Interaction between the coffee plant *Coffea arabica* and *Coffea liberica* and the insect Coffee berry borer *Hypothenemus hampei*

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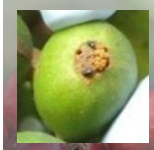
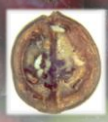
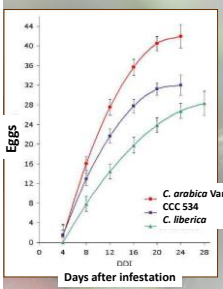
Coffee Berry Borer (CBB) *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae)





- CBB is one of the most destructive insect pest of coffee in Colombia
- It can be found among most coffee producing countries
- In Colombia, CBB has been present since 1988
- Economic losses are due to penetration of the fruit
  - Direct damage of the seed
  - Premature drop of the green fruits
- Infestation of 2% in Colombian – Economic threshold
- Insect control: Integrated Pest Management
  - Cultural Control, Biological Control and Chemical Control
- There is not natural resistance in coffee

Evaluation of Differences in Susceptibility of Coffee Species to the Coffee Berry Borer



- Evaluation of different coffee accession *C. liberica* and *C. arabica*
- A 30% decrease on the number of eggs produced for the CBB females feed on the berries from *C. liberica* compare with *C. arabica* (Romero y Cortina, 2004)

Coffea liberica seeds shown a negative effect on the *CBB* fecundity

OBJECTIVES

To identify and to validate the differentially expressed genes in *Coffea liberica* berries vs. *Coffea arabica* in response to the CBB attack.

Those genes can explain the lower susceptibility of *C. liberica* genotype, the information can be use for developing varieties resistant to CBB.

Infestation of *Coffea liberica* less susceptible genotype and *C. arabica*

Coffea liberica CC1024

Coffea arabica

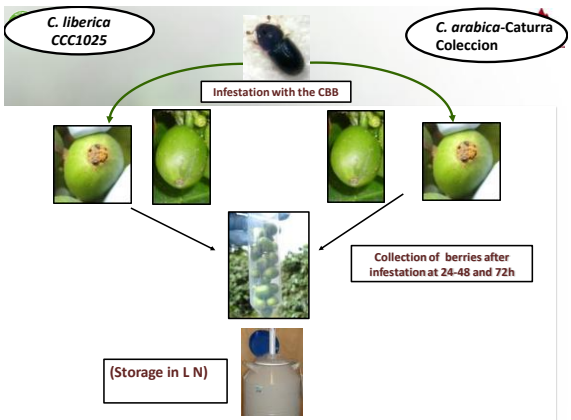
C. liberica: Diploid
C. arabica: Tetraploid

Selection of branches- berries

Infestation of branches with CBB 3:1 (Cages)

Infestation time: 24-48-72 h pos infestation

Dates of infestation: 2005-2006-2007-2008-2009-2010- 2011

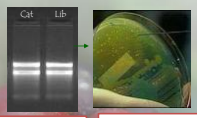


MOLECULAR TECNIQUES

- Identification of a gene pool relate to plant defenses
  Sequencing of cDNA Full length libraries (10.000 sequences – 5.000 from *C. arabica* and 5.000 *C. liberica*). 24h pos infestation.
- Development of Microarrays- Normalized cDNA libraries (33.253 total cDNA sequences. (19.074 from *C. arabica* var. Caturra, 14.189 from *C. liberica*)-
  For identification of Genes expression at 24-72h pos infestation.
- qRT-PCR-To corroborate the gene differential expression and to quantify the levels of expression at 24-48-72h pos infestation.
- Development of Oligoarray- For identification of Genes expressed 48h pos infestation.
- Sequencing of infested berries transcriptome by RNA seq.

cDNA Libraries constructions-Gene pool identification

Berries infested for 24h / *C. liberica* and *C. arbica*



RNA Extracción

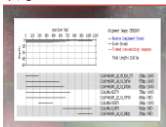
Rneasy® plant Mini kit (Qiagen

The cDNA libraries construction : SMART cDNA Library Construction Kit

Sequencing of the cDNA libraries

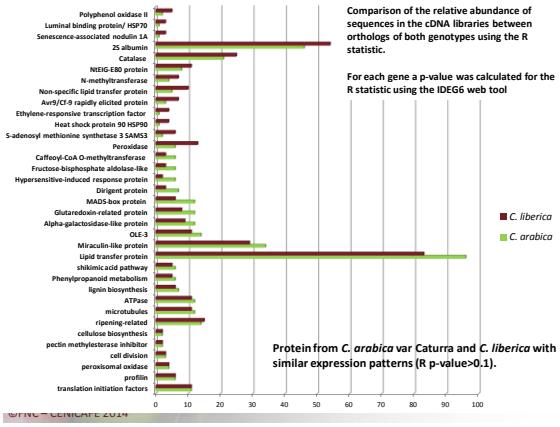
REXAGEN, Seattle, WA, USA
-MACROGEN, Korea

~5,000 sequences per genotype.



Bioinformatic analysis of the sequences- Cenicafé- Coffeebank storage

Target Identifier Report	*C. arabica*	*C. liberica*
Total number of sequences	5405	5198
Full-length sequences	2506	2382
Short full-length sequences	307	320
Ambiguous sequences	351	319
Partial sequences (5'- partial sequence)	946	901
3'- partial sequence	151	100
Full-length sequences and ORF	1815	1514

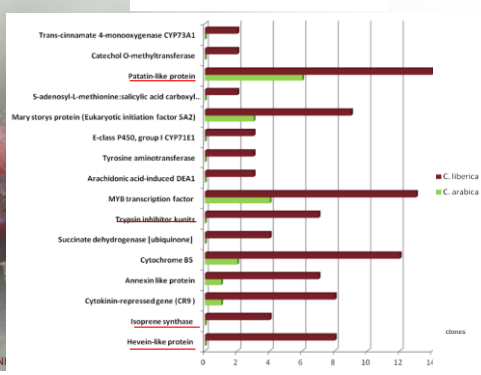


Comparison of the relative abundance of sequences in the cDNA libraries between orthologs of both genotypes using the R statistic.

For each gene a p-value was calculated for the R statistic using the IDEG6 web tool

Protein from *C. arabica* var Caturra and *C. liberica* with similar expression patterns (R p-value>0.1).

Proteins with higher expression in *C. liberica* respect to *C. arabica* (R p-value<0.1)



Microarray/ Data analysis

New cDNAs normalizad lybraries were printed onto glass slides

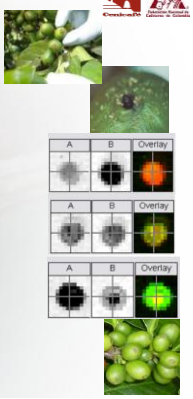
37.632 spots were registered on the array.
19,074- *C. arabica* (5 tissues RNA mixture).
7,169 -*C. liberica* (berries)
7,020 *C. liberica* (leaves)

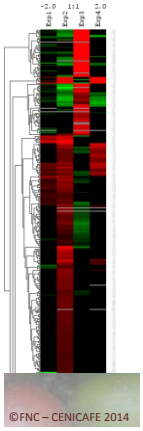
Infested vs non infested berries
At 24h or 72h
2 genotypes.

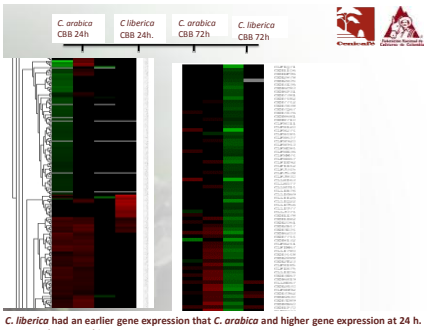
Simultaneous hybridization of Cy3 and Cy5 indirect labeled RNA

4 Biological replicas /2 technical replicas Dye swap

After normalization of intensity data p-values were calculated for each gene





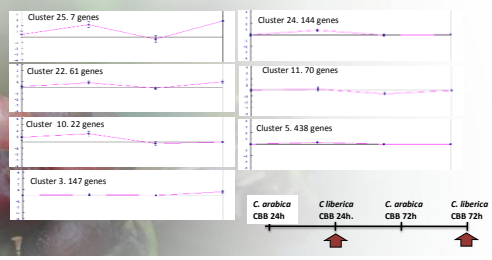


C. liberica had an earlier gene expression that *C. arabica* and higher gene expression at 24 h. compared to *C. arabica*.

At 72 h. there is more expression of genes in *C. arabica* compared to *C. liberica* and there is a differential transcriptional response.

From the total 33.253 cDNA sequences, 2.585 (8%) had the same patterns of expression under insects attack. A 25 different expression patterns were identified.

C. Liberica response to CBB infestation



Seven clusters, containing 520 different transcripts showed over-expression in *C. liberica* at 24h and/or at 72 h after insect attack comparing with *C. arabica*.

Many of the transcripts correspond to enzymes of metabolic pathways related to stress response.

- **The induction of those genes suggests that both genotypes are able to sense the stress produced by the insects.**
- **In general *C. arabica* responds to the CBB expressing genes involved in general biotic and abiotic stress responses and they are not very specific to insect defense, the genes induced are more related to plant-pathogen interactions**
- ***C. liberica* responds with the induction of proteins more specifically related to hervibory and insect attack response (i.e., genes related to the jasmonic acid pathway), such as: a hevein-like protein, isoprene synthetase, a trypsin inhibitor Kunitz, and patatin-like protein that has been reported as specifically induced by insects.**

Selection of candidate genes related to insect defenses

Genes with Insect defense function based on annotation were chosen

cDNA libraries: 70 unigenes sequences

Microarray: 500 unigenes sequences

A group of 26 genes that showed higher differences between *C. arabica* and *C. liberica* were chosen to validate by qRT-PCR

	Gen	Primers	
1	Short-Chain dehydrogenase 1	CTGCACAGATGGCTGGATGT	GAGACTGTGCACCATCGACAA
2	SAM metiltransferasa 1	TGGTAGCAAGGGGTATGGCG	TGGACCTGGAAAACCACCCC
3	Alcohol deshidrogenasa	ACCCTTGCTTGGTCTTCTCA	TCCACCAACCAACTTCCTTC
4	ARF/SAR super family	TGCCGGCAAAACCACCTTGC	TCGGGTACTGCGTTGGCTGATG
5	UP12	CAAACGTGTGACGATTCGGGT	TTGTGTCCAGCCGTTTCCAG
6	Jasmac2	TGGTAGCGCCAGGTTGGATG	AGCCATGACTGTGCTCTCTGC
7	NAM	AGAAACTGACCCGCTTTCAC	CATGGCCTGCAACTTTTCTG
8	Pectinlyasa	ACTCTGTGCACACTTTGGCAGGC	TGCCGCAGCATTGCCAAACAC
9	Inhibidor de Proteinasa 25A	TCGGCTGAAGTGGAAGCTAT	TTTCCACCACCAGTGACAAA
10	SAM descarboxilasa	TGAAGAGAAGGAGCACGGGG	GAAGAGAAGGAGCACGGGGA
11	Plant Lipid Transfer Protein	GAAGTTGCAGTTTGCCTTTGC	TCCTGGAGGAATGGTTCTGC
12	Band 7	AAATGCAGCTCAAAGGCAACAG	CTGCTTTGCAACCCCAACTC
13	Glycoside hydrolase	AATTTGGTTGCGAGGTGGTG	TGAAACTCCGTGAACCTTCCA
14	Ethylene Response	GCGAGCCAACTCGGATAATGA	GCCAGAAGTGTTGGAGCGAAT
15	2 oxo-dehydrogenase	CTGCGACACTGCTGGTGGAA	TGGTAGCGCCAGGTTGGATG
16	Haloacid dehydrogenase	GGGGCGCTTCAGGCTTTGATA	AGGAGCAGCGGAGCGTTGAAG
17	Aspartate	AGGGTTGGAGCTGAGTTTTTGGC	CCAACATGGGGAAACCACCCAAA
18	Glucan synthase	TGGGAAGCACACTTCACTTGCG	CAAACTTCCAGCTGATGCTCCGA
19	Protein inhibitor i4	AAGCCTGCTGAGGTCACCAATG	GGCTGATCTTTGCAAATGCGCT
20	Patatin	CGACTTCGGCTCTGTCGGTT	GTCCCCACTTGGCAACATCCT
21	Glucan synthase	TGGGAAGCACACTTCACTTGCG	CAAACTTCCAGCTGATGCTCCGA
22	Hevelina Like	TGCATGTGGGAGGTGCCTAA	TCTAGCCCACCATTGCTGCAT
23	Kunitz protein	CACGAACCACGCCTTCTTCC	TGCCCTCTGGCAGTTGTTCA
24	Isopren synthase	TCCTCGACGGCGGAGATAGA	TCGGGCAAACTCCTCAGACA
25	Cytochrome P450	CAGGAG	
26	Enoyl CoA	CCTCG	

Gene-specific primers were designed (Primer3plus). Predicted melting temperature of 61-62°C, primer length of 19-23 nucleotides, guanine-cytosine contents of 40-60%, and PCR amplicon lengths of 70-150 bp.

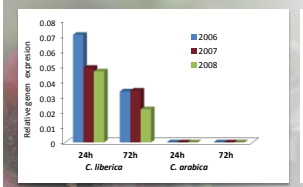
qRT-PCR- Gene Expression in *C. liberica* vs *C. arabica*

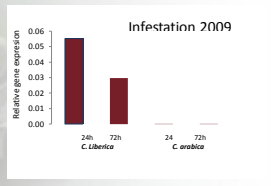
From the 26 genes chossen - 18 genes were evaluated by qRT-PCR

18 genes qRT-PCR evaluated
12 genes showed differential expression

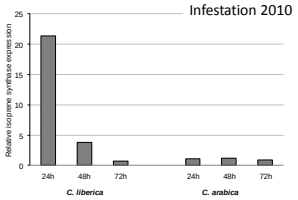
1. **Isoprene synthase gene (ISPS)**
2. **Hevein –like protein**
3. **Trypsin inhibitor Kunitz gene**
4. **Patatin**
5. **S-adenosyl-L-methionine: Salicylic acid carboxyl**
6. **No apical meristem (NAM) protein, induce jasmonic acid**
7. **Membrane protein family Band7,**
8. **Indol acetic acid IAA,**
9. **Aspartate aminotransferase,**
10. **Plant Lipid Transfer Protein-Lipase,**
11. **Glicosido hidrolasa.**
12. **SAM metiltransferasa 1**

Isoprene synthase Gene





Isoprene synthase Gene



Relative expression of isoprene synthase gene CEN71857 by quantitative real–time PCR. qRT_PCR was performed with cDNA from green coffee berries at 24h, 48h and 72h after infestation of coffee berry borer in *C. liberica* and *C. arabica*.

Reported in Poplar. Isoprene synthase is the enzyme responsible for the synthesis of isoprene.

A volatile compound that protects the photosynthetic apparatus of certain plants against high temperatures and oxidative stress.

It is also involved in: -plant-insect interactions, in which the plant acts as a deterrent - repellent (direct defense) (Laothawornkitkul *et al.* 2008a, 2008b).

- affects tritrophic interactions, interfering with the attraction of parasitic wasps to volatile compounds emitted from herbivore-infested plants (indirect defense)

Plant, Cell and Environment (2008) **31**, 1410–1415 doi: 10.1111/j.1365-3040.2008.01849.x

Isoprene emissions influence herbivore feeding decisions

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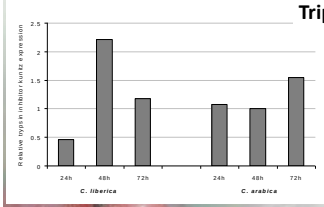
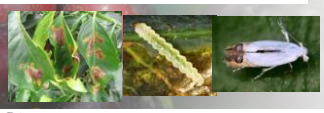
Tripsin inhibitor Kunitz gene



Reported in coffee by Mondego et al. (2005 and 2010), this is the same miraculin-like gene, *CoMir.* - coffee leaf miner (*Leucoptera coffeellum)*

The gene is also up-regulated after coffee leaf miner oviposition in resistant plants *C. racemosa* it is down-regulated during coffee leaf miner oviposition and larvae eclosion (herbivoria) in susceptible plants.

In this experiment *C. liberica* is behaving in a similar way to the resistant genotype.

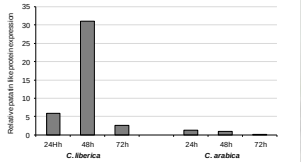


Planta
DOI 10.1007/s00425-010-1284-9

Molecular characterization of a miraculin-like gene differentially expressed during coffee development and coffee leaf miner infestation

Jorge Mauricio Costa Mondego · Melina Pasini Duarte · Edmardo Kiyota · Leandro Martínez · Sandra Rodrigues de Camargo · Fernanda P. De Caroli · Beatriz Santos Capela Alves · Sandra Maria Carmello Guerreiro · Maria Luiza Vilela Oliva · Oliveiro Guerreiro-Filho · Marcelo Menossi

Patatin like protein (lipid acyl hidrolase)



Affects lipids metabolism (Renier et al. 2004) and inhibit the growth of coleoptera larvae (Strickland et al. 1995).

When applied in artificial diets, potato patatin is lethal to Spotted Cucumber larvae and will stunt the growth of survivors so maturations is preventing resulting in no reproduction (Alidhai & Rydel 2010)

(12) **United States Patent**
Alidhai et al.

(10) Patent No.: US 7,662,372 B2
(45) Date of Patent: Feb. 16, 2010

(54) INSECT INHIBITORY LIPID ACYL OXYHASE GENE

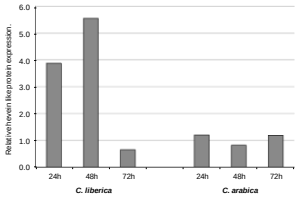
(52) U.S. Cl. 424/94.6; 435/195; 435/197; 536/23.2

(73) Assignee: Monsanto Technology LLC, St. Louis, MO (US)

U.S. PATENT DOCUMENTS

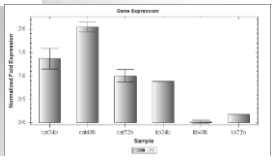
Spotted Cucumber Beetle
Diabrotica undecimpunctata

Hevein like protein



-Type of chitinase –
This gene is involved in the salicylic acid pathway (Medeiros et al. 2010), it has antifungal and antibacterial activity but also it is induced by methyl jasmonate treatment in plants (Kiba et al. 2003), and it can be induce by insects.

Other chitinases are induced in *C. arabica.*



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http://www.informaworld.com/smpp/title~content=t716100758

Sequences and transcriptional analysis of *Coffea arabica* var. Caturra and *Coffea liberica* plant responses to coffee berry borer *Hypothenemus hampei* (Coleoptera: Curculionidae: Scolytinae) attack

Sandra M. Idarraga[a], Ana M. Castro[a], Eliana P. Maeca[a], Alvaro L. Gaitán[b], Luis F. Rivera[b], Marco A. Cristancho[b], Carmenza E. Góngora[a]
[a] Department of Entomology, Cenicafé (National Centre of Coffee Research), Chinchina, Caldas, Colombia [b] Department of Plant Pathology, Cenicafé, Chinchina, Caldas, Colombia

First published on: 21 April 2011

Oligoarray
Expression of 45.000 sequences at 48h CBB pos-infestation

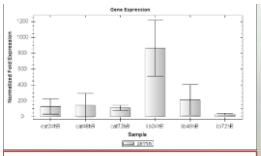
New oligoarray with 45.000 coffee sequences in order to study response of *C. liberica* vs. *C. arabica* after 48h of infestation with CBB.

A 2,500 sequences respond to infestation 900 are highly up-regulated in *C. liberica*, at least 2 times more than in *C. arabica*.

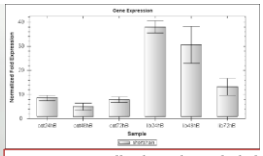
The expression of the genes previously identified was validated- Patatin is the genes that expressed the most in *C. liberica* at 48h

New genes were also identified:
–Glycoprotein from de epidermis EP1 (barrier).
-Berberine enzyme involve in synthesis of deterrence alkaloids.

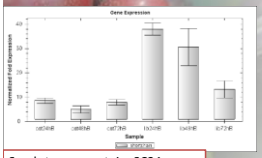
Source	Identification	Anotation	Infection	Infection fold-change (linear)	genoty
liber	liber_CEN32371	Patatin-like protein - Gossypium hirsutum (Upland cotton) (Gossypium mexicanum)	infected-uninfec	22.8	Arabic
liber	liber_CEN73479	Miraculin-like protein - Coffea arabica (Coffee)	infected-uninfec	6.4	Arabic
liber	liber_CEN71857	Isoprene synthase - Populus alba (White poplar)	infected-uninfec	3.7	Arabic
other	liber_CEN71825	Wound-induced protein WIN1 hevein	infected-uninfec	4.0	Arabic
liber	liber_CEN73476	Miraculin-like protein - Coffea arabica (Coffee)	infected-uninfec	4.0	Arabic
liber	liber_CEN32173	Hypothetical protein - Oryza sativa (japonica cultivar-group)	infected-uninfec	3.0	Arabic
liber	liber_CEN79960	Hypothetical protein - Oryza sativa (indica cultivar-group)	infected-uninfec	1.4	Arabic
liber	liber_CEN73479	Miraculin-like protein - Coffea arabica (Coffee)	infected-uninfec	6.4	Arabic
liber	liber_CEN73601	Hypothetical protein T8L23.1 - Arabidopsis thaliana (Mouse-ear cress)	infected-uninfec	1.3	Arabic
liber	liber_CEN73220	NOFOUND	infected-uninfec	2.7	Arabic
liber	liber_CEN74281	ATP binding / kinase/ protein kinase/ protein serine/threonine kinase/ protein-tyros	infected-uninfec	1.3	Arabic



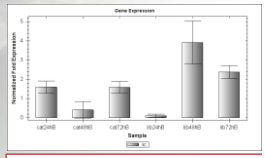
Germin-like storage protein,
Resistance to *Manduca sexta* in Nicotiana.



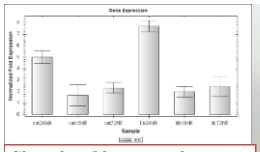
CPRD12 nonmetallo-short chain alcohol dehydrogenases Cowpea, induces under dehydration and abiotic stress conditions.



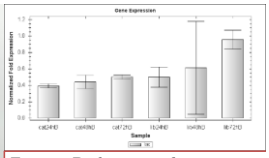
Seed storage protein. SC24
lectin- like



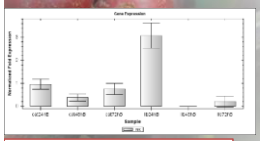
EP1 Glycoprotein from epidermis
It can have a barrier function.



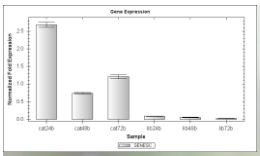
Glyoxalasa/bleomicina, lectin
Specific for manose y glucose.



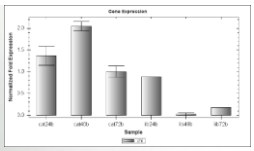
Enzyme Berberin involves in synthesis of deterrence alkaloids.



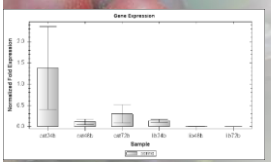
ACC oxidase related to
ethylene synthesis.



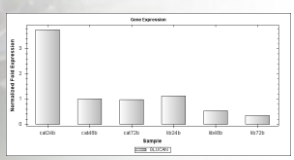
Protein related to senescence



Chitinase

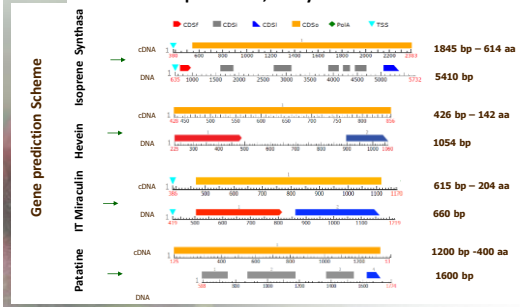


Subtilin endonuclease,



ß-1,3-glucanase

Genes amplification, analysis of structures and functions

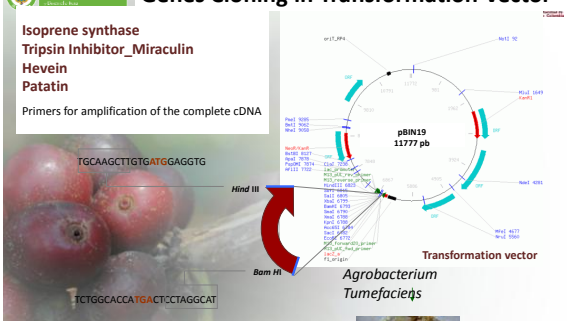


The Four genes in both genotypes *C. arabica* and *C. liberica* are similar-identical at level of DNA and cDNA. *C. liberica* induction in presence of the insect

Genes Cloning in Transformation Vector

Isoprene synthase
Tripsin Inhibitor_Miraculin
Hevein
Patatin

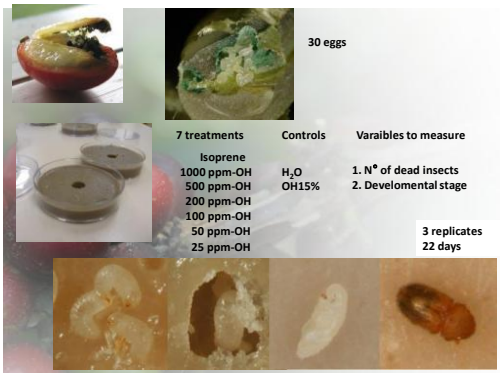
Primers for amplification of the complete cDNA



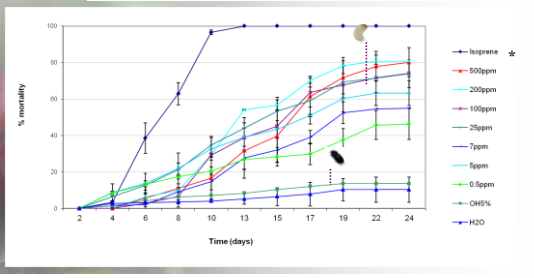
Result: Increase the number of copies of the genes – and level of expression in *C. arabica*

Embriogenic tissu
C. arabica
CUX2178

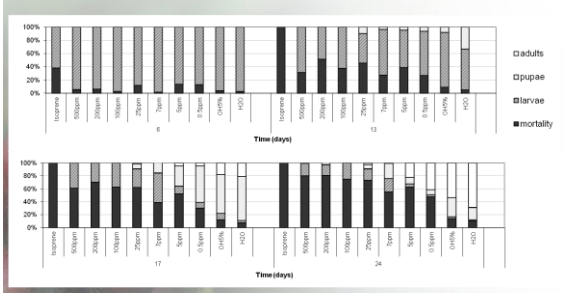
Effect of Isoprene on the growth and development of the CBB larvae



Isoprene effect on the mortality of coffee berry borer



Isoprene effect on the mortality of coffee berry borer evaluated on artificial diets.
The letters A, B and C indicate statistical differences between the treatments according to the Duncan test at 5%.



Coffee berry borer developmental stages on artificial diets with different isoprene concentration and without isoprene up to day 6, 13, 17 and 24 of the experiment

Fruits transcriptome of *C. arabica* and *C. liberica* infested with CBB -RNA seq

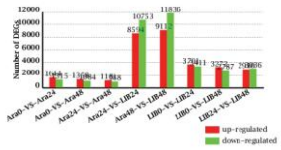
Sequencing of RNA samples (BGI) , 25 millions of short reads (25-300bp) of DNA the reads are aligned with a possible genome.

LIB24: Stat. of Map to Gene

Map to Gene	reads number	percentage
Total Reads	24951321	100.00%
Total BasePairs	1222614729	100.00%
Total Mapped Reads	19871477	79.64%
perfect match	14702488	58.92%
<=2bp mismatch	5168979	20.72%
unique match	6708838	26.89%
multi-position match	13161639	52.75%
Total Unmapped Reads	5079844	20.36%

Gene Expression Difference Analysis

Statistic chart of DEG



Conclusions

- The genes that have been identified in this study increase the understanding of functional genomics.
- The genes over-expressed in the *C. liberica* genotype in response to the CBB attack can be turned into new tools to widen the genetic basis of the cultivable varieties in the effort to improve their resistance to pests and diseases.
- It has been demonstrated that the CBB responds to olfactory stimuli in order to locate and colonize fruits; therefore, it is likely that the plant exploits this fact to defend against and repel the CBB.
- Isoprene represents a compound with toxic and repellent effects that could be used as part of a novel strategy to control CBB infestation.

Perspectives

•To obtain the complete genome sequence from *C. liberica* and *C. arabica*. Search for promoters.

•To continue with transformations of coffee tissue for gene piramidizacion and generation of cisgenic plants.

•To evaluate the contribution of isoprene in the possible thermo-regulation of the plant related to climate change.