynthesis in Indian lac insect. The *in vitro* reconstitution of 9,10-dihydroxyhexadecanoic acid synthesis was successfully achieved in the present study.

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List of Abbreviations and Symbols

AA	Arachidonic acid
CAI	Codon adaptation index
CYP450	Cytochrome P450
DGE	Differential gene expression
DHD	Dihydroxyhexadecenoic acid
DOD	Dihydroxyoctadecenoic acid
EET	Epoxyeicosatrienoic acid
EH	Epoxide hyrolase
EPOX	Epoxygenase
FAD	Fatty acid desaturase
FAME	Fatty acid methyl ester
FOC	Frequency of optimal codons
HETE	Hydroxyeicosatetraenoic acid
HODE	Dihydroxyoctadecadienoic acid
HOME	Hydroxyoctadecenoic acid
HPODE	Hydroperoxyoctadecadienoic acid
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LOX	Lipoxygenase
MONO	Monooxygenase
MP	Master plate
NADPH	Nicotinamide adenine dinucleotide phosphate
PHA	Polyhydroxyalkanoic acid
qPCR	Quantitative PCR
sEH	Soluble epoxide hydrolase