Standards and Trade Development Facility

project SafeNut

http://www.stdf-safenutproject.com/

Fourth Progress Report:

Reporting Period from 1 October 2007 to 31 January 2008

Project coordinators:
Catherine BRABET, CIRAD, France - General Coordinator
Monica OLSEN, NFA, Sweden - Scientific Coordinator

Date of Report: March 2008

CIRAD LIVSMEDELSVERKET SL r-biopharm Ministério da Agricultura, Pecuária e Abastecimento GOVERNO FEDERAL Embrapa

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GENERAL DATA ON THE PROJECT

Donor number: STDF 114

Title: Validation and transfer to the key stakeholders of a sustainable and effective aflatoxin management system in the Brazil nut production chain for recovering and consolidating export markets, particularly in Europe.

Duration: 2 years (1 June 2006 – 31 May 2008)

Executing agency: CIRAD (Centre de coopération internationale en recherche agronomique pour le développement), France

Partners:
NFA (National Food Administration), Sweden
CSL (Central Science Laboratory), United Kingdom
R-Biopharm AG company, Germany – As a sub-contractor of CSL
MAPA (Ministério da Agricultura, Pecuária e Abastecimento), Brazil
EMBRAPA (Empresa Brasileira de Pesquisa Agropecuária), Brazil

Specific objectives:
1. Characterization of the Brazil nut production chain, and formulation of organizational and incentive strategies for safety control.
2. Validation of recommended good practices in the Brazil nut production chain for aflatoxin control.
3. Validation and implementation of a rapid aflatoxin surveillance system for use along the Brazil nut production chain.
4. Knowledge and technology transfer to the key stakeholders.
5. To strengthen the public-private dialogue and partnership in the Brazil nut sector.

PROJECT STATUS

1. Describe briefly the major tasks or activities achieved during this reporting period (attach details on available training material, summary reports, etc...)

1.1. Management project activities

Safenut project extension
An extension of six months, i.e. until 30 November 2008, was requested and agreed by the STDF Working Group.

Updating of the Safenut action plan
At the request of the general coordinator, a synthesis of the main project results, conclusions and plans discussed during the Safenut progress meeting in September 2007, was prepared for each project specific objective (Specific objective 1: MG Piketty ; Specific objective 2: C. Brabet & M. Olsen ; Specific objective 3: John Banks ; Specific objective 4 & 5: C. Brabet). These syntheses were used to update the Safenut action plan and propose a new timetable as part of the request for project extension.

Fund transfer to the Safenut partners
CIRAD has transferred to MAPA 50 % of their total budget after deducing the expenses assumed directly by CIRAD up to the date of transfer, thus completing the transfer to all the Safenut partners of 50 % of their total budget. CIRAD has also transferred to MAPA, EMBRAPA and NFA, 35 % of their total budget, and will transfer to CSL 35 % of their total budget once the protocol/Standard Operating Procedure for complementing the evaluation of the ELISA and LFD kits will be finalized and validated among partners, as well as the laboratory for the execution of this work (LACQSA/MAPA, Brazil or R-Biopharm, Germany) and the associated costs confirmed (see 1.2 Specific Objective 3).

Organization and participation in project meetings
- Rio Branco-Acre, 5-8 November 2007 – C. Brabet and D. Montet, CIRAD + EMBRAPA local team: Follow up of the laboratory and field activities.
- Belém-Pará, 25-29 November 2007 – C. Brabet, CIRAD + J. Garcia and T. Alves de Sá, LACQSA/MAPA + MAPA local team: Organization of the Brazil nut sample collection and analyses in 2008 (sampling plan, time scale for the analysis of aflatoxins at LACQSA, financial and administrative issues…).

Supervised students for project activity support:
- Felicia Maria Nogueira Leite, Brazilian student, UFAC (Universidade Federal do Acre) / SEAPROF (Secretaria de Extensão Agroflorestal e Produção Familiar), MSc in post-harvest technology: Execution of the activities A.2.2 in the state of Acre (project specific objective 2).
- Sarita Maria de Azevedo, Brazilian student, UFAC, engineer in agronomy: Support for Brazil nut sample preparation and analyses at the EMBRAPA Acre (project specific objective 2).

Reporting to the STDF Working Group:
The third progress report was sent for the reporting period June-September 07, and a status report.

1.2. Scientific & dissemination project activities

The major tasks or activities achieved during the reporting period are presented within the corresponding Safenut specific objectives.

Specific objective 1: Characterization of the Brazil nut production chain, and formulation of organizational and incentive strategies for safety control

Further data were collected, in particular on Brazil nut price dynamics, in order to complement the characterization of Brazil nut production and commercialisation in the Brazilian states of Acre and Pará.

Questionnaires were also elaborated to be applied to the different actors of the Brazil nut production chain (associations/cooperatives of producers/extractivists, intermediaries, agro-industries/exporters) during the first semester of 2008, both in the states of Acre and Pará. The objective of these field surveys is to collect the information still lacking for completing the description of the Brazil nut production chain.

Specific objective 2: Validation of recommended good practices in the Brazil nut supply chain for aflatoxin control

A2.2: Brazil nut sample collection and analyses in the states of Acre and Pará

- **Harvest season 2007:**

In the state of Pará:
Brazil nut sample collection in the rainforest, communities of the selected association of producers and one selected processing plant, as well as analyses (water activity, fungi and aflatoxins by using HPLC method) and environmental factor registration (temperature and relative humidity) have been completed until September 2007 (see Safenut Third progress report).
These field data were used to evaluate the results of the laboratory study for the development of a simple predictive model for aflatoxin and fungi production in the Brazil nut production chain (see below A2.3).

In the state of Acre:
Brazil nut sample collection in the rainforest, two associations of producers and one selected processing plant, as well as analyses (water activity and fungi) and environmental factor registration have been completed until January 2008.
The collected samples were sent by EMBRAPA Acre to LACQSA/MAPA, Belo Horizonte-MG, for aflatoxin analysis by HPLC method. At LACQSA, the physical characteristics of these samples, such as homogeneity and particle size, were evaluated according to LACQSA's sample preparation procedures. After a critical evaluation (ISO 15025:2005), each sample was registered and stored at -15°C until aflatoxin analysis.

- **Harvest season 2008:**

The collection of further Brazil nut samples along the production chain (from the rainforest to the storage of the dried unshelled Brazil nuts in the processing plant) was initiated in both states of Acre and Pará in January 2008 (i.e. at the beginning of the harvest season), in order to have a more comprehensive study for identifying the steps in the BnFD the mycotoxin hazard originates, or concentrations increase to unacceptable levels.
The Brazil nut samples are collected in the same selected production chains than in 2007 (see above Harvest season 2007), at the exception of the Acre state where sampling in the rainforest is carried out

only in the association of producers 1 (and not in the association of producers 2) because of the improved production practices applied by the extractivists and better access to the locality.

Brazil nut sample collection began in the rainforest. Samples that could not be collected at some sampling points during the harvest season 2007 (for example, pods recently fallen from the tree) were already collected during the harvest season 2008.

- **Other relevant progress:**

Methodological issues
- The sampling plan to be applied in 2008 was discussed and adjusted as necessary, as well as the instructions for the laboratory work.
- Further ring tests for fungal analyses were performed by the laboratories of microbiology of EMBRAPA Acre and LANAGRO-PA/MAPA in order to verify their laboratory skills before the onset of data collection in 2008. Both laboratories continue to have sufficient precision in these analyses.

Additional human resources
- Virginia de Souza Álvares, PhD in post-harvest physiology, researcher at EMBRAPA Acre since November 2007: support for the Safenut activities, in particular A2.2, in the state of Acre.
- José Delfin de Figueiredo Filho, technician at SEMAGRI (Secretária Municipal de Agricultura), Oriximiná-PA: support for Brazil nut sample collection in the rainforest in the state of Pará.

A2.3: Development of a simple predictive model for aflatoxin and fungi production in the Brazil nut production chain

The experiment with storage of Brazil nuts in a respirometer (March - June 07) was statistically analyzed and a full project report was delivered in November 2007. From this material, a scientific article has been written and submitted to the World Mycotoxins Journal in February 2008 (see Abstract in Annex).

The experimental study aimed at gaining more knowledge of the growth of aflatoxigenic moulds and aflatoxin production in Brazil nuts in relation to humidity conditions and storage time.
For this purpose, fresh unprocessed Brazil nuts in shell were inoculated with an aflatoxin producing fungal strain of *Aspergillus nomius* previously isolated from Brazil nuts. The nuts were stored at 27 °C under three humidity conditions (97, 90 and 80 % relative humidity) in a respirometer for up to 3 months.
The General Linear Model (GLM) was used for evaluation of the effect of water activity (a_w) and time on aflatoxigenic mould levels and on aflatoxin levels, as well as the relation between mould and aflatoxin levels. In addition, a logistic regression model was developed to describe the probability that the European legislative limit of 4 µg/kg for total aflatoxins in nuts will be exceeded in relation to colony counts of aflatoxigenic moulds. The results of the experimental study were evaluated against the results from the field study conducted in the state of Pará, Brazil.

Main conclusions:

- Once the Brazil nuts have been infected with aflatoxigenic moulds, the probability and rate of fungal growth and toxin formation was significantly related to the a_w in the experiment study. During storage at the highest relative humidity (97 %) aflatoxin formation occurred rapidly (a couple of days), whereas storage at 90 % relative humidity resulted in slower aflatoxin formation (the probability for toxin production increased from 25 to 95 % between 5 and 15 days). At the lowest relative humidity (80%), aflatoxin formation occurred sporadically during storage.
- However, the a_w had no effect on the amount of toxins that was formed at a certain mould level. This means that analysis of mould levels may serve as a predictor for toxin levels no matter if all nuts or only a part of the nuts are infected and regardless of the a_w.

- The probability that total aflatoxin levels will exceed the European legislative limit of 4 µg/kg increased rapidly from approx. 30% to above 80% at mould levels between 2 and 3 log cfu/g. The accuracy, i.e. sensitivity and specificity, for a threshold between 2-3 log cfu/g was high in both experimental and field data. The results of the field study indicate that choosing 2 log cfu/g as the threshold for action would mean less than 5 % risk of missing positive cases, compared to 10 % if the threshold is set to 3 log cfu/g. Furthermore, 2 log cfu/g is a critical threshold also for higher maximum limits since the aflatoxin formation increases rapidly after this level. These conclusions are valid if representative sampling is performed with quantification of aflatoxigenic moulds using AFPA.

- The field data also showed that the probability of aflatoxin formation or total aflatoxin levels above the European legislative limit of 4 µg/kg increased rapidly between 40-90 days following collection of the nuts, and before the nuts reached the final drying stage at the processing plant. Moreover, from these data, it seems that significant onset of aflatoxin formation primarily takes place in the village communities. When growth has started, colony counts and aflatoxins will accumulate as long as the a_w and nutrient availability allows it. Growth will cease after drying of the nuts but fungal spores and aflatoxins that have been formed earlier will remain.

- All toxin positive field samples contained B and G aflatoxins and according to the meteorological data from the city of Óbidos, Pará state, Brazil, the average daily temperature for the harvesting seasons (December through May) for all years (2004-2007) was 26.9 °C, supporting the choice of the *A. nomius* fungal strain which is able to produce both B and G aflatoxins and the storage temperature of 27°C in the experimental study.

- Several *A. nomius* strains were recovered from Brazil nuts in the current study and Brazil nut lots imported to Europe and containing G aflatoxins (another project, 2005). In addition, *A. parasiticus* which is also able to produce both B and G aflatoxins was not detected in any samples in the current study. These results indicate that *A. nomius* may be the major aflatoxin producer in natural Brazil nut samples. That should be further investigated if possible.

Further studies are being performed in March 2008 to evaluate the impact of infection dose and temperature and of differences between aflatoxigenic strains/species that are relevant to Brazil nuts on the formation of aflatoxins.

Specific objective 3: Validation and implementation of a rapid aflatoxin surveillance system for use along the Brazil nut production chain

A3.1 / A3.3: Adapt and validate existing rapid ELISA and on-site Lateral Flow Device (LFD) brought in to the project for aflatoxins in Brazil nuts

Based on the two previous delivered reports documenting the fit for purpose studies carried out on Brazil nut samples spiked with aflatoxin B1 (0 & 20 ppb) and naturally incurred with aflatoxins (over a range of about 2 to 300 ppb total aflatoxins), the following three kits brought in to the project by R-Biopharm were selected to be used within the Safenut project for the estimation of aflatoxins along the Brazil nut production chain:
- RIDASCREEN® FAST Aflatoxin (ELISA) for the estimation of total aflatoxins,
- RIDASCREEN® Aflatoxin B1 30/15 (ELISA) for the estimation of aflatoxin B1,
- RIDA®QUICK Aflatoxin LFD for the estimation of total aflatoxins.

Based on the discussions and questions raised (effect of the lower cross reactivity of the antibodies to aflatoxin G1 compared to B1 in the RIDASCREEN® FAST Aflatoxin kit used for the estimation of total aflatoxins; effect of high dilutions) at the Safenut progress meeting organized in September 2007, it was agreed that additional tests would be performed as a double check for completing the evaluation of the

ELISA and LFD kits. As for the previous fit for purpose studies, these tests will consist in a comparison of results obtained from the analysis of Brazil nut samples by both the kits and HPLC.

The general coordinator prepared a work plan proposal that was validated by CSL and R-Biopharm. This work plan includes the following additional tests:

- Evaluation of the kits in samples with high amount of G1 to test the effect of lower cross reactivity of the antibodies to aflatoxin G1 compared to B1;
- Evaluation of the kits in samples with high amount of aflatoxins to test the effect of high dilutions;
- Further comparisons between the ELISA and LFD kits and HPLC as necessary, by analysing Brazil nut samples collected in the states of Acre and Pará, in order to provide a more complete series of data.

A detailed protocol/Standard Operating Procedure is being drafted and must be finalized as well as the estimation of the associated costs before the confirmation of the laboratory for the execution of the work (LACQSA/MAPA, Brazil or R-Biopharm, Germany) and the implementation of the additional fit for purpose study.

A3.2 / A3.4: Set up in Brazil a rapid ELISA and on-site LFD for aflatoxins in the laboratory, the Brazil nut production area and one processing plant, in conjunction with project specific objective 4 (training courses)

No training courses expected during the reporting period.

Further training courses in ELISA and LFD will be organized in 2008 at the same time than the training courses in good practices i) for the Safenut partners for completing the implementation of both methods in the laboratory ii) for the Brazil nut producers and processors for setting up the LFD method in the Brazil nut production area and the processing plant.

Demonstration sessions on the ELISA and LFD kits will be organized at the Safenut final workshop (see project specific objective 5).

Specific objective 4:
Knowledge & technology transfer to the key stakeholders

A4.2: Training courses in ELISA and LFD for aflatoxin analyses in Brazil nuts
No training courses expected during the reporting period (see project specific objective 3 - A3.2 / A3.4).

A4.4: Development of a project specific website
Updating of the project specific website (http://www.stdf-safenutproject.com/)

A4.5: Scientific and specific sector publications

A scientific article has been written and submitted to the World Mycotoxins Journal in February 2008 (see Abstract in Annex).
P. Johnsson, M. Lindblad, A. M. Thim, N. Jonsson, E. A. Vargas, N. L. Medeiros, C. Brabet, M. Quaresma de Araújo, M. Olsen, 2008: Modelling the growth of aflatoxigenic moulds and aflatoxin formation in Brazil nuts.

Specific objective 5:
To strengthen the public-private dialogue and partnership in the Brazil nut sector

No deliverable expected during the reporting period.

A5.3: Final workshop
This workshop will be organized at the end of the project.
Suggested dates: 3-8 November 2008, to be confirmed with the Safenut partners. An email was already sent to them on 3 March 2008.
Suggested locality: Belém, State of Pará, Brazil, to be confirmed with the Safenut local teams.

2. Briefly list any issues / problems which impeded project implementation during this reporting period or which might affect project implementation in the future:

Project specific objective 2
One member of the NFA team, Pernilla Johnsson, has left NFA for another position at the Swedish University of Agricultural Sciences. However, NFA has been able to engage Pernilla for some hours during this spring and thereby perform further experimental study to evaluate the impact of infection dose and temperature and differences between aflatoxigenic strains/species (3 *Aspergillus nomius* and 2 *Aspergillus flavus*, all isolated from Brazil nuts). NFA has also engaged other permanent NFA staff to assist this study.

Project specific objective 3
Based on the two previous delivered reports documenting the fit for purpose studies carried out on Brazil nut samples spiked and naturally incurred with aflatoxins as well as the discussions and questions raised at the Safenut progress meeting in September 2007, it was agreed that additional tests would be performed as a double check for completing the evaluation of the ELISA and LFD kits.
Because of the delay of the completion of the kit validation and consequently their implementation at the laboratory level, the general and scientific coordination agreed that the HPLC method would be used for analysing aflatoxins in the Brazil nut samples collected within the project specific objective 2, rather than the ELISA method (originally scheduled in the Safenut proposal). The budget of Brazilian partners will be used to cover the HPLC costs, while the budget of CSL and R-Biopharm will be used to cover the completion of the kit validation and setting up (training courses and demonstration session).

3. Describe briefly any measurable project impacts in the reporting period (as distinct from project outputs)

Reinforcement of the skills of the local teams in the state of Acre and Pará (EMBRAPA, SFA-PA and LANAGRO-PA/MAPA) for the collection, preparation, analysis, handling and sending of Brazil nut samples, through technical and methodological support.

As one of the main conclusions of the experimental study conducted at NFA (see 1.2 Specific objective 2 – A2.3), analysis of mould levels may serve as a predictor for toxin levels no matter if all nuts or only a part of the nuts are infected and regardless of the a_w. The probability that aflatoxin levels will exceed the European legislative limit of 4 µg/kg increased rapidly between 2-3 log cfu. Choosing 2 log cfu/g as the threshold for action would mean less than 5 % risk of missing positive cases, compared to 10 % if the threshold is set to 3 log cfu/g. Furthermore, 2 log cfu/g is a critical threshold also for higher maximum limits since the aflatoxin formation increases rapidly after this level. These conclusions are valid if representative sampling is performed with quantification of aflatoxigenic moulds using AFPA.

ANNEX: SCIENTIFIC PUBLICATIONS

Scientific article submitted to the World Mycotoxins Journal:

Modelling the growth of aflatoxigenic moulds and aflatoxin formation in Brazil nuts

P. Johnsson[1], M. Lindblad[1], A. M. Thim[1], N. Jonsson[2], E. A. Vargas[3], N. L. Medeiros,[4], C. Brabet[5], M. Quaresma de Araújo[4] and M. Olsen[1]

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Abstract

The present study aimed at gaining more knowledge of the growth of aflatoxigenic moulds and aflatoxin production in Brazil nuts in relation to humidity conditions and storage time.

For this purpose, fresh unprocessed Brazil nuts in shell were inoculated with an aflatoxin producing strain of *Aspergillus nomius* previously isolated from Brazil nuts. The nuts were stored at 27 °C under three humidity conditions (97, 90 and 80 % relative humidity) in a respirometer for up to 3 months.

The General Linear Model (GLM) was used for evaluation of the effect of water activity and time on aflatoxigenic mould levels and on aflatoxin levels, as well as the relation between mould and aflatoxin levels. In addition, a logistic regression model was developed to describe the probability that the European legislative limit of 4 µg/kg for aflatoxins in nuts will be exceeded in relation to colony counts of aflatoxigenic moulds.

The results of the experimental study were evaluated against results from a field study conducted in the state of Pará, Brazil.

During storage at the highest relative humidity (97 %) aflatoxin formation occurred rapidly, whereas storage at 90 % relative humidity resulted in slower aflatoxin formation. At the lowest relative humidity (80%), aflatoxin formation occurred sporadically during storage. The probability that total aflatoxin levels will exceed the European legislative limit of 4 µg/kg increased rapidly from approx. 30% to above 80% for both experimental and field samples at mould levels between 2 and 3 log cfu/g. The field data also showed that the probability of toxin formation or toxin levels above the European legislative limit of 4 µg/kg increased rapidly between 40-90 days following collection of the nuts, before the nuts reached the final drying stage at the processing plant.