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THE ANIMAL PRODUCTION AND HEALTH LABORATORY

EXECUTIVE SUMMARY

The main mandate of the Animal Production and Health Laboratory (APHL) of the Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture is to provide assistance to Member States (MSs) in improving productivity of livestock and preventing and controlling transboundary animal and zoonotic diseases (TAZDs).

The COVID-19 pandemic that emerged in 2020 has clearly demonstrated the impact infectious diseases may have on animal and human health and economies. It also highlighted the importance of early detection, surveillance, and monitoring of known and new pathogens at the animal human interface to reduce the risk for human exposure and for better preparedness, effective control and the eradication of zoonotic diseases.

More than 60% of human infections are caused by zoonotic agents and many of these zoonotic infections are causing diseases in animals and production losses in the livestock sector. It is evident that zoonoses not only have huge impact on public health but also on livestock economies and food security.

According to previous estimates, over 600 million people globally are livestock-dependent; they represent up to 70% of the population in the most marginal areas, where rural communities are more isolated and proper awareness and control are difficult to achieve. Unsurprisingly, there is a huge gap regarding the impact of zoonotic diseases between developed and developing countries. Considering the disability-adjusted life years (DALYs) as a partial indicator for zoonotic disease burden, previous estimates indicated that "in low income countries, zoonoses and diseases which recently emerged from animals make up 26% of the DALYs lost to infectious disease and 10% of the total DALYs lost. In contrast, in high income countries, zoonoses and diseases recently which emerged from animals represent less than 1% of DALYs lost to infectious disease and only 0.02% of the total disease burden".

It is an APHL priority to assist MS veterinary laboratories – particularly those operating in limited resourced settings - in building and strengthening their capacity for early detection and control of zoonotic diseases and preparedness for novel, emerging pathogens such as SARS-CoV2.

APHL is providing valuable assistance to MSs laboratories regarding SARS-CoV2 testing for the rapid identification and confirmation of COVID-19 cases. APHL contributed to the selection, quality control and verification of reagents, laboratory standards and laboratory procedures for SARS-CoV2 diagnosis to be shared with- or shipped to- Member States. APHL, in collaboration with AGES laboratories, conducted an important technical verification and comparison of selected commercially available products for RT-PCR based SARS-CoV2 testing. The final aim of this technical activity was to expand the portfolio available for laboratories located in different part of the world, particularly in limited resourced countries.

Other important work conducted on zoonotic diseases in 2020 were the verification and transfer to two MSs (Botswana and Indonesia) of a newly developed multiplex detection platforms for important zoonotic pathogens causing abortion in ruminants and the development and application of straightforward pipeline for the full gene characterization of Rift Valley Fever virus, an important zoonotic agent in Africa.

Concerning transboundary animal diseases, APHL actively worked to support MSs in facing the emergence of animal infectious diseases such as African Swine Fever (ASF), Lumpy Skin Disease (LSD) and Rabbit Hemorrhagic Disease (RHD), rapidly spreading in areas previously unaffected of Asia and Africa. Interesting research data were obtained on irradiated vaccines and vaccine formulations for priority animal diseases. Regarding ASF, vaccines are not available at present therefore experiments started to explore the application of irradiation technology for vaccine development.

Furthermore, a new assay for the rapid ASF identification and differential diagnosis was optimized and transferred to some MSs and support to MSs was provided for ASF molecular epidemiology and outbreak investigations. Similarly, technical and scientific support was provided for LSD in East and South Asia, where the disease appeared for the very first time in some countries, and for RHD in West Africa, where this acute and fatal virus infection quickly spread across vast areas of the region.

In animal genetics, APHL research and development made significant progress, particularly in developing a multi-species camelid DNA chip for selection and breeding of old and new world camels for increased productivity. The process of validation and field testing of the chip is being continued and will be rolled out in 2021 for the use of camel breeders across Africa, Asia and Latin America. APHL supported national cattle breeding programmes of nine countries to identify animals with high genetic potential for increased milk and meat productivity. Technical support was provided to countries like Argentina, Bangladesh, Peru, Serbia, Sri Lanka and Uruguay to perform genome wide evaluation of their local cattle populations. The purpose of genomic evaluation was to perform genome wide association of genotypes with milk production traits, estimate genetic admixture, assess the level of taurine inheritance in crossbreds and identify selection signatures related to high altitude adaptation in cattle. In 2020, significant achievements were also made towards successful implementation of National Action Plans on Animal Genetic Resources in various Member States. With APHL support and through IAEA technical cooperation projects, effective population size and genetic bottleneck status was evaluated for more than 10 indigenous African cattle breeds/populations.

In addition to R&D, APHL was also involved in capacity building activities in IAEA and FAO Member States. APHL actively supported the establishment or strengthening of molecular genetic laboratories in various countries (Cameroon, Eritrea, Indonesia, Nigeria, Burkina Faso and Mongolia) through provision of necessary equipment and laboratory supplies. However, the training activities to improve technical capacities in MSs took a back seat due to COVID-19 related travel restrictions and regulations. APHL was able to host one fellow from Peru for training on genomic characterization of locally available Criollo cattle breeds. Despite the restrictions imposed by COVID-19, one expert was fielded in Indonesia to support the veterinary laboratory in Bogor in setting up and troubleshoot the diagnostic pipeline for ASF.

Through remote assistance and delivery of laboratory emergency tool boxes, APHL was able to build capacity and transfer technology to several MSs concerning LSD (Bhutan, Indonesia, Myanmar, Nepal, Vietnam), ASF (Indonesia), Peste des Petites Ruminants (PPR - Bhutan, Myanmar; international ring trial), RHD (Burkina Faso, Nigeria, Senegal) and avian influenza (Mongolia, Senegal).

Knowledge dissemination and data sharing has been central to APHL activities, through: (a) APHL involvement and coordination of the VETLAB Network of national veterinary diagnostic laboratories, the membership of which today counts 46 African and 19 Asian countries; (b) sharing of technical data and scientific information with MSs and scientific communities (18 publications in international scientific journals); and (c) resource mobilization to enhance capacity building and technology transfer, with financial support from USA and Japan (IAEA Peaceful Uses Initiative), and South Africa (African Renaissance Fund).

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MAJOR ACHIEVEMENTS IN RESEARCH AND DEVELOPMENT

Animal Health

New animal vaccine prototypes and formulations developed using irradiation technologies

Application of Gamma Irradiation for African Swine Fever virus vaccine development

African swine fever virus (ASFV) is among the most devastating and economically significant disease affecting pig industry. To date, there is no licensed vaccine for ASFV, and current control methods involve quarantine and culling of animals in affected areas and regions. The causative agent, ASF virus (ASFV), belongs to the genus Asfivirus within the Asfarviridae family. ASFV has complicated architecture and a large double-stranded DNA genome (170–193 kb) containing 151–167 genes depending on the strain.

ASFV can cause high morbidity and mortality in domestic pigs and wild boars. When introduced into disease-free regions or domestic pig populations, the disease predominantly shows acute forms with high mortality rates up to 100%. After several years of ASFV presence in endemic areas, mortality rates decline due to virus adaptation to the hosts and infected individuals show subacute forms of the disease or even no clinical signs, complicating even more its detection and eradication.

There are currently 24 genotypes of ASFV based on the major capsid protein p72, and 8 serotypes based on the viral hemagglutinin CD2-like protein (CD2v) and C-type lectin. The virus circulating in Europe, Russia, and China has been identified as a highly virulent, genotype II strain.

ASFV is stable in the environment and can be readily transmitted through infected pork products and contaminated fomites. Thus, ASFV poses a significant threat to the swine industry worldwide, and the need for an ASFV vaccine is of high priority. The pursuit for an effective vaccine against ASFV has been largely unsuccessful. This is due to the complexity of the virus and our limited understanding of ASFV virulence factors and the correlates of protection.

Different vaccine strategies for ASF have been evaluated in the past decades: inactivated vaccines, DNA vaccines, subunit vaccines, and adenovirus-vectored vaccines have been tested and proved to be unsuccessful. Some gene deleted ASFVs have shown potential as live attenuated vaccines, but it is not known if they could convert to more virulent strains during their replication in pigs.

Despite inactivated vaccines being very efficient at inducing antibodies, on occasions capable of blocking the virus in fluids, they are not very efficient at inducing specific cytotoxic CD8+ T cells (CTLs), crucial for elimination of virus-infected cells. Incorporation of new adjuvant formulations and novel, and more innocuous, inactivation procedures might contribute to designing efficient inactivated vaccines in the future. Lately, considerable improvements have been made regarding adjuvants, especially with action towards cellular immunity, which is vital for the protection against ASFV.

A scientific collaboration has been established between the research group at the “Friedrich Loeffler Institut” (FLI), Germany and APHL for testing what dose of Cobalt60-Gamma-irradiation is required to inactivate African Swine Fever Virus, preserving antigenicity and thus the ability to elicit immune response.

This collaboration has in plan to test in vivo whether an irradiated-inactivated African Swine Fever Virus can be efficient as a vaccine in conferring protection to domestic pigs. Three different African Swine Fever Virus strains (WSL-adapted Estonia/2014, Armenia/08 and WT-Estonia/2014) have been

irradiated at 10, 20, 30, 40 and 50 kGy in order to assess the minimum inactivation dose through hemadsorption test (HAD), performed in a BSL3 facility. The hemadsorption test represents the standard method for diagnosis and for virus titration of (hemadsorbing-) ASFV isolates; it is based on the fact that swine macrophages, once infected with ASFV, display on their surface the viral CD2v protein, which is able to bind erythrocytes. When red blood cells bind around the surface of an infected macrophage, it is possible to observe the classical "rosette" shape, clear sign of viral infection (Figure1). With the microscope observation of the rosettes at 48, 72 and 120 h.p.i it was possible to confirm the infection (or in absence, the complete inactivation) and to calculate viral titer. Considering that scientific literature lacks of information whether gamma irradiation is able to impair the expression of CD2v protein (and thus the hemadsorbing capacity) of irradiated ASFV, an immunofluorescence test has been performed in parallel, targeting the major capsid protein p72, to ensure that the fully inactivation indeed occurred. As a result of such tests, the inactivation of the irradiated strains was confirmed and WT-Estonia/2014 was selected as candidate vaccine due to its clearer phenotypical expression of inactivation and consistency throughout the different assays (Hemadsorption and Immuno-fluorescence); its minimum-inactivation dose was established at 10 kGy. Since for the Armenia08 strain, 10 kGy was not enough to inactivate the virus, it was assumed that at 10 kGy some viral particles might still be alive in all strains tested. For this reason, it was decided to use the 20 kGy as a starting point to inactivate the virus batch to produce the candidate vaccine. For safety reason, it was agreed to go one step further and choose the next higher dose, thus say 30 kGy. The higher irradiation dose should be able to guarantee from one hand the full and safe inactivation of every single viral particle; from the other, the preservation of the outer structure and thus of the surface antigens stimulating an immune response.

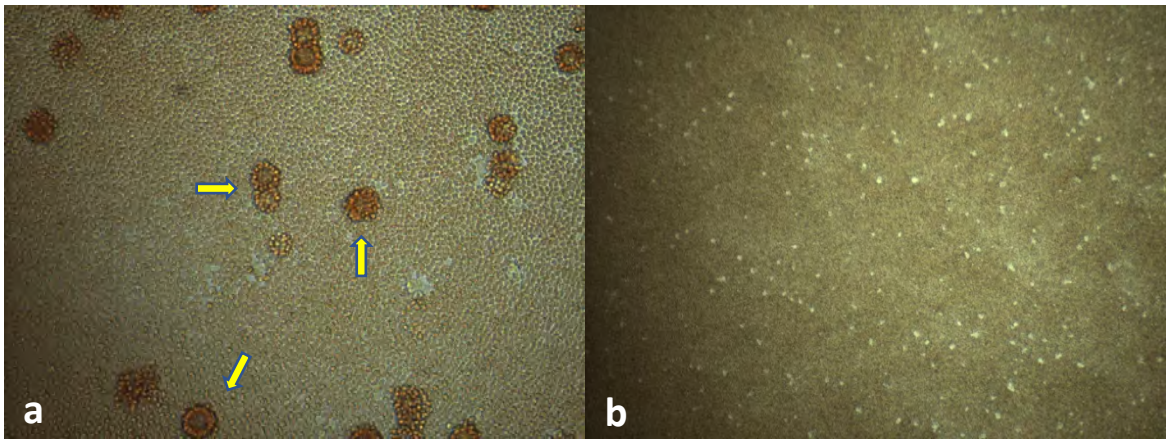


Figure 1. *Swine primary cells (macrophages) were plated in 96 wells-plates with a concentration of 5X10^6/ml; successively, the monolayer was infected with ASFV WT-Estonia/2014. Finally, 1:100 diluted erythrocytes were added to perform the hemadsorption test.* ***a)*** No irradiation /*Positive Control; formation of "rosettes", meaning that viral infection ad replication occurred;* ***b)*** *ASFV irradiated with 10 kGy; no rosettes, displaying full inactivation of the virus.*

Irradiated replication-incompetent lactobacilli preserve the metabolic activity and have immune modulation properties as live bacteria

Probiotics are defined as non-pathogenic live microorganisms that, when administered in adequate amounts, confer a health benefit on the host. These probiotics basically act through changing the gut environment through changing the gut microbiota leading to immunomodulation, preventing the growth of pathogenic organisms, and maintaining the intestinal barrier integrity. However, due to number of reasons the use of live probiotics has certain disadvantageous. This includes translocation of gut bacteria into the circulation, safety issues in immune-compromised populations and transfer of genes from probiotic bacteria to commensals and pathogens leading to anti-microbial resistance (AMR). As such

inactivated probiotic microorganisms have been explored to derive the same benefits yielded by live organisms. The potential of heat-killed microorganisms to provide a benefit to humans or animals has been examined in several studies. On the other hand, Lactobacilli (LAB), a group of bacteria that has been used as probiotics to provide health benefits, has shown to be effective in promoting a healthy microbiome. Because of their immune modulation capacity, they can also be used as either immunostimulants or immune regulators. This property of immune stimulation is especially beneficial to augment or to change the immune pathway that is stimulated. This property of LAB could be used to enhance the efficacy of vaccines. While vaccines can theoretically provide complete protection against infectious diseases, many vaccines that are currently available on the market are suboptimal. This is more prevalent with livestock vaccines as the number of infectious diseases present in numerous species are large. It seems the antigens used for the vaccines are appropriate in providing protection against the disease pathogens, but the breadth and depth of the immunity induced is not enough to provide a long-lasting effect. This phenomenon is evident in killed vaccines as the vaccine antigens do not stimulate the local vaccination environment sufficiently to attract antigen presenting cells. Therefore, many killed vaccine formulations are supplemented with molecules called 'vaccine adjuvants' that augment the vaccine response. Throughout the history of vaccine development, a handful of vaccine adjuvants have been produced and added into commercial vaccine preparations. Ultimately, using such adjuvants adds to the price of vaccines, increasing the cost of a vaccine dose. While live LAB has been explored as vaccine adjuvants in many experiments, due the reasons mentioned earlier, it would be interesting to investigate inactivated LAB for their immune modulation property. In collaboration with the University of Natural Resources and Life Sciences, Vienna (BOKU university), APHL conducted a series of experiments to determine the immune modulation property of LAB. In the first experiment we determined the irradiation dose that is needed to stop the replication of four strains of LAB; L. casei (LB 400), L. acidophilus (LB 401), L. paracasei (LB 402) and L. plantarum (LB 403). This was done by examining the D10 value for each strain. D10 value is the dose of irradiation needed to lower the concentration of an organism by one log10 (Figure 2). The results show that the D10 values are 526.2 Gy, 661.4 Gy, 592.4 Gy and 471.5 Gy for L. casei, L. acidophilus, L. paracasei and L. plantarum respectively; thus, at log 9 concentration minimum dose needed for the inhibition of replication would be 4735.8 Gy, 5952.6 Gy, 5331.6 Gy and 4243.5 Gy respectively.

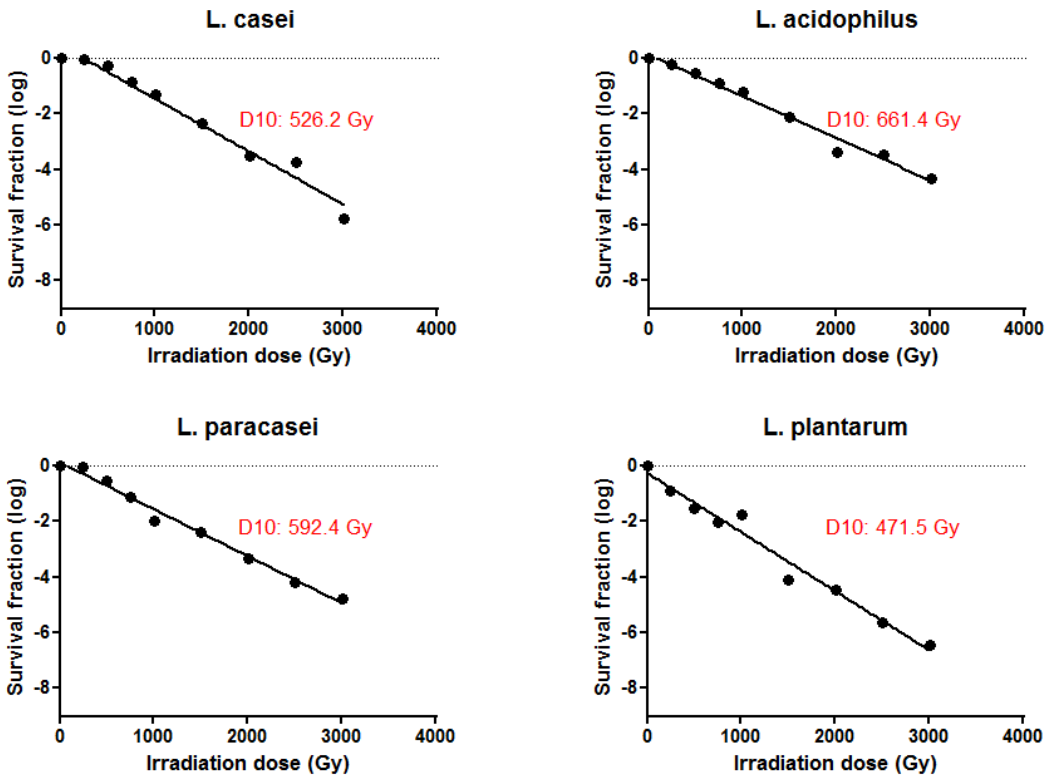
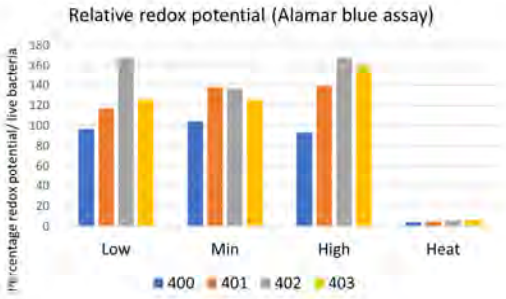
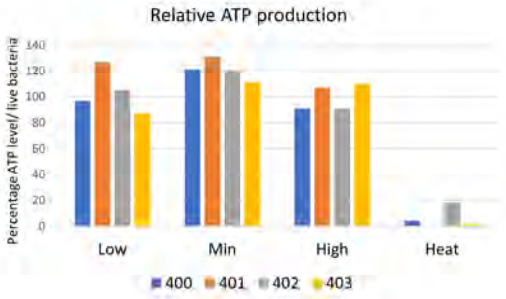
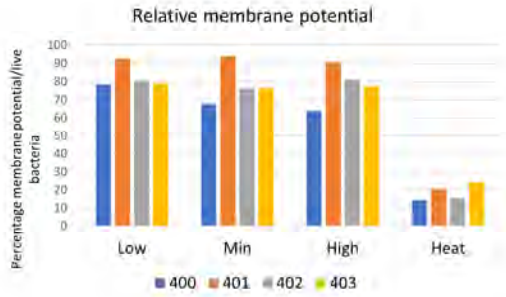


Figure 2. *Determination of D10 values for lactobacilli. L. casei, L. acidophilus, L. paracasei and L. plantarum were formulated with 50% trehalose and gamma irradiated (Co60 source) under frozen conditions. Then, they were plated in MRS agar to find the microbial cell count. D10 value, the dose of irradiation that lowers the cell count by one log is shown in red. (graph from APHL newsletter 72)*

The second experiment was conducted to determine the metabolic activity of the irradiated LAB. Irradiated bacteria were assayed for the metabolic activity after irradiation with a dose that is enough to inhibit the replication (Medium: varies for each species as above) or lower (Low; 5000 Gy) or higher (High; 10000 Gy). Three assays were applied to evaluate the metabolic activity: 1.) Resazurin (Alamar blue) based redox potential assay, 2.) ATP bioluminescence assay and 3.) Assay for membrane integrity using a fluorescent based live/dead dye. Results suggest that metabolic activity was preserved in irradiated LAB (Figure 3). Interestingly, the metabolic activity remained even higher than live bacteria in terms of redox potential and ATP production. Moreover, the metabolic activity was even preserved with higher doses of irradiation. We also included heat-inactivated LAB in this experiment and found that heat-inactivated LAB does not preserve the metabolic activity.







LAB strain		Irradiation			Heat
Strain No.	Strain name	Low	Min	High	
Lb 400	L. casei	3000 Gy	5500 Gy	10000 Gy	95°C 30 minutes
Lb 401	L. acidophilus		7000 Gy		
Lb 402	L.paracasei		6500 Gy		
Lb 403	L.plantarum		5000 Gy		

Figure 3*. Metabolic activity of irradiated LAB following irradiation. Relative metabolic activity of LAB irradiated with various doses as a percentage against nonirradiated (0 Gy) LAB is shown.*

In the third experiment, we assessed the immune modulation by irradiated LAB. In this experiment, peripheral blood mononuclear cells (PBMCs), were isolated from pig blood and they were incubated for two hours with antibiotics to remove any contaminating bacteria, washed, and then were incubated with either live, irradiated or heat-inactivated LAB for another 16 hours. Then, RNA was isolated and assayed for immune marker expression by the swine PBMCs. Our initial results suggest that irradiated LAB modulates immunity similar to live but different from heat-inactivated LAB according to heat map analysis of immune marker expressions (Figure 4). Hierarchical analysis shows the immune marker expression induced by irradiated and live LAB are closely related compared to heat inactivated LAB. Therefore, irradiated LAB could be used in place of live LAB as immune therapeutic interventions and vaccine adjuvants rather than heat inactivated LAB. This would be a safe approach to prevent AMR development and to be used in an immunocompromised host. Moreover, since these irradiated bacteria are not replicating, they would not cause an undesirable effect at the local injection site when if used as a vaccine adjuvant. Current experiments are directed expanding these studies to investigate immune modulation activities by irradiated LAB.

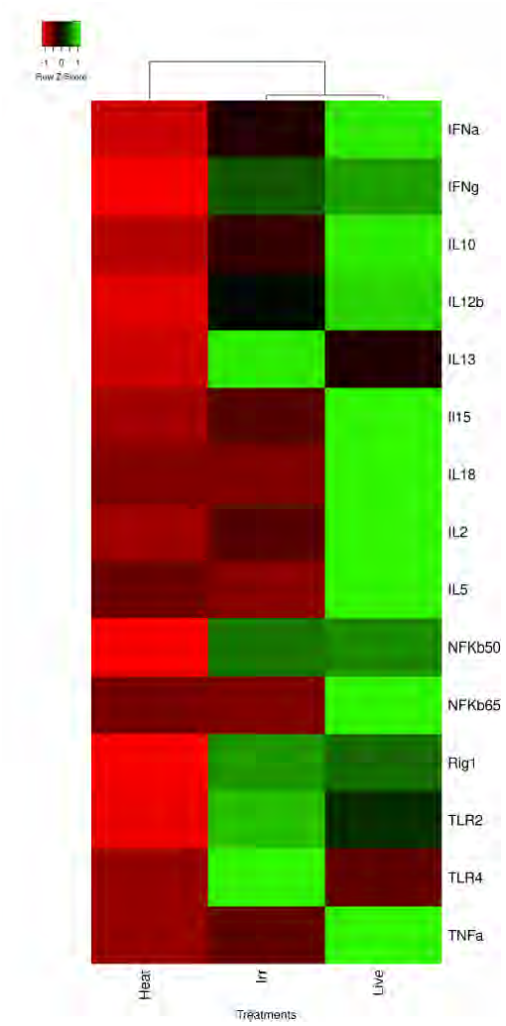


Figure 4. *Log2 fold change heatmap and cluster analysis of 15 immune markers expression by swine PBMC following incubation with either live, irradiated or heat-inactivated LAB 402 (L. paracasei). Log2 fold change calculated based on delta Ct value compared to the control samples and green implies increased expression while red implies decreased expression. Hierarchal clustering was used to generate the cluster dendrogram among three treatments. Heatmap legend: low = scaled value of 0, high = scaled value of 1. Representative diagram from two independent experiments.*

Diagnostic methods for the rapid detection of multiple pathogens

Differential diagnosis of African Swine Fever and other acute hemorrhagic diseases in pigs

In recent years, African swine fever (ASF) has emerged as one of the most significant transboundary diseases in Africa, Asia, and Europe, threatening the global pig industry. The early and accurate detection of ASF is crucial for the implementation of the control measures. Since other pathogens can cause hemorrhagic disease in pigs, the differential diagnosis tools in a single reaction tube are vital. APHL has earlier undertaken the development of a multiplex assay to detect four pathogens: African swine fever virus (ASF), classical swine fever virus (CSF), salmonella, and Erysipelothrix causing hemorrhagic diseases in pigs. To increase the assay's sensitivity for all the four targeted pathogens, APHL has undertaken additional work to update the assay. Of interest, several amplification master mixes were evaluated to develop a proper one-step RT-qPCR. In a comparative study, the Bio-Rad iTaq showed the best performance and was used to optimize the assay. The final assay displayed efficiencies between 100% and 90%, depending on the target. The Limit of Detection was 6.39, 7.28, 7.29, and 49.22 copies/reaction for Salmonella, Erysipelothrix, ASFV, and CSFV, respectively. Based on a

seventy-seven panel, the assay showed a 100% agreement with the King et al., 2003 protocol for detecting African swine fever. The assay was also transferred in the veterinary laboratories of Indonesia and Nigeria in 2020 for use for differential diagnosis during ASF suspected outbreak. The data are currently being compiled for publication.

Rapid detection and differentiation of zoonotic abortifacient infectious agents in ruminants

Abortions cause significant economic losses in livestock. Among abortifacient agents, bacteria such as *Brucella* spp, *Leptospira* spp, *Listeria monocytogenes*, and *Coxiella burnetii* are common agents affecting ruminants, presenting public health importance because of their zoonotic potential. To facilitate the early and accurate detection of abortifacient agents, a multiplex HRM assay for the detection of these four bacteria was developed and tested. This is collaborative research involving APHL and the Botswana National veterinary laboratory. To design the assay, well-established targets for monoplex PCR for detection of *Brucella* spp (IS711), *Coxiella burnetii* (IS1111), *Leptospira spp* (LiPL32) and *Listeria* spp (ssrA) were used. The primers were designed on the conserved regions using the primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/), to produce PCR amplicons of different sizes and different GC content to allow a good separation between the melting temperatures of the four targeted pathogens. In-silico simulation was performed using the uMelt software (https://www.dna.utah.edu/umelt/umelt.html) to predict the expected PCR amplicon's melting temperatures and avoid overlapping or similar Tm between the pathogens. Based on the results of the simulations, the primers were ordered and optimized. The discrimination power was tested using various samples of *Brucella* spp, *Leptospira* spp, *Listeria monocytogenes*, and *Coxiella burnetii*. The analytical specificity was assay by testing non-related pathogens. The results showed that each bacterium is detected at a specific melting temperature with a good separation between the Tm of the four pathogens: Leptospira spp (75.6-75.8), Listeria spp (77.4-77.6), Coxiella spp (80.40-80.60) and Brucella spp (83.0-83.2) (Figure 5). The assay failed to detect pathogens such campylobacter, Stapholocus aureus, Trichomonas, Pseudomona, Salmonella, pasteurella and E. Coli, confirming the specificity. Following the assay's optimization and analytical validation, the assay is undergoing field validation at the BNVL. We also transferred the assay for evaluation at the Research Center for Veterinary Science, Bogor Indonesia.

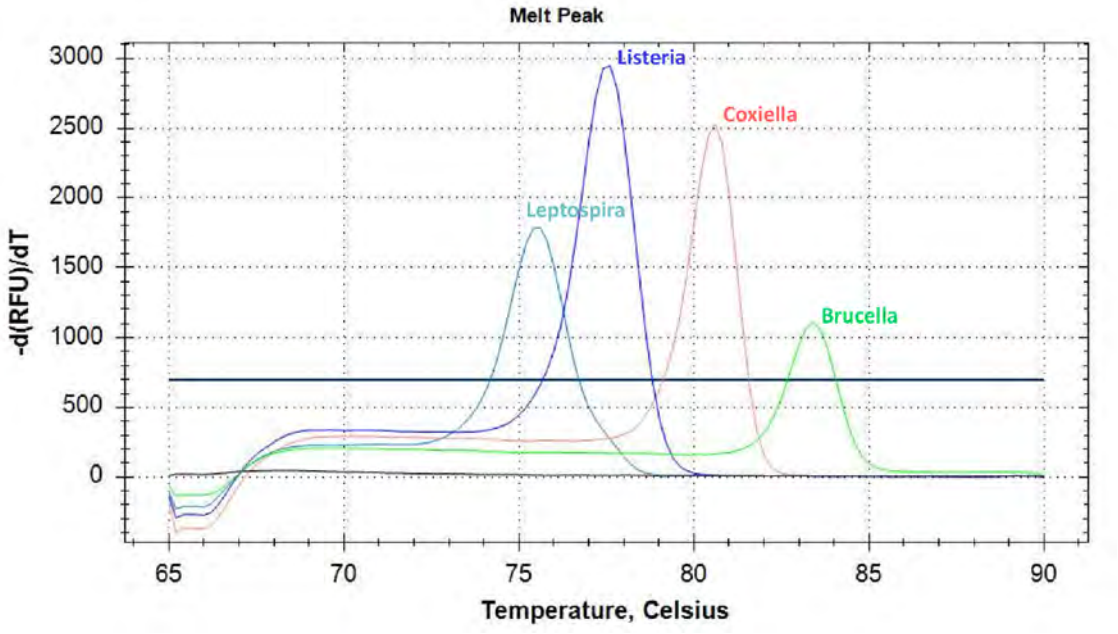


***Figure 5**. Fluorescence melting analysis of 4 bacteria including in the multiplex HRM assay for the detection of abortifacient zoonotic agents.*

Evaluation of the performances of laboratory diagnostic assays for transboundary animal and zoonotic diseases

Evaluation of real time PCR Based Detection Kits for SARS-CoV-2

Transmission mitigation of SARS-CoV-2 requires the availability of accurate and sensitive detection methods. In low resource settings, commercial kits' availability and cost can be a limiting factor to many diagnostic laboratories. In such cases, laboratories need to identify alternative and cheaper reagents. With this in mind, eight commercial qPCR ready mixes from Applied Biosystems, Bio-Rad, Biotech Rabbit, Invitrogen, Promega, Qiagen, QuantaBio, and Takara, were tested together with three ad hoc molecular diagnostic kits [GeneFinder, (Osang Healthcare); Genesig (Primerdesign); and Viroreal, (Ingenetix)]. We calculated the limit of detection for each assay using serial dilutions of a defined clinical sample. We determined the clinical sensitivity against a panel of 178 clinical samples (Figure 6) and specificity against a panel of human betacoronaviruses. Although quantitative differences in the qPCR detection were observed among the tested products, qualitative results (i.e. positive/negative) were comparable. In fact, the inter-assay agreement was assessed using statistical tests (Bland-Altman, Fleiss-Kappa, and Cohen's Kappa) and was excellent to good in all cases. We concluded that all the assays included in this study were suitable for the routine detection of SARS-CoV-2 and that the qPCR Ready Mixes are a valid alternative to ad hoc molecular diagnostic kits.

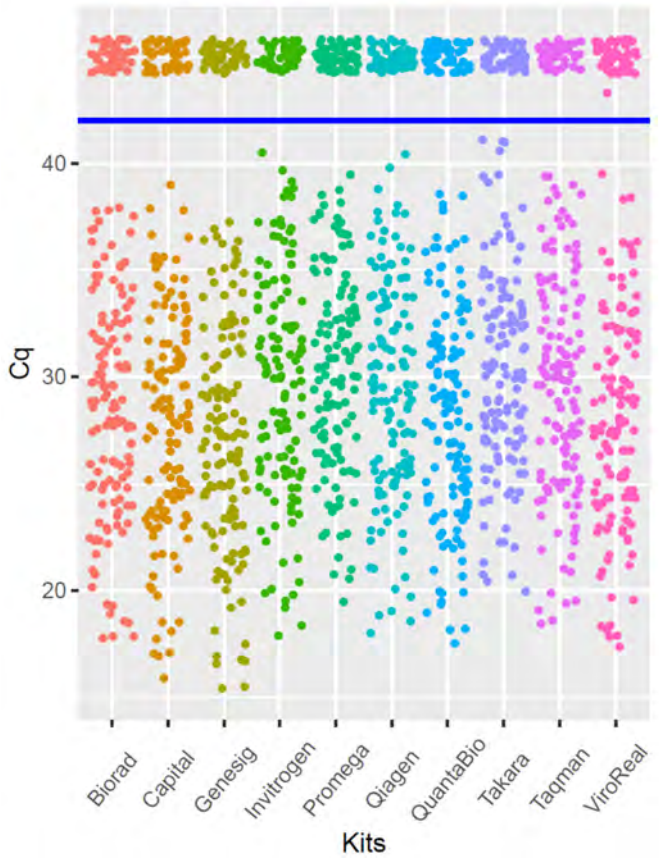


Figure 6. *Graphical visualization of the distribution of the Cq values of 178 samples across the tested kits.*

Evaluation of Commercially Available PCR-Based Detection Kits for African Swine Fever

African swine fever (ASF), a lethal viral hemorrhagic disease of pig caused by African swine fever virus (ASFV), is endemic in most sub-Saharan African countries. ASF has recently re-emerged in Europe and spread into Asia, threatening the most substantial part of the world pig industry and global food security. As the expansion of ASF increases the need for testing, there is a growing number of commercial ASFV detection kits on the market. These kits offer an excellent and stable alternative to in-house tests; however, there is a lack of information that enables the end-users to compare their performance and decide which one would fit their needs better.

APHL conducted a study to compare the commercial kits' sensitivity (Thermo#A28809 Indical#VT281905 and IDVET#IDASF-100) with the OIE recommended protocol (King et al. 2003).

All four tests detected the ASFVs DNA successfully in a panel sample, comprising ten viruses from five genotypes (I, II, IX, XVI, and XXIII). For each of the samples tested, the IDVET kit showed the lowest Cq values, and the Thermo kit had the highest ones (Figure 7). The Thermo kit allows for more amplification cycles, with the highest cut-off value (Cq < 45), as compared to the two other kits and the in-house assay (Cq < 40), therefore, it would efficiently detect ASFV in samples with low viral loads as well. The limits of detection were 10.87, 9.83, 8.28, and 13.54 copies per reaction for IDVet, Indical, Thermo, and reference qPCR assay (King et al., 2003), respectively. Commercially kits present the advantage of including an internal to assess the nucleic acid extraction step and the sample's quality.
Although the IDVET kit showed the smallest Cq values at the individual levels, there was no substantial difference in the sensitivity (the LODs) of these assays and their ability to detect the five genotypes of the panel.

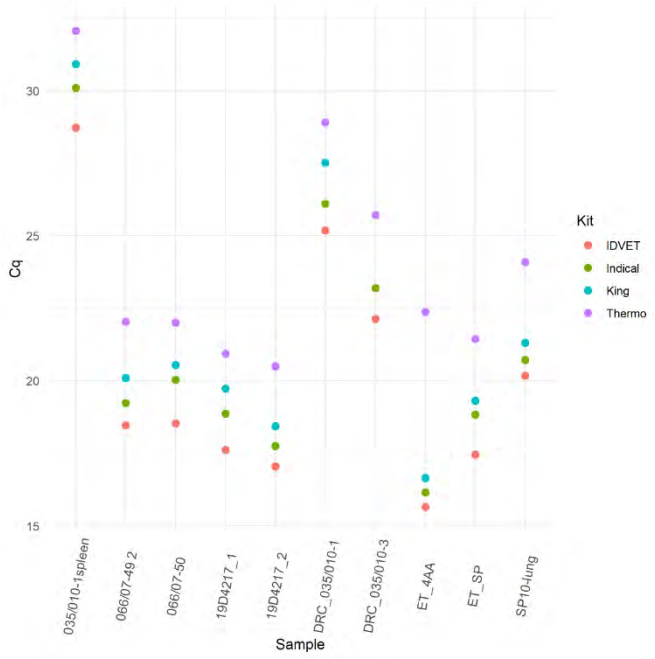


Figure 7. *Comparison of the Cq across different ASFV detection kits.*

Molecular epidemiology for outbreaks' investigations

Rabbit Hemorrhagic Disease 2 (RHD-2) epidemic in West Africa

From March 2020, several countries in West Africa experienced mortality in domestic rabbits. APHL collaborated with the OIE reference laboratory for Rabbit Hemorrhagic Disease (RHD) at the IZSLER, Brescia-Italy, to gather and share Standard Operating Procedures (SOPs). APHL also updated its capacity for support in the virus's characterization. Meanwhile, coordinated by the VETLAB Network, Senegal assisted Nigeria by sharing its updated SOPs, enabling Nigeria to detect the RHDV-2 and notify the disease to OIE on 13/10/2020. Meanwhile, Burkina Faso also requested support for the detection of suspected cases of RHDV-2. APHL assisted Senegal, Nigeria, and Burkina Faso with a more comprehensive characterization of their local isolates. APHL has successfully sequenced the full genome of 2 RHDV-2 samples from Senegal, two from Nigeria, and one from Burkina Faso, all collected during recent outbreaks in the three countries. The samples were sequenced using the Ion S5 sequencing platform. Following the assembly against a reference genome, a quick comparison of the assembled RHDV-2 genomes to publicly available sequences showed some genomic variability between the RHDV-2 from Burkina Faso, Nigeria, and Senegal thought all were more closely related to European RHDV-2 viruses collected between 2016 and 2018 in Poland, Germany, and the Netherlands.

Molecular Characterization of African Swine Fever

African swine fever (ASF) is a severe hemorrhagic disease of domestic and wild pigs caused by the African swine fever virus (ASFV). In recent years, ASF has steadily spread toward new geographical areas, reaching Europe and Asia.

In 2020, APHL supported several VETLAB partners in African and Asia with SOPs, reagents, and controls for ASF diagnosis and assisted them in characterizing the local isolates. Of interest, APHL analysed retrospectively ASFV collected in three West African countries to better understand the virus's evolution. Hence, ASFV samples collected between 1989 and 2016 in Burkina Faso, Mali, and Senegal, were analysed and compared to publicly available ASFV sequences. The C- terminal end of the p72 protein gene, the full E183L gene, and the central variable region (CVR) within the B602L gene were sequenced and analysed in eighteen samples from Burkina Faso, three from Mali, and five from Senegal. The phylogenetic analysis showed that all viruses belong to genotype I, with the ASFVs from Burkina Faso and Mali grouping with genotype Ia and Senegal genotype Ib. The CVR tetrameric tandem repeat sequences (TRS) analysis showed four TRS variants in Burkina Faso, two in Senegal, and one in Mali. The three countries did not share any common TRS, and all CVRs of this study differed from previously reported CVRs in West Africa, except for Senegal. Three of the five isolates from Senegal fully matched with the CVR, p72, and p54 sequences from ASFV IC96 collected during the 1996 ASF outbreak in Ivory Coast, suggesting the spread of the same ASFV strains across countries. This study highlights the importance of continuous monitoring of ASFV at the regional level in West Africa.

In another study, APHL assisted Indonesia to characterize their local isolates. After entering China in 2018, ASF has continued to spread through Asia, reaching Indonesia in September 2019. APHL supported the Research Center for Veterinary Science, Bogor, the VETLAB partner in the country to investigate those outbreaks in backyard pigs in the Dairi and Humbang Hasundutan districts of North Sumatra province and Bogor District, West Java province, by providing the technical assistance and reagents, but also undertaking the molecular characterization of the ASFV in Indonesia. The partial or full-length genes (i.e. p72, p54, pB602L, and CD2v) and a 356-bp fragment between the I73R and I329L genes were sequenced from representative samples.

In the NJ trees of the p72 sequences (Figure 8) and the p54 sequences, the ASFV in samples from North Sumatra and West Java clustered within genotype II, with viruses from Vietnam, Georgia, China, and Belgium. The CD2v ML tree showed that the Indonesian sequences belong to serogroup 8, with ASFVs from Georgia, Russia, Vietnam, and China. The pB602L gene showed a single tandem repeat sequence

(TRS) profile "BNDBNDBNAA" with ten copies of amino acid tetramers in Indonesian samples, similar to the CVR profiles of ASFVs from Vietnam, China, and Russia. The intergenic region between the I73R and the I329L genes was 100% identical in the ASFVs from North Sumatra and West Java, with the insertion of an additional "GGAATATATA" motif in the TRSs similar to ASFVs from domestic pigs in Vietnam, China, Russia, Belgium, Belarus, Estonia, and Ukraine.

Based on the five targets analysed, the ASFVs from North Sumatra and West Java were identical to other ASFV genotype II from domestic pigs in Vietnam, China, and Russia, confirming the transboundary nature of this infection.

Emergence of Lumpy skin disease viruses with unique genetic characteristics in Asia

Lumpy skin disease (LSD) is a contagious viral disease of cattle caused by lumpy skin disease virus (LSDV). LSD has recently spread in Asia following outbreaks in the Middle East and Europe. Between 2019 and 2020, Lumpy Skin Disease Virus was reported in several Asian countries, such as Bangladesh, India, Nepal, China, China Taipei, Bhutan, and Vietnam.

In 2020, APHL assisted VETLAB network partners laboratories in Vietnam, Bhutan, and Myanmar to detect and report LSDV, through sharing SOPs, controls, and reagents. APHL also helped Bangladesh and Vietnam with the molecular characterization of their local isolates.

LSDV emerged in Bangladesh in July 2019, in the Chattogram district, then rapidly spread throughout the entire country. Following the incursion of LSDV, the VETLAB partner in Bangladesh, the Central Veterinary Laboratory, requested the APHL support for the characterization of their local isolates, information that is crucial for a proper implementation and monitoring of vaccination programs.

APHL received and analysed samples from LSD outbreaks in 6 different Bangladesh districts (Chattogram, Dhaka, Gazipur, Narayanganj, Pabna and Satkhira). A multitarget approach was used for the molecular characterization of Bangladesh isolates, exploring the full RPO30 and GPCR genes and the partial EEV glycoprotein gene.

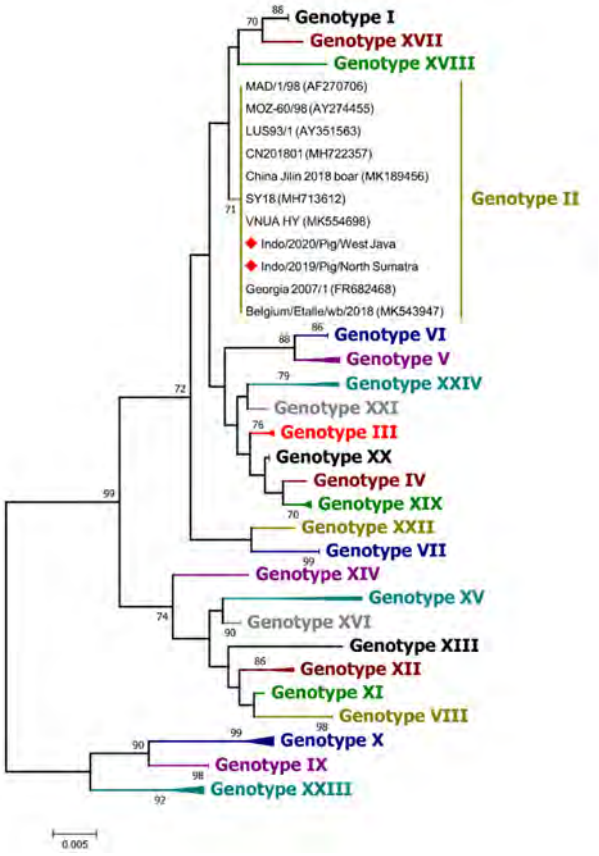


Figure 8. *Neighbour-joining tree of the partial p72 gene sequences. The tree shows the genetic relationships of the Indonesian 2019 and 2020 ASFVs with representatives of the 24 known ASFV genotypes. The evolutionary distances were computed using the Maximum Composite Likelihood method. Bootstrap values greater than 70% are shown. The isolates from this study are highlighted with red diamonds.*

The Bangladesh LSDVs showed 100% identity among each other on all the analysed targets.
On the RPO30 tree, Bangladesh isolates clustered tightly with LSDV KSGP 0240 (KX683219), Indian LSDVs, and two recombinant LSDV field isolates from Russia (LSDV Russia/Udmurtiya/2019 and LSDV Russia/Saratov/2017), segregating from commonly circulating field isolates from Africa, the Middle East, and Europe. The third subgroup of isolates consisted of LSDV Neethling derived vaccine strains, the historical field LSDV RSA/54 Haden, and two LSDV field isolates from China. On the GPCR tree (Figure 9), the Bangladesh LSDV isolates clustered together within a subgroup containing LSDV SGP_O-240 (KJ818288), LSDV NI-2490 (AF325528), LSDV Kenya (MN072619), common LSDV field isolates from Africa, the Middle East, and Europe and two LSDVs from China. A second subgroup of the GPCR consisted of LSDV Neethling derived vaccines, LSDV RSA/54 Haden (FJ869376), and three recombinant LSDVs from Russia (LSDV Russia/Udmurtiya/2019, LSDV Russia/Saratov/2017, and LSDV Dergachevskyi). The multiple sequence alignments of the GPCR gene showed that the Bangladesh LSDV contained the 12-nucleotide insertion (Figure XXX). This deletion is commonly present in LSDV vaccines (LSDV KSGP 0240 and LSDV Neethling) and a few historical field isolates (collected before 1960), in recombinant LSDVs from Russia (LSDV Russia/Udmurtiya/2019, LSDV Russia/Saratov/2017, and LSDV Dergachevskyi), and recent the LSDV isolates from China.

Alignment of the EEV glycoprotein gene sequence showed a 27-nucleotide insertion in all LSDVs from Bangladesh (Figure 9), which is characteristic of common field isolates and also present in the LSDV KSGP-0240 derived vaccines and historical LSDVs, LSDV NI2490 (1958) and LSDV Kenya (1950), both from Kenya.

Taken together, the analyses of all three targets suggest that the Bangladesh LSDVs were more related to LSDV KSGP-0240, LSDV NI-2490, and LSDV Kenya. They differed from all recent LSDV field isolates, including the LSDVs from China and the recombinant LSDVs described in Russia, and the LSDV Neethling vaccine strain. These results show the importance of continuous monitoring and characterization of circulating strains and the need to continually refine the strategies for differentiating vaccine strains from field viruses. The findings were published in the BMC Veterinary Journal (*BMC Vet Res* **17,** 61 (2021). https://doi.org/10.1186/s12917-021-02751-x). APHL has also sequenced the full genome of one virus from Bangladesh, and the results suggest the high similarity to the above-mentioned viruses from Kenya.

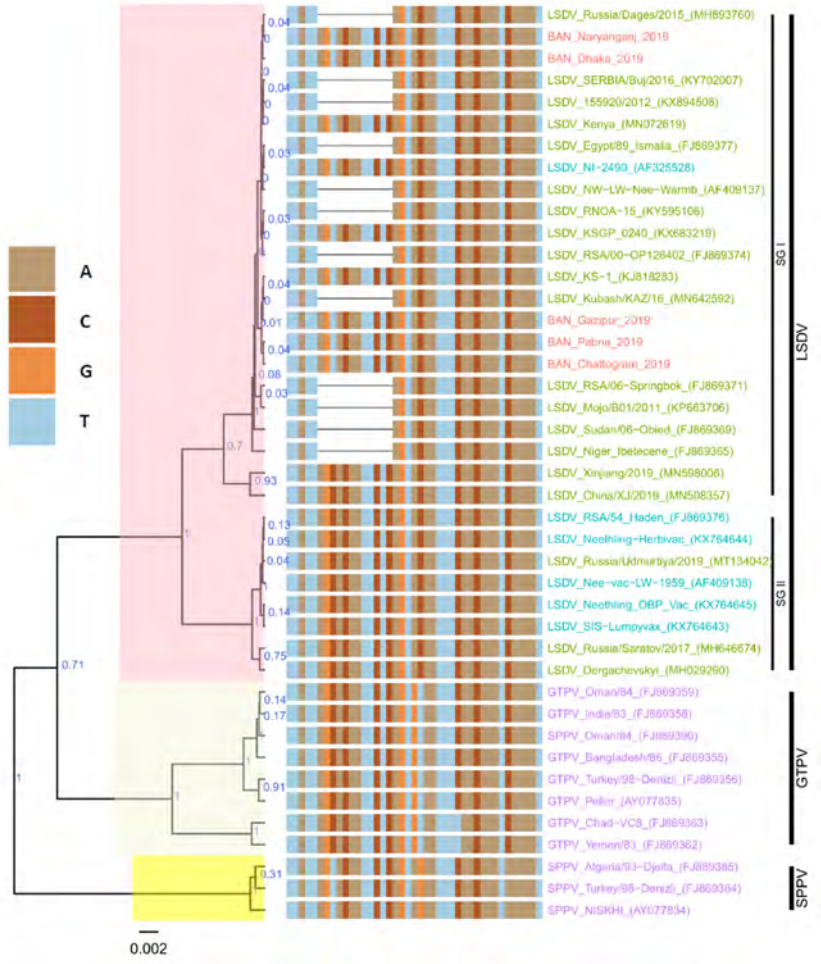


Figure 9. *Maximum clade credibility (MCC) tree based on the complete GPCR gene sequences of Capripoxviruses, plotted together with multiple sequence alignment. Only the portion of the alignment between positions 80 and 120 is shown. The posterior probabilities are plotted as respective nodes labels. LSDVs from Bangladesh are highlighted in red and reference sequences are represented with their accession numbers.*

Porcine circoviruses 2 (PCV2) identified in Namibia for the first time

Porcine circovirus 2 (PCV-2) is a swine pathogen of global importance. It is the causative agent of porcine circovirus diseases (PCVD) which are an assortment of several disease conditions including postweaning multisystemic wasting syndromes (PMWS) [now known as PCV-2-systemic disease (PCV-2-SD)], porcine respiratory disease complex, porcine dermatitis and nephropathy syndrome, and reproductive failure. PCV-2 belongs to the genus Circovirus of the family Circoviridae and is among the smallest viruses known [4]. Currently, four species of PCVs have been identified namely, PCV-1 to PCV-4, although the information available on PCV4 is still limited to a few studies. All have a similar genome organization consisting of two open reading frames (ORFs) (ORF1 and ORF2) that are orientated in opposite directions on a small circular genome ranging in size from 1758 to 2001 bp and encode a replication-associated protein and capsid protein, respectively. Currently, and using the most

recent classification methodology, eight genotypes (i.e. a to h) have been identified based on the comparison of the ORF2 gene from a wide collection of PCV-2 sequences. PCV-2a, PCV-2b, and PCV-2d are widespread and similarly virulent in pigs while the clinical significance of the remaining genotypes is unknown. The prevalence of PCV-2 in many African countries is currently unknown, with reports of molecular characterizations only available from Mozambique, South Africa and Uganda. As there is currently no information on PCV-2 in Namibia this study was undertaken to determine its presence in commercial pigs and warthogs (Phacochoerus africanus). The phylogenetic analysis revealed that the samples contained PCV-2 DNA belonging to three separate genotypes namely PCV-2b, PCV-2c and PCV-2d (Figure 10). More specifically, seven of the viruses identified in pigs belonged to genotype PCV-2b and six to genotype PCV-2d. All ten of the viruses from warthogs were genotype PCV-2c. The ORF2 sequences of the PCV-2b viruses showed the highest similarity (99.72%) to a PCV-2 identified in South Africa in 2015. They were collected from two separate farms located 10 km from each other and which regularly import pigs from South Africa. This epidemiological link indicates a potential shared origin of the viruses which should be further investigated. PCV-2d, was identified in two regions of the country, with most of the positive samples (n=5) coming from a commercial pig farm of approximately 600 animals close to the capital Windhoek. The remaining PCV-2d-positive sample was obtained from a single pig at a commercial farm of approximately 300 animals located 320 km north of Windhoek. The animal stock in both farms were all of Namibian origin and there was no evidence of contact or sharing of animals between the two farms. The comparison with worldwide available PCV-2d sequences demonstrated a close genetic similarity (>99% at the nucleotide level) with Chinese strains sampled between 2011 and 2013. However, no importation of live pigs from China has been reported. The ORF2 nucleotide sequences of the ten positive warthog samples clustered with previously identified PCV-2c genotypes from Brazil and Denmark. The detection of PCV-2c in warthogs is of particular interest, especially since it is the first time that PCV-2 of any genotype has been identified in warthog. In addition, it is the first report of PCV-2c in Africa thereby providing new information that could help in the reconstruction of the history, ecology and evolutionary paths of PCV-2 and a better understanding of the causes behind its emergence.

Molecular Characterization of Rabies Viruses from Two Western Provinces of the Democratic Republic of the Congo (2008 to 2017)

Although rabies is enzootic in the Democratic Republic of the Congo, there is very little molecular epidemiological information about the viruses circulating in animals. For this study samples were collected by both public and private veterinary services from two western provinces of the DRC namely Kongo Central and Kinshasa City between 2008 and 2017. Brain tissue was collected during necropsy performed for confirmation of rabies by direct fluorescent antibody (DFA) test and RT-PCR Central Veterinary Laboratory (CVL) of Kinshasa.

The DFA test was used according to the recommendations of the OIE Terrestrial Manual 2018.
For RT-PCR analysis, brain tissue (1g) from each DFA positive smear was homogenized in 5ml of sterile PBS, pH 7.2 using a mortar and pestle. The homogenate was clarified in a refrigerated centrifuge at 5000 x g for 10 minutes. Total RNA was then extracted from 200µl of supernatant using the QIAamp Viral RNA Mini kit (Qiagen, Germany) following the manufacturer's instructions. RT-PCR for N gene detection according to DeBenedictis et al. (2011) [Lyssavirus detection and typing using pyrosequencing. J Clin Microbiol 49:1932-1938].

Twenty-one positive DFA test samples were processed by RT-PCR generating the expected 603bp amplicons. The amplicons were sent for sequencing using standard Sanger methods at LGC genomics (Berlin, Germany). Sequences have been deposited in GenBank under accession numbers MN264691 to MN264711.

The sequences generated were highly similar to each other (99.3 to 100% identity) and clustered together with a single sequence from a rabies virus obtained from a canine brain sample collected in the Republic of Congo in 2014.

This study was confined to the western part of the DRC. Therefore, little is still known about the distribution of RABV in the rest of the country. Gathering representative samples from various provinces throughout the country could result in a more complete understanding of the RABVs circulating in the DRC.

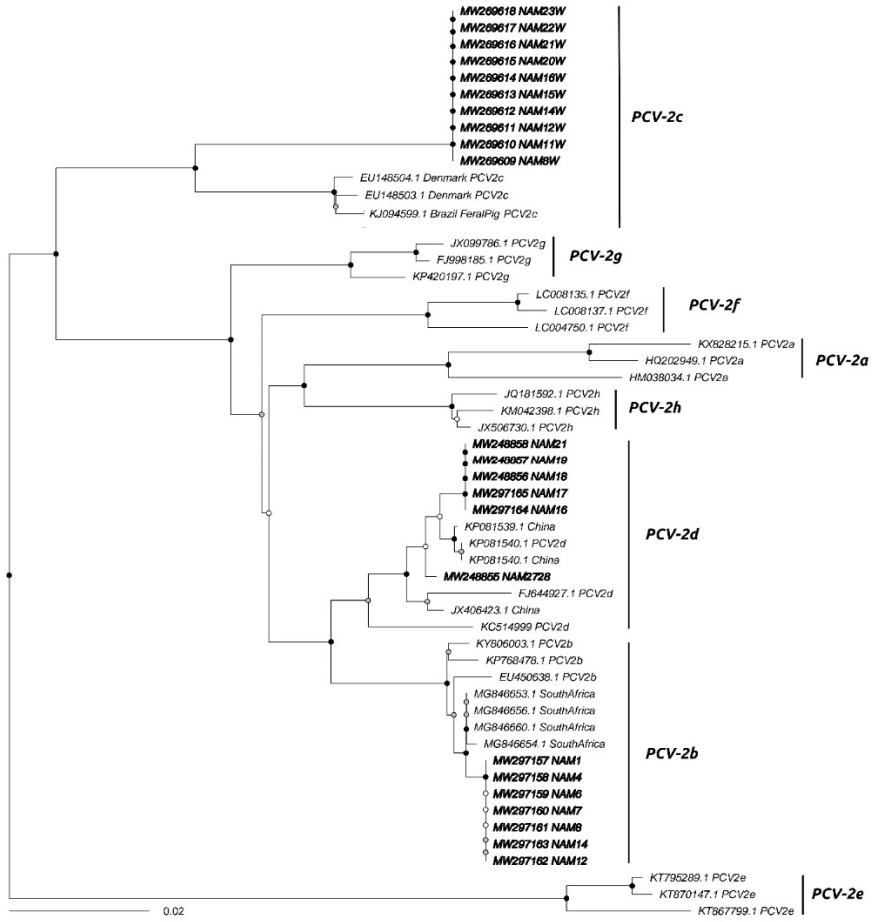


Figure 10. *Neighbour-joining phylogenetic tree employing the p-distance model of nucleotide substitution and 1000 bootstrap replications of the ORF2 gene sequence from PCV2s sampled in Namibia combined with similar sequences available in GenBank. The sequences from this study are shown in bold.*

Full Gene Sequencing of Rift Valley Fever Virus from Botswana

Rift Valley Fever (RVF) is a mosquito-borne zoonotic viral disease caused by the RVF virus (RVFV). RVFV is an arbovirus in the genus Phlebovirus within the family Bunyaviridae. RVFV possesses a single-stranded, segmented RNA comprising a large (L), medium (M), and small (S) segments.

The virus affects both wild and domestic ruminants, especially sheep, cattle, and goats, and humans. In susceptible animals, RVFV infection induces high fever, abortion storms, and high mortality in new-born animals.

The disease is widespread in sub-Saharan Africa, with outbreaks also reported in Egypt and the Arabian Peninsula. In Botswana reported the first case of RVFV in livestock in 2010, with subsequent outbreaks

in 2013 and 2017. To better understand the diversity of RVFV involved in outbreaks in Botswana, the APHL, in collaboration with the VETLAB partner in Botswana, the Botswana National Veterinary Laboratory, is undertaking the molecular characterization of the isolates recovered from outbreaks in between 2013 and 2017 in Botswana.

Out of fourteen RVFV positive samples, three segments, of a 2018 isolate from goat, were amplified and sequenced using the Ion S5 sequencer. The sequences were assembled against a reference genome using bowtie 2.

The genome was compared to existing RVFV using sequence similarity searches and multiple sequence alignment. In addition, a maximum likelihood tree was constructed using MEGA 7. The results showed that Botswana RVFV virus is closely related to RVFVs recovered from sheep and cattle in South Africa between 2009 and 2018.

The phylogenetic reconstructions showed that the Botswana virus belongs to viral lineage E based on all the three segments. Further analysis and sequencing using additional samples are ongoing to assess the diversity of RVF viruses in Botswana.

MAJOR ACHIEVEMENTS IN RESEARCH AND DEVELOPMENT

Animal Genetics

Development of a multi-species array for breeding and improvement of camelids

Camel is one of the most popular domestic species in regions experiencing harsh climatic conditions. Camels are raised for milk, meat, fibre (wool and hair), transport and work. There is growing recognition of the value and benefits of camel products that provide a rich source of income to nomadic herders in Asia and Africa. During 2020, Animal Production and Health Laboratory (APHL) in collaboration with Veterinary Medical University (Austria) and International Camel Genome Consortium, developed a multi-species camelid DNA chip for selection and breeding of high producing camel and increase productivity. Whole genome sequences from nine dromedary (C. dromedaries) and seven Bactrian (C. bactrianus) (NCBI BioProject PRJNA276064) were utilized to identify at least 60000 single nucleotide polymorphisms (SNPs) in each of these old-world camelid species. Similarly, 56 whole genome sequences from Llama, Alpaca, Guanco and Vicugna (14 from each species provided by Pablo Orozco-Terwengel, School of Biosciences, Cardiff University, United Kingdom) were utilized to identify ~60000 SNPs in new world camelid species. The finalized multi-species camelid array (Figure 11) was developed on Affymetrix-Axiom platform and consisted of ~200K SNPs with at least >60K from each of dromedary, Bactrian and new world camelid species. The process of fabrication of custom camelid array was completed and delivered to APHL, Seibersdorf for technical validation and field testing.

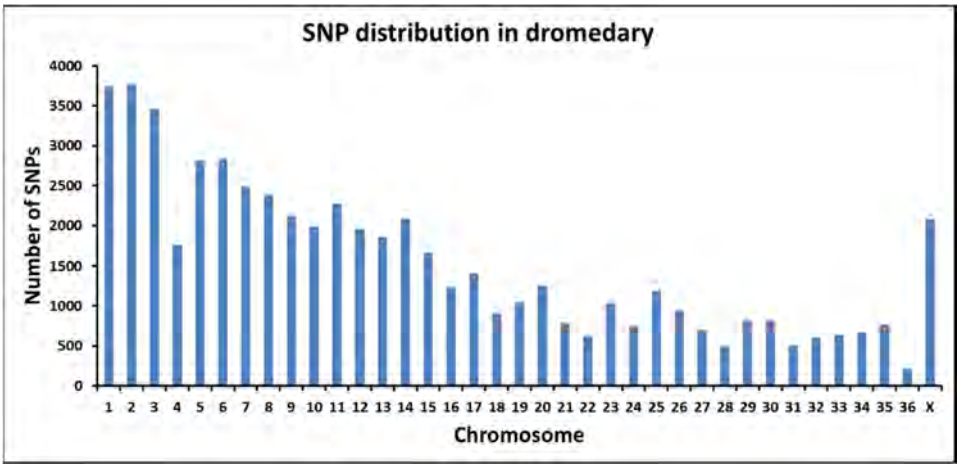


Figure 11. *Chromosome-wise distribution of dromedary SNPs in the multi-species camelid array.*

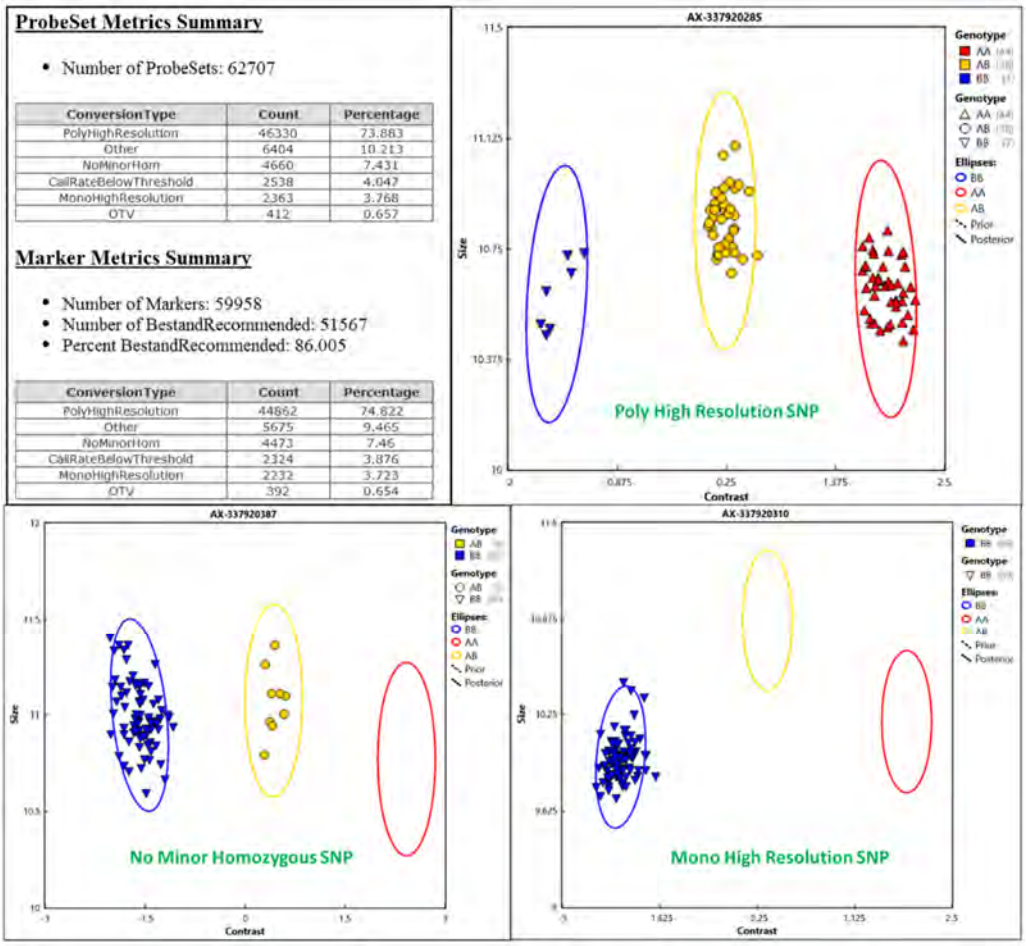


Figure 12. *Probe and marker metric summary with sample SNP cluster plots (Poly High Resolution, No Minor Homozygous & Mono High Resolution) for dromedary camel.*

As a first step of validation, the array was tested on a panel of 96 dromedary camel samples to generate library files that can convert signal data into genotypes. The process of validation of 60K dromedary SNP panel was successful with extraction of genotypes at ~86% loci under highly stringent thresholds of quality control parameters (DQC> 0.82, SNP QC call rate >97%, average call rate for passing samples ≥ 98.5 and percent passing samples ≥ 95). The array consisted of 62707 probe sets covering 59958 genome wide SNP markers specific to dromedary camel. Of these, a total of ~51500 markers were successfully genotyped that included ~44860 PolyHigh Resolution (presence of both homozygotes

and heterozygotes), 4660 NoMinor Homozygotes (absence of minor allele homozygotes) and ~2360 MonoHigh Resolution (monomorphic) SNPs (Figure 12). The successful validation of 60K dromedary SNPs has now enabled genetic and genome wide evaluation of dromedary populations. The process of validation and field testing is being continued for other camelid species (Bactrian camel, Alpaca, Llama) and the array will be rolled out in 2021 for the use of camel breeders across Africa, Asia and Latin America. The array is first of its kind for camelids and will be an important genomic tool to aid breeding and improvement of these important livestock species for increased productivity.

PCR based phenotyping of Trypanosomosis in dromedary camel

Camel is relatively less susceptible to many devastating diseases that affect livestock species, such as rinderpest, contagious pleuropeumonia and foot and mouth disease. However, Trypanosomosis, predominantly caused by *Trypanosoma evansi* is a major threat to dromedary camels. The protozoan pathogen is transmitted mechanically by biting and sucking insects although vertical, horizontal, iatrogenic, or per-oral transmission are also possible depending on the host and the geographical area. There has been anecdotal evidence on differences in susceptibility to infection among dromedary populations located in different regions, possibly having an inherent genetic basis for disease resistance. During 2020, APHL initiated a study on genetics of resistance to Trypanosoma infection in dromedary camel and to evaluate genome-wide association with relevant phenotypes. Several phenotypes (PCR based detection of Trypanosoma species, clinical signs, packed cell volume, red blood cell count, differential count of white blood cells and immunoglobulins) were identified to assess the susceptibility, resistance and/or tolerance to disease.

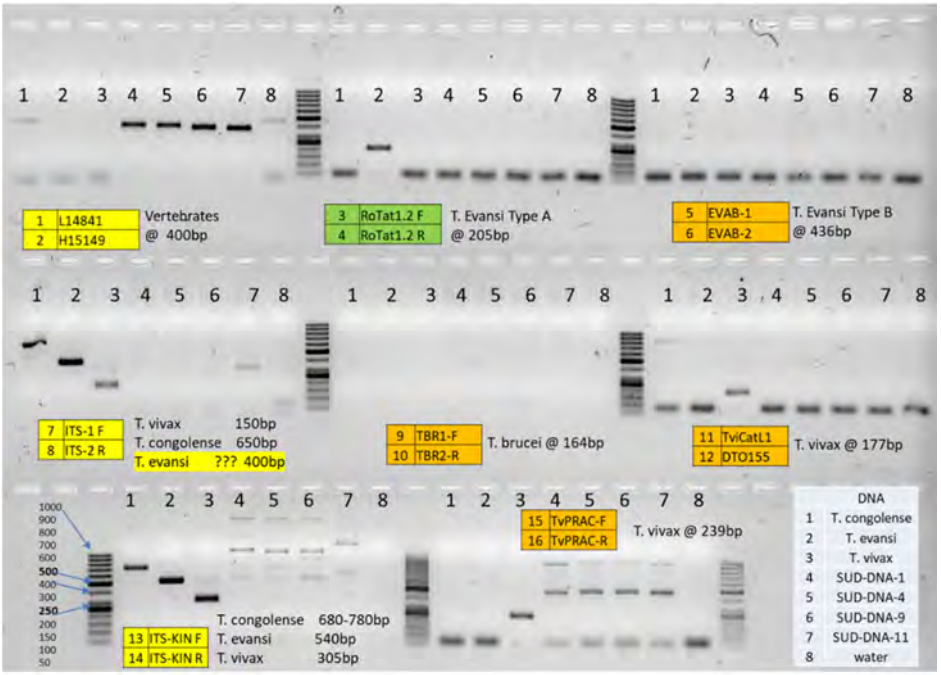


Figure 13. *Optimization of PCR for detection and differentiation of Tryps infections in dromedary camel.*

As a first step, the process of PCR (polymerase chain reaction) based phenotyping of Trypanosoma infection in dromedary camel was initiated. A battery of PCR primers that are conventionally used for molecular epidemiological screening were tested to optimize a panel of markers for detection and differentiation of Trypanosoma species infecting dromedary camel (Figure 13). Three major species of Trypanosomes were targeted that included *T. evansi*, *T.vivax* and *T.congolense*. PCR markers were identified for (i) first level screening to detect Trypanosoma infection (ii) differentiating T. evansi subtypes (iii) confirmatory diagnosis of T. vivax and T. congolense. Further optimization is currently underway following the initial results obtained.

Genome-wide evaluation of cattle to estimate genetic admixture and association with milk production traits

Improvement of cattle for increased milk production occurs mainly through selective or cross breeding schemes. Genome wide evaluation using SNP microarray can help in improving the efficiency of both the breeding schemes. The Animal Production and Health Laboratory (APHL) provided technical support to Argentina, Bangladesh, Peru, Serbia, Sri Lanka and Uruguay in performing genome wide evaluation of their respective local cattle. Axiom bovine (BovMDv3) array consisting of 63648 markers was used to genotype more than 1900 samples from these six countries (~180 from Argentina, ~370 from Bangladesh, ~290 from Sri Lanka, ~570 from Peru and ~360 from Serbia). The purpose of genomic evaluation was to (i) perform genome wide association of genotypes with milk production traits (Serbia) (ii) estimate genetic admixture and assess the level of taurine inheritance in crossbred cattle (Bangladesh, Sri Lanka) (iii) identify selection signatures related to high altitude adaptation (Peru) and assess genetic biodiversity in local cattle (Argentina and Uruguay). Upstream analysis of genome-wide data has been completed to generate data on SNP genotypes for each marker loci, chromosome number, strand, dbSNP ID, flanking region sequence, reference allele, associated gene or genomic region, etc. Further downstream bioinformatics analysis of genotype dataset is currently underway.

Development of baseline information and genetic evaluation of West African cattle

Advanced nuclear and genomic technologies play an important role in improving efficiency of dairy cattle improvement programs for increased milk productivity. Genomic tools can help estimate levels of genetic admixture in crossbred cattle, verification of genetic purity in purebred cattle and match performance data with appropriate genetics to select superior stocks for breeding. Under CRP D3.10.28, APHL embarked on developing baseline genetic information and evaluation of African cattle using classical and genome-wide DNA markers. More than 700 cattle from 17 breeds across West Africa (Benin, Burkina Faso, Mali and Niger) were genotyped and evaluated. The history of cattle breeding in West Africa involved the colonization of African taurine cattle followed by a wave of zebu introgression with varying levels of admixture in native cattle breeds. Hence, the investigated breeds included West African taurine, West African Zebu, their crossbreds, Asian Zebu and European taurine cattle types.

The results, illustrated in Figure 14, showed 5.4% of total genetic variation in West African cattle was due to between breed differences. Dynamic changes in demography and breed ancestry were observed in West African cattle with significant zebu introgression in Kouri cattle, a traditional West African taurine breed from Niger. Similarly, zebu admixture was significantly higher in Bourgou cattle from Benin and Gourounssi breed from Burkina Faso. Genetic purity was better maintained in Lagunaire and Lobi cattle (taurine) and Bororo Maaaoua (zebu) cattle. These findings point to the need of applying appropriate breeding strategies, specific to the requirements of different regions for improving efficiency and productivity.

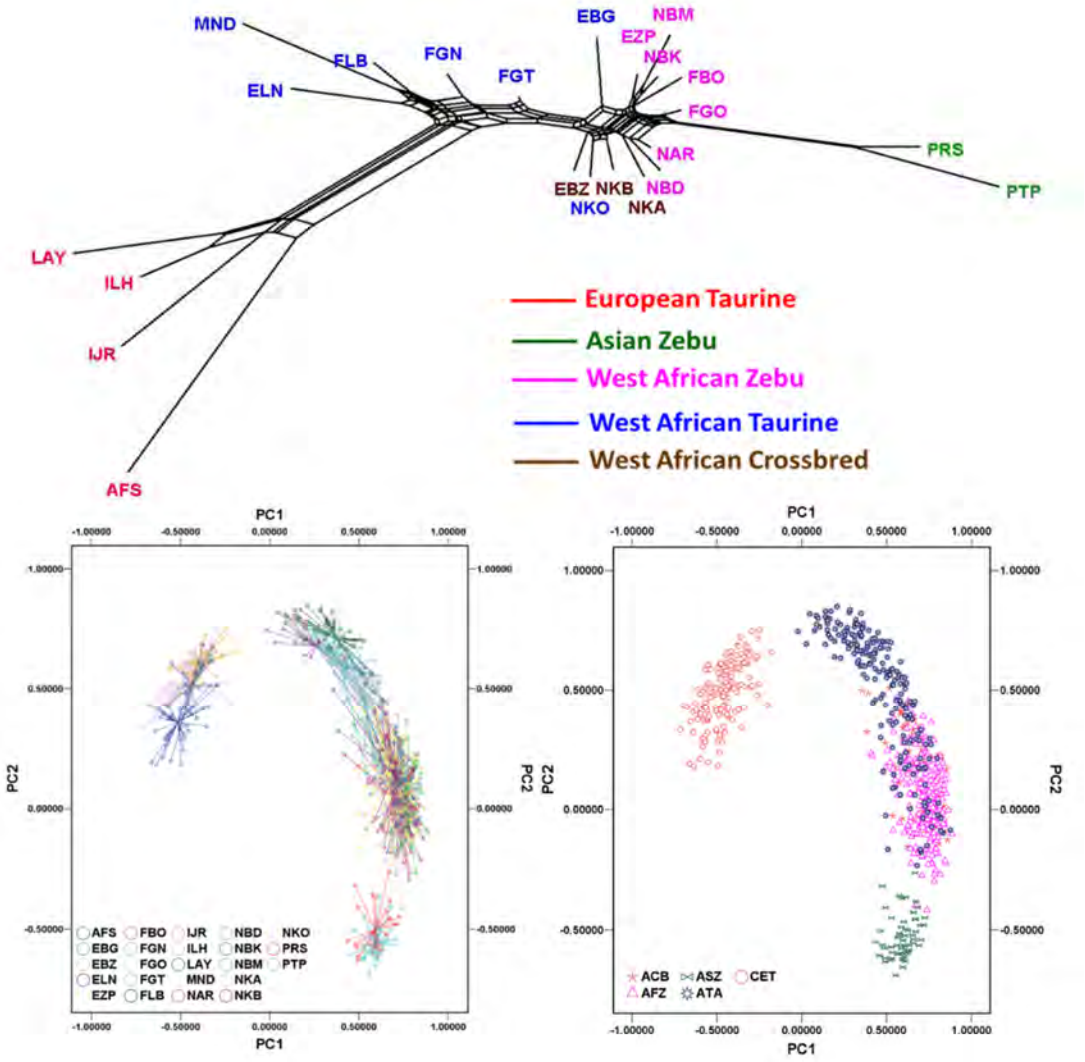


Figure 14. *NeigborNet showing relationship among West African cattle (top) and Principal components analysis based genetic structure among West African zebu, taurine and crossbred cattle (a) Breed-wise (Bottom left) (b) Species-wise (Bottom Right).*

Implementing Global Plan of Action for Animal Genetic Resources (AnGR)

In continuation of Joint FAO/IAEA efforts towards implementing Global Action Plan on animal genetic resources (AnGR), APHL supported member states in at least two major strategic priority areas: breed characterization and capacity building.

Genetic characterization of Cambodian native cattle breeds

Livestock in Cambodia, including cattle, supports 1.4 million smallholder farmers and accounts for 20.9% of agricultural gross domestic product. Rearing of cattle is largely dominated by smallholders and is predominantly kept for draught power in agriculture activity with recent interests focusing on market-oriented beef production. Cambodian cattle are predominantly zebu type (*Bos indicus*), consisting mainly of indigenous breed called Gor Srok or Gor Khmer or Gor Kdarm. Based on morphology and coat colour, two kinds of Cambodian native cattle are widely distributed: Gor Kdarm Red and Gor Kdarm White. To improve beef production, native cattle are subjected to crossbreeding with imported breeds like Brahman and Hariana. This resulted in varying levels of genetic admixtures and dilution of indigenous germplasm. Improving native cattle breeds through genetic selection and artificial insemination would ensure native breeds retain their adaptability to local environment and tolerance to local diseases. The International Atomic Energy Agency, through its Technical Cooperation

programme (KAM5003: Supporting Sustainable Livestock Production) collaborated with the Animal Production and Research Institute of Cambodia to launch a national artificial insemination and genetic evaluation program for improvement of Cambodian cattle. APHL provided technical support to generate baseline genetic information and evaluate population structure, admixture and genetic relationships among Cambodian native and imported cattle breeds. The results (Figure 15) showed high levels of genetic variability in Cambodian native cattle as compared to zebu cattle breeds located near the centre of domestication. Genetic structure analysis revealed significant introgression of Brahman into native Gor Kdarm White cattle while limited genetic admixture levels was observed in Gor Kdarm Red cattle. The findings will play an important role in formulating effective strategies for breeding and improvement of Cambodian cattle.

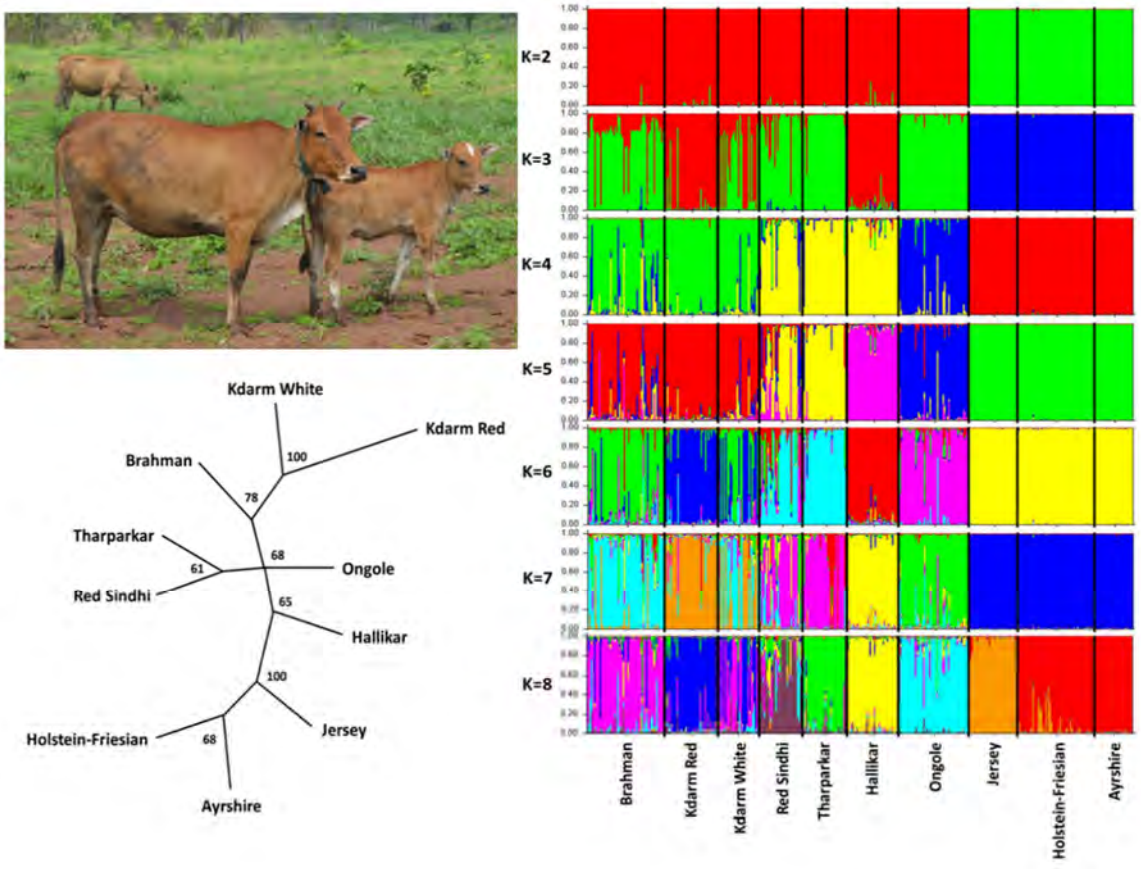


Figure 15. *Gor Kdarm Red cattle of Cambodia (top left); genetic relationship (bottom left), population structure and genetic admixture (right) in Cambodian native cattle breeds.*

Neutral genetic variability and population structure in Eastern European cattle

Under the FAO technical cooperation programme RER3604 'Conservation of dual-purpose cattle in Eastern Europe in Armenia, Georgia and Ukraine', APHL provided technical support and services to implement 'Genomic analysis of Caucasian and Carpathian Brown cattle'. In continuation of this, APHL conducted assessment of neutral genetic variability in Caucasian Brown, Carpathian Brown and Bulgarian cattle breeds. A total of 182 cattle from six breeds (Bulgarian rhodope, Rhodope shorthorn (Bulgaria), Bulgarian Brown (Bulgaria), Caucasian Brown (Armenia), Caucasian Brown (Georgia) and Carpathian Brown (Ukraine) cattle) were genotyped and evaluated. All the six breeds were compared with commercial European taurine cattle that included Jersey, Holsteins, Fleckvieh-Simmental and Ayrshire cattle (Figure 16). The Bulgarian native cattle were closely related to Jersey and Fleckvieh-Simmental breeds as compared to Caucasian Brown and Carpathian Brown cattle. Significant gene-flow was observed among the six Eastern European cattle breeds. The results on evaluation of neutral genetic variability will be utilized to further improve the efficiency of conservation and genetic improvement programs targeted for development of cattle in Armenia, Georgia, Ukraine and Bulgaria.

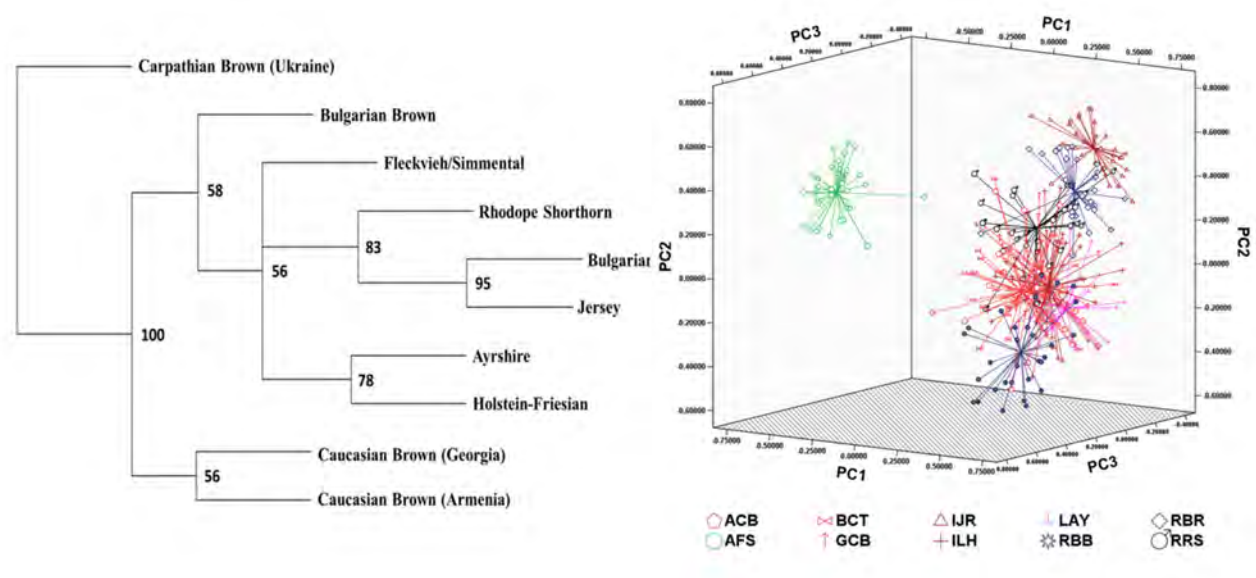


Figure 16. *Phylogeny and genetic relationships among East European cattle breeds (Right: Neighbour-Joining tree derived from pairwise Cavalli-Sforza and Edwards chord distance; Left: principal components based genetic structure analysis).*

Evaluation of mutation drift equilibrium to detect cryptic genetic bottleneck in indigenous West African taurine and zebu cattle populations

West African cattle display a unique genetic feature of adaptation to the prevailing climate and livestock production system in the region. Several factors are threatening West African cattle breeds, particularly declining population size, unsupervised crossbreeding and the prevailing harsh environmental conditions. The decline in effective population size can affect within breed genetic variability and could lead to loss of many rare alleles. Reduced genetic diversity and increased inbreeding are bound to affect the viability of small populations due to their inability to withstand extreme selective pressures. Therefore, an investigation was carried out to assess West African cattle breeds for the risk of recent genetic bottleneck using multi-locus short tandem repeat markers. A total of 453 samples belonging to seven taurine and seven zebu cattle breeds located across four West African countries (Benin, Burkina Faso, Niger and Mali) were investigated. Among all the investigated breeds, at least three (Arabe Pur, Kouri Pur, Gourounssi Nahaouri) showed statistically significant ($P<0.05$) heterozygosity excess when tested with at least one of the three statistical methods under the assumption of infinite alleles model of microsatellite mutation. However, such a heterozygosity excess was not observed in these populations when assumed under stepwise or two-phase mutation models. The qualitative graphical test for distorted distribution of allele frequencies revealed all West African cattle populations except N'dama showing a normal L shaped distribution. The study thus revealed no concrete evidence for the occurrence of a recent genetic bottleneck in West African taurine and zebu cattle populations.

PUBLICATIONS

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CAPACITY BUILDING

Strengthening laboratory infrastructure

APHL continued its efforts to improve the laboratory capacity of member states and enable implementation of advanced DNA based technologies for efficient management of locally available animal genetic resources. Institutional and technical support were provided to at least six countries (Cameroon, Eritrea, Indonesia, Nigeria, Burkina Faso and Mongolia) for establishing/strengthening molecular genetic laboratories through provision of necessary equipment and laboratory supplies under the framework of national and regional technical cooperation projects. APHL provided technical support in setting up a new sequencing/genotyping facility at Veterinary Services Division, Asmara, Eritrea.

Expert missions

The VETLAB laboratory in Indonesia (Indonesian Research Center for Veterinary Science - IRCVS, Bogor) was visited by one APHL expert (10-14 February 2020). The visits aimed at reviewing the setup of molecular biology laboratory, transfer and troubleshoot the qPCR and multiplex assay technology for detection of transboundary animal diseases. Eight participants from IRCVS were trained on handling multiplex assays on two different real time PCR platforms. Participants were trained on multiplex assays for detection of hemorrhagic diseases of swine, respiratory diseases in small ruminants and abortifacient pathogens in cattle. The participants were able to simultaneously detect African swine fever virus and salmonella in ASF suspected samples. Installation of new real time PCR machine and troubleshooting of existing qPCR was illustrated to the technical staff. Workflow for characterization of African swine fever viruses from amplification to sequencing data analysis to phylogenetic analysis was demonstrated.

Fellowship and internship training

In 2020, due to safety and health regulation related to the COVID-19 pandemic, APHL hosting capacity was limited to 1 fellow and 2 interns.

Name	Country	Status	Duration	Topic
Marcela Mora	Peru	Fellow	1 month	Genomic evaluation of Peruvian cattle using DNA microarray technology
Dingrong Xue	China	Intern	6 months	Molecular epidemiology of transboundary animal and zoonotic diseases
Hanifati Subki	Indonesia	Intern	12 months	Implementation of the iVetNet platform

VETLAB NETWORK

The Veterinary Diagnostic Laboratory (VETLAB) Network, coordinated by the Animal Production and Health Section (APH) and supported through IAEA and FAO programmatic activities as well as by South Africa through the African Renaissance Fund (ARF) and by the USA and Japan Peaceful Uses Initiative (PUI), consists of national veterinary diagnostic laboratories located in 46 African and 19 Asia

and Pacific Member States. In 2020, the VETLAB Network has been instrumental to technically supporting partner laboratories in countries affected by COVID-19 pandemic. In some of these countries, VETLAB laboratories are supporting medical laboratories for SARS-CoV2 RTPCR testing. Through the network, emergency support was delivered to the national veterinary laboratories appointed by the respective national health authorities to conduct COVID-19 testing. To date, 42 national veterinary laboratories in three continents were directly supported. The emergency packages delivered to these laboratories contained all needed reagents, reference material, consumables as well as major equipment to safely run recommended diagnostic tests based on RT-PCR, including biosafety cabinets and real time PCR platforms. Support was also provided to MS laboratories for the detailed and rapid characterization of SARS-CoV2 isolates by genetic sequencing. Furthermore, the VETLAB Network has provided strong support to partner laboratories in Asia facing Lumpy Skin Disease, Avian Influenza and African Swine Fever (ASF) epidemics to strengthen their diagnostic capacity, preparedness and rapid response actions. Efforts concentrated on procuring reference material such as positive controls, equipment and reagents for the rapid implementation and expansion of early diagnostic and confirmatory tests.

Although delayed due to restrictions and limitation imposed by the COVID-19 epidemic, the Network has organized the yearly interlaboratory trial for the serological and molecular detection of Peste des Petites Ruminants (PPR) virus. Thirty-nine laboratories in 34 countries confirmed their participation in the 2020 ring trialthat will be concluded in early 2021. Countries at-risk for PPR virus introduction were also supported for their laboratory preparedness plan.

For the same restrictions and limitations, the VETLAB Directors meeting and VETLAB laboratory training courses could not take place in 2020. APH is issuing on a regular basis the VETLAB Network Bulletin in the hope of providing a forum for participating laboratories and other stakeholders to communicate and exchange knowledge/information, to showcase achievements and to share expertise within the VETLAB Network.

THE FOOD AND ENVIRONMENTAL PROTECTION LABORATORY

EXECUTIVE SUMMARY

The Food and Environmental Protection Laboratory (FEPL) of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture provides assistance to Member States in implementing food control systems to ensure the safety and quality of the food supply, safeguarding consumer health and helping to facilitate international trade. Technical support is provided for food provenance and authenticity determination and for the control of residues and contaminants in food. This support underpins food safety and control systems and helps to safeguard human health and to combat economic loss through food fraud - the illegal production and marketing of counterfeit and adulterated products. Activities include applied research and the development, validation, and transfer of nuclear and related methods for testing foods. The application of these technologies and methods in Member States is supported by the development and provision of technical protocols, advice and guidance, training, and contributions to the development of international standards.

Research and development outputs were maintained in 2020 despite disruption caused by the COVID-19 pandemic. Method development encompassed both rapid screening methods and more sophisticated techniques, in order to provide Member States with the options needed for their food control systems. A major development in 2020 was the transition of FEPL to new premises in the Yukiya Amano Laboratories. Although laboratory outputs were, inevitably, impacted during the year by the transition as well as the COVID-19 pandemic, the capability of FEPL to assist Member States in the future has been greatly enhanced by the improved facilities.

Research and development work in 2020 included the investigation of analytical methodology for the authentication of foods labelled as organic, a major area of food fraud. Methods were developed using Fourier-transform infrared spectroscopy and metabolomics by high-resolution mass spectrometry, with associated chemometrics, to discriminate between organic and conventionally produced orange juices. A home-based experiment using a near-infrared portable sensor was performed during COVID-19 lockdown, demonstrating its potential for use outside the laboratory to differentiate between organic and conventional tomatoes. The capabilities of FEPL were enhanced by the installation of a new bench-top nuclear magnetic resonance (NMR) spectrometer, which has many potential applications for food authenticity screening. Proof of principle was demonstrated for the differentiation of Arabica and Robusta coffee beans by NMR. A new method was developed for the determination of the geographical origin of rice by microwave assisted hydrolysis of starch to monosaccharides and analysis of the non-exchangeable hydrogen isotopes. The control of agrochemical residues and natural contaminants in food remains a key concern for Member States. Response to Member States needs in this field included the development and validation of a multi-residue method covering 85 pesticides in orange juice by gas- and liquid chromatography-mass spectrometry.

The FEPL coordinated and provided technical input to two coordinated research projects on food authenticity, involving approximately thirty countries.

Personnel from FEPL presented at four virtual international conferences, webinars or podcasts, and the FEPL was represented in the scientific committees for two major international conferences on food safety and authenticity and an advisory panel for a food industry forum dealing with food system resilience issues resulting from the COVID-19 pandemic. The FEPL contributed to international efforts to develop food fraud controls through participation in the European Commission's CEN Technical Committee (460) 'Food Authenticity' Working Group 6, 'Stable isotope Analysis' and as a member of the UK's Food Authenticity Methodology Working Group.

The FEPL provided capacity building for Member States through technical management of twelve national and three regional technical cooperation projects in 2020. Although many planned

technology and knowledge transfer activities were disrupted due to the COVID-19 pandemic, human resource capability was enhanced in Member States through the training of more than 700 scientists, analytical chemists and laboratory personnel by means of virtual training workshops, the development of distance learning courses, and webinars implemented via networks such as the Red Analítica de Latinoamérica y el Caribe (RALACA). The FEPL hosted one fellow, one cost-free expert, one intern and two PhD consultants.

The project, 'Enhancing Capacity in Member States for Rapid Response to Food Safety Incidents and Emergencies', funded by the government of Japan under the 'peaceful Uses Initiative', continued in 2020 with method development and training activities.

Fifteen papers with FEPL staff as co-authors were published in peer-reviewed scientific journals in 2020.

STAFF

Name	Title
Cannavan, Andrew	Laboratory Head
Kelly, Simon	Food Safety Specialist
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Nakaya, Shuichi	Food Safety Specialist
Mihailova, Alina	Analytical Chemist
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Abrahim, Aiman	Laboratory Technician
Maxwell, Florence	Laboratory Technician
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Mletzko, Joanna	Team Assistant
Permetov, Serik	Laboratory Attendant
Liang, Ying	Cost-free Expert
Rezende, Sofia	PhD Consultant (food contaminants)
Migues, Ignacio	PhD Consultant (food authenticity)

MAJOR ACHIEVEMENTS IN RESEARCH AND DEVELOPMENT

The IAEA, including the Joint FAO/IAEA Centre for Nuclear Techniques in Food and Agriculture, helps its Member States to build capacity for food safety control through the technical cooperation programme and with upstream adaptive research at Seibersdorf and through coordinated research projects. Further to those mainstream activities, there has been a recent focus on enhancing resilience in Member States and improving their abilities to respond to crises such as the COVID-19 pandemic that affect the food supply chain, human resource availability and the capacities of food control laboratories to implement regulatory testing. The pandemic has highlighted the importance of the project 'Enhancing Capacity in Member States for Rapid Response to Food Safety Incidents and Emergencies', which is funded by the government of Japan under the 'Peaceful Uses Initiative' mechanism and implemented by the Joint FAO/IAEA Centre's Food and Environmental Protection Laboratory (FEPL). This project enables applied research in FEPL to develop analytical methodology for the detection of food contaminants and adulterants and to verify the geographical origin of foods. The capacity to effectively respond to incidents and situations compromising food safety is developed in Member States through enhancement of their knowledge and understanding of the potential underlying food safety problems and the tools available to control the problems. The application of analytical methods, that provide reliable food safety and authenticity data, enables governments, the food sector and consumers to make informed and science-based decisions to manage such situations. Effective technology and knowledge transfer are achieved through training courses, the development of databases and other online resources, and networking.

Analytical method development in the FEPL encompasses both sophisticated techniques capable of providing essential information such as the identity and amount of food contaminants present or the probable origin or production technique of a food product that allow follow-up actions to deal with the issue, and cost-effective, screening, 'point of contact' methods that can be deployed in the field (on the food production line or supply chain) to provide rapid answers regarding the safety, quality or authenticity of food raw materials or products. A combination of these techniques provides Member States with the options needed for their food control systems, both under normal circumstances and when the systems are challenged by crises or emergencies.

In 2020, seven novel analytical methods and associated method protocols were developed in FEPL. Four instrument or workflow operating procedures were prepared to standardize operations in FEPL and for use in training and technology transfer to Member States. A proficiency test was initiated for CRP and TCP participants using ten food matrix reference materials for stable isotope analysis that were previously characterised in FEPL as part of an interlaboratory study.

Some of the main research activities and results are presented below.

Food authenticity

Food fraud and adulteration, though driven by economic gain, can also present a significant risk to human health. There have been many examples of this throughout history. In recent times, notable examples include melamine in milk powder, Sudan dyes in chilli powder and methanol in counterfeit spirits. The global occurrence of such incidents may cause negative impacts on international trade, reputational damage to companies or entire food sectors and, at worst, serious illness or fatalities to consumers.

Analytical methods for the differentiation of organic and conventionally produced foods

Over the past decade organic food production has been rapidly increasing worldwide. Organic food products are usually sold at premium prices compared to their conventional counterparts. Whilst organic crops are subject to safety and quality checks, there are no officially recognized end-product tests to verify organic labelling claims. This is a major gap in the quality control of organic produce since economic incentives to fraudulently mislabel conventionally cultivated crops as organic remain

high. The average price premium for organic products is between 20 and 50%, but price differentials can be as high as 300% for some specific products, such as tea and coffee. This has led to a significant incidence of fraud in recent years, at a cost of many millions of dollars.

The authentication of organic foods relies predominantly on certification processes and regular farm inspections – currently they are not routinely tested for authenticity before reaching the consumer. This creates an incentive for unscrupulous producers and retailers to mix organic and conventional produce, or to substitute organic produce with conventional, for financial gain. A significant number of cases of fraud, where conventional produce was mislabelled and passed off as organic, have been reported in recent years worldwide. The issue concerns the whole agri-food sector, from small local farmers' markets to supermarket chains and global retailers. To support the integrity of the whole organic food and drink supply chain, it is therefore of great importance that, in addition to traceability and certification schemes, the authenticity of organic foods can be verified in an objective and independent way using analytical tests.

Various analytical techniques have been investigated and applied over the past decade for the authentication of organic products. Stable isotope analysis and elemental analysis have been the most widely tested techniques. Stable isotopes have proven to be good indicators of authenticity for both plant- and animal-derived food products; however, complete discrimination between organic and conventional foods is often not possible based solely on stable isotope analysis. Current thinking is that the authentication of organic food products is unlikely to be achieved by the measurement of a single or only a few selected markers.

There is, therefore, a significant need for efficient, rapid and easily deployable methods for verifying the authenticity of organic food. The development of such methods is a current focus of FEPL work, in support of coordinated research projects D52040, 'Field-deployable analytical methods to assess the authenticity, safety and quality of food' and D52042, 'Implementation of Nuclear Techniques for AuthentiCaTion of Foods with High-Value Labelling Claims (INTACT Food)'.

Some of the method development work carried out in FEPL in 2020 is described below.

Differentiation between organic and conventional fruit juices using FTIR spectroscopy

In 2020 FEPL commenced work on the development of methods for differentiating organic and conventional orange juices using a limited number of authentic samples from Mexico, supplied by the fruit juice industrial association, Sure-Global-Fair (SGF). Initial analyses were carried out using Fourier-transform infrared spectroscopy (FTIR). Spectra were acquired in the range 450 to 4000 cm^{-1}. Data were centred and pre-processed using multiplicative scatter correction (MSC) and the 1^{st} derivative functions. The goodness of fit (R2X (cum)) and the predictability (Q2(cum)) values of the PCA model generated were 0.79 and 0.59, respectively. R2X (cum), R2Y(cum) and Q2(cum) values of the cross-validated OPLS-DA model were 0.56, 0.96 and 0.68, respectively.

The dataset was divided into a test set (2/3 of the samples) and a prediction set (1/3 of the samples). An OPLS-DA model (Figure 1) was constructed for the test set and was subsequently used for the prediction of the origin of the samples in the prediction set. The prediction was performed three times using three randomly generated test and prediction sets. The OPLS-DA model correctly predicted 100% of organic samples, and on average 86.7% of conventional samples.

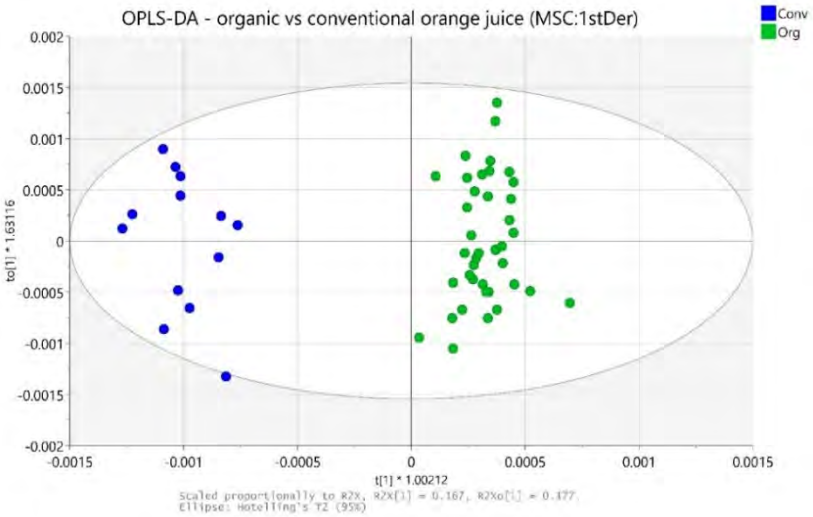


FIG. 1: *OPLS-DA model of organic (n=40) and conventional (n=16) orange juice samples from Mexico*

The major signals responsible for the differentiation of organic and conventional juices were examined using the spectral images generated from the samples analysed (Figure 2) as well as VIP and S-plots (Figure 3) from the generated OPLS-DA model. The major differences were observed in the FTIR spectral region corresponding to major sugars.

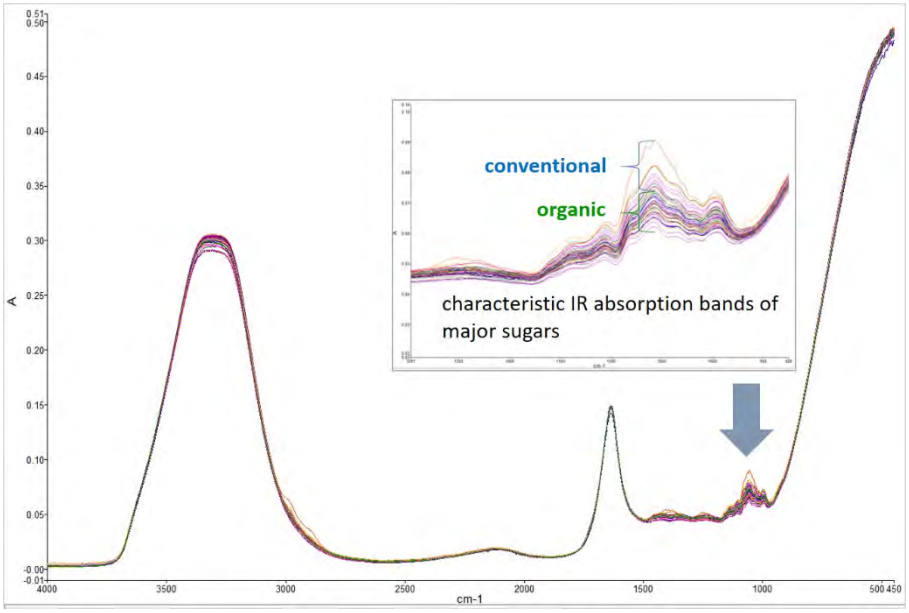


FIG. 2: *FTIR spectra of organic (n = 40) and conventional (n = 16) samples*

The results to date, using a limited number of guaranteed authentic samples, indicate the potential of FTIR as a rapid method to differentiate between organic and conventionally produced fruit juices. However, the methodology must be validated using a much larger sample set, which was not possible in 2020 due to disruption to the supply of authentic samples by SGF caused by the global COVID-19 pandemic. Further work on the model validation will continue in 2021 when more authentic samples are available.

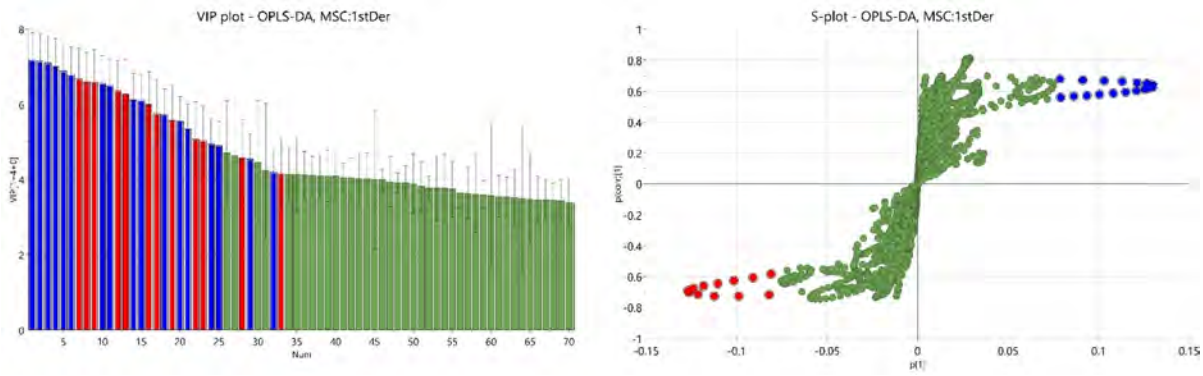


***FIG. 3**: OPLS-DA model of organic (n = 40) and conventional (n = 16) orange juice samples from Mexico: VIP plot (left) and S-plot (right)*

Untargeted metabolomics analysis for the discrimination between organic and conventional orange juices – method development work

A growing number of studies on the use of untargeted metabolomics report that this analytical approach has potential for differentiation between organically and conventionally grown crops. A wide range of primary and secondary metabolites, including phenolic compounds, organic acids and amino acids, have been reported to differ significantly between organic and conventional crops and could serve as potential discriminant markers. Additional, well-designed studies are required to assess the applicability of untargeted metabolomics for organic food authentication.

In 2020, FEPL worked on the development and optimization of an untargeted metabolomics method using ultra-performance liquid chromatography – quadrupole time of flight mass spectrometry (UPLC-QTOF-MS) for the authentication of organic orange juices from Mexico. This work supports CRP D52042, 'Implementation of nuclear techniques for authentication of foods with high-value labelling claims'.

Method development and optimization were performed using commercial organic (n=2) and conventional (n=2) juice samples. Several different sample extractions and UPLC elution methods were tested and compared. All extraction methods involved centrifugation of the extract at 25,000 rpm for 10 min and filtration (0.22 µm PTFE membrane filter) prior to analysis. UPLC settings were optimized by comparing different elution parameters, e.g., different elution gradients, flow rates and column temperatures. The MS settings, e.g., capillary, sampling cone and extraction cone voltages were also optimized.

The data acquired using all methods were compared and assessed for run alignment, drift, total number of compound ions, repeatability, the discriminative power and goodness of fit of the principal component analysis (PCA) models. Methanol extraction and elution with acetonitrile gave the highest number of compound ions, best alignment of the sample runs and best repeatability. An example of MS chromatograms of conventional (A) and organic (B) samples in negative electrospray (ESI-) mode is presented in Fig. 4. The organic, conventional and QC samples extracted and analysed using the optimized method were well separated using PCA and the two organic and two conventional juice samples were also well separated from each other. Though this stage was for method development only, using only the two authentic organic and two conventional samples that were available, it is surmised that the separation between organic and conventional samples is likely to be attributed to, but may not be limited to, the cultivation system. The separation between two types of organic (as well as two types of conventional) samples may be related to the differences in the country of origin and orange fruit variety of the commercial juice samples used for method development. This may be a limiting factor for the application of the untargeted approach when analysing real-life samples and will need to be assessed during further work, including the analysis of large numbers and varieties of samples.

To investigate the applicability and robustness of the methodology, the optimised method will be applied for the analysis of authentic organic and conventional orange juice samples from Mexico to be supplied by the fruit juice industrial association, SGF. This was not possible in 2020 due to the COVID-19 pandemic.

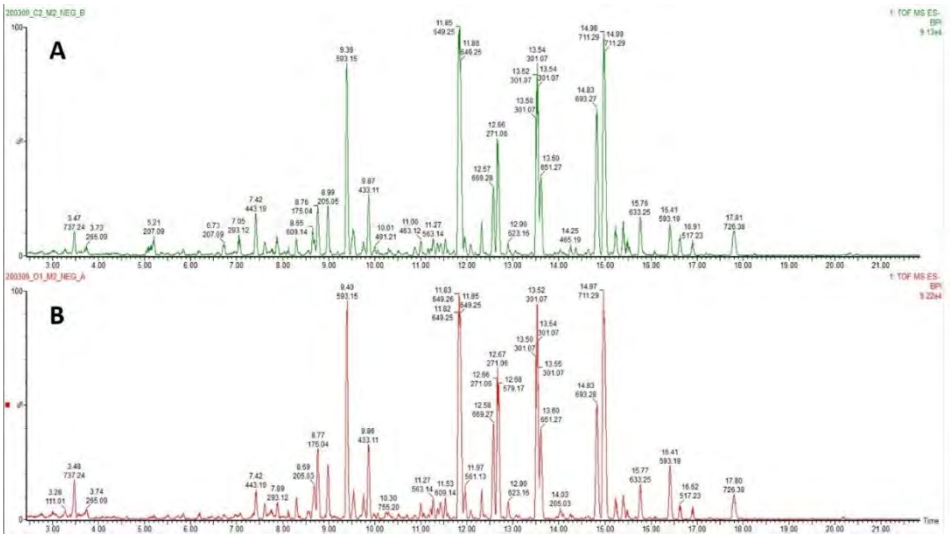


FIG. 4: *MS chromatograms (ESI- mode) of organic (A) and conventional (B) orange juice*

Is it organic? A home-based feasibility study with the SCiO pocket molecular sensor to distinguish between organic and conventional tomatoes

Research and development in FEPL were disrupted in 2020 due to restrictions imposed by the COVID-19 pandemic. This was especially true during the first lockdown in Austria, from mid-March until the end of June, when the laboratory was effectively closed. Although work continued from home on data analysis, writing up reports and papers etc., this unprecedented situation also provided the opportunity for some home-based experiments utilizing hand-held equipment, such as that being investigated in coordinated research project D52040, 'Field-deployable analytical methods to assess the authenticity, safety and quality of food', for point of contact (POC) use in Member States.

FEPL staff undertook a short home-based feasibility study during the lockdown period to investigate the capability of the low-cost SCiO™ near-infrared (NIR) pocket molecular sensor to provide information on the authenticity of tomatoes labelled as 'organic' from the market. Tomatoes were chosen for this study because they have a global production of over 182 million metric tons and were recorded in 2018 by the FAO as the most commonly produced vegetable crop in the world. The SCiO™ device was used to acquire the spectral data of intact tomato samples in the wavelength range 740–1070 nm with a sampling interval of 1 nm. The SCiO™ Lab online application was used for data collection and storage and for statistical analysis of the spectra. The prediction of the tomato quality attributes was based on the overall state of the fruit captured by the sensor, which can be indicative of various physical and chemical properties such as skin colour, stage of ripening, surface scattering characteristics and near-surface sugar content.

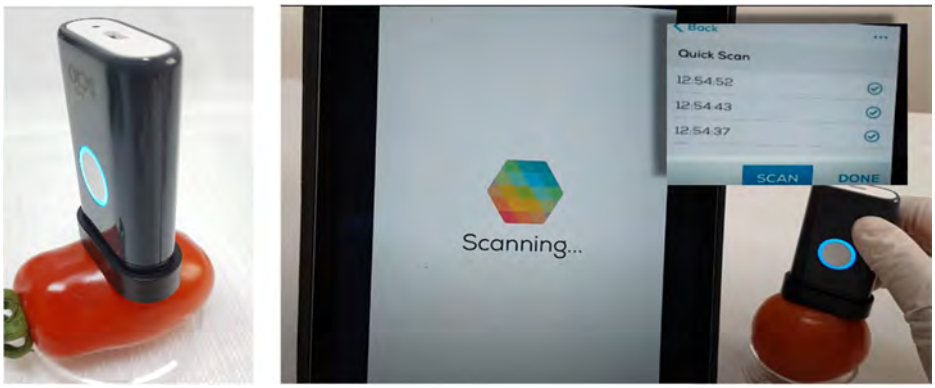


***FIG.5**: Near Infrared (NIR) hand-held microspectrometer (SCiO™) collecting spectra from a tomato sample and scanning using the "SCiO™ Lab" Web application*

For this feasibility study, nine samples of conventional tomatoes and six samples of tomatoes labelled as 'organic' were purchased from different retail outlets and their NIR spectral data acquired to develop predictive statistical models. In addition, another 5 unique (3 conventional and 2 organic) retail samples were purchased on different days and analysed as challenge samples for external validation of the predictive statistical models. The performance of the SCiO™ sensor for quality prediction was assessed by developing classification models using the SCiO™ Lab online application, assuming that the tomatoes were authentic examples of their target-class, as labelled. Four randomly selected tomatoes from each packet were tested and each tomato was scanned three times in different positions, approximately 120° apart, around the circumference of the fruit, as shown in Fig. 5. This measurement approach produced a total of 60 measured samples and 180 scans.

Various spectral data pretreatments were investigated using SCiO™ Lab. The best performing principal component analysis (PCA) statistical model produced correct classification rates of 92% for organic tomatoes and 96% for conventional tomatoes (Fig. 6). To further challenge the models, another test collection was created for an additional set of tomatoes bought from the market on a different day, as new anonymized samples. The model predicted 92% of the organic tomatoes correctly and 72% of the conventional tomatoes correctly, based on the tomato labelling information provided.

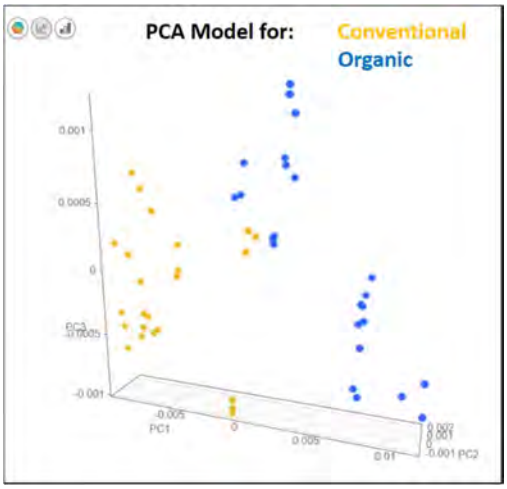


***FIG. 6:** PCA plot of the multivariate model developed for conventional and organic tomatoes*

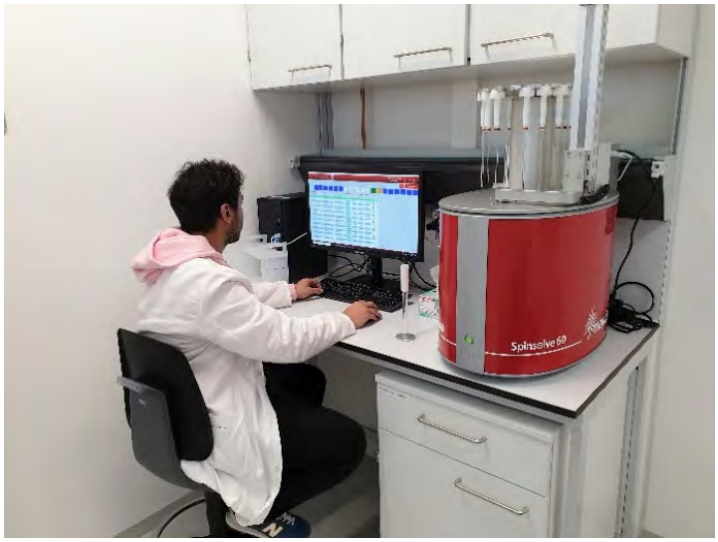
This feasibility study successfully demonstrated the potential of using a low-cost, handheld, NIR device for POC use, to test market samples outside the laboratory environment. In combination with on-line statistical tools, and with the limited sample set available, it was possible to distinguish organic tomatoes from conventional ones with acceptable success rates in home-based experiments. This preliminary study suggested that, with larger sample sets of organic and conventional tomatoes of confirmed provenance, it should be possible to develop databases in Member States permitting more accurate, reliable and robust models for routine testing. The SCiO lab data processing option offers the potential for immediate POC food authenticity, quality or safety testing in real time.

New food authenticity screening applications at FEPL using benchtop nuclear magnetic resonance (NMR) spectroscopy: a preliminary study on the differentiation of Arabica and Robusta coffee beans

Various spectroscopic and mass chromatographic techniques have been applied for food authenticity testing. There is, however, an ongoing need to develop faster, cheaper and more efficient methods to

complement current techniques. Nuclear magnetic resonance (NMR) spectroscopy is a non-destructive analytical technique that provides detailed information about the molecular structure of a sample, enables quantification of molecules even in complex mixtures and allows the direct observation of chemical reactions. Combined with chemometrics, it can be a useful tool for untargeted food metabolomics and rapid food adulteration and/or contamination screening. NMR spectroscopy has been used for a wide range of applications in the areas of chemistry, biology, medicine, pharma, food and feed, forensics and process control. In the area of food safety and authenticity, NMR has been applied for the quality control, authentication and traceability of fruit juices, honey, coffee, spices, edible oils, wine, meat, fish and other food commodities.

The most common types of NMR are proton (1H) and carbon (13C) NMR spectroscopy, but the technique is applicable to any sample that contains nuclei possessing spin. The resolution of an NMR spectrometer depends directly on the strength of the magnetic field used. High-resolution NMR spectrometers (300-1200 MHz), which have a large superconducting magnet, can enable structural elucidation and thus can be used as a powerful metabolomics tool. However, the main drawback of these instruments is their very high cost in terms of initial investment, consumables (liquid helium), maintenance (hardware) and operation (skilled personnel). This led to the introduction in recent years of benchtop NMR spectrometers with permanent magnets (42–100 MHz), which have a small footprint, a 5-20 × lower cost, almost zero maintenance and easy operation. Although the resolution and sensitivity of benchtop NMR systems are significantly lower than those of high-resolution NMR, the former still offer sufficient performance for a wide range of applications, including food safety and authenticity. Some examples of food safety and authenticity applications using low resolution benchtop NMR systems include the detection of cheap refined edible oils as adulterants in extra virgin olive oil or cold pressed rapeseed oil, pork or horse meat in beef, cheaper Robusta coffee in Arabica coffee, and synthetic chemical compounds or cheap edible oils in Patchouli and other essential oils.



Spinsolve 60 benchtop NMR spectrometer in FEPL

Benchtop NMR spectroscopy offers an untargeted multi-analyte screening capability as well as low operational costs. This makes the technique suitable for authenticity screening, complementing the other analytical approaches that are being developed and used at FEPL and transferred to the Member State laboratories under CRP D52042 'Implementation of Nuclear Techniques for Authentication of Foods with High-Value Labelling Claims'. To facilitate research and development in this field, a Magritek Spinsolve 60 benchtop NMR spectroscopy system was commissioned in FEPL in September 2020.

One of the first food authenticity applications being developed at FEPL using benchtop NMR spectrometry is the differentiation of Arabica and Robusta coffee beans. Cultivated coffee beans are one of the most widely traded commodities in the world and are frequently targets for fraud. The two main species of cultivated coffee are Coffea arabica and Coffea canephora (C.robusta). Arabica beans, which account for around 60% of global coffee production, are prized for their superior smooth flavour, but the plants are susceptible to disease. Robusta plants are more disease-resistant, but produce beans that yield a stronger, harsher and more bitter drink, and thus command lower prices than Arabica beans. This price differential offers the potential for unscrupulous traders to make economic gain by partially or

wholly substituting Arabica beans with Robusta. Analytical methods are required for the reliable identification of both coffee species and for the estimation of their contents in coffee products.

Coffee contains a complex mixture of hundreds of different organic compounds, primarily carbohydrates, amino acids and lipids. Potentially more characteristic of the individual species, however, are minor components such as the diterpenes of the kaurane family. These include cafestol, found in both bean types, and kahweol, found in Arabica beans and in some, but not all, Robusta beans. Diterpene 16-O-methylcafestol (16-OMC), is found exclusively in Robusta beans, and has been proposed as a reliable marker for distinguishing between the two bean types. There is an official method for the determination of 16-OMC in roasted coffee by high performance liquid chromatography, however it is laborious and requires a time-consuming sample preparation (DIN 10779: 2011-03). The development of alternative, rapid, low-cost methods, would significantly improve the capabilities of Member States to screen coffee samples for authenticity.

High-resolution 1H NMR spectroscopy has been previously applied for the analysis of coffee. It was shown that many minor components, including kahweol and 16-OMC, produce clearly identifiable peaks in 400 MHz spectra, which can be used to estimate the amount of Robusta in coffee blends with an approximate detection limit of 1-3% w/w. Several studies demonstrated that low-field NMR spectroscopy can offer sufficient specificity and sensitivity to distinguish between Arabica and Robusta coffee samples, and further, to quantitatively characterize mixtures of the two.

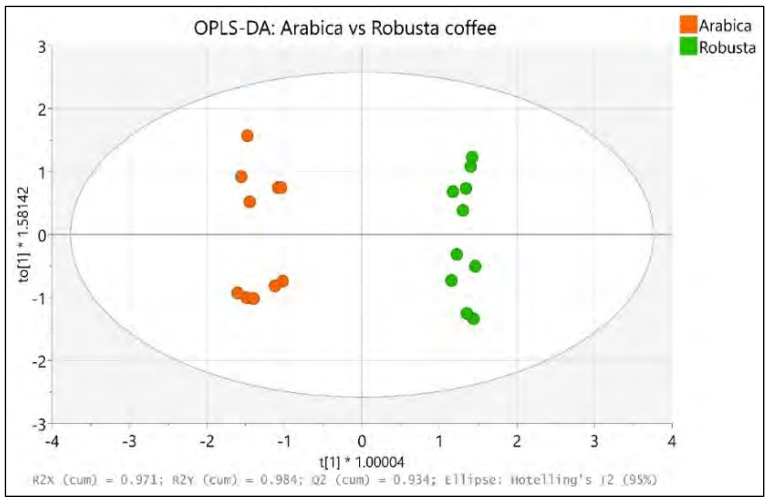


FIG. 7: *An example of an OPLS-DA model of Robusta and Arabica coffee generated using NMR spectral data obtained in FEPL*

In FEPL, different sample extraction protocols and instrumental conditions were tested and optimized to produce an analytical method for the differentiation between Arabica and Robusta coffee samples using the benchtop NMR spectrometer. Initial results demonstrated good discrimination of commercial Arabica and Robusta coffee samples, with goodness-of-fit (R2X (cum) and R2Y (cum)) and predictability (Q2 (cum)) values of the OPLS-DA model (Fig. 7) being 0.971, 0.984 and 0.934, respectively. Further work on the model validation and the characterization of Arabica and Robusta coffee mixtures will continue in 2021 using a larger sample set.

A new method for determination of the geographical origin of rice by stable isotope analysis

Polysaccharides, particularly cellulose, are the most abundant biopolymer on earth. They are long chain monosaccharides linked by glycosidic bonds and play important roles in plants, such as energy storage (e.g. starch and glycogen), structure and growth (e.g. cellulose and pectin). The determination of carbon bound non-exchangeable (CBNE) hydrogen isotope ratios in polysaccharides is of great interest to a broad range of research areas, as they contain intrinsic information about the metabolic pathway and geographical origin of the plant, derived from water incorporated during photosynthesis. This information can be used, for example, to differentiate between rice samples of different geographical origins.

Measuring non-exchangeable hydrogen isotope ratios in polysaccharides is challenging, employing methods such as the dual-water isotope equilibration technique that are labour intensive due to the chemical and physical properties of polysaccharides. A new strategy to determine non-exchangeable hydrogen isotopes in polysaccharides is to hydrolyse the biopolymer into monosaccharides, followed by conversion into volatile trifluoroacetamide (TFA) sugar derivatives for analysis by the gas chromatography – chromium silver/ high temperature conversion - isotope ratio mass spectrometry (GC-CrAg/HTC-IRMS) (Fig. 8) method previously developed in FEPL.

The method developed in FEPL employs a rapid microwave assisted hydrolysis stage followed by freeze drying and derivatization of the sugars with N-methyl-bis-trifluoroacetamide (MBTFA). The mono- and disaccharide TFA derivatives are separated by gas chromatography and converted to hydrogen gas by reductive pyrolysis in a GC-CrAg/HTC-IRMS system, and the hydrogen isotopic ratios measured.

The new method is simpler and more rapid than alternative, currently available methods and should allow CBNE hydrogen isotope analysis to be more easily and widely used for applications such as confirmation of the declared geographical origin of rice.

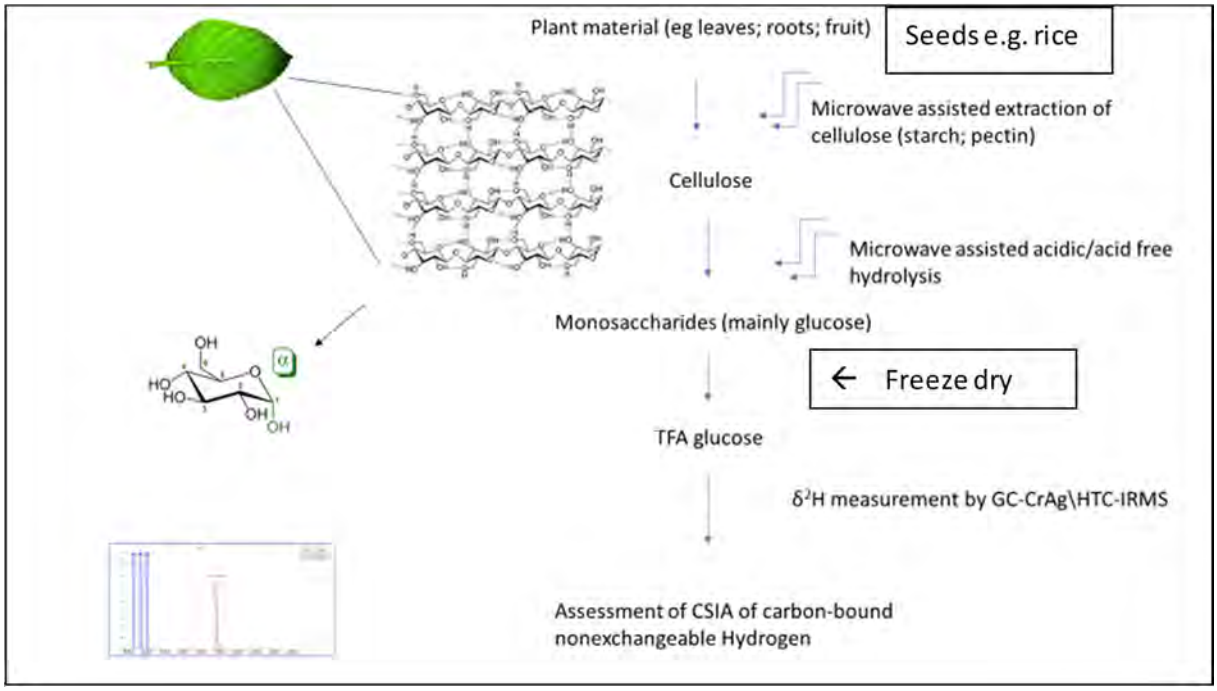


FIG. 8: *Strategy for determination of non-exchangeable hydrogen isotopes in biopolymer by Gc-CrAg/HTC-IRMS*

Coordinated research on food authenticity

In 2020, FEPL coordinated and provided technical input to two coordinated research projects (CRPs) in the field of food authenticity.

Field deployable analytical methods to assess the authenticity, safety and quality of food (D52040)

In May 2020, the Agency's Committee for Coordinated Research Activities in Nuclear Applications formally reviewed the mid-term progress of CRP 'Field-deployable Analytical Methods to Assess the Authenticity, Safety and Quality of Food' and approved its continuation to complete the planned 5-year duration. A significant number of feasibility studies have been completed to make preliminary assessments of all of the portable nuclear and complementary techniques identified at the outset of the project, resulting in ten peer reviewed scientific publications to date. The project outputs have

already been used to improve national capacities in rapid screening technologies, e.g., to control the illegal use of recycled palm oil for cooking in Malaysia. This CRP is also putting legacy outputs in place through a technical contract with the Walloon Agricultural Research Centre in Belgium, which includes the fabrication and distribution of sealed glass units of vegetable oils and milk powder for inter-laboratory spectral calibration, to ensure reproducibility in measurements, and the development of an on-line spectral library of authentic products, which will be used by CRP participants and other Member States for food authenticity and safety control. Eleven papers have been published from this CRP in 2020.

Implementation of Nuclear Techniques for AuthentiCaTion of Foods with High-Value Labelling Claims (INTACT Food) (D52042)

This project started in 2019, the first research coordination meeting (RCM) having been held at the IAEA Headquarters in Vienna from the 13-17 May 2019. The project participants comprise 12 research contract holders (from China, Costa Rica, India, Indonesia, Jamaica, Malaysia, Morocco, Myanmar, Slovenia, Thailand and Uruguay), six research agreement holders (from Denmark, Germany, Italy, Japan, New Zealand and Spain) and 5 observers representing Imprint Analytics (Austria), the Oil Crops Research Institute (China), the Tentamus Global Center for Food Fraud (Germany) and Organic Services (Germany). The workplans for the first phase of project are proceeding as well as possible under the constraints of COVID-19. One scientific paper has been published so far in the peer-reviewed scientific literature. Due to disruption caused by the COVID-19 pandemic, the 2nd RCM has been delayed and will be held in Jamaica in November 2021.

Control of residues and contaminants in food

A multi-class analytical method for pesticide residues in orange juice by LC-MSMS

A wide variety of pesticides is applied to crops in conventional agricultural practices, to maintain high yields and quality in the production of fruits. Frequently, after harvest, pesticides can remain on the crops as residues. It is the role of the analytical testing laboratories within regulatory control systems to detect the occurrence of residues in food and provide evidence of compliance of residue levels with the maximum residue levels (MRLs) established by national and international regulations. Accurate, reliable, cheap, rapid, robust and selective multiresidue analytical methodologies must be optimized and validated at the laboratory to ensure that the measurements can be implemented efficiently at the trace levels required and that unequivocal evidence can be provided to confirm the identity and quantity of any pesticide residues detected. Chromatographic techniques coupled to mass spectrometric detection are well suited for this purpose.

Based on Member State needs, the FEPL initiated a study on the optimization and validation of a multiresidue method for the detection of pesticide residues in orange juice using liquid chromatography coupled to tandem mass spectrometry (LC-MSMS). During the second part of 2020 the method was validated according to Codex guidelines. The Swedish ethyl acetate sample preparation method (SweET method) was adopted for its simplicity, speed, and the selectivity afforded by the low solubility of sugars and proteins in ethyl acetate. The validation experiments were designed to ensure that the method is fit for purpose; that the analytes can be accurately quantified at trace levels (typically µg/kg) and that unequivocal evidence can be generated to confirm the identity of any pesticide residue detected.

Ethyl acetate extracts, cleaned up using the SweET method, were directly injected into the GC and LC systems. In GC-MSMS the inlet and oven temperature programme were optimized to achieve the best quantitative transfer of analytes in vapour phase to the column and sufficient separation of close eluting analytes. In LC-MSMS chromatographic separation was achieved using an optimized gradient elution profile. Both GC-MSMS and LC-MSMS were operated in scheduled multiple reaction monitoring (MRM) to achieve the required sensitivity and selectivity. The experimental design included the analysis of reagent blanks, matrix blanks, fortified samples at three different concentration levels (0.010, 0.020 and 0.050 mg/kg), each in 5 replicate samples, with repeated analysis on 3 different days to establish the within laboratory reproducibility.

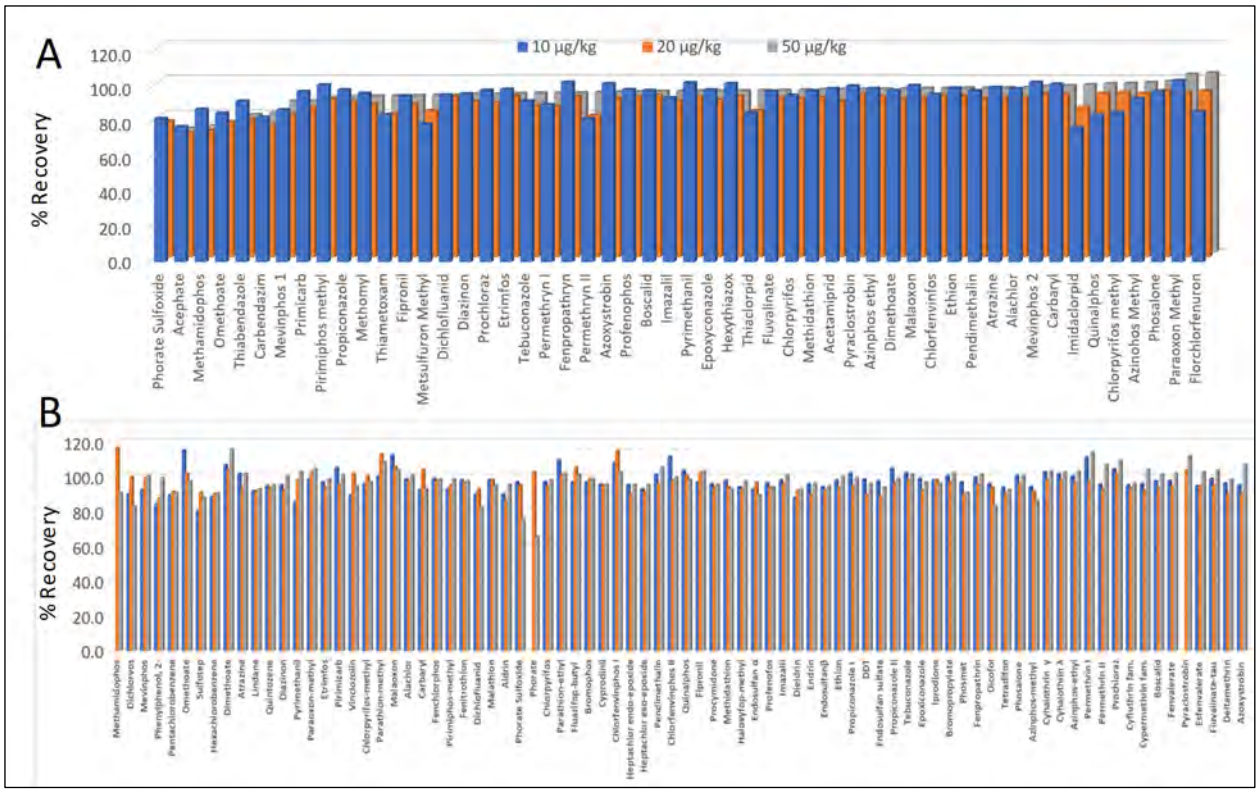


FIG. 9: *The average recoveries obtained at three fortification levels (0.01,0.02,0.05 mg/kg) for:* ***A****, the 52 pesticides in the scope of the LC-MSMS method;* ***B****, the 82 pesticides in the GC-MSMS method*

The key method performance parameters investigated were specificity, linearity, trueness, within laboratory repeatability and reproducibility, limit of detection, limit of quantitation, matrix effects and qualitative criteria for confirmation. From an initial proposed scope of 85pesticides (GC and LC amenable), only 82 analytes in GC-MSMS and 52 analytes in LC-MSMS achieved the desired performance criteria according to the Codex Alimentarius Guidelines on Good Laboratory Practice in Pesticide Residue Analysis (Codex guideline CAC/GL 40-1993. Rev.1-2003). Recoveries for the pesticides in the validated method ranged from 60 to 116%, and the RSDs were lower than 20% for all the pesticides studied. The analytes included in the scope of the method and the average recoveries obtained at the three fortification levels for LC-MSMS and GC-MSMS are shown in Fig. 9. Examples of the elution profiles of the method in a fortified sample, a matrix matched calibrator and a blank sample by LC-MSMS are shown in Fig.10. In the LC-MSMS method the blank sample produces some interferences. The large peak present at approximately 6 minutes, as observed in Fig. 10 (C), causes signal saturation of malathion, excluding it from the final validated method. In addition, fenthion (in both LC-MSMS and GC-MSMS), phosmet (in LC-MSMS only) and acephate (in GC-MSMS only), failed to achieve the qualitative criteria for ion ratios specified in the Codex Guidelines and therefore were excluded from the respective validation scopes. In both LC-MSMS and GC-MSMS, fenthion had

pronounced matrix effects (about 300%) and could not be precisely quantified even using matrix matched calibration strategies.

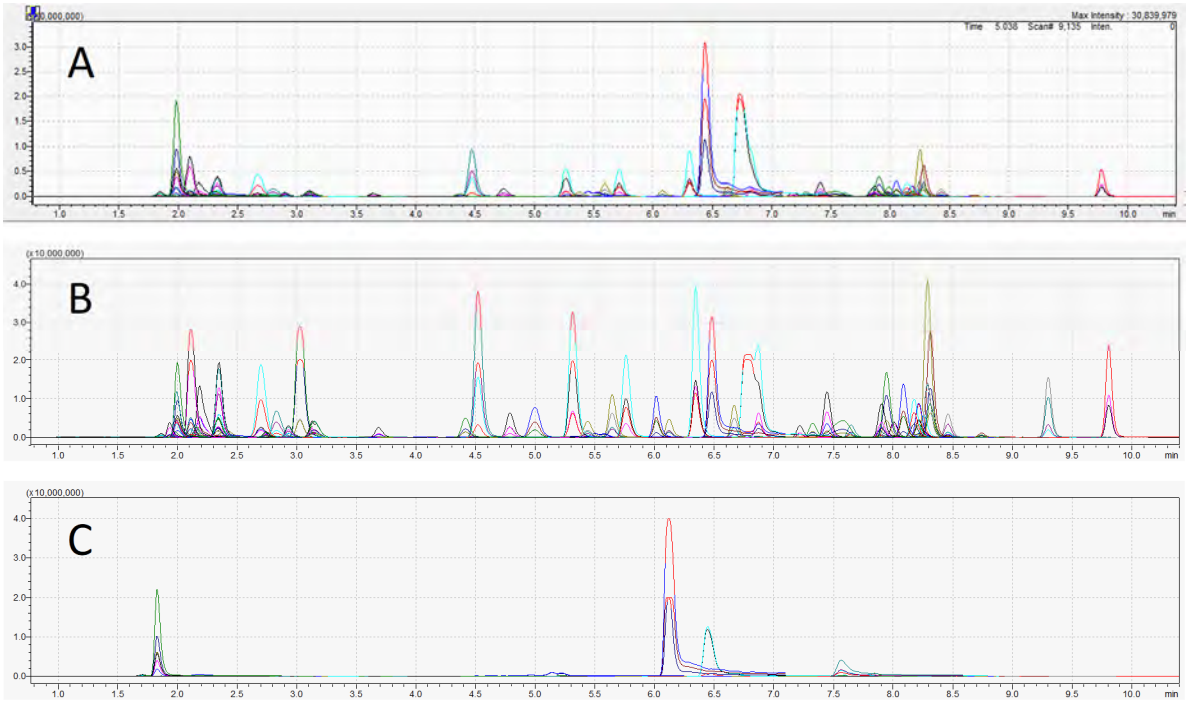


***FIG. 10**: The elution pattern of the method in a fortified sample at (A), 0.05 mg/kg, (B) in a matrix matched calibrator at 0.05 mg/kg, and (C) a blank sample.*

Dissemination of research results

The results of the research and the methods developed or adapted and validated in the FEPL are generally made available to Member States through various mechanisms, including training courses, workshops, publications in the scientific literature and via the internet, public outreach events, conferences and symposia. The 'Food Contaminant and Residue Information System' (FCRIS, http://nucleus.iaea.org/fcris/) provides useful data on food contaminants and residues and includes analytical methods databases. The methods databases for veterinary drug residues and for pesticide residues were developed in response to requests from the Codex Committees on Residues of Veterinary Drugs in Food and on Pesticide Residues.

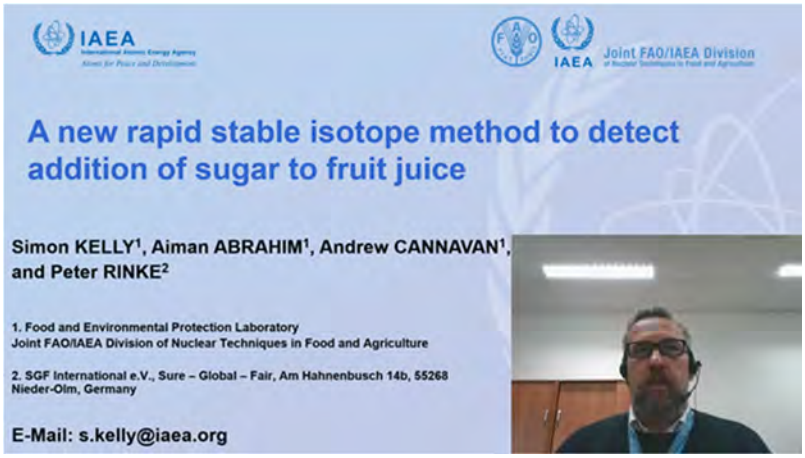
In 2020, many of the planned events were either postponed or cancelled due to travel restrictions and quarantine requirements imposed by the COVID-19 pandemic. Some events were held virtually or through distance learning.

Conferences & Webinars

Sixth International Forum on Food Authenticity Technology and Industry Development, Beijing, China, 01-02 November 2020. The International Forum was organized by the Chinese National Centre of International Research on Food Authenticity Technology, in affiliation with the NHC Key Laboratory of Food Safety Risk Assessment, and Sinolight Food Inspection and Certification Company. One hundred and sixty delegates attended in person and over 4800 attended through on-line participation. The forum was designed to address the requirement for the development of new methods and techniques to verify the quality, authenticity, and safety of food. This requirement is largely driven by the frequency of major food scandals, the globalisation of food sales and the increasing length and complexity of international supply chains. It also reflects changing consumer preferences in China and

the growing number of added-value claims that are attached to food production, such as organic, locally produced, natural, authentic, and Geographically Indicated foods, but also new analytical challenges and new risk factors. Mr Simon Kelly (FEPL), gave a pre-recorded scientific lecture on "The use of stable isotope and trace element (SITE) analysis to verify the origin of foods with geographical indication (GI) status" in the session, "Food integrity: integrating quality, safety and authenticity" and participated in a 30-minute live question and answer session with Dr Carsten Fauhl-Hassek from the German Federal Institute for Risk Assessment.

International Fruit and Vegetable Juice Association (IFU) Technical Webinar: Authenticity, 15 October 2020. This Webinar was organised as a replacement for the IFU Technical Workshop in 2020, which was cancelled as a live event due to the COVID-19 pandemic. IFU Technical Workshops are the meeting point for the national and international juice industry, including research and development, quality, laboratories, suppliers and universities to discuss the latest developments in methodology related to the safety, quality, and authenticity of fruit juice. More than one hundred and fifty delegates attended the Webinar on authenticity.



Mr Kelly giving an invited lecture at the International Fruit and Vegetable Juice Association (IFU) Technical Webinar: Authenticity, 15 October 2020

Mr Simon Kelly gave a pre-recorded scientific lecture on a new rapid method using hydrogen stable isotope analysis to detect undeclared addition of sugar to fruit juice. The improved procedure was developed in the FEPL and utilizes a simple one-step chemical reaction that makes sugars sufficiently volatile to be separated and measured by gas chromatography coupled to isotope ratio mass spectrometer. The new procedure has advantages over methods using nitro-sugar derivatives, sugar degradation products and fermentation in terms of ease of use, analysis time and sensitivity. The potential of the technique for detecting economically motivated adulteration of foods and beverages was discussed, with illustrations of the differences between the isotope abundance of the non-exchangeable hydrogen in sugars from authentic pineapple juice and those of beet and cane sugars/syrups, which permits the presence of these potential adulterants to be rapidly detected.

Mr Kelly also participated in a 30-minute live question and answer session with experts from Sure Global Fair (SGF) Producers Association and Gesellschaft für Lebensmittel-Forschung (GfL) GmbH.

Virtual Panel discussion on 'Isotope fingerprints: the common thread between forensic applications - from food and beverage adulteration to crime scenes investigations', 06 October 2020. This open access Webinar was organised by ThermoFisher Scientific and had panellists from the University of Mexico, Imprint Analytics GmbH, Austria, and Mr Simon Kelly (FEPL). More than one hundred and twenty registered delegates attended the panel discussion through on-line participation. The webinar was designed to provide background information on the use of stable isotope analysis in forensic

studies covering biogeochemical cycles, archaeological/criminalistic materials, foods and beverages, officially recognised methods for foods, criminal forensics and Interlaboratory data comparison, drawing on expertise from private laboratories, governmental agencies and academic research institutes. Mr Kelly gave a pre-recorded scientific lecture on the principles of light-element stable isotope systematics in biogeochemical cycles and how these underpin the application of stable isotope analysis in forensic studies. Mr Kelly also presented information about new food matrix stable isotope reference materials that were characterised by a consortium including FEPL and officially recognised stable isotope methods from the European Committee for Standardization (CEN), the Association of Official Analytical Chemists (AOAC), the International Organisation of Vine and Wine (OIV) and the international Codex Alimentarius Standard 234. Mr Kelly then participated in a 20-minute live question and answer session with the other panel members. the webinar is free to view on-demand at:

https://event.on24.com/wcc/r/2665786/9158FE201606FA6C0EFE250445CB04FD/1565634?mr=s

FAO Podcast in association with Lloyds Register Foundation on Food Safety. This panel discussion was part of the podcast 'How to feed the world, safely', published on 28th May 2020 (FAO - LRF-global-safety-podcast), an edition of the Lloyds Register Foundation podcast series 'The Global Safety Podcast'. Mr Simon Kelly (FEPL) was a panel member discussing the question, 'When rising to the challenge of feeding a growing world population, with over 7 1/2 billion hungry mouths, how can we do so safely without putting live at risk?' The panel discussed solutions that can help reduce the deaths, illnesses and malnourishment across the globe.

CAPACITY BUILDING

The FEPL provided the main technical management for twelve national and three regional technical cooperation projects in 2020. Many planned technology and knowledge transfer activities were disrupted due to the ongoing pandemic. However, support was maintained through the provision of advice and guidance, procurement, and in some cases through the development of alternative training methods, such as virtual training courses and webinars, greatly assisted by the laboratory networks such as RALACA. This enabled the training of more than 700 Member State scientists, technicians and regulators.

Virtual training on "Detection and Control of Organic Contaminants in Food"

The training course "Detection and Control of Organic Contaminants in Food – targeted analysis" was planned to be held in FEPL in April 2020, with funding from Japan under PUI project "Enhancing Capacity in MS for Rapid Response to Food Safety Incidents and Emergencies". Because of travel and quarantine restrictions and other impacts of the COVID-19 pandemic, it was necessary to postpone and reformat the training course. In order to maintain outputs and continue to assist Member States, a preliminary course was held from 1-4 December 2020, based mainly on recorded lectures that could be viewed offline, focusing on the theoretical and background aspects of the detection and control of organic residues and contaminants in food. There will be a follow-up laboratory-based course at Seibersdorf in June 2021 for those participants who have satisfactorily completed the virtual training.

The course consisted of lectures by guest lecturers and included a live, online question and answer session on 7 December and a follow-up quiz/examination based on the contents of the course. The course had 24 participants from institutes in 24 developing countries (Algeria, Argentina, Bangladesh, Brazil, Chad, Ecuador, Ethiopia, Ghana, India, Jordan, Lao P.D.R, Lebanon, Myanmar, Namibia, Oman, Pakistan, Paraguay, Seychelles, Sri Lanka, Thailand, Tunisia, Uruguay, Uzbekistan and Viet Nam). All

participants successfully completed the training and will be invited to the hands-on laboratory component in 2021.

In tandem with the training event, distance learning modules were developed and made available to course participants via IAEA NUCLEUS. The training modules comprise 19 lectures in 4 topics: organic contaminants in food, testing methodologies, analytical instrumentation and planning of experiments, method validation, and quality control measures. The distance learning course will be made openly available through registration in NUCLEUS at a later date.

RALACA Laboratory Network

The Red Analítica de Latinoamérica y el Caribe (RALACA) network is active in more than 54 institutions in 21 participating countries in the Latin American and Caribbean region. The network's objective is to help with efforts to improve food safety and quality and enhance environmental sustainability. RALACA was legally registered as a foundation in May 2020, greatly enhancing its sustainability.

Meetings and training activities were disrupted in 2020 due to the COVID-19 pandemic. Nevertheless, since May 2020, RALACA held 10 webinars and 2 virtual training events to help the analytical community. The webinars addressed a series of different topics that are relevant to the Latin America and the Caribbean region. The presentations are available at http://www.red-ralaca.net/2-uncategorised/482-webinar-2020. With an average participation of about 60 members at the webinars, a record 139 participants was logged for the 2-day training on the future of data sharing, a learning opportunity presented by the European Food safety Authority (EFSA) to the LAC region through RALACA.

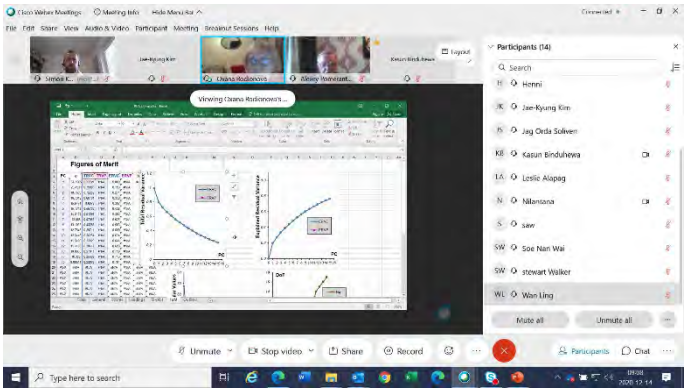
Since May 2020, the network has created two new committees, the Data Sharing Committee (DSC) and the Academy. The DSC incorporates counterparts from 19 countries in the LAC region that will start collecting and managing a common repository of food safety related data under the framework of a regional ARCAL project on Strengthening the Regional Collaboration of Official Laboratories to Address Emerging Challenges for Food Safety (ARCAL CLXV; TCP RLA5080). The RALACA Academy has been created to promote capacity building activities aimed at providing new knowledge to the countries of the Latin American and Caribbean region. The first virtual training course of the Academy will take place in the first quarter of 2021.

Ms Maestroni (FEPL, top left) participating in a virtual meeting of the RALACA Board during the COVID-19 lockdown

To make training proposals please contact the email box: academiaralaca@gmail.com or for general enquiries contact the RALACA board at ralacaboard@gmail.com .

Enhancing Food Safety and Supporting Regional Authentication of Foodstuffs through Implementation of Nuclear Techniques (RAS5081)

One regional training course on 'the use of the 'Chemometrics Add-in for Excel (CAFE)' software and its application was held virtually under this project, from 14th to 18th December 2020, for 20 participants. A second regional training course, 'food authentication toolbox and training resource centre', which was planned to be held in Singapore in September 2020 was postponed until 2021 because of restrictions imposed by the COV-19 pandemic.



Virtual training on 'Chemometrics Add-in for Excel'

Seminar on data sharing - IAEA TCP RLA5080, 'Strengthening the Regional Collaboration of Official Laboratories to Address Emerging Challenges for Food Safety' (ARCAL CLXV)

The IAEA technical cooperation project RLA5080 "Strengthening the Regional Collaboration of Official Laboratories to Address Emerging Challenges for Food Safety (ARCAL CLXV)" started in January 2020. The goals of this regional project are to contribute to improving food safety through risk-based policies to ensure public health and environmental protection, promote cooperation between reference laboratories in the region, harmonize the risk monitoring and evaluation methodology, facilitate the generation of analytical data through collaborations between reference laboratories and establish a data network as part of the regional infrastructure in food safety. The expected outcome of the project is the availability in the region of consolidated data, in the form of a database, that represents the starting point for evidence-based risk assessment and subsequent decision-making by the competent authorities.

Despite a difficult first year marked by the COVID-19 pandemic, the project managed to create functional synergies among the counterparts, brought together different partners in the region and, most importantly, paved the way to create sustainability in the region through the creation of a RALACA Data Sharing Committee (RALACA-DSC). An event on the future of data sharing, was held from 27-29 October 2020, in which delegates from the European Food Safety Authority (EFSA) discussed the benefits of data sharing, the modalities implemented at the EU level and the challenges and lessons learned from the European process of establishing a data portal for evidence-based risk assessments.

Advice and Information Exchange

In 2020, staff of the FEPL were members of the scientific or organising committees of two international conferences. Mr Andrew Cannavan served on the committee for EuroResidue IX: Current issues and emerging trends in residue control, which was planned to be held in The Netherlands in May 2020 but was postponed due to COVID-19 restrictions. The conference will now be merged with the 8th International symposium on Hormone and Veterinary Drug Residue Analysis and is planned for May 2022, venue to be decided. Mr Cannavan also serves on the scientific committee for the ASSET 2022 Belfast Summit on Global Food Integrity, UK, and on the Advisory Panel for the ASSET 2021 Food Industry Forum, dealing with food system resilience issues resulting from the COVID-19 pandemic. Mr Simon Kelly participates in the European Commission's CEN Technical Committee (460) 'Food Authenticity' Working Group 6, 'Stable isotope Analysis' and the UK's Food Authenticity Methodology Working Group. Ms Britt Maestroni serves on the Board and provides advice through various committees of the RALACA network.

Fellowships and Interns

Name	Country	Status	Duration	Topic
Binduhewa, Kasun Madusanka	Sri Lanka	Fellow	1 month	Isotope ratio mass spectrometry and chemometrics
Liebisch, Beatrix	Austria	Intern	12 months	Analytical methods for food control

PUBLICATIONS

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EXTRABUDGETARY SUPPORT

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THE INSECT PEST CONTROL LABORATORY

EXECUTIVE SUMMARY

In the Genetics and Molecular Biology (GMB) group of the IPCL, work has continued to develop genetic sexing systems for fruit flies and mosquitoes. The naturally occurring morphological white pupae mutation of *Ceratitis capitata* occurs spontaneously and has now been isolated not only for *C. capitata* but also for *Bactrocera dorsalis and Zeugodacus cucurbitae.* Classical and modern genetic approaches were used to identify and characterize the white pupae genetic locus in the three species. The white pupae gene was shown to be present in many insect species, suggesting that it can potentially be used as a generic selectable marker for the development of genetic sexing strains for SIT applications against several insect pest species and human disease vectors.

Two different GSSs were developed for the mosquito *Aedes aegypti*, i.e. the Red-eye GSS and White-eye GSS, in which the males are heterozygous with wild-type eyes (black) and females are homozygous with mutant eyes (red or white). The data for productivity and lifespan clearly showed that the Red-eye GSS was more prominent and, in addition, more genetically stable than the White-eye GSS.

An inversion was incorporated in the Red-eye GSS and White-eye GSS using low dose radiation. Screening of consecutive generations indicated reduced recombination frequencies compared to the original GSSs. Although the inversion had a cost and reduced productivity and male flight ability, it significantly reduced the probability of female contamination in the male-release batches.

Staff of the Livestock Pest (LP) group has mainly continued working on the potential use of near infrared (NIR) light to separate male from female tsetse pupae. NIR can be used to record the maturation of the tsetse flies inside the puparium by looking at the darkening (melanization) of some of the structures, e.g. eyes and wings. As females develop faster than males, the melanization process becomes visible one day earlier in female pupae than in male pupae, opening avenues for the separation of the sexes. A new Near InfraRed Pupae Sex Sorter (NIRPSS) was developed that can separate male from female tsetse pupae five days before the emergence of the adults with an accuracy of 89%. Preliminary evaluation of the effect of NIRPSS on the quality of the males indicated that no significant wing damage could be found and that there was no negative effect on the male's ability to fly.

Before being released, sterile male tsetse flies are marked with fluorescent dye to distinguish them from unmarked wild males in the traps. Sand remains the recommended medium to apply fluorescent dye to tsetse, but the preliminary results of directly dying tsetse pupae look promising and will prompt further investigation.

An assessment was made of the prevalence of *Spiroplasma* infections in natural tsetse populations and the results indicate that the prevalence significantly varied with tsetse species and location. Colony tsetse flies infected with *Spiroplasma* showed reduced mating ability and productivity. The genotyping of *Spiroplasma* infection to determine its population genetics is ongoing.

Two other viruses, i.e. the iflavirus and negevirus, first detected in *Glossina morsitans,* were found in many tsetse species except *Glossina pallidipes.* Preliminary results indicate that both viruses are present in the brain, milk glands, salivary glands, testes and ovaries of adult tsetse flies. Their presence in the salivary glands suggests the potential of horizonal transmission through *in vitro* blood feeding, but their presence in the testes, ovaries and the milk glands indicates the potential of vertical transmission from mother to offspring.

Microsatellites, often considered as ideal markers for population genetics studies, were developed for the two tsetse species *Glossina austeni* and *Glossina brevipalpis*. Using the available sequences for these species, 200 pairs of primers were selected and tested for amplification of short sequences. Primers that met the selection criteria were selected for further analysis.

In the Human Disease Vectors (HDV) group, an automatic *Aedes* mosquito pupae sex sorter was evaluated and proved to be a promising instrument to standardize the separation process and hence, increase time efficiency and reduce manpower.

Pupal age, oxygen-poor atmospheric environments and irradiation source were the most important variables that caused significant effects in irradiation dose responses of mosquitoes. Factors that affected dose response to a lesser degree were ambient temperature, life stage, and differences in handling protocols.

A review was made of semi-field and open-field mosquito trials that were conducted over the last decade. The results indicate that irradiated male mosquitoes can be competitive if produced under appropriate conditions. Observed reductions in the quality of the produced sterile male insects are more related to mass-rearing, handling, marking and release processes, rather than radiation *per se*. When all these processes are mastered, a good competitiveness of sterile male mosquitoes can be obtained, i.e. the Fried index C was above 0.2 in all reported studies, which is considered the lower threshold for cost-effective projects.

In the Plant Pest (PP) group, work continued under the USDA/FAO/IAEA agreement of phytosanitary treatments for exotic fruit flies. Results showed that fruit fly third instars irradiated with approved phytosanitary irradiation doses in either hypoxia or severe hypoxia failed to emerge as adults. Hence, it was concluded that it is safe to apply phytosanitary irradiation under modified atmospheres for treatments targeting tephritid fruit fly species.

Different ingredients and recipes have been tested to identify economically viable formulations that could be easily adapted to mass-rearing conditions for *Drosophila suzukii*. A very promising formulation that uses potato powder or sweet potato powder as a bulking agent was first developed at the Instituto de Sanidad y Calidad Agropecuaria de Mendoza (ISCAMEN), Mendoza, Argentina and is now being tested at the IPCL.

The Raycell MK II, a commercial X ray irradiator for blood treatment, was characterized and its performance and dosimetry assessed. The primary findings showed that the dose variation in a vertical way inside the irradiation container is very small, with a dose uniformity ratio of 1.2.

Females of the standard VIENNA-8 GSS of the Mediterranean fruit fly have a slower development rate as compared with males. Using classical genetic approaches, a strain was developed which showed faster larval female development, enabling the females to complete their larval development simultaneously with the males. This allows optimization and cost reduction of the mass-rearing process through a reduced quantity of larval diet and rearing space.

In 2020, the IPCL hosted 2 cost-free experts, 7 consultants (of which 3 were PhD students), 9 interns and 5 TC fellows.

The GMB/PP groups carried out 49 fruit fly shipments to 21 institutions in 10 countries and 4 shipments of preserved fruit flies. The LP group carried out 71 tsetse shipments to 6 institutions in 5 countries. The HDV group carried out 12 mosquito shipments to 5 institutions in 4 countries.

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MAJOR ACHIEVEMENTS IN RESEARCH AND DEVELOPMENT

Genetics and Molecular Biology

Discovery of the white pupae gene in three tephritid species

The Oriental fruit fly *Bactrocera dorsalis*, the Mediterranean fruit fly (medfly) *Ceratitis capitata*, and the melon fly *Zeugodacus cucurbitae* are major horticultural pests affecting crop and revenue yields worldwide. During the last decades, their populations have been successfully managed using area-wide integrated pest management (AW-IPM) approaches with a sterile insect technique (SIT) component. SIT applications rely predominantly on mass releases of sterilized male insects and they have gained significant efficacy and cost-effectiveness boosts due to the development and use of genetic sexing strains (GSS) of these three tephritid species.

The development of any GSS is ruled by two fundamental elements: a selectable marker, either phenotypic or conditionally lethal, and the linkage of the wild-type allele of this marker to the male sex, ideally as close as possible to the male determining region. Males of a GSS are heterozygous and express the wild type phenotype while females are homozygous for the mutant allele and they can be subsequently separated. Pupal colour-based mutants were initially used as a selectable marker for the construction of GSS. In *C. capitata, B. dorsalis*, and *Z. cucurbitae* the typical puparium colour is brown. The naturally occurring morphological mutation for white pupae occurs spontaneously and it has been isolated for all three species and used as a selectable marker to develop GSS (Fig. 1). The insect puparium is sclerotized and melanized by pathways that are inter-connected and biochemical studies in medfly have shown that the white pupae mutants lack catecholamines for tanning the cuticle.

FIG. 1. White colour pupae of the Mediterranean fruit fly Ceratitis capitata
(Photo credit: Georgia Gouvi – FAO/IAEA)

The white pupae phenotype in medfly is due to a recessive mutation in an autosomal gene (*white pupae – wp*) located on chromosome 5, however the gene was unknown till recently. Extensive genetic and cytogenetic studies in medfly have shown that the *wp* gene is localized in the region 59B and 76B on chromosome 5 in the trichogen cells and salivary gland polytene chromosome map, respectively. In addition, a chromosomal inversion called D53, which spans a large region of chromosome 5, has been induced and integrated into the medfly VIENNA 8 GSS. The *wp* locus is located inside the

inversion, close to its right breakpoint, as shown by cytogenetic analysis, but the exact position of the right breakpoint remained unknown.

In a recent study [Ward et al. (2021). White pupae phenotype of tephritids is caused by parallel mutations of a MFS transporter. Nature Communications 12:491 | https://doi.org/10.1038/s41467-020-20680], classical and modern genetic approaches were used to identify and characterize the white pupae genetic locus in *C. capitata, B. dorsalis*, and *Z. cucurbitae*. In *C. capitata*, long read sequencing data, cytogenetic analysis and *in situ* hybridization confirmed the overall structure of the D53 inversion and allowed the identification of the chromosomal breakpoints. Locating the breakpoints of the D53 inversion led to the narrowing down of the target region, and identification of *wp* candidate genes. Further genome sequencing and comparative transcriptomic analysis revealed a single candidate *wp* gene in all three species. In *C. capitata*, the third exon of the gene is disrupted by 8150 bp insertion. In *B. dorsalis*, a 37 bp frame-shift deletion was detected in the first coding exon of the gene, introducing a premature stop codon while in *Z. cucurbitae*, a deletion (13 bp) also introduces also a premature stop codon but this time in the third exon of the gene.

In all three tephritids, the candidate white pupae gene was linked with a metabolite transport protein that contains a Major Facilitator-like superfamily domain. Interestingly, the white pupae gene was shown to be present in many insect species, suggesting that it can potentially be used as a generic selectable marker for the development of genetic sexing strains for SIT applications against insect pest species and human disease vectors.

Aedes aegypti *genetic sexing strains based on eye colour mutations for SIT applications*

Aedes aegypti is one of the major transmitters of arboviruses like Zika, dengue, chikungunya and yellow fever. Most vector control programs have largely relied on insecticide applications and removal of breeding sites, but these approaches have repeatedly failed to reduce *Ae. aegypti* populations efficiently and sustainably. The SIT can be applied as part of an AW-IPM suppression strategy to manage mosquito populations and the diseases they transmit. However, mosquito population suppression requires the development of efficient sex separation systems to limit the risk of releasing potentially pathogen-transmitting females. An efficient sex separation system will accurately separate males from females and eliminate the risk of releasing females. *Aedes aegypti* presents an evident sexual dimorphism at the pupal stage with females being larger in size than males. Using mechanical tools, *Ae. aegypti* is sex separated based on this trait, but the efficiency of this technique is highly variable since it depends to a great extent on the rearing conditions.

Staff of the IPCL has developed two GSS for *Ae. aegypti* using classical genetic approaches [Koskinioti et al. (2020). Genetic sexing strains for the population suppression of the mosquito vector Aedes aegypti. Philosophical Transactions B Royal Society (Phil. Trans. R. Soc. B) 376: 20190808. http://dx.doi.org/10.1098/rstb.2019.0808]. *Aedes aegypti* males are characterized by a dominant male-determining locus (M-locus) that resides on chromosome 1 and defines male development. Two eye colour genes, *red eye* (*re*) and *white eye* (*we*), are located on chromosome 1 and the inheritance of both mutant phenotypes is controlled by sex-linked, recessive genes. Two different strains, Red-eye GSS and White-eye GSS, were developed in which the males are heterozygous with wild-type eyes and females are homozygous with mutant eyes (red or white instead of black which is the wild-type colour) (Fig. 2). The recombination frequency in males was shown to be at the same level to the frequency in females, and therefore a filter rearing system was developed that was based on sorting at different stages. Combining different sex-specific or sex-linked characteristics at different stages, the recombinant progeny was removed in each generation, mainly at the pupal stage, and allowed the maintenance of pure Red-eye GSS and White-eye GSS colonies.

FIG. 2. Aedes aegypti *pupae: (a) black eye male, (b) red eye female and (c) white eye female. (Photo credit: Lucia Duran de la Fuente - FAO/IAEA)*

The quality control analysis of the two GSS involved evaluation of the genetic stability and rearing efficiency including productivity, immature development duration, sex ratio, and lifespan. Although there were no significant differences in sex ratio and immature development duration, the data for productivity and lifespan clearly showed that the Red-eye GSS is more prominent and, in addition, more genetically stable than the White-eye GSS.

Two of the most critical aspects for a successful SIT application are the flight ability and the mating competitiveness of the released sterile males. In order to evaluate both factors in the two GSS, a proper irradiation dose had to be selected beforehand that would be used as the reference value for all downstream experiments. Therefore, an irradiation dose-response curve was developed to determine the optimal dose for induced sterility. The selected dose was 90 Gy and it was shown that the irradiated Red-eye GSS was better than the irradiated White-eye GSS and the control strain regarding flight ability and similar to the control strain regarding male mating competitiveness. Accordingly, the evaluation of biological quality parameters and mating competitiveness of the irradiated males ranked the Red-eye GSS higher in the list of key performance indicators required for a successful GSS.

Encouraged by the great potential of the Red-eye GSS, we tested the ability of the irradiated males to suppress a target population in laboratory cage experiments. Two suppression cages and one fertile control cage were set up and bi-weekly releases of irradiated males proved to be sufficient to fully suppress a target laboratory cage population within six weeks.

Given that the red-eye phenotype is evident in as early as first instar larvae and the promising results of the Red-eye GSS mentioned above, it is clearly shown that figuring out a strategy that will eliminate female larvae based on the eye colour at early developmental stages is a worthy investment. Elimination of larvae early in the mass-rearing process can improve the rearing efficiency money- and time-wise.

Suppression of recombination in Aedes aegypti *genetic sexing strains using irradiation-induced inversions*

The development of any GSS requires genetic stability which is achieved either by the lack of recombination events in males (a common phenomenon in many Diptera) or by chromosomal inversions that can alter the recombination frequencies or suppress recombination events. In *Aedes aegypti*, recombination frequency in males is not suppressed and the genetic stability of a GSS faces the threat of the quick accumulation of recombinants and eventual collapse of the colony. Inversions that occur either naturally in the genome or are induced by irradiation can assist in the genetic stability of genetic sexing strains.

In a recent study performed at the IPCL [Augustinos et al. (2020). Irradiation induced inversions suppress recombination among the M locus and morphological markers in Aedes aegypti. BMC Genetics 21(Suppl 2):142], irradiation was used to induce inversions on the M chromosome of a wild type *Ae. aegypti* strain and the recombination rates between the M locus and the mutated loci that lead to the red-eye and white eye phenotypes were checked (re -M and the w-M, respectively) (Fig. 3). Screening of several lines resulted in the isolation of inversion lines that could significantly suppress recombination both in the re-M and the w-M regions. These inversion lines could be maintained in the laboratory under the normal rearing procedures, suggesting that the chromosomal rearrangements involved did not negatively impact the fitness of *Ae. aegypti*.

The re-M inversion line (Inv35) was incorporated in the Red-eye GSS and White-eye GSS and massive screening for consecutive generations of the eye phenotype and its linkage to the sex demonstrated extremely reduced recombination frequencies compared to the original GSSs. The biological quality of the Red-eye GSS with and without the inversion was evaluated and indicated that the inversion has a cost, reducing productivity and male flight ability both with and without irradiation. In a cage population suppression experiment, the inversion line of the Red-eye GSS managed to fully suppress the targeted laboratory population, thus further promoting our results for the incorporation of an *Ae. aegypti* GSS in an SIT application. The introduction of the irradiation-induced inversion in the Red-eye GSS strain has significantly reduced the probability of female contamination in the male release batches and all the above data converge to conclude that the irradiated Red-eye GSS males can be used in SIT applications to suppress or even locally eliminate *Ae. aegypti* populations.

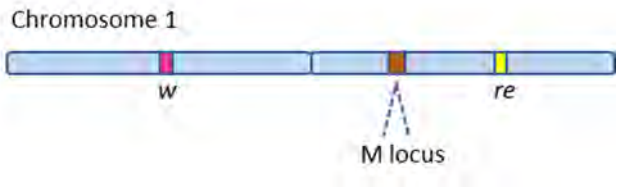


FIG. 3. M locus, re *and* w *genes on chromosome 1 of* Aedes aegypti

Livestock Pests

Tsetse colonies

The IPCL maintains colonies of tsetse to meet the needs and requirements of the various Member States. At present, colonies of seven tsetse species, i.e. *Glossina pallidipes, Glossina morsitans morsitans*, *Glossina morsitans submorsitans*, *Glossina morsitans centralis*, *Glossina brevipalpis, Glossina fuscipes fuscipes* and *Glossina palpalis gambiensis* and six strains are maintained at the IPCL.

Colonies of *G. pallidipes* and *G. morsitans* have been maintained for several years to support research on tsetse symbionts and viruses. This work significantly contributed to the continuous improvement in tsetse-rearing techniques to produce high-quality males needed for the successful implementation of the SIT for the control of tsetse in Africa.

The colony of *Glossina palpalis gambiensis* is the largest at the IPCL (Fig. 4) and has been maintained since 2009 to conduct research in support of efforts to create a tsetse-free zone in the Niayes area in the north of Dakar, Senegal. The colony is currently maintained at a level of 30 000, producing females to provide research material and supplying operational sterile males currently deployed in the tsetse eradication campaign in Senegal.

Recent interest in the use of the SIT for the control of the human African trypanosomiasis vector, *Glossina fuscipes fuscipes,* in Chad led to an increase of research activities on this species. To respond

to this increase in the need of research material, the colony of *Glossina fuscipes fuscipes* was enlarged and is currently maintained at a level of 5,000 producing females.

FIG. 4. A Glossina palpalis gambiensis *mating pair from the IPCL colony (Photo credit: Thomas Wallner)*

Evaluations of the new Near Infrared Pupal Sex Sorter (NIRPSS)

The success of the SIT depends on the release of competitive, sterile males into the natural habitat of the species targeted for control. Systems that efficiently separate males from females during mass production are needed as the release of sterile females will not contribute to the sterility in the field population and the females are needed for production. This is even more important in the case of tsetse. In contrast to most insects, female tsetse flies only produce a single larva in the uterus per ovulation cycle (10 days), resulting in a very low productivity. As a result of this low fecundity of tsetse, all the females are needed for colony production and a non-destructive method of sex separation is needed.

In most tsetse SIT programmes thus far, the adult flies are chilled, and the females separated from the males by hand. Hand separation is a very tedious and time-consuming task and the chilling adversely affects the quality of the flies. Other means of sex separation are possible. Tsetse females emerge from the pupae first and by manipulating the temperature conditions and the timing of emergence the sexes can be separated with an accuracy of 99%. However, the sexes can only be separated with this system at the end of the pupal developmental period and the accuracy very much depends on stability of the pupal incubation and emergence conditions. A further constraint arises when long distance shipment of irradiated male pupae is required, as this requires emergence of the female flies first and keeping the remaining male pupae at low temperatures (8-10 °C) for an extended time to prevent emergence during transportation. This lengthy exposure to these low temperatures has a significant negative effect on the quality of the males.

In the past, many attempts were made to separate the apparently identical male from female pupae. Differences in the shape, weight loss, density, floatation and electric capacitance were considered. Of the various techniques evaluated, near-infrared imaging provided the most promising results. By using near infrared light (NIR) to record the maturation of the tsetse pupae inside the puparium it was revealed that, because females develop faster than males, the darkening (melanization) of some of

the structures, e.g., eyes and wings of the flies in the ventral position of the pupae, become visible one day earlier in female pupae than in male pupae. On this particular day of pupae maturation, male and female pupae can be separated under near infrared light. This discovery led to the development of the Near InfraRed Pupae Sex Sorter (NIRPSS) (Fig. 5).

Within the NIRPSS the pupae are fed with the aid of vibration onto a moving belt into tracks. The belt movement and vibration cause the puparia to roll and move along the track. The NIRPPS captures high speed video data of the pupae under near infrared light that passes through the puparium shell. The pupae rotate as they move along the channel under the camera, so that a set of images in every relative position is analysed for each pupa. Based on the video images, a melanization index is calculated in real time by analysing the percentage of dark pixels in the different positions of the pupa. This melanization index ranges from 0 (un-melanized pupa) to 1 (fully melanized pupa). Based on this index, the pupae are sorted by a mechanical actuator at the end of the channel resulting in sex separation. The NIRPSS can sort males from female tsetse pupa with 89% accuracy five days before the emergence of the adults. Preliminary evaluation of the effect of NIRPSS on the quality of the males indicated that no significant wing damage could be found and that there was no negative affect on the male's ability to fly.

The NIRPSS is currently in operation at the IPCL and at Scientica in Bratislava, Slovakia. It is used for daily sorting of pupae that are shipped long distance to the tsetse eradication programme in Senegal. As the pupa can now be sorted five days before adult emergence, the male pupae can be shipped long distance at the normal developing temperature of 22-23 °C which has resulted in a 20% increase in the quality of the male flies that have been shipped to the Senegal programme.

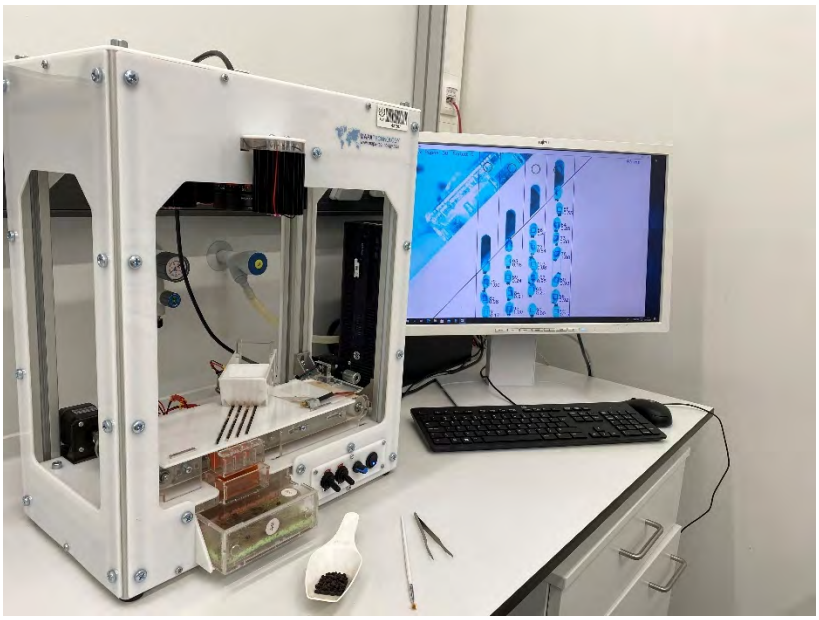


FIG. 5. The Near Infrared Pupae Sex Sorter (NIRPPS) and the images obtained as displayed on the computer screen

Tsetse pupae marking with fluorescent dyes

The sterile males that are released in SIT programmes must ideally be marked before release to monitor parameters that include their dispersal and survival, and sterile to wild male ratios. Currently a mixture of sand and fluorescent dye is used to mark the emerging tsetse flies. Sand is thoroughly mixed with the dye at a concentration of 0.25% to 0.50% (up to 1%) depending on the type of sand

used. Tsetse pupae are placed into a container and covered with at least 5 mm of the sand/dye mixture just before emergence. When the adult fly emerges, it breaks open the puparium with the ptilinum, a balloon-like structure in the head which pulsates so that it can also be used to maneuverer its way to the surface of the sand. During this manoeuvre the ptilinum become coated with dye. Once the tsetse is free from the sand, the ptilinum is withdrawn inside the head capsule and as the cuticle hardens the ptilinum and dye is permanently sequestered inside. These dye particles can be observed with a UV microscope in the crevice around the edge of the ptilinum (Fig. 6).

The possibility of using other mixing substrates such as sawdust, as well as dying the pupae directly, was investigated. Pupae were placed in sand or sawdust mixed with dye at a concentration of 0.20%. *Glossina palpalis gambiensis* pupae (n=650) were gently mixed with 0.08 g or 0.02 g of dye by rolling the pupa in the dye. After the adults emerged from the pupa, they were observed with a UV microscope camera for fluorescent marking with special attention given to the presence of the dye in the head capsule. For the tsetse that emerged from the sand mixture, 65% of the head capsules were adequately marked and only 25% of the flies had excessive dye on their bodies. The dye particles in the coarse sawdust mixture were not evenly distributed, resulting in only 10% of the head capsules being adequately marked. The flies that emerged from the pupae directly marked with 0.08 g of dye were all excessively marked. Of the flies that emerged from the pupa marked with 0.02 g of dye, 85% have had well-marked bodies but only 25% of the flies' head capsules were marked. Therefore, sand is still the recommended medium to apply fluorescent dye to tsetse, however the preliminary results of direct dying of tsetse pupae looks promising and prompts further investigation.

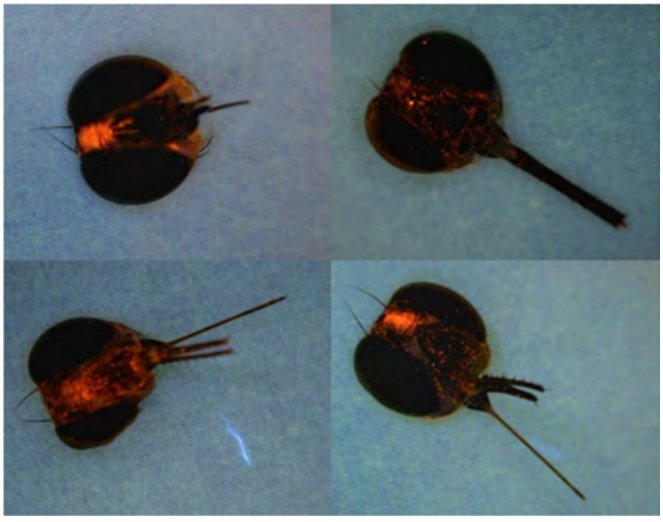


FIG. 6. Fluorescent dye particles inside Glossina palpalis gambiensis *head capsules*

Irradiation under hypoxia conditions reduced the negative impact of irradiation on Glossina morsitans morsitans

The sterile insect technique was successfully used to eradicate a population of *Glossina austeni* from Unguja Island, Zanzibar, United Republic of Tanzania in 1994-1997. Thereafter, there were several attempts to implement the SIT in other countries in mainland Africa, i.e. Ethiopia and Senegal. The quality of the sterile males remains a key factor for the success of any SIT programme, and therefore attempts to reduce the negative impact of the irradiation treatment used to sterilize the release males is required. Ms Caroline Mirieri, a PhD consultant from Kenya, assessed the feasibility of irradiation under low oxygen conditions (hypoxia) and its impact on the quality of the sterile males. The results indicated that *G. m. morsitans* males irradiated with several irradiation doses under hypoxia

conditions produced more pupae when mated with virgin females, as compared with females mated with males irradiated under normal conditions (normoxia) (Fig. 7). Although the quality of the sterile males seems to have improved, the residual fertility of the males sterilized by irradiation under hypoxia conditions might be an issue. Therefore, the dose needed to obtain 95% sterility in irradiated males and evaluating the residual fertility of the sterile males is in progress.

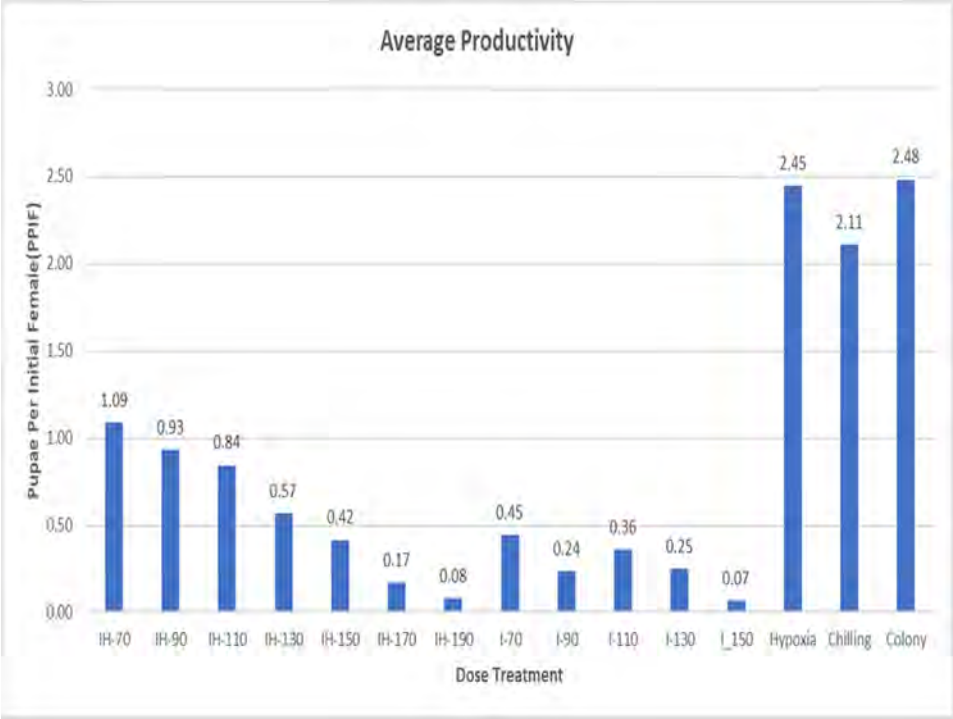


FIG. 7. Productivity of Glossina morsitans morsitans *virgin females mated with irradiated males under normoxia (I) or hypoxia at the late pupal stage (IH). Mated females were maintained under laboratory condition for 40 days after mating*

Prevalence of the tsetse symbiont Spiroplasma *in natural tsetse populations*

Tsetse flies are harbouring four symbiont bacteria that play an important role in tsetse biology including productivity, performance and their susceptibility to trypanosome infections. The symbionts bacteria are *Sodalis*, *Wigglesworthia*, *Spiroplasma* and *Wolbachia*. *Spiroplasma* was recently detected in some tsetse species including *G. f. fuscipes, G. tachinoides and G. p palpalis.* The impact of *Spiroplasma* infection on tsetse colony performance and the flies' biology has so far remained unknown. In addition, there is no knowledge on the prevalence of *Spiroplasma* infection in natural tsetse populations. Mr Mouhamadou Dieng, a PhD consultant from Senegal, assessed the prevalence of *Spiroplasma* infections in natural tsetse populations and the results indicate that the prevalence significantly varied with tsetse species and location (Fig. 8). The genotyping of *Spiroplasma* infection to determine its population genetics is ongoing.

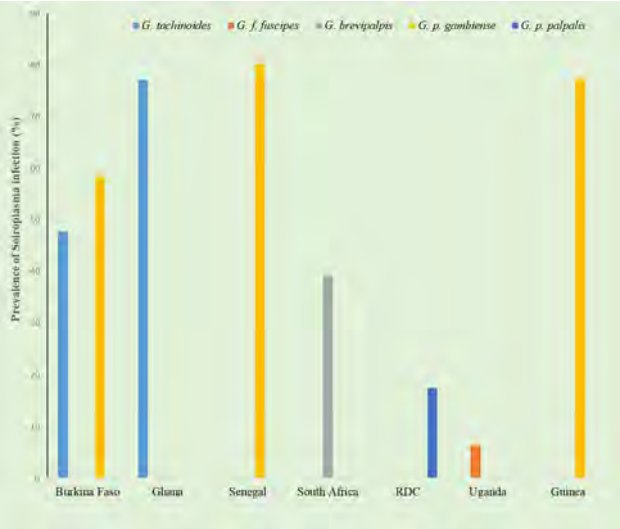
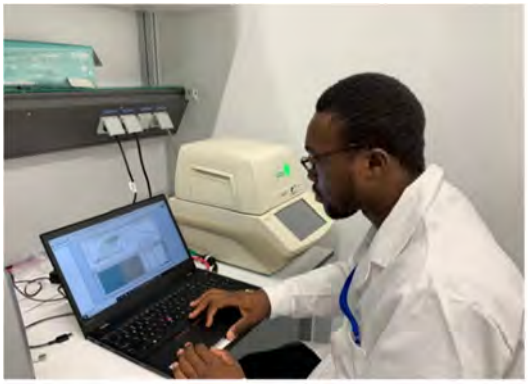


FIG. 8. Prevalence of Spiroplasma infection *in natural tsetse populations*

The impact of Spiroplasma *infection on* Glossina fuscipes *colony performance*

Mr Kiswenda-Sida Mikhailou Dera, a fellow from The Insectary of Bobo Dioulasso (IBD), Bobo Dioulasso, Burkina Faso, assessed the dynamics and the impact of the *Spiroplsama* infection on *G. f. fuscipes* performance. This work was carried out in collaboration with Prof. Serap Aksoy and Dr Brian Weiss from Yale University, USA. The results indicate that *Spiroplasma* infection seems to reduce the mating ability and the productivity of the females (Fig. 9). *Spiroplsma* seems to be mainly transmitted maternally (from females to offspring), however, paternal transmission cannot be excluded.



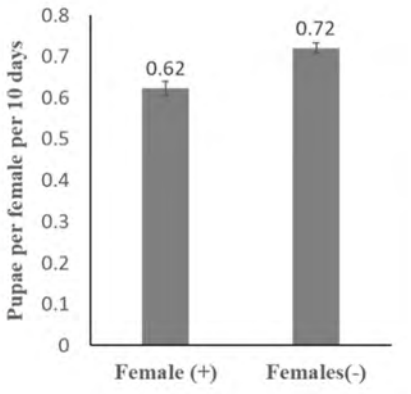


FIG. 9. (left) Mr Kiswenda-Sida Mikhailou Dera analysing the Spiroplasma *density in* Glossina fuscipes fuscipes *flies using qPCR. (right) Productivity of G. f. fuscipes females infected (+) and not infected (-) with* Spiroplasma

The impact of iflavirus and negevirus infection on tsetse fly performance

In addition to the four symbiont bacteria, tsetse flies harbour three groups of viruses that may play an important role in tsetse biology including productivity and performance. So far, three viruses have been detected in tsetse colonies, i.e. (1) the salivary gland hypertrophy virus (SGHV) which has infected many species but only causes severe fecundity problems in *G. pallidipes* colonies, (2) the iflavirus and negevirus first detected in *G. m. morsitans* and later found in many tsetse species except *G. pallidipes*. Ms Hannah Huditz, a PhD consultant from Austria, is currently investigating the impact of iflavirus and negevirus infection on tsetse performance. This research is done in collaboration with

Prof. Monique van Oers from Wageningen University, the Netherlands and Prof. Wolfgang Miller from the Medical University of Vienna, Austria. The preliminary results indicated that both iflavirus and negevirus are present in different tissues of tsetse adults including the brain, milk glands, salivary glands, testes and ovaries. The presence of these viruses in salivary glands might suggest the potential of horizonal transmission from infected flies to uninfected flies through *in vitro* blood feeding. The presence of the virus in reproductive tissue (testes and ovaries) and the milk glands might indicate the potential of vertical transmission from mother to offspring (Fig. 10).

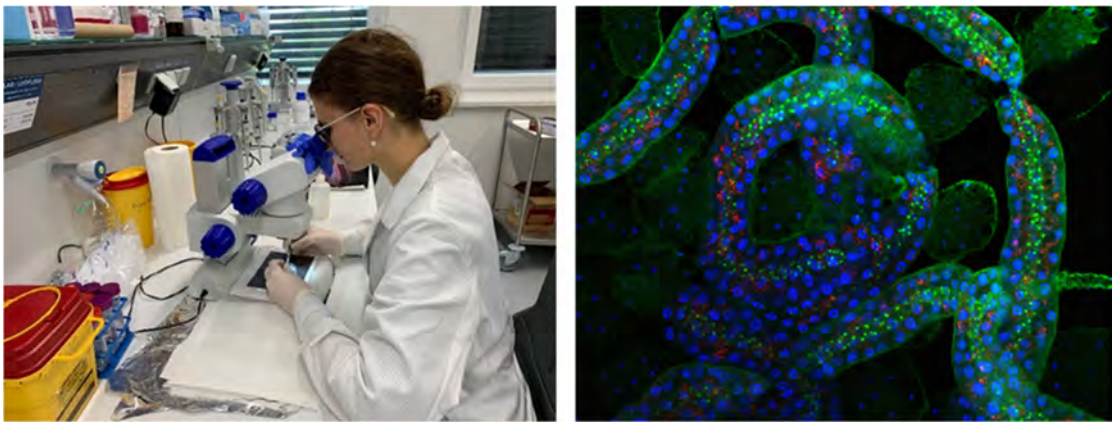


FIG. 10. (left) Ms Hannah Huditz, a PhD consultant from Austria, assessing the prevalence of virus infections in tsetse tissues, and (right) the presence of both iflavirus and negevirus in milk glands and body fat of 30-day old female G. m. morsitans *(red=iflavirus, Green= F-actin, Blue= Nucleus - Magnification 20x)*

Microsatellites for Glossina austeni *and* Glossina brevipalpis

The SIT is a species-specific control method that needs to be applied on an area-wide basis, i.e. against an entire insect population. Knowledge on the degree of isolation of the targeted insect populations is therefore crucial to be able to develop appropriate intervention strategies. Population genetics studies that assess gene flow between different populations has been used extensively in the past to determine the degree of isolation of tsetse populations, e.g. *G. p. gambiensis* in Senegal.

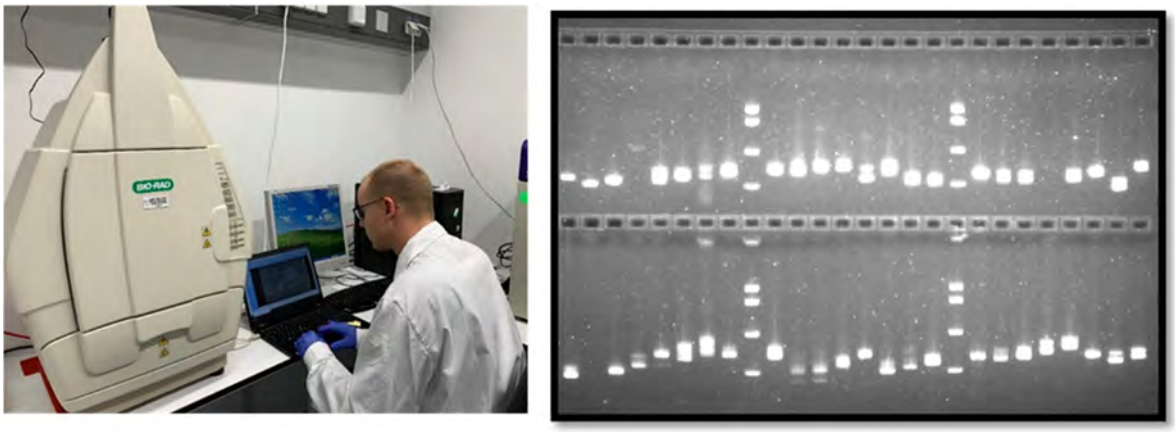


FIG. 11. (left) Mr Fabian Gstöttenmayer screening the potential microsatellites using 4% gel electrophoresis. (right) Gel electrophoreses of different primer pairs of Glossina brevipalpis *colony flies*

Microsatellites are often considered as ideal markers for these studies, but these were lacking for the two species, *Glossina austeni* and *Glossina brevipalpis*. Mr Fabian Gstöttenmayer, an intern from Austria, assisted with the selection of primers flanking multiple repeat regions that might serve as

specific microsatellites for these species. Using the available sequences for these species, 200 pairs of primers were selected and tested for amplification of short sequences. Primers that met the selection criteria (amplifying the expected product size, produce different bands with haploid and diploid forms) were selected for further analysis (Fig. 11).

Human Disease Vectors

The work of the Human Disease Vectors group of the IPCL has focussed on improving the various steps of the SIT package against mosquitoes and to transfer knowledge, protocols and training materials to support capacity building in Member States towards pilot suppression trials in selected field sites.

Efficiency of an automatic mosquito pupae sex sorter for Aedes *mosquitoes*

For SIT-based approaches or any other population suppression approach, the elimination of female mosquitoes prior to male releases is mandatory for its application. However, separating millions of male mosquitoes per day for large-scale release operations is challenging. Existing methods such as metal sieving plates and the Fay-Morlan glass separator are time-consuming or have so far failed to eliminate all the females in order to achieve male-only releases for SIT or other related applications. Therefore, there is a need to develop automated and efficient sorting methods to ensure reasonably consistent sex separation with an acceptable level of female contamination for large-scale release operations. The automatic mosquito pupae sex sorter developed by the Chinese company Wolbaki Biotech (Fig. 12) is presently being used successfully to separate the sexes of a *Wolbachia*-infected *Ae. albopictus* strain based on pupal size dimorphism. The machine was loaned to the IPCL and its efficacy evaluated against the manual sorting method using the Fay-Morgan glass plate separation for both *Ae. albopictus* and *Ae. aegypti*. The results were encouraging, with less than 1% female contamination in male pupae in both species together with a significant reduction of manpower and increase in time efficiency. In the conditions of present mosquito rearing, the automatic separator is able to separate 100 000 *Ae. albopictus* males in 1 h 30 m and the same number of *Ae. aegypti* males in 2 h. In comparison, 5 to 6 hours are needed to do so with the manual glass sorter. This automatic mosquito larval counter is a promising instrument to standardize the separation process and therefore increases time efficiency and reduces manpower for sex sorting of *Aedes* mosquitoes.

FIG 12. The automatic mosquito pupae sex separator

Advances in the understanding of mosquito irradiation and the factors that affect dose response

The SIT against mosquitoes relies on the reliable induction of sterility in the target insect population. For this, dependable irradiation protocols are required to ensure constant and high levels of induced sterility, whilst maintaining the highest possible quality of the sterile males. With the aim to standardize irradiation and pre-irradiation handling protocols, it is important to identify critical factors that affect dose responses in mosquitoes. Biological factors that were investigated include the geographic origin of the mosquito strains, pupal size and age at which they are irradiated, and life stage of the mosquito. Physical factors included ambient temperature, ambient atmosphere, and irradiation source and dose rate. Possible variations in handling procedures, and data collection methods and their effects on sterility results were also investigated. The variables that were found to be most important, causing significant effects in dose response were pupal age, oxygen-poor atmospheric environments, and irradiation source. Factors affecting dose response but to a lesser degree were ambient temperature, life stage, and differences in handling protocols. Geographic origins of mosquito strains and pupal size did not have any significant effects. Guidelines for the routine sterilization of mosquitoes at pupal stage have been formulated, as were protocols for assessing dose-response curves in *Aedes* and *Anopheles* spp.

A new aerial release device for sterile male mosquitoes

The SIT is an effective genetic control method to manage insect populations. However, it is crucial to release sterile mosquitoes by air to ensure homogeneous coverage, especially in larger areas. The FAO/IAEA has recently reported on a successful trial that released sterile mosquitoes with a drone in Brazil. However, given the restrictive regulations in terms of size and weight of drones allowed in urban areas, a smaller version of a release device for chilled sterile *Aedes* male mosquitoes was designed (weight <250g including 50k mosquitoes). The device is mainly composed of an insulated mosquito-holding chamber with a layer of mosquitoes less than 45 mm to avoid mechanical damage by compaction. The device has a release mechanism that accepts pre-set release plans based on a list of geo-referenced waypoints and has its own release rate control. The device was loaded with batches of chilled/marked/compacted/irradiated/ male mosquitoes and their quality was tested under two room temperature conditions (5°C and 26°C) in terms of survival, flight ability and mechanical damage according to the level of compaction (top, bottom) within the release device. The quality was significantly impacted by room temperature whereas no difference was observed between males at different positions (top, bottom) within the holding chamber and the control (non-released males). The chilled release machine will be tested in the field to assess whether it can be used both for ground and aerial releases of chilled mosquitoes.

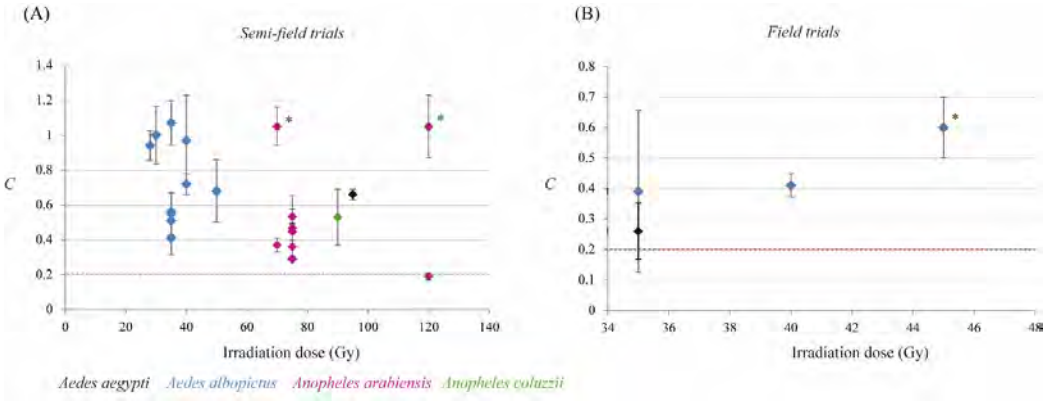


FIG.13. Competitiveness (Fried index) of irradiated male mosquitoes measured in semi-field trials (A) and field trials (B), as a function of the irradiation dose (Source: Bouyer & Vreysen, 2020)

Competitiveness of irradiated male mosquitoes

The competitiveness of a sterile male is the odds of a wild female mating with a sterile male compared to a wild male when exposed to both in equal numbers and it is generally measured through the Fried index. Adequate sexual competitiveness of sterile males is a prerequisite to apply the SIT. We reviewed the semi-field and open field trials conducted over the last decade, which demonstrated that irradiated male mosquitoes can be competitive if produced in appropriate conditions.

A reduction in quality of the produced sterile male insects may be observed but is generally more related to mass rearing, handling, marking and release processes rather than radiation *per se*. When all these processes are mastered, a good competitiveness of sterile male mosquitoes is observed and the C value was above 0.2 in all reported studies, which is considered the lower threshold for cost-effective projects (Fig. 13). A guideline on Mark-Release-Recapture of sterile male mosquitoes was formulated to provide Member States with protocols to assess field competitiveness before launching a control campaign, as recommended in the phased conditional approach for testing SIT against mosquitoes.

Plant Pests

Phytosanitary treatments under the FAO/IAEA/USDA agreement

The tolerance of quarantine fruit fly species to phytosanitary irradiation and cold treatments was evaluated under the FAO/IAEA/USDA agreement on "Harmonization of phytosanitary treatments for exotic fruit flies". This agreement has made substantial contributions to the development and validation of cold and irradiation treatments.

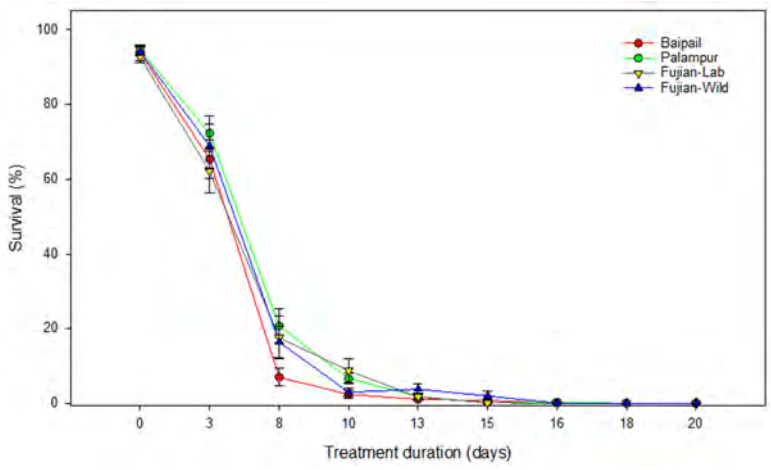


FIG. 14. The effect of sublethal doses of cold treatment (< 1.7°C) on the survival of Zeugodacus tau *third instars from Baipail, Palampur, and Fujian*

Experiments assessing the cold tolerance of *Zeugodacus tau* populations from Bangladesh (Baipail), China (Fujian-Wild and Fujian-Lab), and India (Palampur) were completed. As shown in Fig. 14, our results suggest that third instars of *Z. tau* from Bangladesh, China, and India responded differently to the cold treatment of < 1.7°C only at sublethal doses (up to 15 days). Only one larva of a wild population from China survived after 20 days of cold treatment. Therefore, additional research has been conducted to evaluate the tolerance of the wild population from China to a cold treatment of 22 days (Fig. 15). Our goal is to provide scientific evidence supporting a potential treatment schedule of 22 days at < 1.7°C that could be used as a stand-alone option to ensure quarantine security against all *Z. tau* populations. Results from this research have the potential to support an official cold treatment proposal against *Z. tau* infesting oranges, which can benefit Member States of the FAO and IAEA.

We have published a study evaluating the effect of low oxygen treatments on the efficacy of phytosanitary irradiation treatments. Our results showed that fruit fly third instars irradiated with approved phytosanitary irradiation doses in either hypoxia or severe hypoxia failed to emerge as adults. Hence, we have concluded that it is safe to apply phytosanitary irradiation under modified atmospheres for treatments targeting tephritid fruit fly species. This conclusion supports the ink amendment to remove the restriction of phytosanitary irradiation application under modified atmosphere (low O_2) against fruit flies from 7 PTs in ISPM 28. With the rescheduling of the fifteenth session of the Commission on Phytosanitary Measures (CPM-15) due to the COVID-19 pandemic, that ink amendment will be virtually evaluated on March 16, 2021. The removal of restrictions from phytosanitary irradiation treatments can increase the applicability of nuclear technology in agriculture, reduce quarantine restrictions, and facilitate agricultural trade among FAO and IAEA Member States.

The IPCL is currently evaluating the effect of radiation source and dose rate on the survival of *Ceratitis capitata* third instars treated with several radiation doses, including doses approved as phytosanitary treatments. Research on fruit fly tolerance to vapour heat treatment using a controlled atmosphere temperature treatment system (CATTS) chamber are planned to begin in the second semester of 2021.

FIG. 15. Ms Inajara Viana Gomes and Mr Fabio Luís Galvão, interns from Brazil, dissecting fruit infested by Zeugodacus tau *after exposure to cold phytosanitary treatment*

Drosophila suzukii

The rapid spread of the Spotted Wing Droshophila *Drosophila suzukii* and the subsequent economic losses of the affected areas has encouraged the development of different approaches for the efficient management of this pest. The SIT can potentially be integrated in area-wide integrated pest management (AW-IPM) approaches to manage this pest under confined environment systems such as greenhouses. Staff of the IPCL have been working on the development of the SIT package for *D. suzukii* including determination of the optimum sterilization dose, development of effective mass-rearing procedures and quality control protocols as well as the assessment of their mating behaviour. To date, the conventional larval diet for rearing the Mediterranean fruit fly, *Ceratitis capitata*, has been used to rear *D. suzukii*. However, this larval medium is not optimal for *D. suzukii* and efforts have

been undertaken to develop a more specific larval diet that allows improved larval rearing efficiency as well as facilitating separation of larvae from pupae in the diet (Fig. 16).

FIG. 16. Larval diet for Drosophila suzukii *based on potato powder*

Different ingredients and recipes have been tested to identify economically viable formulations that could be easily adapted to mass-rearing conditions for *D. suzukii*. A very promising formulation that uses potato powder or sweet potato powder as a bulking agent was first developed at the Instituto de Sanidad y Calidad Agropecuaria de Mendoza (ISCAMEN), Mendoza, Argentina and is now being tested at the IPCL.

FIG. 17. Ms Paloma Guazzelli Della Giustine, a fellow from Brazil performing irradiation experiments to determine irradiation dose-response curves for Anastrepha fraterculus

Anastrepha fraterculus

The South American fruit fly, *Anastrepha fraterculus* (Diptera: Tephritidae) is a pest that has a major impact on the fruit industry of Brazil and all other countries in South America. The SIT can be an additional control tactic to manage this pest on an area-wide basis. Significant advances in the domestication and artificial rearing technology for *A. fraterculus* have been made. As reported in previous newsletters, an *A. fraterculus* GSS based on a colour mutation of the pupae (black pupae) was developed from a laboratory population of *A. fraterculus* morphotype 1, distributed in the north and south of Argentina. Ms Paloma Guazzelli Della Giustine, a fellow from Brazil, was involved in the assessment of the biological characteristics of the strain during a 10-month TC fellowship. One main

outcome of this work was the development of the complete radiation dose response curve for both males and females (Fig. 17). This achievement will greatly facilitate the selection of the appropriate irradiation dose for SIT application in field action programmes against this important pest. The black pupae GSS was transferred to the Centre of Nuclear Energy in Agricultura (CENA) and the University of Sao Paulo in Piracicaba, Sao Paulo, Brazil, where a colony has been established to be used in a future male-only release pilot project in the south of Brazil.

Raycell MK2 Blood Irradiator for SIT

Mr Yeudiel Gomez Simuta, a consultant from the Moscafrut Program, Mexico, was recruited to characterize and assess the performance and dosimetry of a "Raycell MK2", an X ray blood irradiator produced by Best Theratronics Inc. (Canada). The irradiator consists of two units: one containing the two X ray tubes, a control system, a power supply and the canister, and the other containing the heat-exchanger for cooling the tubes (Fig. 18). X rays are produced by bombarding targets with electrons, which are accelerated in a vacuum through a high-voltage electrical field. The dose is delivered by two opposing X ray tubes, with the sample canister located centrally between them. The two cone-shaped beams ensure that a tight dose is delivered to the product and good dose uniformity is obtained. Dose delivery is controlled by setting and monitoring the irradiation time, based on the central dose rate. Irradiation time is controlled by two independent microprocessors. The advantage of X ray generators is that dose variation effects can be reduced, once the energy is kept constant and the range of variation can be obtained through dose-mapping knowledge. The variation can be reduced through adjusting the parameters of the equipment and/or configuring the volume of the irradiation products. The primary findings in the characterization of this equipment showed that the dose variation in a vertical way inside the irradiation container is very small, with a DUR of 1.2.

FIG. 18. The Raycell MKII

This data helps to ensure that all insects in the irradiation chamber will receive the dose between the specific range, fulfilling one of three main process control elements recommended by the SIT to avoid release of insects that are significantly under-dosed. These three elements which control various steps in the irradiation process are: Sterility Testing, Routine Dosimetry and Radiation Sensitive Indicators and thus, they complement each other. Another advantage of the incorporation of ionizing radiation produced by electric energy is that the delivered dose is not affected by the decay factors, e.g. as with gamma rays. For comparison, the absorbed dose obtained from the MK2 X ray generator is similar to what would be received by exposing a sample for 4.2 minutes in a cobalt-60 gamma source type Gammacel-220 with an activity of 1 000 Curies (3.7 x 1013 Bq); however, in a cobalt-60 gamma source, it would be necessary to increase the exposure time each year with 12% in order to get the same dose, whereas the X ray generator will keep the same irradiation time during the whole year. In addition, the X ray machine can be turned off when not being used. In conclusion, the findings of the small dose variation within the irradiation chamber of the Raycell MK2-ray blood irradiator support the possibility that this X ray generator could be used in pilot or medium-scale SIT projects as an alternative to self-shielded gamma irradiators.

New improved Mediterranean fruit fly VIENNA-8 genetic sexing strain

In the standard VIENNA-8 GSS of the Mediterranean fruit fly, males emerge from brown pupae and are resistant to high temperatures, while females emerge from white pupae, are sensitive to high temperatures and can be eliminated at the embryonic stage. Females, however, have a slower development rate as compared with males. Recently it was demonstrated that slower development observed during the larval stage is driven by a gene that is not associated with the *temperature sensitive lethal* (*tsl*) phenotype. Using classic genetic approaches, a novel white pupae and temperature sensitivity lethal strain "fast development" (*wp tsl* FD) was isolated which showed faster larval development in addition to differences in its temperature sensitivity compared with the standard *tsl* strain.

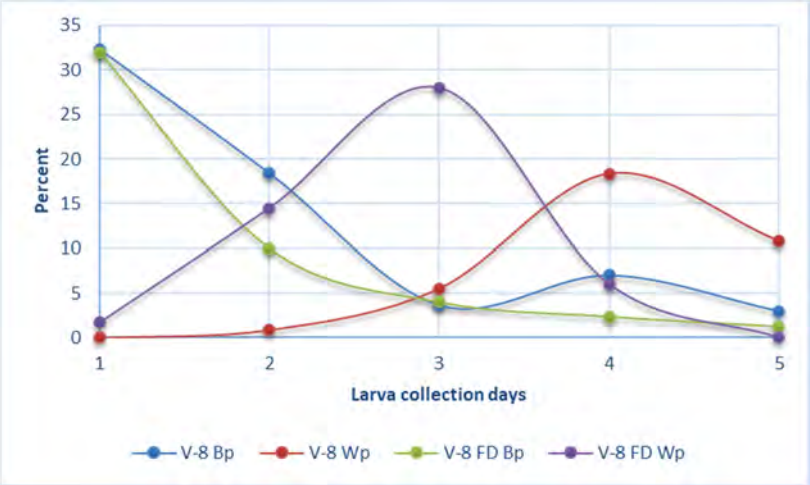


FIG. 19. Percent male (brown pupae, Bp) and female (white pupae, Wp) of larvae of the Mediterranean fruit fly GSS Vienna-8 (V-8) and VIENNA-8 FD (V-8 FD) strain collected during a 5 day-collection period

The introgression of this novel *wp tsl* FD phenotype into the VIENNA 8 strain, resulted in a new strain (VIENNA-8 FD), where females showed a shorter larval development time, differences in the thermal sensitivity and productivity profiles. Under standard mass-rearing conditions, the females of the *tsl* strain VIENNA-8 need 8 to 9 days to complete their larva development, which is 2 or 3 days more than the males. A faster development of females would entail that the females complete their larval development simultaneously with the males, which would allow optimization and cost reduction of the mass-rearing process (Fig. 19) through a reduction of the quantity of larval diet and rearing space.

The strain is ready to be tested in mass-rearing facilities and interested Mediterranean fruit fly SIT action programmes should contact Marc Vreysen, Head of the Insect Pest Control Laboratory, to request the strain. More information on the recent publication:

(https://academic.oup.com/jee/advancearticle/doi/10.1093/jee/toaa220/5918279?searchresult=1)

CAPACITY BUILDING AND SERVICES

In 2020, the IPCL hosted 2 cost-free experts (CFE) and 7 consultants (C) (of which 3 were PhD students), 9 interns and 5 fellows (F). The fellows were funded by the IAEA'S Department of Technical Cooperation.

Name	Country	Status	Duration	Topic
DA SILVA, Fabio	Brazil	Intern	4 mth	Phytosanitary treatments
ESPINAL PEREZ, Tania	Mexico	Intern	3 mth	Characterizing GSS of fruit flies
LIMA, Inajara	Brazil	Intern	12 mth	Phytosanitary treatments
CARAVANTES, Luis	Guatemala	Intern	1 mth	Phytosanitary treatments
SANCHEZ AGUILAR, Jhonatan	Mexico	Intern	10 mth	Characterizing GSS of fruit flies
GOMEZ Simuta, Yeudiel	Mexico	C	12 mth	Characterizing new X ray machine
SIMOES DIAS DE CASTRO, Vanessa	Brazil	C	4 mth	Phytosanitary treatments
GUAZELLI DELLA GIUSTINA, Paloma	Brazil	F	3 mth	Fruit fly rearing and quality control
TIENDRBEOGO, Antoine	Burkina Faso	F	2 mth	Fruit fly rearing and quality control
MIRIERI, Caroline	Kenya	C (PhD)	12 mth	Impact of stress factors on tsetse flies
DIENG, Mouhamadou	Senegal	C (PhD)	12 mth	Tsetse symbionts and refractoriness to trypanosome infection
NAWAZ, Arooj	Pakistan	C	12 mth	Maintenance of a tsetse production unit for the support of the tsetse SIT in Senegal

Name	Country	Status	Duration	Topic
HUDITZ, Hannah	Austria	C	6 mth	Analysing the role of Iflavirus and Negavirus presence in tsetse colonies for more cost-effective mass rearing
GSTÖTTENMAYER, Fabian	Austria	Intern	5 mth	Development of microsatellite markers for population genetics studies of *Glossina brevipalpis* and *Glossina austeni*
TANG, Zhaoyang	China	Intern	5 mth	Detection of mosquito-borne viruses in mosquito colonies
CANIC, Sumejja	Bosnia & Herzegovina	Intern	5 mth	Impact of NIRPSS for tsetse pupae
TAQI, Syeda	Pakistan	Intern	1 mth	Development of tsetse SIT packages through colony maintenance
KISWENDA-SIDADERA, Mikhailou	Burkina Faso	F	11 mth	Analysis of tsetse population genetics
MOLEFE Moyaba, Percy	South Africa	F	3 mth	Tsetse rearing
GOUVI, Georgia	Greece	C (PhD)	12 mth	Cytogenetics in tephritid species
SOLLAZZO, Germano	Italy	CFE (PhD)	12 mth	Temperature-sensitive lethal genes in tephritid species
GRIGORIOU, Maria-Eleni	Greece	CFE	5 mth	White pupae gene in *Bactrocera correcta* and *Bactrocera olaea*

Name	Country	Status	Duration	Topic
NTOYI, Nonhlanhla	South Africa	F	3 mth	Assessment, management and maintenance of *Anopheles arabiensis* sexing strains

In 2020, the Genetics and Molecular Biology (GMB) group maintained 11 species of fruit flies (130 strains/colonies/populations) and 3 species of mosquitoes (66 strains/colonies/populations in total). The Plant Pests (PP) group maintained 17 species of fruit flies (65 strains/colonies/populations), the Livestock Pests (LP) group maintained 7 tsetse species (6 strains) and the Human Disease Vectors (HDV) group maintained 3 mosquito species (11 strains).

The GMB and PP groups carried out 49 fruit fly shipments to 21 institutions in 10 countries (Italy, Senegal, Kenya, Belgium, Tunisia, USA, France, Austria, Spain and Germany), and 4 shipments of preserved fruit flies to the USA and Belgium. The LP group carried out 71 tsetse shipments of 60,767 pupae (54,067 *G. palpalis gambiensis* pupae to Senegal) to 6 institutions in 5 countries (Senegal, France, UK, USA and Italy). The HDV group carried out 12 mosquito shipments to 5 institutions in 4 countries (France, UK, Sweden and Austria).

In 2020, the IPCL received 91 visitors from 25 countries.

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THE PLANT BREEDING AND GENETICS LABORATORY

EXECUTIVE SUMMARY

Conventional mutation-assisted breeding using physical or chemical mutagenesis techniques has produced superior crops with higher yields, tolerance to plant pests and diseases and abiotic stresses such as drought, salinity and heat. Crop mutation breeding has had a global impact on agricultural productivity worldwide with a track record of safety for food, feed and the environment.

Research at the Plant Breeding and Genetics Laboratory (PBGL) is focused on integrating innovations in science and technology with conventional crop mutation breeding approaches to increase the efficiency, speed and precision of crop mutation breeding. Under the overall umbrella of Functional Genomics for Trait Utilization, PBGL is leveraging advances in genomics technologies, big data analysis and innovative genotyping tools for genome-wide mutant trait discovery and targeted mutant selection. In addition, innovations in plant cell tissue culture are combined with mutation induction techniques to improve mutation breeding of vegetative crops and trees which have lagged, behind the annual seed crops.

In 2020, PBGL's R&D on transboundary pest and disease resistance continued, focusing on Fusarium Wilt in desert banana caused by the fungus *Fusarium oxysporum* Tropical Race 4 (TR4); *Striga* (witchweed) in rice and sorghum; and, leaf rust disease in arabica coffee caused by the fungus *Hemileia vastatrix*. According to FAO, the economic losses attributed to the parasitic weed *Striga* in Africa amount to USD 7 billion annually. Similarly, coffee leaf rust has created recent epidemics in Central and Southern America while Fusarium Wilt TR4 is threatening Cavendish banana production in top banana exporting countries such as Ecuador, following the spread of TR4 to Colombia in 2019.

R&D on the development/adaptation and validation of glasshouse and laboratory screening protocols for resistance to *Striga* in cereals under the *Striga* CRP (D25005) continued in 2020. The laboratory-based extended gel assay to investigate the *Striga* resistance mechanism was improved, enabling quantitative analysis of the resistance mechanism in the mutant sorghum lines. Using this protocol two key steps in the *Striga* infection pathway, i.e. *Striga* seed germination and the formation of haustoria, could be quantified in a single bioassay. This improved bioassay was applied to 13 *Striga* resistant sorghum lines and can be used to link distinct *Striga* resistance mechanisms with underlying genetic variants in subsequent genetic mapping and marker development studies.

In 2020, a computational workflow for Next Generation Sequence (NGS) analysis of induced mutations in plant genomes was completed. The workflow enables genome-wide analysis of NGS data sets and the identification of informative sequence variants, Single Nucleotide Polymorphisms (SNPs) and Insertion/Deletions (InDels) in particular. The workflow ties together publicly available data analysis tools into a single package, intended to mainstream advanced genomics tools to non-expert users in Member States. At PBGL, the computational workflow has been successfully used for genome-wide characterization of induced mutations in sorghum, cowpea, and for exome capture data in arabica coffee.

To convert DNA sequence variations into a usable format for plant breeders, additional tools are required. A fluorescence-based medium-throughput genotyping platform was established at PBGL to tie specific traits, phenotypes and functions to specific DNA variants, genes or genome regions for Marker-Assisted Selection (MAS). The genotyping platform is based on differential PCR amplification of mutant versus wild type alleles (KASP assay) and has been successfully applied for rapid introgression of the barley Orange Lemma feed quality trait into three different genetic backgrounds through MAS. In addition, two new markers have been developed that are closely linked (ca 5-6 cM) to the early maturity/semi-dwarf trait in sorghum using this assay.

In 2020, laboratory protocols for efficient mutation induction of single cell cultures of arabica coffee using the chemical mutagen EMS were developed under the coffee and banana disease CRP (D22005). The method allows to establish a kill curve within 3-4 weeks and determine optimal dose(s) for mutation induction. Some 50 EMS mutant plantlets have been regenerated which are expected to be homohistont, i.e. non-chimeric. Chimerism presents a challenge for most vegetative crops and trees given their long reproductive cycle and their heterozygous nature. In addition, the protocol can be significantly scaled up for the cost-effective production of large mutant populations and can serve to establish novel, *in vitro* based selection schemes.

Further, an exome capture kit was developed for arabica coffee and successfully tested at pilot-scale using M_1 mutant coffee plants developed at the PBGL. The kit allows cost-effective genome-wide mutant analysis in arabica coffee and is commercially available for wider use by Member States.

In 2020, staff of the PBGL published eight scientific papers in peer-reviewed articles and drafted 13 protocols as contributions to three CRP protocol books: 'Efficient Screening Techniques to Identify mutants with TR4 Resistance in Banana'; 'Mutation Breeding for Resistance to Striga in Cereals'; and, 'Mutation Breeding in Coffee with Special Reference to Coffee Leaf Rust' plus a laboratory protocol 'A Software Workflow for Automated Analysis of Genome (Re-)Sequencing Projects'.

In 2020, the PBGL hosted six interns and two fellows for training in conventional mutation breeding techniques, *Striga* precision phenotyping, barley genotyping and MAS and analysis of big data in genomics. The PBGL delivered 24 shipments to 19 Member States covering a total of 124 samples. The number of irradiation treatments and fellows hosted at the PBGL in 2020 was reduced compared to 2019 due to COVID-19 related restrictions on international travel.

STAFF

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Luvai, Faith[10]	Intern

[1]left in December 2020; [2]left in May 2020; [3]May to December 2020; [4]joined in December 2020; [5]May 2019 to March 2020; [6]June 2019 to June 2020; [7]joined in September 2020; [8]October 2019 to July 2020; [9]joined in October 2020; [10]joined in December 2020

MAJOR ACHIEVEMENTS IN RESEARCH AND DEVELOPMENT

An improved extended bioassay for quantitative analysis of gamma-ray induced Striga *resistance mechanisms in advanced sorghum mutants*

The parasitic weeds *Striga asiatica* and *Striga hermonthica* are major biological constraints to cereal production in most of Sub-Saharan Africa and semi-arid tropical regions of Asia. According to FAO estimates, up to 50 million hectares of crop land are infested in Africa, causing an annual loss in cereals in excess of USD 7 billion. The control or management of the *Striga* pest is particularly challenging because it infects the roots of its host plant and remains invisible until the time when it emerges from the soil. By then, the damage to the host plant is already inflicted. Because the damage occurs underground, conventional weed control measures cannot be applied.

The main objective of the CRP D25005 on 'Mutation Breeding for Resistance to *Striga* Parasitic Weeds in Cereals for Food Security' is to develop laboratory, screenhouse and field screening protocols of mutant populations of sorghum and upland rice for resistance to *Striga asiatica* and *Striga hermonthica*. In addition, the CRP focuses on technologies such as rapid cycling of generation of crop plants, doubled haploid techniques and molecular markers to enhance the efficiency of mutant identification and accelerate delivery of resistant varieties.

In 2020 the PBGL continued the R&D activities on the development and improvement of laboratory bioassays for the identification and quantification of *Striga* resistance mechanisms in advanced, *Striga* resistant mutant sorghum mutants. The extended laboratory gel-assay protocol was optimized to enable prolonged co-culture of *Striga* seeds with seedlings of resistant mutants compared to wild parents. Cleaned *Striga* seeds were settled in solidifying agar (0.8%) in plastic petri-dishes. Improvements included the use of larger petri-dishes which allowed longer incubation. After 1-2 weeks the distance between the furthest germinated *Striga* seeds and the root was scored. In addition, after three weeks haustorium formation could be scored simultaneously with *Striga* seed germination. The principle is that the higher the germination stimulant produced by the host plant root the longer the distance to *Striga* seeds stimulated to germinate by the root as illustrated in Fig 1. The protocol was applied to 13 advanced sorghum mutants.

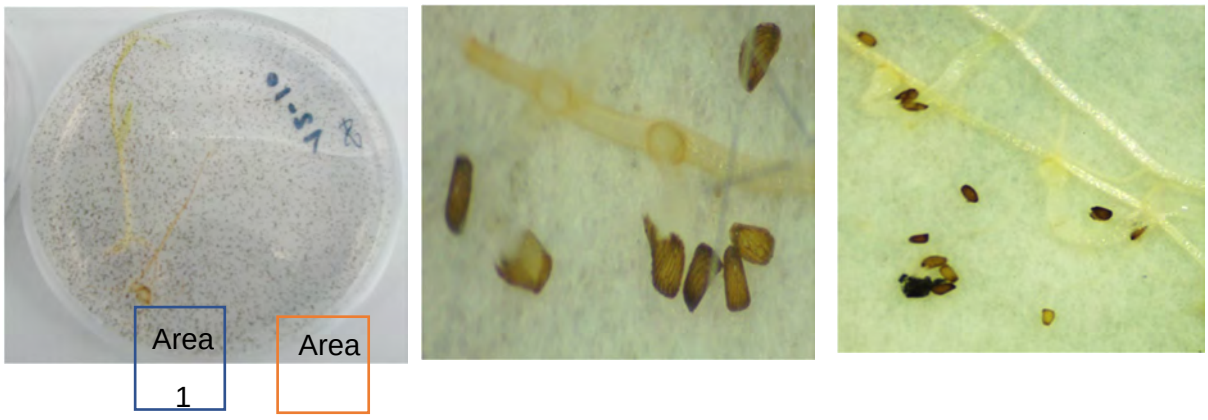

Fig. 1. Example extended gel bioassay protocol illustrating quantification of two key steps in the Striga infection pathway: Germination of Striga seeds (left) and haustorium development (middle and right)

A typical result from a comparative quantitative analysis between susceptible control, resistant control and resistant mutant sorghum is summarized in Table 1. As shown, the resistance phenotype of the mutant sorghum is confirmed. In the example, the BF3 sorghum mutant shows significantly reduced *Striga* seed germination and significantly reduced haustorium development compared to its parental line.

Genotypes	Max. Germ. Distance (cm)	Germ. % in Area 1- after 3 days	5 days	Germ. % in Area 2- background control	Haustoria %	Germ. index (Area 1 5D/Area 2 5D)
Framida (R control)	0.3 ± 0.1 b	2.0 ± 0.9 b	39.1 ±1.7 b	62 ± 1.9 b	5.5 ± 1.4 b	0.62 ± 0.0 b
BF3 (R mutant)	0.45 ± 0.1 b	1.6 ±0.2 b	22.6 ±1.9 c	58 ± 1.8 b	9 ± 0.9 b	0.39 ± 0.0 c
Parent	1.4 ± 0.3 a	7.4 ±0.6 a	66.6± 1.8 a	70.6 ± 0.6 a	28.5 ±1.52a	0.93 ± 0.0 a

Table 1. Comparative analysis of resistance response of the control Striga resistant sorghum variety Framida with the Striga resistant mutant BF3 and its parental line which is susceptible to Striga. Means followed by different letters are significantly different at 0.05 probability

New markers for the gamma-ray induced early maturity/semi-dwarf trait in sorghum

Over 90% of the traits induced through random mutagenesis such as gamma irradiation are recessive, Mendelian traits or major QTLs. For recessive traits, molecular markers offer significant advantages for the introgression or gene pyramiding compared to phenotypic selection in Marker-Assisted Backcross schemes. In 2020, work at PBGL on the development of molecular markers for the gamma-ray induced early maturity/semi-dwarf trait in sorghum continued. Previously, some 7 sequence variants had been identified through whole genome (re)sequencing and genetic mapping of F2 populations derived from a cross between the mutant sorghum and its parent. The sequence variants were located on chromosome 4 near the centromere in a highly repetitive region. *In silico* analysis of the sequences flanking the variants indicated the presence of short stretches of DNA sequences that were less repetitive. Laboratory experiments confirmed that specific regions could be amplified resulting in unique PCR products. Following this, KASP marker assays could be developed for two SNPs. Marker-trait association studies were conducted using these two markers and two F2 populations segregating for the early maturity/semi-dwarf trait. The result illustrated in Fig 2 confirms close linkage ($\geq$90% or about 5 cM) between the mutant trait and the marker.

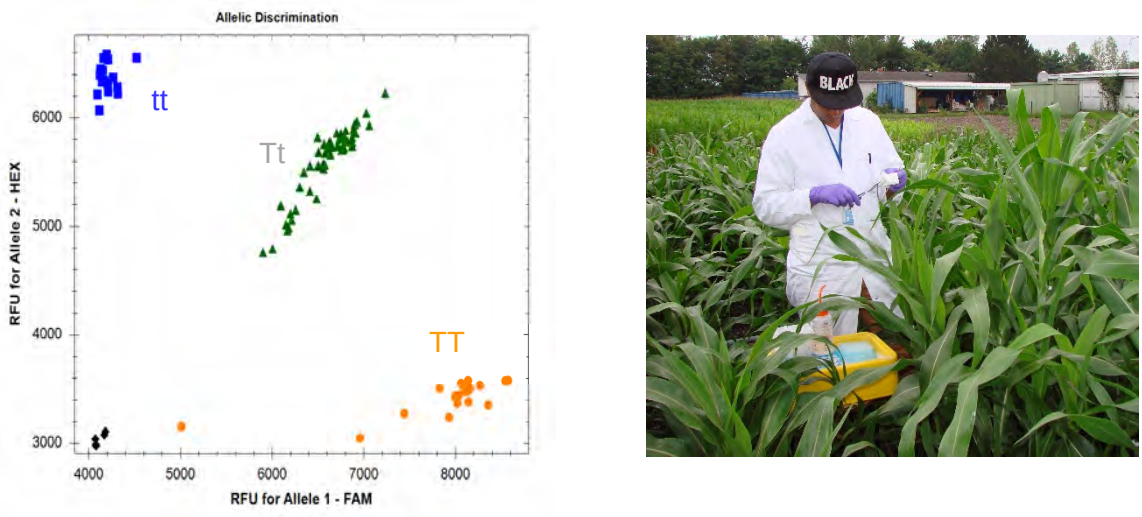

Fig. 2. Left: KASP genotying assay of an F2 population segregating for the early maturity/semi-dwarf trait in sorghum. Clustering of the three possible genotypes (tt, Tt and TT) and marker-trait linkage analysis confirmed that the marker is closely linked to the mutant trait. Right: field phenotyping and sample collection for DNA extraction

Single-cell mutation induction and in vitro regeneration of *Coffea arabica*

To date, many plant species can be regenerated from individual somatic cells using *in vitro* tissue culture techniques. Combined with efficient mutation induction, these technologies can provide solutions to avoid or reduce the presence of chimeric tissues which are produced when multicellular tissues such as stem cuttings, seed or in vitro shoot tips are used as source explants for mutation induction. *In vitro* plant tissue technologies thus offer potential to shorten the mutation breeding cycle of crops such as coffee and banana as well as new opportunities for lab-based selection schemes.

In 2020, experimental procedures were developed for mutation induction of *Coffea arabica* suspension cultures using the chemical mutagen EMS. Dose response experiments were conducted to optimize the optimal dose(s) for mutation induction of *in vitro* suspension cells/cell clusters. Following mutagenesis, *in vitro* plantlets were regenerated. The EMS dose-response of suspension cells takes 3-4 weeks and is illustrated in Fig 3. The dose response curve shown in Fig 4 indicates that LD_{30} is ca 0,5% EMS.

Fig. 3. Dose-response of arabica coffee cell suspensions 4 weeks after EMS mutagenesis. From left to right: 0%, 0.2%, 0.5%, 0.8%, 1.5% and 2.0% EMS

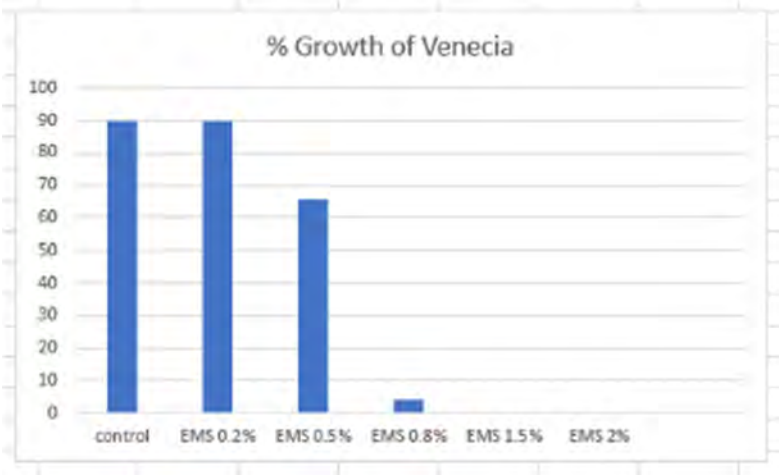

Fig. 4. Dose-response curve of arabica coffee cell suspensions 4 weeks after EMS mutagenesis

PBGL Mutation Detection software workflow is publicly available

Genomics approaches are revolutionizing the biological sciences. PBGL strives to enable Member States to fully participate in the genomics revolution and thus enhance their Mutation Breeding projects.

Mutation discovery through whole genome re-sequencing is very cost-effective and DNA sequencing capacity is accessible around the world. Modern Next (second) Generation Sequencing (NGS) machines turn out hundreds of millions of sequencing reads per run, which in principle allow for in-depths views of crop genomes. However, handling such large amounts of data is challenging. In fact, PBGL identified the challenges of genome data analysis as one major obstacle for adoption of genomic approaches by Member States.

Turning the raw sequencing data into meaningful information for the plant breeder requires a series of computational steps: quality control and trimming followed by alignment of each of the millions of sequencing read to a closely related reference genome assembly, removal of PCR duplicates, consolidation of the alignments by realigning small insertions and deletions, and, finally, traversing through the alignments and finding statistically significant differences between the sequenced samples. This process yields a long list of genetic variants that then need to be further filtered and, ideally, visualised. For all steps in the process there exists a multitude of publicly available software tools. Even with the individual software tools available off-the-shelf, using them requires substantial bioinformatics expertise.

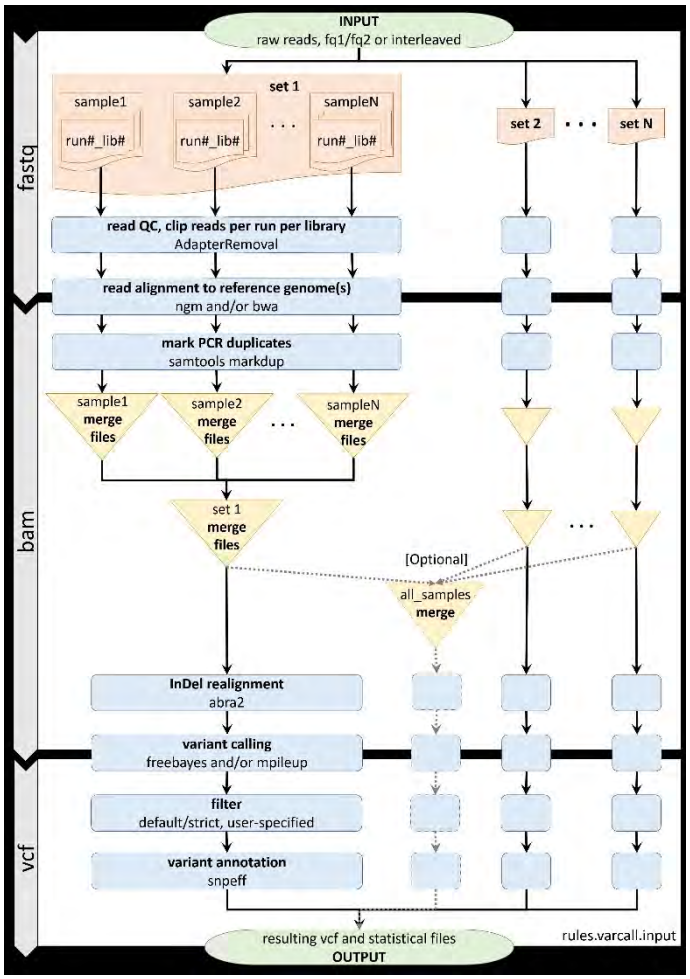

Fig. 5. Mutation detection computational workflow for automated re-sequencing analysis of whole genomes

PBGL has been using NGS enabled genomics approaches since 2017 and has re-sequenced over 500 genomes in crops ranging from sorghum, rice, cowpea to tomato and coffee. We hence understand the challenges. We have automated the in-house analysis process by building a computational workflow that chains together state-of-the-art, open source analysis tools into a coherent pipeline to efficiently find genetic variants and extract novel mutations (Fig 5). For building the workflow we opted to use snakemake, a workflow specification language based on python (*https://snakemake.github.io/*), and we manage all software dependency through conda environments. The workflow starts from raw sequence reads (fastq), aligns reads against one or several reference genome(s) to produce alignment files (bam). From those alignment files SNPs and InDels will be called and recorded in variant call format files (vcf). If a genome annotation for the reference genome is available, the PBGL workflow can annotate their predicted impact on gene function. The user has the choice between different alignment programs (bwa mem/nextgenmap) and variant callers (freebayes/mpileup) and can use multiple reference genomes simultaneously.

Using the workflow is a great time saver and abstracts much of the complexity of the analysis from the user, empowering non-experts to embrace genomics projects. As additional advantage, using workflows makes the documentation of every analysis, step-by-step, fully implicit. Each individual analysis is hence easily shared and fully reproducible.

PBGL is now making the analysis pipeline along with a detailed documentation publicly available to Member States: free and open source. We provide a workflow template that users will configure for their datasets and crop. Input data are the raw fastq file as they come off the sequencing machines and the workflow will return vcf files that list genetic variants/mutations between the input samples in comparison to the desired reference genome. If the user provides a gene annotation for the reference genome annotation, the workflow can also determine the putative effects of the variants/mutations on gene function.

Currently the workflows are available from PBGL upon request. Researchers from Member States can contact PBGL and we will guide them through the process. PBGL is planning to host training courses for researchers from Member States on genomics approaches in Plant Mutation Breeding including the use of our workflow.

Screening all Coffee Genes for New Mutations by Exome Capture Sequencing

One objective of CRP D2205 on coffee and banana disease is to develop mutation induction and screening techniques in coffee that allow production and large-scale screening of mutant plants to identify rare valuable mutants. PBGL has successfully induced mutations in coffee and more than 500 M_1 mutant coffee plants are growing in the greenhouse in Seibersdorf, Austria.

Fig. 6. M_1 mutant coffee plants from three Coffea arabica varieties growing in the PBGL shaded greenhouse

Given the low probability of a beneficial mutation and the high-cost and long waiting times for assessing the coffee plants and beans, it is desirable to pre-screen mutant plantlets before evaluating them in the greenhouse or field. Focusing the phenotyping efforts on plants with promising genotypes, i.e. mutations in candidate genes and pathways, will reduce space and cost and hence increase efficiency.

For population-scale genome wide characterization of coffee plants, PBGL is pioneering the so-called exome capture sequencing technique for arabica coffee. Exome capture sequencing is a cost-effective approach to characterize all genes. Target capture techniques exploit the property that complementary DNA strands hybridize with each other to form a double strand. Using a set of synthesized oligonucleotides, complementary target DNA molecules can be captured and in turn extracted from a mixture; hence the general name ‘target capture’. In our case, the targets are exons of all genes, hence the name ‘Exome Capture’. Subsequent sequencing of only the exons significantly reduces the sequencing cost per sample compared to sequencing the whole genome.

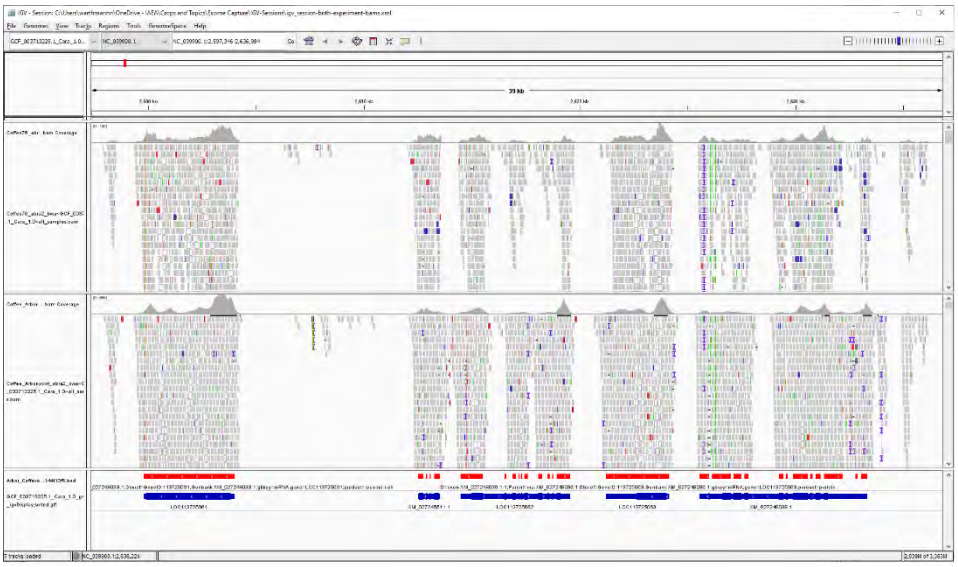

Fig. 7. Exome capture pilot experiments on Coffea arabica. From whole genome sequencing libraries only the DNA fragments complementary to target regions (red) are extracted prior to sequencing. Target regions were designed to cover the exons of all coffee genes (blue)

Together with a commercial provider, Arbor BioScience (USA), PBGL has developed an Exome Capture kit for *Coffea arabica*. With 275,000 targeted exon regions, the kit reduces the sequencing space from 825 Mbp down to 110 Mbp of coding sequence. In two pilot experiments we achieved above 90% of reads on targets (see Fig 7). Compared to whole genome sequencing, this reduces the amount of required sequence by 8-fold. This is bringing population scale sequencing of coffee mutant populations within reach for Member States. The kit is commercially available from Arbor BioScience (Ann Arbor Michigan, USA) for broad community use (Fig 8).

Fig. 8. Coffea arabica Exome Capture Kit

TECHNOLOGY TRANSFER, CAPACITY BUILDING AND SERVICES

Data Science Internships at PBGL

'Big Data' has truly arrived. Plant Mutation Breeding is no exception. Analyzing whole genome-sequencing experiments is 'Big Data' science. As the genomics revolution sweeps through the plant sciences, the global shortage of data science experts that can help breeders turning this data in relevant knowledge has become rate limiting. PBGL is directly addressing this shortage by offering data science internships. During 2020, PBGL hosted two data science interns: Anza Ghaffar (Pakistan) and Anibal Morales Zambrana (USA). Recent graduates from computational disciplines get the opportunity to work in PBGL for up to one year. The interns receive hands-on training and learn how to apply data science in Plant Mutation Breeding.

Fellowships and Interns

In 2020, the PBGL hosted two fellows (F) and six interns (I) (the fellows were funded by the IAEA's Department of Technical Cooperation) in the following areas:

Name	Country	Status	Topic	Duration
Mr Phillipe NIKIEMA	Burkina Faso	F	Mutant population development *Striga; Striga* screening protocol	4 months
Mr Mokhtar BARAKET	Tunisia	F	Barley low-cost genotyping protocol for feed quality	2 months
Ms Yuling YUE	China	I	Drought/*Striga* screening protocols; marker development in rice and sorghum	3 months
Ms Li ZHU	China	I	Drought/*Striga* screening protocols; marker development in rice and sorghum	6 months
Mr Anza GHAFFAR	Pakistan	I	Visualization tools (R) and mutation catalogue dashboard (python)	7 months
Mr Anibal MORALES ZAMBARA	Spain	I	Computer programming; analysis of genomic datasets and automating the laboratory's liquid-handling robot	9 months
Ms Susu ALKIER	Austria	I	Field, glasshouse and laboratory experiments for phenotyping, mutant characterization and marker development for agronomically useful mutants in cereals	12 months
Ms Faith LUVAI	Kenya	I	In vitro cellular and molecular tools for barley feed quality and banana TR4 screening protocol	12 months

Crop Irradiation Services

In 2020, the PBGL received 24 requests for plant irradiation from 19 Member States and irradiated 124 accessions/varieties. Requests were received in the context of CRPs or Technical Cooperation (TC) projects while the remaining requests were from stakeholder institutions from Member States, as summarized in the table below. The total number of irradiation requests now stands at 1647.

Request	Country	CRP/TC	Species
1623	Ireland		eucalyptus
1624	Germany		ornamental
1625	Hungary		ornamental
1626	The Netherlands		ornamental
1627	Zambia	CRP	groundnut, cowpea
1628	Tanzania	TC	rice
1629	Eswatini (Swaziland)	TC	cowpea
1630	Niger	TC	rice
1631	Zambia	CRP	cowpea, soybean
1632	Serbia	TC	wheat, barley
1633	PBGL		coffee
1634	Germany		ornamenthal
1635	Namibia	CRP	cowpea
1636	Kenya	CRP	cowpea
1637	Senegal	CRP	cowpea
1638	Uruguay		rice
1639	The Netherlands		ornamental
1640	Croatia	TC	wheat
1641	Germany		ornamental
1642	Nigeria		yam
1643	Cameroon	TC	cowpea, maize, watermelon
1644	Togo	TC	rice
1645	Cyprus	TC	barley
1646	Zambia	CRP	cowpea
1647	Namibia	CRP	groundnut

PUBLICATIONS and INFORMATION DISSEMINATION

Conference Abstracts and Posters

Warthmann N, Ghanim AMA, Ali A, and Ingelbrecht I. Mutation Breeding Creates Desired Traits for African Sorghum –Semi-Dwarf and Early Maturing. PE0810. Plant and Animal Genome XXVIII, 11-15 January 2020, San Diego, USA.

Warthmann N, Ghanim AMA, Ali A, and Ingelbrecht I. Mutation Breeding Creates Desired Traits for African Sorghum – Semi-Dwarf and Early Maturing. International Symposium of the Society for Plant Breeding (GPZ), Digital Breeding, 11–13 February 2020, Tulln, Austria.

Peer-reviewed articles

Cespedes R, Arrieta N, Barquero M, Abdelnour A, Nielen S, and Ingelbrecht I. Determination of Radiosensitivity of Coffea arabica var. Venecia Seeds to Gamma-ray Irradiation. Chapter 7, Section 3. Mutation Induction Techniques for Enhancing Genetic Variation. Proceedings of the FAO/IAEA International Symposium on Plant Mutation Breeding and Biotechnology. (in press)

Maghuly F, Jankowicz-Cieslak J, and Bado S. Improving coffee species for pathogen resistance. CAB Reviews 2020, 15, 009, pp 1-18.

Hawliczek A, Bolibok L, Tofil K, Borzęcka E, Jankowicz-Cieślak J, Gawroński P, Kral A, Till B, and Bolibok-Brągoszewska H. Deep sampling and pooled amplicon sequencing reveals hidden genic variation in heterogeneous rye accessions. 2020. BMC Genomics 21:845.

Jankowicz-Cieślak J, Goessnitzer F, Datta S, Viljoen A, Ingelbrecht I, and Till BJ. Induced Mutations for Generating Bananas Resistant to Fusarium Wilt Tropical Race 4. Chapter 9, Section 4. Mutation Breeding in the Post-Genomic Era. Proceedings of the FAO/IAEA International Symposium on Plant Mutation Breeding and Biotechnology. (in press)

Mba C, Abang M, Diulgheroff S, Hrushka A, Hugo W, Ingelbrecht I, Jankuloski L, Leksien D, Lopez V, Muminjanov H, Mulila Mitti J, Nersisyan A, Noorani A, Piaor Y, and Sagnia S. (2020). FAO Supports Countries in the Implementation of the Second Global Plan of Action for Plant Genetic Resources for Food and Agriculture. Acta Hortic. 1267, 197–208. DOI: 10.17660/ActaHortic.2020.1267.30.

Nikièma MP, Yonli D, Rabefiraisana HJ, Ali A., Ouédraogo N, Traoré H, Yanogo HYA, Dao K, Sawadogo K, Jankuloski L, Ingelbrecht I, and Mukhtar Ali Ghanim A. Induced Resistance to *Striga hermonthica* in sorghum by gamma irradiation. American Journal of Plant Sciences. 2020. 11:1545-1561.

Rabefiraisana HJ, Ghanim AMA, Adrianjaka A, Rasoamampionona B, Jankuloski L, Razafindraso MA, Ravelonjana, Ravelonjanahary NH, and Rakotoarisoa. Impact of Mulch-Based Cropping Systems Using Green Mulch and Residues on the Performance of Advanced Mutants Lines of Maize (Zea mays (L.)) Under Infested Field with the Parasitic Weed Striga asiatica (L.) Kuntze in Madagascar. Chapter 18. Section 2. Mutation Breeding in Crop Improvement and Climate-Change Adaptation. Proceedings of the FAO/IAEA International Symposium on Plant Mutation Breeding and Biotechnology. (in press)

Roy S, Ghanim AMA, Nunekpeku W, Rashidul I, MD Rasel and MD Mahmud Al Noor (2020). Phenotyping of Lentil Genotypes for Drought Tolerance Using Polyethylene Glycol. Indian Journal of Natural Sciences ISSN: 0976–0997; Vol.10, Issue 58, February 2020.

PBGL success stories

Homegrown Soybeans are Making a Comeback in Indonesia Thanks to New Varieties Developed Using Irradiation (5 October 2020)

https://www.iaea.org/newscenter/news/homegrown-soybeans-are-making-a-comeback-in-indonesia-thanks-to-newvarieties-developed-using-irradiation

Using nuclear technology to fight a devastating parasitic plant in Africa
International Year of Plant Health 2020 | FAO | Food and Agriculture Organization of the United Nations

THE SOIL AND WATER MANAGEMENT & CROP NUTRITION LABORATORY

EXECUTIVE SUMMARY

The Soil and Water Management & Crop Nutrition Laboratory (SWMCNL) of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture assists Member States in the development and transfer of isotopic and nuclear technologies to improve the resilience of farmers' communities to climate change through climate-smart agriculture, including soil and water conservation and optimization of soil, water and nutrient management practices. The SWMCNL also helps Member States to be better prepared in responding to nuclear emergencies affecting food and agriculture, as well in remediating the impact of these events on soil and agricultural water resources.

In 2020, the SWMCNL conducted a wide range of activities: (i) it developed robust and affordable isotope, nuclear and related conventional techniques for climate-smart agriculture through R&D; (ii) supported the improvement of nuclear emergency preparedness and response in food and agriculture, (iii) trained technical staff and scientists from Member States in the use of nuclear and related techniques to develop climate-smart soil and water management practices and improve remediation of radioactive contamination in agriculture; (iv) carried out isotope analyses for research and development (R&D); and (v) provided quality assurance services to Member States.

Despite the fact that the year 2020 has been a year full of challenges, due to the COVID-19 pandemic, many R&D activities were implemented at the SWMCNL including novel applications of isotope and nuclear techniques for remediation of radioactive contamination in agriculture or climate-smart cassava and banana production. Approaches were developed for producing high-resolution soil moisture map with Cosmic-Ray Neutron Sensor and Sentinel-1 satellite imagery data for temperate and semi-arid environments. Significant steps were made to characterize bacterial and fungal community structure and diversity to complement soil erosion information derived from $^{239+240}$Pu determination. Important progress was also made in the development of protocols on the use of nitrogen process inhibitors for improved fertilizer management, or machine learning to improve prediction of soil properties which are relevant for remediation of radioactive contamination in food and agriculture. All these activities are essential in supporting the implementation of the five Coordinated Research Projects (CRP) of the SWMCN Subprogramme, two of which are coordinated by the SWMCNL, i.e. CRP D1.50.17 on *'Nuclear Techniques for Better Understanding of the Impact of Climate Change on Soil Erosion in Upland Agro-ecosystems*' and CRP D1.50.19 on *'Monitoring and predicting radionuclide uptake and dynamics for optimizing remediation of radioactive contamination in agriculture'*.

A major component of the work of the SWMCNL is its significant contribution to training and capacity building in Member States. In 2020, the SWMCNL focused on capacity building through the training and guidance of three PhD, two MSc students (through IAEA internships), three interns from four countries in the use of nuclear and isotope techniques for climate-smart agriculture and nuclear emergency response.

R&D information was further communicated to Member States through 38 publications as guidelines, protocols, books, book chapters, conference papers and publications in international peer-reviewed journals, including two books and three TECDOCs on the use of isotope and nuclear techniques for climate-smart agriculture and nuclear emergency response.

The SWMCNL analysed a total of 4123 and 200 samples for stable isotopes and fallout radionuclides, respectively. Most analyses were carried out in support of R&D activities in the SWMCNL, focusing on the design of isotope and nuclear techniques to optimize soil and water management practices. Emphasis was also put on ^{13}C-CO_2 and ^{15}N-N_2O measurements using the laboratory-based laser isotope analysers.

STAFF

Name	Title
Dercon, Gerd	Laboratory Head
Mabit, Lionel	Soil Scientist
Said Ahmed, Hami	Soil Scientist
Heiling, Maria	Senior Laboratory Technician
Resch, Christian	Senior Laboratory Technician
Weltin, Georg	Senior Laboratory Technician
Gruber, Roman	Laboratory Technician
Toloza, Arsenio	Laboratory Technician
Jagoditsch, Norbert	Technical Attendant
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Eguchi, Tetsuya	Consultant
Fesenko, Sergey	Consultant
Mirkhani, Rayehe	Consultant
Van Laere, Jonas	Consultant
Vantyghem, Mathilde	Consultant
Dengra i Grau, Francesc Xavier	Intern
Ding, Yang	Intern
Jabbarimalayeri, Hoda	Intern
Willems, Elden	Intern

MAJOR ACHIEVEMENTS IN RESEARCH AND DEVELOPMENT

The Soil and Water Management & Crop Nutrition Laboratory (SWMCNL) assists Member States in the development and transfer of isotopic and nuclear technologies to improve the resilience of farming communities to climate change by optimizing soil, water and nutrient management practices. These efforts are supported by a new generation of robust and affordable isotope and nuclear techniques that can be used *in situ* at plot (on-farm) or area-wide level.

The SWMCNL also supports Member States to be better prepared in responding to nuclear emergencies affecting food and agriculture, as well as in remediating the impact of such events on soil and agricultural water resources.

Climate-Smart Agriculture

Climate change is a major threat to global food security. Changes in weather patterns, with increasing severity of storms, floods, droughts and extreme temperatures, impact sustainable agricultural production. These increasingly amplify soil erosion, land degradation and crop failures worldwide. Agriculture can further accelerate climate change due to the greenhouse gas it emits. The need to sustain agricultural production in these challenging conditions has never been greater. Consequently, there is an increasing demand from Member States for technical assistance and training in developing soil and water management packages for climate-smart agriculture.

Producing high-resolution soil moisture map with Cosmic-Ray Neutron Sensor and Sentinel-1 satellite imagery data for temperate and semi-arid environments

In 2020, the SWMCN laboratory developed a method for producing high spatial and temporal resolution soil moisture maps based on satellite Sentinel-1 imagery and Cosmic-Ray Neutron Sensor (CRNS) data. Two case studies were used for this method development, with one located in a temperate (Austria) and one in a semi-arid environment (Kuwait).

Recently, active microwave remote sensing Synthetic Aperture Radar (SAR) imaging has emerged as an effective tool to estimate surface soil moisture. The Sentinel-1 C-band (SAR) satellite shows great potential for soil moisture monitoring and for producing soil moisture maps, and this at a spatial and temporal resolution of at least 10 m by 10 m, and 5-day interval, respectively. As Cosmic Ray Neutron Sensors (CRNS) have the capability to continuously measure, providing real-time field-scale soil moisture (SM) in large areas up to 20 to 30 ha, CRNS technology can be used for calibration and validation of the remote sensing imagery predictions at field and area-wide level.

The first results from the case study in Austria showed that soil moisture measured by the CRNS and the radar backscattered signal have a similar trend (Figure 1 a). Therefore, a simple linear regression model could be made (R^2=0.81) (Figure 1 b). In addition, the model was tested in Kuwait and showed a similar trend (R^2=0.77).

Once calibrated, the linear model was integrated into Google Earth Engine (GEE) to convert the VV polarization radar data into soil water content maps. In a next step, a web application was created for easy access of soil moisture estimation based on the satellite imagery data. Further, Normalized Difference Vegetation Index (NDVI) data from Sentinel-2 and rainfall data also derived from satellite imagery (Climate Hazards Group InfraRed Precipitation with Station - CHIRPS) were integrated in the prediction to consider soil moisture and vegetation dynamics due to irrigation.

In Figure 2, an example of a derived high-spatial resolution soil moisture map and related soil moisture timelines, visible in the developed web application, is shown for Kuwait. In this example, the impact of irrigation can be seen; soil moisture decreases down to a certain threshold (15 or 20%), and it increases again to 25 or 30% after irrigation. Such information is useful for agricultural water management and can help the farmer decide when and how much to irrigate.

This study is a major step in soil moisture monitoring at high spatial and temporal resolution by combining remote sensing data and the CRNS based nuclear technology. The CRNS technology bridges the critical gap between satellites and point-scale ground sensors and enables the calibration of satellites, such as Sentinel-1, to improve soil moisture data estimated by remote sensing.

However, the model is sensitive to vegetation density and snow, and therefore further research will be conducted to incorporate this important parameter using advanced mathematical techniques, such as machine learning. The developed web application is an important tool not only for agricultural water management, but also for hydrology, drought, and flood prediction, and may be even useful in desert locust preventive management in the future.

This work has been conducted under CRP D1.20.14, on Enhancing Agricultural Resilience and Water Security using Cosmic-Ray Neutron Sensor. We acknowledge the collaboration of the Kuwait Institute for Scientific Research (KISR).

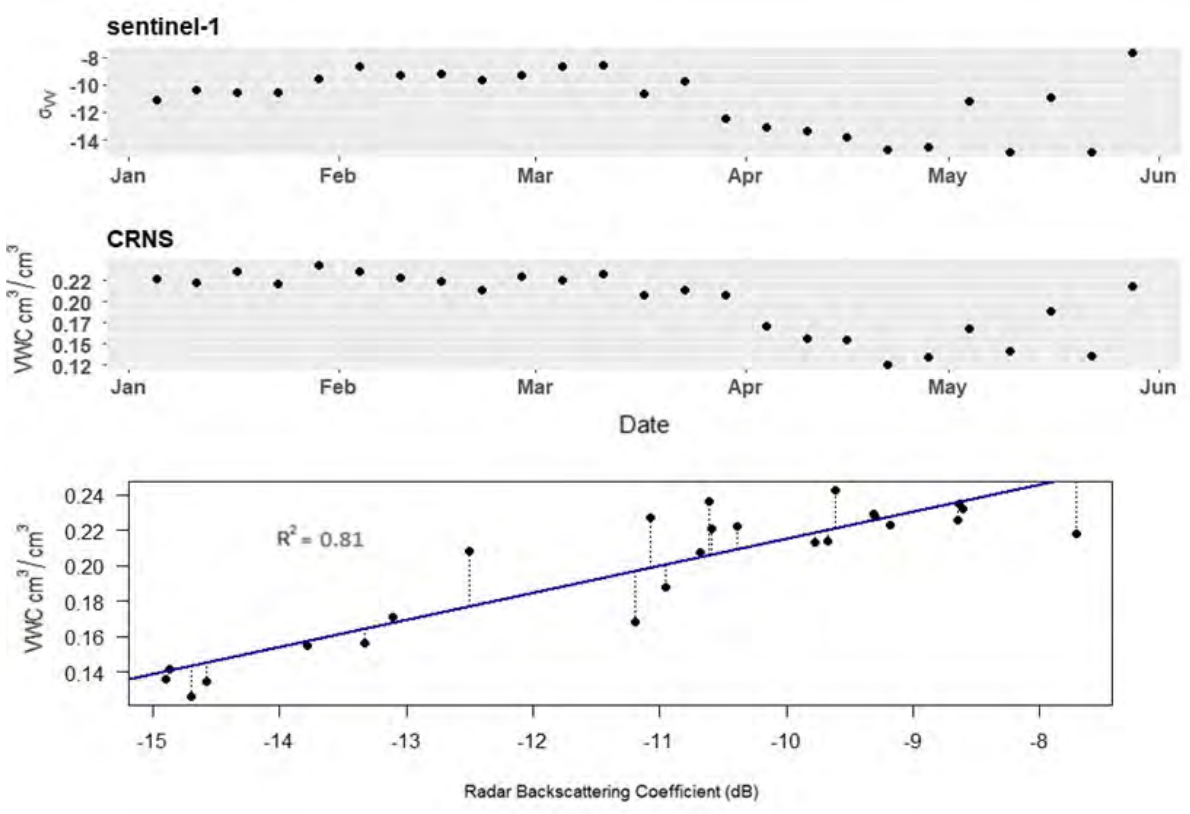

Figure 1. (a) Sentinel-1 radar backscattered coefficient (σVV) and soil moisture measured with Cosmic Ray Neutron Sensor in the case study located in Petzenkirchen (Austria) and (b) Linear regression model between σVV and soil moisture for the same case study.

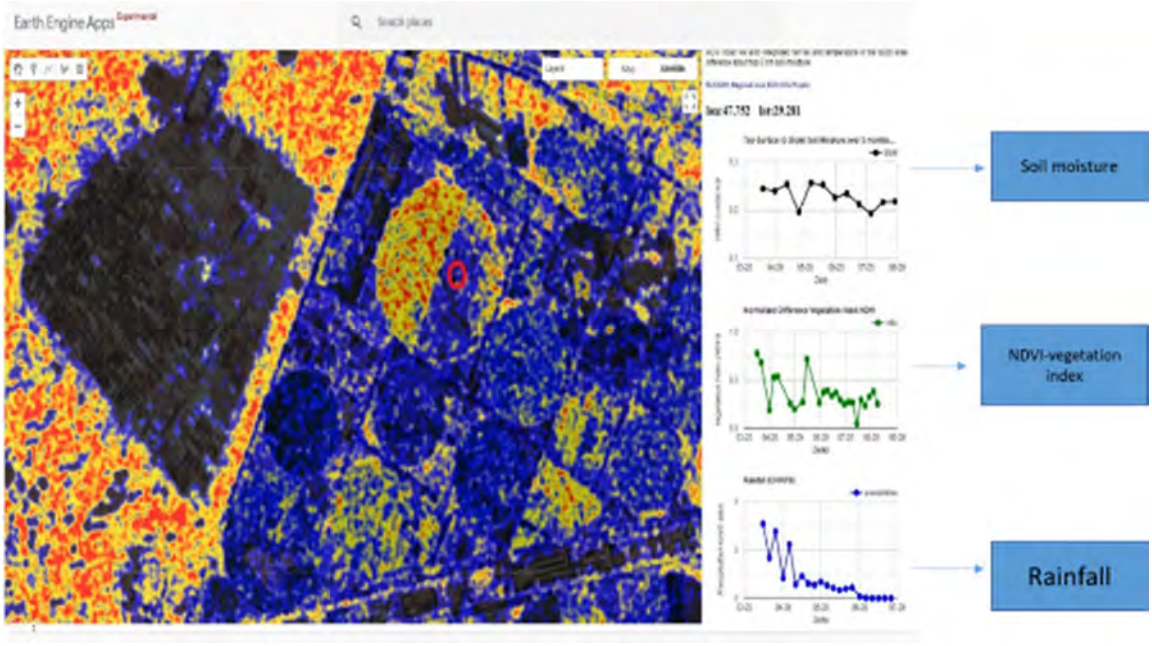

Figure 2. Screenshot of the web application for estimating spatial and temporal soil moisture data based on satellite imagery information, calibrated by cosmic ray neutron sensor technology, from the case study in Kuwait.

The SWMCN Laboratory also contributed to a new infographic video on 'Cosmic Ray Neutron Sensor (CRNS): From cosmic ray to soil moisture!', explaining the principle of CRNS through a short video. The video can be found on: https://youtu.be/odZd3aPG8f4.

Influence of nitrogen process inhibitors on maize yield

In the summer of 2020, a field experiment was established at the SWMCN laboratory in Seibersdorf, Austria to determine the effect of different Nitrogen (N) fertilizers coated with N process inhibitors on maize yield and ammonia emission in the summer of 2020.

Nitrogen (N) fertilizer management is challenging due to the many factors that influence N use efficiency (NUE). Nitrogen losses from the soil reduce plant yield as well as have negative impacts on the environment. Nitrogen processes inhibitors, such as urease and nitrification inhibitors, are chemical compounds which reduce urea hydrolysis and nitrification, respectively. Urease inhibitors (UI) (also known as 2-NPT or N-(2-nitrophenyl) phosphoric acid triamide) inhibit the hydrolytic action of the urease enzyme on urea, and nitrification inhibitors (NI) inhibit the biological oxidation of ammonium to nitrate. By coating ammonium based chemical fertilizers with N process inhibitors allows N to stay in a more stable form of ammonium (NH_4^+), thus minimising N losses as well as improving NUE and consequently enhancing crop yield. NI is further divided into MPA or N-[3(5)-methyl-1H-pyrazol-1-yl) methyl] acetamide (abbreviated as NI-1), and DMPP or 3,4-dimethylpyrazole phosphate (abbreviated as NI-2).

Three combinations of N fertilizer (urea or NPK) with N process inhibitors (UI and/or NI)) were tested and compared with a control treatment (without N fertilizer) and a urea application without any inhibitor. All treatments received 60 kg ha^{-1} P_2O_5 and 146 kg ha^{-1} K_2O. The amount of nitrogen added to each treatment receiving N fertilizer was 120 kg N ha^{-1}. The inhibitors used were (i) UI, (ii) NI-1, and

(iii) NI-2. DMPP, a nitrification inhibitor, was used in combination with NPK fertilizer, as the fertilizer contains besides nitrate-N also ammonium-N.

A randomized complete block design with four replications was used in this study. Treatments were: T1 (control treatment - without N fertilizer), T2 (Urea only), T3 (Urea + UI), T4 (Urea + UI + NI-1), and T5 (NPK + NI-2). Urea was applied through two split applications in the T2 treatment (at 20 DAP and 34 DAP). In T3, T4, and T5 treatments, N fertilizers were applied only once (at 20 DAP). Supplemental irrigation was only applied in the early stages of growth, to ensure that the crop could establish. Harvest was carried out at 98 days after planting (DAP), before full maturity. Hence yield is expressed as dry matter (DM). The Seibersdorf field site is characterised by a moderately shallow Chernozem soil with significant gravel content.

The preliminary results are shown in Table 1. The yield data showed that different fertilizer treatments had a significant ($p \leq 0.01$) effect on maize yield (dry matter production). There was no significant difference between treatments 4 and 5, which had the highest yield. A second group, with intermediate yield, was formed by the treatments 2 and 3. As expected, the yield under the control treatment, with only phosphorus and potassium but without N application, was the lowest. Based on the preliminary results, it can be concluded that nitrogen process inhibitors play a significant role in improving maize yields. In particular, the comparison between T2 and T3 shows that the application of a urease inhibitor avoids the need for a split application of urea, which decreases labour costs. Adding NI-1 (under T4) further increases the yield. Also, the package of NPK, a common choice by farmers in Austria, in combination with the nitrification inhibitor NI-2 showed equally good results as urea combined with two inhibitors. Further analyses are now being carried out to understand the effects of different inhibitors on soil ammonia (NH_3) emission losses. Finally, a cost-benefit analysis will be carried out.

Table 1. Effect of different nitrogen fertilizers on maize dry matter yield, including the results of a Tukey's test (DM yields with similar letter indicate that there is no significant difference between the yields); results are expressed as mean ± SD.

	Treatment	**Yield DM (t/ha)**
T_1	Control treatment (without nitrogen fertilizer)	6.5 ± 0.7 [c]
T_2	Urea (in two splits)	8.2 ± 0.9 [b]
T_3	Urea + UI (2-NPT)	9.3 ± 1.0 [b]
T_4	Urea + UI (2-NPT) + NI-1 (MPA)	11.0 ± 0.6 [a]
T_5	NPK + NI-2 (DMPP)	11.6 ± 0.6 [a]

* 2-NPT: N-(2-nitrophenyl) phosphoric acid triamide (UI)
* MPA: N-[3(5)-methyl-1H-pyrazol-1-yl) methyl] acetamide (NI-1)
* DMPP: 3,4-dimethylpyrazole phosphate (NI-2)

Characterizing bacterial and fungal community structure and diversity to complement soil erosion information derived from $^{239+240}$Pu determination

In 2020, a study was completed by the SWMCN Laboratory in Grabenegg, 100 km west from Vienna, to investigate how information on the diversity and structure of bacterial and fungal communities in soil, obtained through high throughput DNA sequencing analysis, can be linked with and can complement data on soil quality and soil erosion. To achieve this objective soil quality parameters,

such as pH, organic carbon (C_{org}, %), total nitrogen (N, %), aggregate stability, stable isotope analysis (i.e. $\delta^{13}C$ and $\delta^{15}N$) were analysed and soil erosion rates derived from $^{239+240}Pu$ were calculated.

To figure out the responses of soil microbial communities to soil erosion processes, topsoil samples (i.e. 0-15 cm) were collected along a 60 m long transect, with a slope gradient of 9.1° located in an experimental agricultural field with Cambisols and Luvisols in Grabenegg, Lower Austria.

The analysis showed that the bacterial richness and diversity found in the soil at the top of the slope are significantly higher than at the lower positions, whereas no difference for the fungal communities was observed (see Figures 2 and 3).

Based on principal coordinate analysis (PCoA) of the five sampling points along the studied slope, the bacterial community structure is negatively correlated with pH, and positively correlated with soil aggregate stability indicators (i.e. Mean Weight Diameter [MWD] and Geometric Mean Diameter [GMD]).

The bacterial structure can be explained with pH, MWD and GMD by 65%. As it is the case for the bacterial community, the fungal community structure is also negatively correlated with pH, and positively correlated with soil aggregate stability (MWD, GMD), which, however, explained up to 90% of the fungal community structure.

In the same experimental field, soil loss rates derived from $^{239+240}Pu$ study, carried out in 2019 by the SWMCN Laboratory, are significantly negatively correlated with bacterial diversity ($p < 0.05$), but not correlated with fungal diversity.

The findings obtained under our experimental conditions and for the soils characterizing the region at the northern foot slopes of the Alps highlight that bacterial diversity can be a potential reliable soil quality parameter to investigate soil loss and its impact of soil degradation associated with soil erosion processes.

This study was conducted under CRP D1.50.17 on “Nuclear Techniques for a Better Understanding of the Impact of Climate Change on Soil Erosion in Upland Agro-ecosystems”.

Continuous ^{15}N labelling study: implications for nutrient management in cassava production systems

With the goal of increasing cassava production, the SWMCN laboratory in Seibersdorf is developing stable isotope techniques for assessing water use efficiency (carbon-13 and oxygen-18) and fertilizer use efficiency (nitrogen-15) in collaboration with the Consortium for Improving Agriculture-based Livelihoods in Central Africa (CIALCA, www.cialca.org). These techniques will be implemented in the field to advise on variety selection and fertilizer application, amongst other practices. To increase production in Africa from an average of 9 tons of fresh roots per hectare (FAOSTAT, 2018) to its potential of over 40 tons per hectare, best agricultural practices related to water and nutrient management are needed.

In 2020, laboratory analyses were carried out to assess nitrogen uptake and cycling in cassava, using ^{15}N isotopes, based on samples which were taken during an earlier experiment focusing on the application of potassium to alleviate drought stress carried out in the SWMCNL greenhouses. Cassava plants, originating from the Democratic Republic of Congo, were grown in sand with nutrient solution either high (+K; 1.437 mM K^+) or low (-K; 0.359 mM K^+) in potassium. Additionally, sodium nitrate

($NaNO_3$), enriched in ^{15}N, was added to both solutions to continuously label the plants with ^{15}N during the whole experiment. Plants were harvested 59, 60, 69 and 83 days after planting (DAP), divided into different parts and analysed for ^{15}N.

A summary of the data can be found in Figure 3. Although plants were continuously supplied with the solution containing the enriched nitrate (being the only nitrogen source in the solution), the plant material was not homogenously labelled. We can see a significantly decreasing trend in ^{15}N in the mean shoot from the upper parts (youngest fully expanded leaves at 59 DAP; 0.55 ± 0.04 atom% ^{15}N) to the lower parts (lower leaves at 59 DAP; 0.50 ±- 0.03 atom% ^{15}N). Secondly, storage roots, which were only visually distinguishable from fibrous roots at the last harvest, had a ^{15}N signature of 0.53 ± 0.02 atom% ^{15}N significantly different from the fibrous roots with a ^{15}N signature of 0.65 ± 0.04 atom% ^{15}N. Figure 4 shows a significant effect of the cutting weight on the ^{15}N signatures of the different plant parts. The heavier the cutting, the more diluted the ^{15}N in the plant parts. However, the effect was not significant for the fibrous roots (on day 59 and 60), lower leaves (on day 59, 60 and 68) and for the middle leaves (on day 59).

These results are the basis for improved use of ^{15}N techniques to assess nitrogen use and cycling in cassava, in particular during the critical first months of the plant.

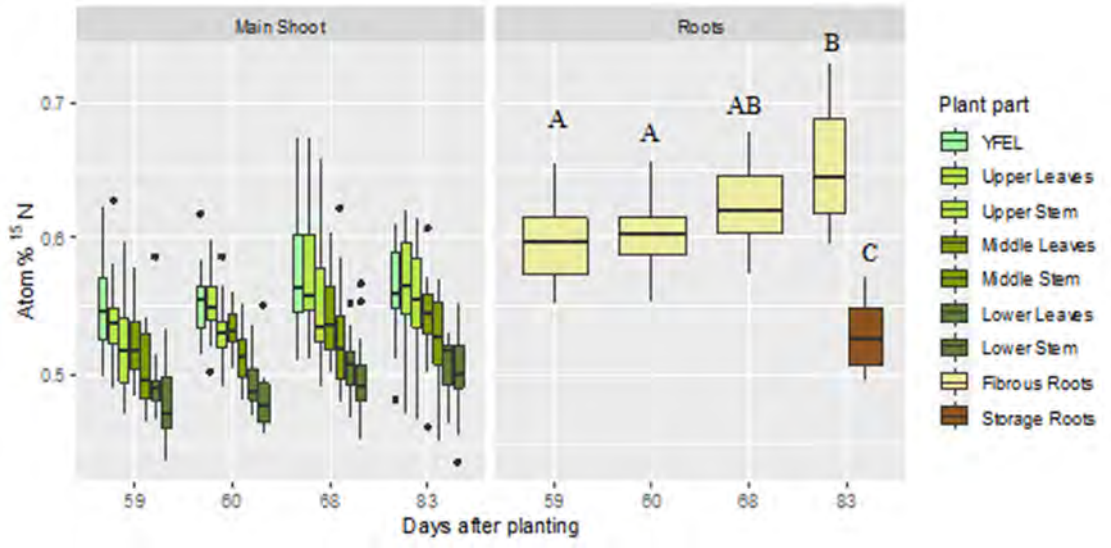

Figure 3. ^{15}N distribution (in atom% ^{15}N) in different parts of cassava plants at four different harvest times (YFEL = youngest fully expanded leaf; 59, 60, 68 and 83 DAP). Left: Data for the main shoot; Right: Data for roots (Groups with a same letter in the label have no significantly different means).

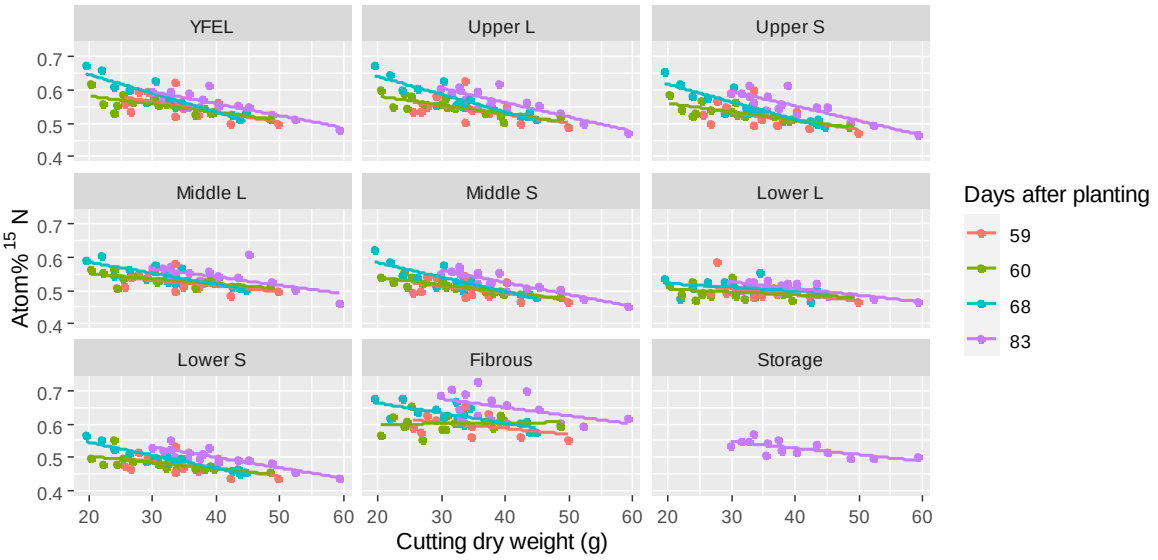

Figure 4. Influence of cutting weight on ^{15}N signatures (atom% ^{15}N) for the different plant parts (YFEL = youngest fully expanded leaf; L= Leaf; S = Shoot); colours correspond to different harvesting dates (days after planting); lines through the data points represent the linear regression.

Refining drought stress measurement methods for banana – leaf temperature and $\delta^{13}C$ of leaf and phloem sap

Under the PUI (Peaceful Uses Initiative) project on 'Enhancing climate change adaptation and disease resilience in banana-coffee cropping systems in East Africa', an experiment was set up in 2020 in close collaboration with the KULeuven (Belgium) to improve our understanding of the $\delta^{13}C$ signal as a proxy for water use efficiency and water stress in banana, under progressing drought conditions (what, when and how to sample?). For this purpose, the open-ground greenhouse of the KULeuven (Belgium) was used, which is a partner under the PUI project.

An optimal and a deficit irrigation (drought) treatment were applied to nine mature banana plants (cv. Cavendish). Treatments were initiated at the end of June 2020. The soil moisture availability was followed up with TDR sensors for every individual plant and the micro-environment (air temperature (°C), relative humidity (%) and light intensity (µmol $(m^2s)^{-1}$) were also monitored (Figure 5). During the progressing drought, daytime leaf temperature was measured once per week with an infrared thermometer. Leaf growth was tracked, and all leaves developed during the drought treatment, were sampled for $\delta^{13}C$ analysis through leaf perforation. Finally, phloem sap samples were taken in accordance to a method developed at the SWMCNL early 2020 under this PUI project. Phloem sap contains recently assimilated sugars and could therefore prove to be a short-term measure for drought stress than bulk leaf $\delta^{13}C$. Moreover, the combination of leaf and phloem sap samples may allow better understanding of the carbon dynamics in a banana plant. About 870 analyses were conducted at SWMCNL. We looked at carbon discrimination in different fractions of the leaf samples, namely the water-soluble organic matter (WSOM) and of the α-cellulose. The $\delta^{13}C$ values of the water-soluble organic matter (WSOM) should express current stress levels and correspond to the $\delta^{13}C$ measured in phloem sap, whilst the $\delta^{13}C$ values of the α-cellulose should express the stress level of the plant at time of the leaf development.

Figure 5. Experimental set-up with TDR sensors inserted in the soil at different depths and RH and air temperature sensor attached to the plant.

Figure 6 shows some preliminary results on the relationships of these different fractions. It becomes clear that $\delta^{13}C$ values of the α-cellulose fraction in the leaf strongly correspond to the bulk leaf $\delta^{13}C$ values. This is in accordance with our expectations. We assumed that a bulk leaf mostly consists of non-mobile carbon in a leaf (such as α-cellulose) and therefore represents the stress level at time of leaf development. What is however surprising, is the relationship between phloem $\delta^{13}C$ and α-cellulose and bulk leaf $\delta^{13}C$. One would expect that generally, phloem $\delta^{13}C$ values at noon are less negative than in the morning, as more photosynthesis occurs, while this should not vary in bulk leaf or α-cellulose. This is indeed the case. The relationship between phloem $\delta^{13}C$ and bulk leaf or α cellulose at noon is shifted upwards compared to the morning. However, we would expect this difference between morning and noon to be the largest in the more stressed plants, with a less negative $\delta^{13}C$ value (upper right corner). Here, we see the opposite, namely that the difference between morning and noon is largest for non-stressed plants (more negative $\delta^{13}C$, left bottom corner). One possible explanation could be the time lag of the phloem $\delta^{13}C$ value. Transportation to the phloem takes time, and therefore the signal at noon might represent the stress level of the morning. Further analysis of our data should allow us to elaborate on this, as well as the currently ongoing repetition of the experiment under field conditions in Tanzania. The obtained information will help finetune stable

carbon isotope techniques for assessing drought stress in banana, in particular how to interpret measured $\delta^{13}C$ values in plant material.

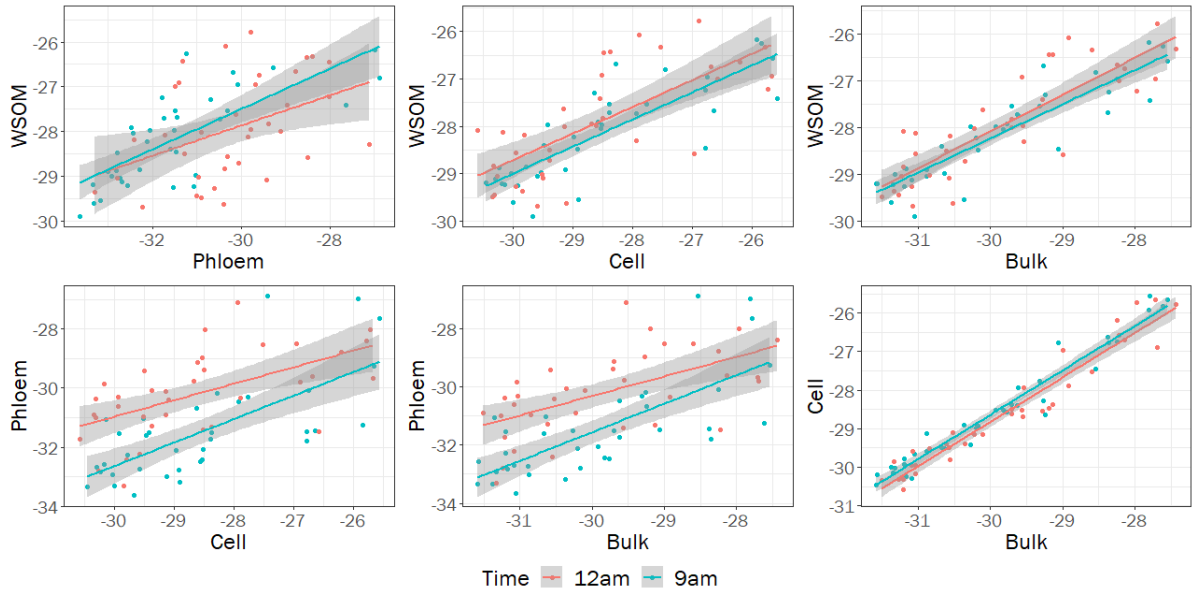

Figure 6. Relationships between the $\delta^{13}C$ values (‰) of different carbon fractions (Water soluble Organic Matter in the leaf –WSOM; Phloem sap – Phloem; α-cellulose in the leaf – Cell; Bulk leaf – Bulk) in the banana plants, at 2 times in the day.

Nuclear Emergency Preparedness in Food and Agriculture

Member States are increasingly interested in improving the capacity to respond to nuclear emergencies affecting food and agriculture due to the growing number of nuclear power plants built. Lessons learned from the Chernobyl and Fukushima Daiichi Nuclear Power Plant accidents identified critical areas for improvement and this includes data collection (sampling and analysis), data management, data visualization to make decisions swiftly, allowing food control and health authorities to respond and disseminate information to all relevant stakeholders appropriately. Further emphasis is also put on the optimization of remediation of radioactive contamination in agriculture.

Evaluation of the use of zeolite amendments and potassium addition on radiocaesium selectivity in Japanese soils

Under the Coordinated Research Project D1.50.19 on Remediation of Radioactive Contaminated Agricultural Land, the SWMCN Laboratory aims to improve remediation of radioactive contamination in agriculture to ensure food safety in the aftermath of a nuclear emergency. One of the key radionuclides that poses a concern for food safety is radiocaesium (RCs). Currently, potassium (K) fertilisation is being applied in the Fukushima Prefecture to further reduce the uptake of RCs by plant roots. Besides, zeolite minerals, which are economically affordable and progressively releasing K to the soil solution, are considered to decrease soil solution caesium, following topsoil removal. However, until present, there is some uncertainty on the specific role of zeolites in the uptake of RCs in Japanese soils.

Through the SWMCN Laboratory, in close collaboration with the National Agricultural Food and Research Organization of Japan, we analysed the RCs behaviour in Japanese soils with major clay mineralogy differences: (i) a Cambisol rich in vermiculite that was chosen because of its ability to

strongly retain monovalent cations such as potassium (K) and caesium (Cs); (ii) an Andosol with very low 2:1 phyllosilicate content and low K and Cs affinity; and (iii) a lowland smectitic Gleysol with high clay content and water holding capacity. We treated the Andosols with increasing doses of zeolites (cation exchange capacity of 130-180 meq.100g^{-1}) and all soils were also fertilised with K, at different levels, and further incubated. K and stable caesium (Cs-133) were analysed in soil solution and solid phase.

The results provide evidence that zeolite addition diminished the soil solution caesium levels (Cs_{SS}), but also reduced the K levels in the soil solution (K_{SS}) in allophanic Andosols. The K and Cs selectivity of the soil increased by zeolite addition, and hence K_{SS} (a key parameter in the uptake of RCs) decreased. A crucial finding is that the effectiveness of K fertilisation to reduce RCs uptake was diminished when combined with zeolite application. Zeolite amendments expect to reduce the Transfer Factor (TF) when K fertilizer was not applied (K target level of 0 in Figure 7). However, when K fertilizer was applied, higher TFs were expected in soils with zeolite (K target levels of 100 to 500 in Figure 7). In that way, it could be concluded that the effectiveness of zeolite application relies on the soil K status to release this cation to the soil solution or being adsorbed on its outer surface.

These novel findings are already being considered in the Fukushima Prefecture, given its practical importance in the field. Our new insights will enhance the accuracy in the fertilizer recommendations for farmers and to develop new actions to remediate radioactively polluted soils. Our next step is to enlarge the datasets with other samples to keep improving the quality in prediction models for different soils from across the world.

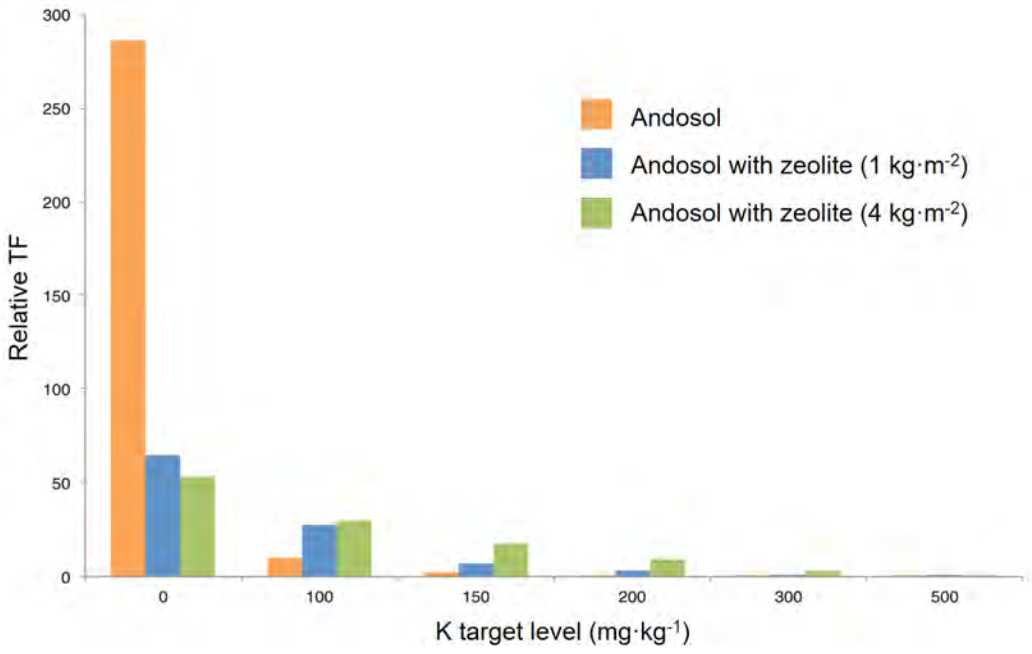

Figure 7. Expected increase in transfer factor (TF), relative to TF of soil with K target level of 200 mg $K_2O \cdot kg^{-1}$ (without zeolite application, value is 1), for Andosol with and without zeolite.

Machine Learning-based Soil Property Prediction for Remediation of Radioactive Contamination in Agriculture

Within the CRP D1.50.19, the high-throughput characterization of soil properties and the estimation of soil-to-plant transfer factors of radionuclides are of critical importance.

For several decades, soil researchers have been successfully using near and mid-infrared spectroscopy (MIRS) techniques to estimate a wide range of soil properties (e.g. Carbon, Nitrogen, CEC, Clay, Sand, pH). In recent years, soil science researchers are increasingly shifting their focus from traditional modelling techniques such as PLSR (Partial Least Squares Regression) to new classes of algorithms, such as Ensemble Learning (e.g. Random Forest, Boosting) or Deep Learning (Convolutional Neural Networks), that have proven to outperform PLSR on most (if not all) soil properties prediction in a large data regime.

Indeed, until recently, only small (~100 samples) and region-specific MIR spectra libraries of soils were accessible. It is now realistic to envision a global MIR spectral library to characterize the world's soils, thanks to the United States Department of Agriculture - Natural Resources Conservation Services (USDA-NRCS), which is maintaining a large and growing library (KSSL library) of MIR-scanned soil samples (~80K as of today), and the FAO GLOSOLAN – Soil Spectroscopy Workgroup (see: http://www.fao.org/global-soil-partnership/glosolan).

In 2020, the SWMCN Laboratory obtained from the USDA-NRCS the KSSL database and reproduced the state-of-the-art modelling and cutting-edges MIR modelling techniques using advanced Machine Learning techniques.

Among the many interesting outcomes, these new classes of algorithms trained on the KSSL large database (all soil taxonomic orders included ~ 50K samples) make it possible to reach a quality of prediction for potassium so far unsurpassed with an acceptable Residual Prediction Deviation (RPD) around 3. Potassium is known for its difficulty of being predictable (RPD~1.5 on small and local datasets) but remains extremely important for the remediation of radioactive contamination after a nuclear accident. Potassium can help reduce the uptake of radiocaesium by crops, as it competes with radiocaesium in soil-to-plant transfer, as demonstrated in the previous section.

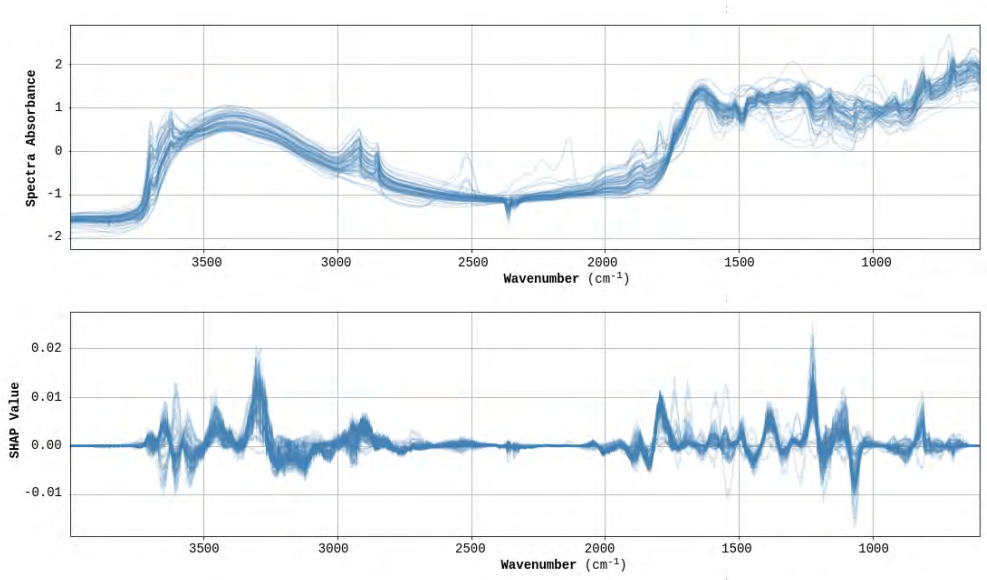

Figure 8. Identifying critical spectral regions for predicting the level of Potassium (high-low) through Mid-Infrared Spectroscopy using Shapley Additive exPlanations (SHAP) values.

As part of our research activities, thanks to these new MIRS-soils library and types of prediction algorithms, we aim to further address the challenges of predicting soil properties relevant to remediation and in particular: (i) Evaluate the performance of prediction algorithms in a simpler classification setup (e.g. low, middle, high levels) and (ii) Improve the interpretability of models developed by explaining individual predictions using methods such as Shapley Additive exPlanations (SHAP) values (i.e. how much specific MIRS wavenumber regions contribute to the prediction) (Figure 8).

SWMCNL is now a member of the GLOSOLAN network, which helps to enhance the usability of MIRS for soil monitoring worldwide. SWMCNL is further developing training packages on the use of traditional and advanced mathematical techniques to process MIRS data for predicting soil properties. Such training packages have been tested in October 2020 with thirteen staff members of the FAO/IAEA Laboratories in Seibersdorf, Austria.

DSS4NAFA - New Developments

Under the CRP D1.50.19 focusing on the optimization of remediation of radioactive contaminated agricultural land, one important objective is to update the existing decision support system DSS4NAFA to support data management during remediation activities in the aftermath of a nuclear emergency affecting food and agriculture. During remediation, the need exists for keeping an overview of the remediation activities and where, when, and how these activities are carried out. Further, it is imperative to keep track of the efficiency and effectiveness of remediation activities (e.g. reduction of soil and food contamination). At this moment the workflow for the remediation module is being developed in close collaboration with the CRP D1.50.19 research partners from across the world for addressing the above-mentioned needs for enhanced data management during remediation.

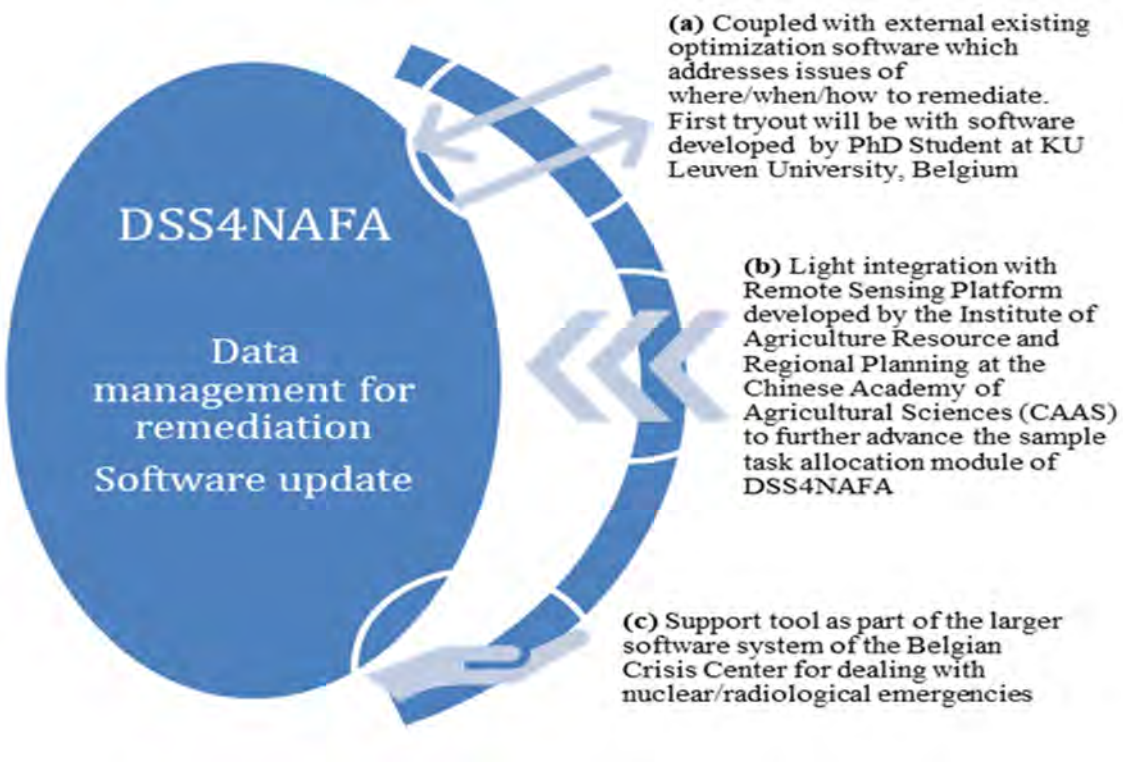

Figure 9. Envisioned activities to enhance and further develop DSS4NAFA, for improving sampling task assignments and the follow-up of remediation intervention in case of radioactive contamination in food and agriculture.

Further, as shown in Figure 9, DSS4NAFA is being coupled with external modelling tools that help with the specific decision of where/when/how to remediate and will be based on expert judgments and multiple stakeholders' preferences (e.g. decision-makers, farmers). The principles of modularity and complementarity are at the heart of DSS4NAFA design choices. Therefore, this new tool, heavily involving various mathematical optimization techniques and other methods is being developed as a new external module of the DSS4NAFA ecosystem. This tool is being developed by the Belgian Nuclear Research Centre in collaboration with the University of Leuven (Belgium).

A further activity related to DSS4NAFA is a new collaboration with the Chinese Academy of Agricultural Sciences (CAAS) (Figure 9). Through assistance provided by the Chinese Institute for Agriculture Resource and Regional Planning at the CAAS, a remote sensing platform is being developed for identifying land use. The focus of this development is to assess the integration of large-scale land use maps into DSS4NAFA to further optimize food and soil sampling during a nuclear emergency response. Possibilities of enhancing the currently existing advanced sample and task allocation (ASTA) module in DSS4NAFA are tested, to be further implemented under the CRP.

Finally, software packages in the core of DSS4NAFA are actively maintained, and each interested Member State is further welcomed to try out DSS4NAFA. Currently, DSS4NAFA has been adapted for the Belgian situation (through the PUI project on Global Networking for Improved Radiological and Nuclear Emergency Preparedness and Response in Food and Agriculture). To meet the Belgian needs, some developments had to be done to provide interoperability between the different applications used for the emergency preparedness and response in Belgium as well as to propose a user-friendly interface for the field extension of DSS4NAFA.

CAPACITY BUILDING

In 2020, due to COVID-19, training activities were drastically scaled down. The SWMCNL focused on the training and guidance of three PhD, two MSc students (through IAEA internships), three interns from four countries in the use of nuclear and isotope techniques for climate-smart agriculture and nuclear emergency response. Further four virtual training courses were organized for a total of 54 trainees from 14 countries on the use of cosmic ray neutron sensor technology and nitrogen-15 isotopes for improving agricultural water and soil fertility management.

A new format for virtual training courses in times of COVID-19

Due to the COVID-19 outbreak, most training courses were held in virtual mode. To accomplish these online courses, a new approach was adopted, based on a flipped classroom format. Such format is an instructional strategy, aiming to increase student engagement and learning. Students were asked to watch the lectures and training videos ahead of the planned course activities. This way the participants could watch the material at their leisure or multiple times for increased comprehension. In addition, English Closed Captioning was provided to help overcome any language barriers. During the planned online course times the participants received a quick summary of the lectures and activities, followed by more interactive discussion and active learning periods. The discussion periods for each day were limited to 2-3 hours to eliminate "zoom fatigue". Finally, all discussion periods were recorded and made available to the participants after the training course. Course surveys indicated that the format was highly effective and enjoyed by the participants despite the challenging circumstances. The online training material will be a valuable resource during the pandemic as well as in future trainings where international travel may be minimized.

ANALYTICAL SERVICES

Laboratory analyses

In 2020, 4123 samples were analysed for stable isotopes and 200 samples were measured for fallout radionuclides, respectively in the SWMCN Laboratory. Most analyses (i.e. 96%) were carried out for supporting Research and Development activities at the SWMCNL focused on the design of affordable isotope and nuclear techniques to improve soil and water management in climate-smart agriculture. An additional analytical focus of the SWMCN Laboratory was on ^{13}C-CO_2 and ^{15}N-N_2O measurements using the laboratory-based laser isotope analysers.

External Quality Assurance: Annual Proficiency Test on ^{15}N and ^{13}C isotopic abundance in plant materials

The worldwide comparison of stable ^{15}N and ^{13}C isotope measurements provides confidence in the analytical performance of stable isotope laboratories and hence an important tool for external quality control.

The 2020 Proficiency Test (PT) on ^{15}N and ^{13}C isotopic abundance in plant materials, organized by the University of Wageningen, the Netherlands, and funded by the SWMCN Laboratory has been successfully completed. The Wageningen Evaluating Programs for Analytical Laboratories (WEPAL, http://www.wepal.nl) is accredited for the organization of Inter-Laboratory Studies by the Dutch Accreditation Council.

Every year, one FAO/IAEA ^{15}N-enriched and three non ^{15}N-enriched test samples are included in one round of the WEPAL IPE (International Plant-Analytical Exchange) programme. A special evaluation report for IAEA participants on the analytical performance in stable isotope analysis is issued by the SWMCN Laboratory and sent to the participants together with a certificate of participation in addition to the regular WEPAL evaluation report. The participation fee for one round per year is covered by the IAEA.

In total seven stable isotope laboratories participated in the PT-round 2020: Africa (1): Morocco, Asia (1): Pakistan, Europe (3): Austria, Belgium and France, Latin America (1): Brazil and South Pacific (1): New Zealand.

Due to the COVID-19 situation most laboratories were locked down for several months, therefore the deadline for reporting of results was extended. The laboratories, who were able to send the results before the new deadline, performed well regarding the C and N elementary analysis. However, as shown by the results below (Table 1), the ^{15}N and ^{13}C analyses were slightly below expectation, and less good than in other years. Nevertheless, it may be explained by the extended closure of the laboratories and the higher difficulty of isotope analysis. The lessons learned in these difficult times are of high importance to see what such extended closure may cause regarding the quality of isotope measurements.

Table 2. Number of results out of control limits.

Sample	$\delta\ ^{15}N$	$\delta\ ^{13}C$	C-elementary	N-elementary	Number of reporting labs
203	0	0	0	0	7
235 (Enriched ^{15}N)	0	4	1	0	6
248	3	1	1	0	7
250	2	0	0	0	7

GUIDELINES AND INFORMATION PUBLISHED IN 2020

Guidelines published in Journal of Environmental Radioactivity

In 2020, a set of guidelines were published in the Journal of Environmental Radioactivity. These guidelines provide background information, as well as generic non-country specific guidance about approaches for sampling and analysing soils, plants and food to scientists, policy-makers and decision makers at different stages of the response phase during and after the nuclear emergency. They are intended to promote standardized and efficient techniques in supporting large scale emergency response in food and agriculture. Specifically, they provide past studies and best practise examples on collecting samples, as well as promote future outlook and guidance on innovative methods. The work was conducted under the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, funded under an IAEA CRP on "Response to Nuclear Emergency affecting Food and Agriculture" (CRP D1.50.15), from 2013 to 2019. The guidelines can be downloaded from:

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2. FESENKO, S., SHINANO, T., ONDA, Y., DERCON, G. (2020). Dynamics of radionuclide activity concentrations in weed leaves, crops and of air dose rate after the Fukushima Daiichi Nuclear Power Plant accident. Journal of Environmental Radioactivity, 222, 106347. https://doi.org/10.1016/j.jenvrad.2020.106347
3. HACHINOHE, M., SHINANO, T. (2020). Large-scale sampling and radioactivity analysis of agricultural soil and food during nuclear emergencies in Japan: Variations over time in foodstuffs inspection and sampling. Journal of Environmental Radioactivity. Volume 218, 106262. https://doi.org/10.1016/j.jenvrad.2020.106262
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5. KHOMUTININA, Y., FESENKO, S., LEVCHUK, S., ZHEBROVSKA, K., KASHPAROV, V. (2020). Optimising sampling strategies for emergency response: Soil sampling. Journal of Environmental Radioactivity. Volume 222, 106344. https://doi.org/10.1016/j.jenvrad.2020.106344
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Open Access new Springer book "Measuring Emission of Agricultural Greenhouse Gases and Developing Mitigation Options using Nuclear and Related Techniques"

This open access book provides insights on how nuclear and related techniques can facilitate the measurement of greenhouse gas emission and help develop mitigation options. It was published through the Soil and Water Management and Crop Nutrition Section, in Springer.

This book comprises of eight chapters covering GHG emission from soil fauna and plants, CH_4 production in ruminant animals, non-isotopic and micrometeorological methods, laboratory and field techniques, isotopic techniques to measure GHGs and identify their sources, and climate-smart agriculture practices for GHGs mitigation. The material presented in this book should be useful for both, beginners in the field, to obtain an overview of the current methodology, and experienced researchers who need a hands-on description of current methodologies. The methods described in this book are written in such a way that they are easy to understand and applicable to a range of users with different expertise and backgrounds. This book can be downloaded from: https://www.springer.com/gp/book/9783030553951

Sharing our research progress and connecting with international researchers through the European Geosciences Union General Assembly 2020, Vienna, Austria

About 22 376 scientists from 134 countries attended the online European Geosciences Union (EGU) 2020 General Assembly held on 4-8 May 2020. The SWMCN Laboratory's activities were reported in 16 presentations covering topics on radionuclide tracers for soil erosion investigations, area-wide soil moisture monitoring, climate-smart crop production, remediation of radioactive contamination of agricultural land and use of stable isotopes as tracer of pollution or indicator for drought stress in under-explored crops such as cassava and banana. The SWMCN CRP D1.50.17 "Nuclear Techniques for a Better Understanding of the Impact of Climate Change on Soil Erosion in Upland Agro-ecosystems", also hosted one EGU session on "Soil erosion and driving factors of soil carbon distribution: A worldwide threat", which had 24 presentations.

The links to all contributions from the SWMCN Laboratory can be found in this annual report under the publication list, at the end of the SWMCNL contribution.

OPENING OF THE NEW SOIL AND WATER MANAGEMENT AND CROP NUTRITION LABORATORY

In 2020, the SWMCN Laboratory moved into the new Yukiya Amano Laboratories (YAL), in honour of the late Director General Yukiya Amano. The Yukiya Amano Laboratories hosts three out of five laboratories of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture. One of the three laboratories is the **Soil and Water Management and Crop Nutrition Laboratory (SWMCNL).**

The new SWMCNL, which has an area of approximately 1800 m^2, has greater capacity to carry out R&D on the development of methodologies and guidelines for improving climate-smart agricultural practices, maximizing crop yields, conserving soil and water resources, to conduct training and training courses, as well as to provide analytical services.

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ALBINET, F., LEE ZHI YI, A., SCHMITTER, P., TORRES ASTORGA, R., DERCON, G. (2020). Exploring the Applicability of Deep Learning Methods in Mid-infrared Spectroscopy for Soil Property Predictions. EGU General Assembly 2020. Abstract EGU2020-4659. https://doi.org/10.5194/egusphere-egu2020-4659

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AN UPDATE ON THE RENUAL PROJECT: THE FAO/IAEA AGRICULTURE & BIOTECHNOLOGY LABORATORIES

The modernization of NA laboratories began in 2014 with the Renovation of the Nuclear Applications Laboratories (ReNuAL) project, which consisted of new building construction to provide modern laboratory space, the acquisition of new laboratory equipment and infrastructure upgrades. A follow-up to ReNuAL, called ReNuAL Plus (ReNuAL+), provided for further construction and modernization of laboratory facilities. As a result of the ReNuAL/ReNuAL+ initiative, two new buildings were built (Insect Pest Control Laboratory building and Yukiya Amano Laboratories) housing a total of four NA laboratories, and a new linear accelerator facility was added to the existing Dosimetry Laboratory.

Yukiya Amano Laboratories Building Officially Opened

Director General Grossi and Austrian Foreign Minister Alexander Schallenberg formally opened the new Yukiya Amano Laboratories (YAL) building on 5 June 2020. The event marked the completion of all major construction begun since the launch of the ReNuAL/ReNuAL+ initiative. The YAL building is home to the Animal Production and Health Laboratory, Food and Environmental Protection Laboratory, and the Soil and Water Management & Crop Nutrition Laboratory. All three laboratories have moved into the building and are fully operational.

ReNuAL2

In September 2020, Director General Grossi briefed Member States on plans for addressing the NA laboratories not yet renovated under the ReNuAL/ReNuAL+ project. The DG outlined the Agency's approach for the final phase of the project, calling it ReNuAL2, which will complete the modernization of the laboratories at Seibersdorf and fulfil the vision of providing laboratory facilities to meet current and emerging needs.

The three main elements of this phase are 1) construction of a new laboratory building, Flexible Modular Laboratory 2 (called FML2), to serve as home for three remaining NA laboratories; 2) refurbishment of the Dosimetry Laboratory in its current location; and 3) replacement of aging greenhouses.

The FML2

The new facility will house the Terrestrial Environment Laboratory, the Plant Breeding and Genetics Laboratory, and the Nuclear Science and Instrumentation Laboratory. The detail design phase for this building is currently in progress and the target date for launching construction is January 2022, with completion projected for late 2023.

Rendering of the FML2 Building

DOL Refurbishment

The Dosimetry Laboratory (DOL) supports radiation dosimetry through the provision of services to IAEA Member States, including dosimetry calibration and dose auditing. As part of the ReNuAL/ReNuAL+ initiative, this laboratory has increased and enhanced its support for Member States by obtaining a new Linear Accelerator Facility, opened in June 2019.

Since the DOL is located in one of the newest wings of the existing NA laboratory facilities and since it has recently received a new linac facility, this laboratory will undergo refurbishment in its current location.

The Greenhouses

The new greenhouses will provide optimal and efficient greenhouse facilities while providing the reliability to protect top level research, training and demonstration requirements that take place there. The three NA laboratories whose work depends on the high quality greenhouses include: the Plant Breeding and Genetics Laboratory; the Food and Environmental Protection Laboratory; and the Soil and Water Management & Crop Nutrition Laboratory.

ReNuAL Resource Mobilization Update

A total of 42 Member States (and some individuals and institutions) provided over €39.7M to the ReNuAL/ReNuAL+ project and have been recognized on the donor wall installed in the new Insect Pest Control Building.

Five Member States have already announced contributions to the ReNuAL2 phase of the project during and since the 64th General Conference in September 2020. These and all subsequent ReNuAL2 contributions will be recognized on a new donor display that will be permanently installed in the FML2 laboratory building upon its completion.

In addition to funding mobilized to date, the Secretariat is seeking to raise €8.9M in extrabudgetary funds this year to provide for a timely launch of construction of the new FML2 building in early 2022 in order to help hold down costs and minimize risk of construction delays.