WP3. Determination of the predictive value of *Hevea* genetic resources in Indonesia.

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The objective of this WP is to class the rubber clones according to their agronomic and physiologic capacities in order to facilitate the selection of material for further in-farm large-scale trials, which will be established after the present project. The traits will be selected for matching with the cropping systems identified by stakeholders (WP1) and with a potential tolerance to predicted environmental constraints (WP2). The *Hevea* germplasm consists of wild Amazonian core-collection of 100 accessions, 14 recommended clones and progenies under selection. The predictive value of rubber clones will be determined from data of long-term large-scale clone trials in various contrasting areas. Besides, the current GA-TPD project deals with the phenotyping of a segregating population of 200 genotypes (clone PB 260 ♀ x clone SP 217 ♂), which is being analyzed in controlled conditions and in a small-scale clone trial (5 replicates of 2 copies = 2,000 trees = 5 ha) planted in November 2016. A fine genomic and phenotypic characterisation of a progeny under selection will provide new candidate clones based on traits not characterized above such as physiological parameters related to latex production, tolerance to abiotic and biotic stress as well as rubber and wood properties. Genetic and genomic analysis of these traits will enable further marker-assisted selection applications.

Activity 3.1. Agro-physiology typology of recommended rubber clones and clones under large-scale evaluation in Indonesia. Up to now, the predictive value of rubber clones is determined after long-term large-scale clone trials (LSCT). IRRI breeding programme has led to recommend 14 rubber clones with permit of planting from the Indonesian government, and some clones from IRR 300 (22) and IRR 400 (10) series are under LSCT in North and South Sumatra. The characterization consists of agronomic and latex diagnosis parameters. The data of all these previous trials will be collected and organized in the current Agro-physiology database. An agro-physiological characterization and clonal typology will be analyzed according to the model developed by the UMR ABSys at CIRAD.

Activity 3.2. Genotyping and genomic analyses

This activity will be carried out by the Genotyping Platform (AGAP) and private sequencing companies (Gentyane Platform in Clermont-Ferrand, Eurofins Genomics).

Activity 3.2.1 Genetic conformity of the plant material phenotyped in trials

The genetic conformity of the individuals in the trials will be carried out with 8 SSR markers

CIRAD has developed a methodology of clone recognition and test of legitimacy based on the use of eight SSR markers associated to a database of more than 2,000 reference patterns. The 200 clones of the GA-TPD trial were previously tested for their legitimacy in budwood garden and their reference pattern with the eight SSR markers were established. Leaflets from the 2,000 trees of the trial will be collected for DNA extraction and their DNA will be pooled in batches of 10 individual samples for each clone. The conformity and the homogeneity of the 200 DNA batches will then be checked with the eight markers. For the batches including some impurities, the identity of each individual tree will then be checked in order to eliminate from data analysis the trees that are not true-to-type.

Activity 3.2.2 Construction of a high-density map (Genotyping Platform, AGAP). In addition to the available SSR map, a high-density linkage map will be constructed based on SNP. A genotyping-by sequencing (GBS) approach will be employed on F1 population to develop high-density molecular marker map. First, Total genomic DNA extractions will be performed from dried leaves by an automated method adapted from [10] on Biomek FXP (Beckman Coulter, CA, USA) and using the NucleoMag Plant Kit (Macherey–Nagel, Germany). DNA samples will be quantified with a Fluoroskan Ascent FL fuorometer (Thermo Fisher Scientifc, Waltham, MA, USA). Genomic DNA quality will be checked using agarose gel electrophoresis. A reduction genomic library will be prepared using PstI-MseI restriction enzymes with a normalized 200 ng quantity of DNA per sample on 96 well. The GBS procedures published by [11] are adapted as described in [12] to large heterozygote genome. The GBS library will be sequenced on Illumina Hiseq 3000 lanes per 96 plex sample. After sequencing step, raw sequence data will be demultiplexed with GBSX v1.2 software [13]. Cutadapt v1.9 [14] is used to trim adapters and SNP calling is conduct with the process_reseq.1.0.py (Python2) program followed by site pre-filtering using the VcfPreFilter.1.0.py (python2) program with the default parameters. Both programs are part of the VcfHunter package [15] (available at https://github.com/SouthGreenPlatform/VcfHunter/). The genotyping matrix obtained will be served to pairwise LOD scores and pairwise recombination frequencies matrices with JoinMap. Linkage group data will be computed with Scaffhunter to order in a single step SNPs and scaffolds.

Activity 3.2.3 De novo sequencing of reference parent PB 260 & resequencing of the male parent clone SP 217 genome

Parental clone PB 260 reference genome. Available genomic data on the parental PB 260 clone are only scaffolds (Zhang et al 2019). With the help of PACBIO technology, a reference genome will be sequenced with very long reads, allowing assembly up to the level of pseudo-molecules, corresponding to the chromosomes. This step is important to anchor the sequences on the physical/genetic map and to analyze the genes underlying the QTLs.

Parental clone SP 217 resequencing and annotation. Leaf nuclear DNA from clone SP 217 will be will be resequenced with a combination of long (PACBIO) corrected by short reads, using Illumina pair-end sequencing (2 x 150 bp). Genomic sequences will assembled through alignment against the clone Reyan7-33-97 reference genome (Tang et al. 2016). The Bowtie tool (Vicient and Casacuberta 2017) will be used to build a Burrows-Wheeler transform index with specific parameters [bowtie -f -v 1 -p 10 -a -m 50 --best --strata (Langmead et al. 2009)].

As already done for clone PB 260, EGN transfer (Foissac et al. 2008) and EGN-EP (Sallet et al. 2019) will be used to transfer annotations from the reference genome from clone Reyan7-33-97 to clone SP 217 and to add de novo annotations, with the help of expression data (SP 217 transcriptomes db and NCBI Euphorbiaceae EST db) and polypeptide data (Swiss-prot db, Reyan7-33-97_protein.faa (Tang et al. 2016) and TrEMBL db). The annotation results will be then evaluated by a BUSCO analysis (Waterhouse et al. 2017).

A website (Hevea Genome Hub), under development, containing the fine annotations of the PB 260 parental clone is already available at Cirad. Genomic data from 7 clones are also publicly available (Reyan 7-33-97, Wenchang11, RRIM600, PR107, Yunyan774, Reyan8-79, PB 260). Genomic sequences from clone SP 217 will be added for the Single Nucleotide Polymorphism (SNP) calling. All accessions reads were processed as follows: 1) reads were aligned against the clone Reyan7-33-97 reference genome (Tang et al. 2016) using BWA v0.7.15 with the mem

algorithm (Li 2013). Reads aligning at several positions were removed using samtools v1.3 (Li et al. 2009). 2) Redundant reads were removed using MarkDuplicate from Picard Tools v2.7.0. 3) Reads were locally realigned around indels using the IndelRealigner tool of GATK v3.3 package (McKenna et al. 2010). 4) For each accession, at each covered position, all mapping bases with a mapping quality ≥10 will be counted with the bam-read count program (https://github.com/genome/bam-readcount). The complete process described here will be performed using the process_reseq_1.0.py custom python script available at (https://github.com/SouthGreenPlatform/vcfHunter).

Activity 3.3 Genetic analysis of complex traits in a biparental population. The population is phenotyped by IRRI researchers in a SSCT for growth, latex production and disease resistance (Project GA-TPD), in bio-assay for several leaf disease pathogens, and controlled conditions for determining the drought factor index (PhD thesis, Andi Nur Cahyo, SEARCA, PHC Nusantara). The present proposal aims at enhancing knowledge on genetic basis of several crucial traits. The phenotypic information will be used for estimating the heritability, and detecting QTLs and underlying candidate genes. The identification of QTLs will be performed using identified using MapQTL6 software.

3.3.1 Genetic analysis of physiological traits related to latex production. Several factors are related to the latex production: sucrose, inorganic phosphorus, thiols, plugging index, dry cut length, bark thickness, and number of laticifer mantels. These parameters will be phenotyped during the first three years of production. The first year of tapping is dedicated to the determination of the rubber yield potential of each genotype by measuring the initial latex production (P_0) and the sucrose content in laticifer. Combined with the inorganic phosphorus content, the data can be used to predict a standard harvesting system for a given clone. Dry cut length (DCL) and thiol content will be monitored after the intensification of the tapping and ethephon stimulation in the second and third year in order to induce early TPD occurrence in TPD-susceptible genotypes. Starch is a carbon reserve that can be remobilized for latex production. These additional parameters will be analyzed in wood and bark (Biochemical Phenotyping Platform, UMR AGAP).

The latex biochemical parameters will be measured in a latex sample of 1 mL harvested during the first five minutes after tapping and collected in acidic medium to coagulate rubber particles. The methods used were developed by CIRAD (Jacob et al, 1989.). At different moment during the seasons, starch content will be analyzed by sampling with a wood auger (0.5 cm diameter). The core is 5 cm long (4 cm of wood and 1 cm of bark). The samples will be immedBiowooebly immersed in liquid nitrogen, then freeze dried. After blending and soluble sugars extraction in ethanol 80%, starch will be quantified by enzymatic way (Chantuma et al., 2009).

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Chantuma P., Lacointe A., Kasempsap S., Thanisawanyangkura S., Gohet E., Clement A., Guillot A., Ameglio T., Thaler P. 2009, 29, 1021-1031.Carbohydrate storage in wood and bark of rubber trees submitted to different level of C demand induced by latex tapping.

3.3.2 Genetic analysis of traits related to the resistance to major pathogens of rubber plantations in Indonesia (Alchimi Putri (Sembawa RC, IRRI), Budi Setyawan (PhD, SEARCA, UGM), Siti Subandiyah (Biotec RC, UGM), Fetrina Oktavia (Sembawa RC, IRRI), Valérie Pujade-Renaud (AGAP, CIRAD)

Activity 3.3.2.1 Collection and preservation of strains for major pathogens (Colletotrichum, Corynespora, and Pestalotiopsis)

Collection of strains for major pathogens (*Colletotrichum* sp., *Corynespora cassiicola*, and *Pestalotiopsis microspora*) is taken from plants with disease symptoms. Plant samples for the collection of *Colletotrichum* sp. taken in the form of leaves that have leaf symptoms that are wrinkled, curled, and there are small spots that appear. The plant samples for the collection of *C. cassiicola* taken in the form of leaves that have spots on the bones of the leaves that looked pinnate like fish bones, while the plant samples for the collection of *P. microspora* have a symptom of light brown to dark brown round spots. Isolation of pathogenic fungi from diseased plants will be carried out on potato dextrose agar (PDA) medium.

Activity 3.3.2.2 Bio-assay on detached leaves

−Bio-assay on detached leave for Colletotrichum leaf fall disease is carried out by producing pathogenic conidia. Production of conidia *Colletotrichum* sp. will be carried out by taking pathogenic conidia from pure culture aged 7 days using a brush. Application of conidia *Colletotrichum* sp. will be carried out on light green rubber leaves 3 weeks old by giving each leaf as much as 4-6 drops of conidia. The concentration of conidia used 10^6 conidia/ml of water. This concentration is the optimum concentration of conidia that can cause disease development in rubber leaves (Sangu & Muid, 2016). Furthermore, the leaves are incubated at room temperature for seven days. Observations were made by calculating the intensity of disease attack on the leaves after 7 days of treatment. Observations using an attack scale of 0 to 4, namely 0 (no attack), 1 (1% -25% of the leaf area contains fungal mycelium or yellow-brown leaves), 2 (26% -50% of the leaf area contains fungal mycelium or yellow-brown leaves), 3 (51% -75% of the leaf area contains fungal mycelium or yellow-brown leaves), and 4 (76% - 100% of the leaf area contains fungal mycelium or yellow-brown leaves) (Pawirosoemardjo, 1984). The results of measuring the attack scale are included in the disease intensity formula. Based on the percentage of disease intensity, the resistance level of each genotype was further grouped as in scale of disease intensity, namely 0-20% (highly resistance), 21%-40% (resistance), 40%-60% (moderate), 61%-80% (susceptible), and 81%-100% (very susceptible) (Pawirosoemardjo, 1984).

Bio-assay on detached leave for Corynespora leaf fall disease is carried out by immersing rubber leaves in pathogenic toxins. Observations are made by calculating the percentage of leaf wilt after 48 hours of treatment. Differences in the susceptibility of each leaf were described by counting leaf weight loss 48 hours after *C. cassiicola* toxin treatment. Based on the percentage of leaf wilt, classification of rubber genotype resistance level is divided into: 0-12% (highly resistant), 13%-24% (moderate resistant), 25%-36% (moderate susceptible), and > 37% (very susceptible) (Situmorang, 2002).

Bio-assay on detached leave for Pestalotiopsis leaf fall disease is carried out by producing pathogenic conidia. Inoculation of *P. microspora* using 5 mm^2 pieces of pure cultured mycelia. Pathogen inoculation is done by attaching pieces of mycelia culture to the injured part of the

leaf. The leaves used are light green leaves 2 weeks old. Observations are made every day until the spotting symptoms appeared.

Activity 3.3.2.3 Identification of QTLs and underlying candidate genes from SSCT field trial and bio-assay

QTLs identification linked to rubber diseases is carried out through a combined analysis of phenotyping data on disease observations of F1 populations from parent clones PB 260 and SP 217 in the SSCT field trial and bio- assay in Laboratory with genotyping data of genetic linkage maps from of the population.

3.3.3 Analysis of rubber film mesostructure with SECMALS to forecast some properties of raw rubber (Sherly Haniparianty (IRRI), Frédéric Bonfils (UMR BIOWOOEB))

Latex will be collected for each genotype of the GA-TPD population. The rubber film will be prepared and dried at the Sembawa RC. Ms Sherly Haniparianty will come at the Rubber Technology laboratory (UMR BIOWOOEB) for 2 months to analyse the rubber properties under the supervision of Mr Frédéric Bonfils.

To analyze natural rubber (NR) properties, at least 100 g of rubber sample is needed. In the framework of this project, knowing only 2-3 trees per genotype will be available, it will be difficult to measure NR properties (ie Wallace plasticity or Mooney viscosity) with normalize methods (ISO 2000). A good way to forecast some NR properties is to characterize NR meso-structure by size exclusion chromatography coupled to multi-angular light scattering (SECMALS) [1-2]. SECMALS characterization can be done with only 30 mg of rubber sample. This technic allows determining the inherent molar mass distribution (MMD0) of NR samples, this MMD_0 varying with the clonal origin of trees [3]. We already showed that it is possible to forecast some NR properties knowing the MMD_0 [3].

References

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Activity 3.3.4 Phenotyping of traits related to the architecture typology of trees and wood properties (Sherly Haniparianty (IRRI), Fetrina Oktavia (IRRI), Jean Gérard (UMR Biowooeb), Frédéric Do (UMR Eco&Sols).

Tree architecture and wood properties influence wind tolerance and wood quality. Pruning is an effective method of preventing wind damage. Rubber wood has a low natural durability but specific rubber wood processing has been established and led to its commercial exploitation.

The major phenotyping parameters are the wood density observed on wood coring (Pressler auger, volumic mass), the estimate biomass as well as some criteria at the tree level (diameter, roundness, straightness, slenderness, height of trunk without branching).

Rubber trees traits to consider for phenotyping applied to further wood processing fall into three main categories: (1) quality of standing trees; (2) intrinsic wood quality; (3) level of growth stresses (linked to wind damage).

A multi-trait typology of the GA-TPD population will be evolved from the results of these measurements:

* **Quality of standing trees.** The traits to be determined give information on ability to primary process -sawing, peeling or slicing- and assessment of recovery from these operations. The traits concern the shape of the boles: height/volume of bole free of branches, conicity, roundness / cylindrical form, straightness / flexuous shape of the bole, slenderness. These traits can be determined with simple measuring tools. Moreover, general evaluation of bole quality considers possible defects of the boles such as local fungi or insects attacks, bumps, longitudinal cracks, and any type of damage. These defects are caused by external or internal factors.

* **Intrinsic wood quality.** Many traits have to be determined in order to completely qualify and quantify wood quality, from physics and mechanics up to chemistry and resistance to biological degradation. In order to simplify this task, only specific gravity (= density) will be considered. It is a basic technological characteristic, the first to be determined when qualifying wood. This property is more or less closely related to the wood's main physical and mechanical properties and with some working characteristics. Average values and variations along the radius, from pith to bark, will be determined on increment cores removed on standing trees. That will indicate general level and heterogeneousness of wood quality.

* **Growth stresses**. Risks of wind damage (wind breakage) can be predicted from growth stresses measurement at the surface of standing trees; growth stresses are measured using a special device designed by Cirad.

Activity 3.4 Development of high throughput phenotyping methods (Anne Clément-Vidal, Gilles Chaix (UMR AGAP), Afdholiatus Syafaah, Sigit Ismawanto (IRRI, Tri Rini Nuringtyas (UGM))

3.4.1 Attempts for rapid methodology for latex diagnosis in multiplate spectrophotometer, sucrose content using test strip

The using of a multiplate spectrophotometer to carried out the biochemical parameters of the latex diagnosis will save times and reagents. Indeed, the plates contains 96 wells of 300µL, the absorbance reading is instantaneous in all wells. This equipment requires developments concerning the volume ratio between the various reagents and samples and the training of technicians to use the multichannel pipets. Inorganic phosphorus and thiols will be easily quantified with this equipment; however, sucrose quantification will require more times because this assay is carried out with high temperature. Then, it will be necessary to heat the reactive medium outside plates and introduce it just before the reading or otherwise use another method to quantify sucrose.

Mainly for sucrose content test strip will be developed using a reflectometer. The reagents are impregnated on the strip and consist in enzymes and dyes, which react selectively with sucrose.

The intensity of the resulting staining is proportional to the sucrose content. The strip is introduced inside the reflectometer and the reflected light from strip is measured. The difference in intensity of emitted and reflected light allows a quantitative determination of the concentration of sucrose. The reflectometer is previously calibrated. The latex will be probably pre-treated but various solutions are available. It will be possible also to test other type of strip to quantify inorganic phosphorus, antioxidant, magnesium, potassium

3.4.2 Application of the NIRS to estimate parameters of latex diagnosis. Application of the NIRS to monitor latex in a perspective of monitoring in-farm trials in the second step of the programme.

Near-infrared spectroscopy has been recognized as a powerful analytical technique for rapid determination of various constituents in many agricultural and raw materials. The approach involves the acquisition of a reflectance/transmittance spectrum after near-infrared radiation. The resulting NIR spectral information is then calibrated against measurements obtained using conventional analytical techniques using linear regression (PLS).

There is few scientific references on Nir dedicated on the quality of latex, coagulum, natural rubber as final product. The parameters studied are total solid content and dry rubber content (Inagaki et al. 2013, Cornish et al. 2004, Suchat et al. 2013 and Kopicky 2014 in Guayule, Kumar 2009), the moisture content of the rubber (Rittiron and Seehalak 2014), the water content of the coagulum (Suchat et al. 2015), and the mechanical properties of the rubber (Pornprasit et al. 2016).

In the frame of this proposal, we plan to evaluate the feasibility of Near infrared (Nir) spectroscopy for latex quality evaluation in the field. We'll compare quality prediction between spectra data from handled Nir spectrometers (low-cost, <100 g, limited range, etc.) vs Nir spectrometers (>50 kUS$, 15 kg, full range). This requires different steps: Nir field measurement on fresh latex from different contrasted genotypes (min 15), trees (min 100), database building and data analysis. We selected handled spectrometer: MicroNir Viavi from Agap-PPB, NanoNir Texas Instrument to buy, ASD Bonzai Electronics from Agap-PPB. The reference values will be produced by IRRI biochemistry lab. We'll use diferent software for chimiometrics analysis as R and rnirs package, The Unscrambler, Matalab.

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