FELLOWSHIP SUMMARY REPORTS

- Your name: **Dr. Miodrag Grbic**

- The subject title and theme number of your research fellowship:
  Natural nano-material derived from spider mite silk: genomics of agricultural pest leads to novel biomaterial
  Theme III: Transformational technologies and innovation

- Your host institution: **Instituto Murciano de Investigación y Desarrollo Agrario y Alimentario (IMIDA)**

- The name of your host collaborator: **Dr. Jose Luis Cenis Anadon**

- The dates of your fellowship: **June 1 2019-October 1 2019 (16 weeks)**

- Your consent to your report being posted on the Co-operative Research Programme's website, or alternatively, a short paragraph about your fellowship which could be used anonymously.
  Consent to publish granted

What were the objectives of the research project? Why is the research project important?

Nanomaterials are one of the fastest developing sectors of industry and technology. Their application ranges from medicine (drug carriers, cancer treatment), the food industry (nanoparticles as biosensors, removal of toxic molecules from food, anti-microbial food packaging), agriculture (antimicrobial agents, targeted carriers of "smart" pesticides and fertilizers, nanocapsules for vaccine delivery), cosmetics (carriers of cosmetic products, sun creams), to industrial applications such as the textile industry, nano catalysis and engineering of new materials. Most of currently used nanomaterials are inorganic nanomaterials such as iron (Fe) nanoparticles, aluminium (Al), copper (Cu), gold (Au), silver (Ag), silica (Si), zinc oxide (ZnO), titanium dioxide (TiO_2), and cerium oxide (Ce_2O_3), or chemically synthesized nanomaterials such as carbon nanotubes or polystyrene. However, utilization of these synthetic nanomaterials raises significant health concerns. In this application, we propose to develop novel biological nano-material derived from spider mite silk (spider mites are major pests in agriculture) using combination of genomics, cell and molecular biology and biotechnology. Our aim is to develop environmentally safe nanomaterial (biocompatible, biodegradable, non-toxic for humans and animals) that have potential in multiple sectors, ranging from agriculture and biomedicine, to the food industry following current EU initiative to develop "green" and bio-based economy.

Main objectives of this proposal were:

I To sequence *T. lintearius* genome using Oxford Nanopore long range sequencing and annotate fibroin genes.

II. To augment production of *T. lintearius* silk at semi industrial level to obtain quantity of silk necessary for biomaterial production and characterization.

III. To characterize various materials obtained from *T. lintearius* silk and their effects to mammalian cell lines.

IV. Dissemination and knowledge transfer

1. Were the objectives of the fellowship achieved?

All objectives were achieved. **Objective 1**: The genome of *T. lintearius* was re-sequenced with Oxford nanopore technology and we have determined 30 fibroin genes in the genome which is 3 times more compared to other related species. This proliferation of fibroin genes accounts for order of magnitude larger silk production of *T. lintearius* relative to other silk-producing spider mites. We investigated Young's module of the silk (relationship of strength and elasticity) using atomic force microscopy(AFM) to show that is in the range of previously determined parameters of related species, *T. urticae*. Silk AFM measurements were performed as described in Hudson et al. 2013. Briefly Trenches were fabricated on silicon substrates by photolithography and reactive ion etching at the Western Nanofabrication Facility (London, Canada), using a custom designed mask produced at the University of Alberta NanoFab facility (Edmonton, Canada). Silk fibers were deposited by allowing adult and larval T. lintearius spider mites to walk on the silicon wafers for two to eight hours. AFM measurements were made using a multimode atomic force microscope with a Nanoscope IIIa controller. NP-S silicon nitride cantilevers (Veeco Instruments) with nominal spring constants of 0.06N/m and 0.35 N/m were used for measurements on larval and adult fibers, respectively. This represents one of most strong biomaterials determined so far with fibre diameter of 50 nm, representing natural bio-nano material. **Objective 2**: We optimized the silk production by developing silk producing modules where we were able to produce sufficient silk for experiments planned in objective 3 (summarised briefly here): *T. lintearius* was reared on its natural host *Ulex europaeus* at the ICVV, Logrono, La Rioja, in a rearing chamber at the temperature 24°C, 16h light:8 dark cycle, and humidity of 60%. Rearing modules consisting of

U. europaeus plant cuttings placed in tube racks and tray filled with water were used for silk mass production (Fig. 1). Silk was collected from the plants using toothpick (silk is wrapped on the toothpick in similar manner as sugar wool, threads adhere to each other) and used for nanoparticle preparation. **Objective 3** We developed protocol to dissolve *T. lintearius* silk where we dissolved silk in LiBr 9.3 M at 60 °C during 2h. Then the resultant dissolutions were dialyzed against distilled water for 3 days to remove the LiBr, filtered through Miracloth paper and centrifuged to remove the insoluble fragments and dust. After this last step the dissolutions were concentrated by dialysis against PEG (10000 Da) for 9 h obtaining three replicates of aqueous TS (1.3 % w/v) and stored at 4 °C until use. In order to compare the effectiveness of *T. lintearius* silk to assemble into nanoparticles compared with the silk fibroin from *B. mori,* we followed the protocol for the preparation of *Tetranychus* silk nanoparticles (TSNs) based on the method described previously by Zhang *et al*. [2] for *B. mori* silk fibroin nanoparticles, with modifications. Briefly, the freshly prepared TS aqueous solutions (1.3 %wt) were slowly dripped (~1 drop every 2 seconds) in cold methanol gently stirred. Methanol proportion in the final mixture was kept over 70 % (v/v) in order to achieve an efficient conformation change of silk from random coil to □-sheet. After a few drops, turbid amber-like suspension appeared, and the mixture was allowed to reach room temperature while stirring for 2 hours. Then, the particle suspension was transferred to Falcon tubes and centrifuged at 10,000 *g* for 15 minutes, at 8 °C. Supernatant was discharged and equal volume of dry methanol was added in order to wash pigments or contaminants adsorbed onto the particles. After sonication, centrifugation and decantation of Methanol supernatant, particles were washed in 30 mL of MilliQ water (3x) and kept in water suspension at 4 °C until used or lyophilized for longer storage. In order to analyze the potential use of *T. lintearius* as a biocompatible material, *T. lintearius* silk films and *T. lintearius* nano particles were produced and tested *in vitro* using *L929* cell line. Murine fibroblasts (*L929* cell line, ECACC Nº 85011425) were chosen for the cell culture study as they are highly stable, fast growing and commonly used for cytotoxicity and biocompatibility experiments. Viability and cell number were determined by trypan blue staining in a Neubauer chamber and the cells were tested for the absence of mycoplasma before performing the experiments. All the chemicals used for cell culture were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Gibco (Paisley, UK); Nunc (Roskilde, Denmark) provided the culture plates. The L929 cells were seeded in 25-cm^2 flasks at a density of $5{\cdot}10^3$ cells$\cdot cm^{-2}$ in DMEM expansion medium (supplemented with 10% FBS, 100 U$\cdot mL^{-1}$ penicillin and 100 µg$\cdot mL^{-1}$ streptomycin) at 37 °C and 5 % CO_2. The medium was carefully replaced twice a week and cells were allowed to grow until the culture reached 80 % confluence. To test the biocompatibility of TS films, the cells were detached using 0.05 % trypsin/EDTA and seeded on the films at a density of at $5{\cdot}10^3$ cells$\cdot cm^{-2}$ with 1 mL of DMEM expansion medium. Tissue culture polystyrene substrates (TCPS) were also seeded to be used as positive controls for adhesion and biocompatibility. Cell proliferation was measured 48 h, 5 days and 7 days after seeding using PrestoBlue (PB) reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), a resazurin-based membrane permeable solution which does not require cell lysis. In the case of *T. lintearius* nano particles, *L929* cells were seeded at a density of at $15{\cdot}10^3$ cells$\cdot cm^{-2}$ with 1 mL of DMEM expansion medium on a nude 48-well tissue culture plate and incubated 24h to achieve cell adhesion and expansion. After this period, *T. lintearius* nano particles were added to the culture at 10, 50, 100 and 200 µg$\cdot mL^{-1}$ to evaluate its potential cytotoxicity using the PB assay after 24 h exposure to the *T. lintearius* nano particles. We observed a lower proliferation rate of *L929* fibroblasts on *T. lintearius* films

comparing to cells growing on the control polystyrene wells, nevertheless this lower cell proliferation on *T. lintearius* films represents a 65-66 % at 48 h and 7 days of study respect to the optimal conditions for cell culture of control wells. No cytotoxic or cytostatic effect of *T. lintearius* silk was observed, as relative fluorescence units of PB assay with TS films increased at the end of the study. *T. lintearius* nano particles were also evaluated *in vitro* by exposition of *L929* cultures to different concentrations of study. 10, 50 and 100 $\mu g \cdot mL^{-1}$ of *T. lintearius* nano particles showed a statistically similar cell proliferation than cells growing on DMEM expansion medium (control). Only 200 $\mu g \cdot mL^{-1}$ of *T. lintearius* nano particles showed a lower cell proliferation but representing a 90 % respect to control. These *T. lintearius* nano particles results, together with those obtained from *T. lintearius* films indicate a good biocompatibility and no-cytotoxicity of the *T. lintearius* silk. Such properties make the silk from *T. lintearius* a potential biomaterial with potential in biomedical and pharmacological applications.

2. What were the major achievements of the fellowship? (up to three)

1. Development of the protocol for semi-industrial silk production
2. Characterization of silk physical properties, demonstrating to be one of strongest natural bio-materials
3. Showing that this silk is biocompatible, opening possibilities for application of this material in pharmacology and biomedicine

3. Will there be any follow-up work?

- Two publications are planned from this work to be published in international open-access journals
- Is your fellowship likely to be the start of collaboration between your home institution and your host?
  Yes, we see a long-term collaboration between IMIDA, Spain and our lab at UWO in Canada in area of biomaterials
- Is your research likely to result in protected intellectual property, novel products or processes?
  IP protection is under the consideration and our findings are evaluated by IP expert.

4. How might the results of your research project be important for helping develop regional, national or international agro-food, fisheries or forestry policies and, or practices, or be beneficial for society?

Please express this in terms of environmental/food security/food safety/economic/health (human and livestock and plant) benefits, etc.

The *T. urticae* (spidermite) genome sequencing project allowed isolation of fibroin gene sequences, however due limited amount of silk production by *T. urticae* and its fine structure it was not possible to characterize this promising biomaterial. To overcome silk quantity limitation, we established culture of related species, gorse spider mite, *Tetranychus lintearius* (Tl), that produces copious amount of silk and using semi-industrial production generated sufficient amount of silk for biochemical and physical characterization. We have shown that Tl silk has similar thickness and physical characteristics as *T. urticae* silk. We produced and characterized nano-particles generated from Tl silk as well as biofilm and demonstrated that rat fibroblasts can successfully grow in culture in presence of Tl derived silk biofilm and nanoparticles. Finally, we fluorescently labelled Tl nanoparticles and demonstrated that they can enter cultured cells. These experiments suggest that Tl silk is new biocompatible material with potential for various applications, including agriculture, pharmacology and biomedicine representing environmentally safe nanomaterial with variety of potential applications.

5. How was this research relevant to:

- The objectives of the CRP?
- *"The objectives of the OECD's Co-operative Research Programme is to strengthen scientific knowledge and to provide relevant scientific information and advice that will inform future policy decisions related to the sustainable agriculture. In particular, the OECD's Committee for Agriculture needs to be informed about scientific developments that are likely to have a medium and longer term impact on policy settings."* The proposed project directly supported the mandate of the programme through the advancement of scientific knowledge in the area of innovative development of new bio-materials facilitated with cutting-edge genomic technology and genome sequencing. Currently the field of nanomaterials is not regulated. However, there are ongoing preparations for the regulation and legislation in this area in EU and world-wide. We envisage the upcoming regulation will favour nanomaterials that are environmentally safe, sustainable, biodegradable and biocompatible, thus quite advantageous for our technology developments. Our proposal generated results that are highly relevant for development of these policies

- The CRP research theme?

  This theme III addresses "*Novel and innovative technologies that achieve a step change*". Our proposal focuses on cutting-edge genomic technologies to produce in an innovative manner new biomaterial, spider mite silk, from unlikely source: main agricultural pest, spider mite. This approach opens a new field of biomaterial-based genomics where genome sequences are becoming source for discovery of novel materials from biological systems. These materials are likely to be biocompatible and environmentally safer than currently used inorganic or synthetic materials providing a strong change in the field.
  In addition, theme III focuses on "smart uses of fertilisers, water, or pesticides etc.". Our proposed research has a potential to generate novel silk-based nano-material that could be used as smart/targeted pesticide carrier that will reduce pesticide application, allow precision pest control and substitute currently used nano-silver as pesticide component, that is known to have adverse effects to environment, humans and livestock.

  In this theme "There is a growing demand from the private sector in bio-products derived from biologically based feedstocks and bioprocessed on an industrial scale to generate high value products as part of the developing bioeconomy." In proposed program, our main long-term aim is to develop bio-product, nano-particles and biofilm from spider mite silk that will represent novel bio-product attractive for industry and bio-based economy. The silk sequence coming from ON sequencing has a high potential for new IP and patent and this material will be highly attractive for different industrial sectors, ranging from agriculture and food to pharmacological industry

6. Satisfaction

- Did your fellowship conform to your expectations?

Yes

- Will the OECD Co-operative Research Programme fellowship increase directly or indirectly your career opportunities? Please specify.

Yes, it allowed transition in the new field of bio-nano-materials that will expsand research in my laboratory.

- Did you encounter any practical problems?

No

- Please suggest any improvements in the Fellowship Programme.

It may be useful to provide initial funding for IP protection and patent filing in the proposals that have a potential to develop new IP.

7. Advertising the Co-operative Research Programme

- How did you learn about the Co-operative Research Programme?

Via OECD newsletter

- What would you suggest to make it more "visible"?

More advertisements in different media

- Are there any issues you would like to record?

That it is an excellent vehicle to initiate novel research and foster international collaboration.