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Kraków, on August 13, 2020

**Review of the  
Ph.D. Dissertation**

**Author: André Loução Bongo**

**Title of the dissertation:**

**“Biodiversity of symbiotic nitrogen fixing rhizobia isolated from arable and virgin soils  
from Huambo province, Angola”**

**Promoter: prof. dr hab. inż. Stanisław Jerzy Pietr**

**Auxiliary promoter: dr Małgorzata Patrycja Oksińska**

The dissertation was prepared at the Laboratory of Agricultural Microbiology of the Department of Plant Protection, Faculty of Natural Sciences and Technology, University of Life Sciences in Wrocław

The review was prepared in response to a letter PD0000004100.7.2020 from prof. dr hab. Marcin Kozak, chairman of the Agriculture and Horticulture Discipline Council, University of Life Sciences in Wrocław of August 3, 2020, which informed me that by the decision of the Discipline Council I was appointed for reviewer of the abovementioned dissertation.

**Research issues**

The vegetable protein is a very important ingredient for both human foods and animal feed. Protein is one of the essential nutrients. Its supply is necessary for proper functioning of the body, because proteins in various ways participate in virtually all processes taking place in the body - they are used to rebuild worn-out tissues, are the basic component of blood, lymph,

milk, hormones and enzymes, they are part of the immune bodies, maintain proper pH of body fluids, they are a carrier of some vitamins and minerals and are involved in regulating blood pressure.

Protein deficiency in the diet can inhibit the growth and development of the body, prevent tissue reconstruction, reduce resistance to diseases and impede wound healing. It also disrupts thought processes and brain function. Far-reaching protein malnutrition causes kwashiorkor and is still seen in the Far East, Africa and Latin America.

Due to the shortage of nutritious, full-value animal proteins, plant proteins should be appreciated, because although they are less absorbed - in a well-balanced diet, they can be well used by the organisms. Among plants, the seeds of legumes - lentils, peas, beans and soybeans - are of particular importance. An excellent source of vegetable protein are *Fabaceae* - family of plants that provide 25-35% of the worldwide protein intake. Around the world the common bean (*Phaseolus vulgaris*) is the third crop in the *Fabaceae* family, supplanted only by soybean (*Glycine max*) and peanut (*Arachis hypogaea*). At present, common beans are the basic nutritional food for over 100 million people in Sub-Saharan Africa (SSA) countries as well as the primary source of dietary protein providing over 65% of the protein, and 35% of the caloric intake.

The yield of these plants is dependent and inextricably linked to symbiotic nitrogen fixing bacteria.

The undertaken research topic has a significant contribution to the development of knowledge in the field of environmental microbiology, agriculture and food production as it concerns soil microorganisms - Plant Growth Promoting Rhizobacteria - directly affecting plant yield and soil fertility. The study was focused on the evaluation of the presence of symbiotic nitrogen fixing bacteria in different soils in central Angola. The effective strains of rhizobia were selected and their efficiency was tested in pot and plot - field experiments. Under the field condition at Gongoinga (Huambo, Angola) the ability of isolates to improve the grain yield of the common bean in comparison to the uninoculated plants grown on unfertilized and fertilized by NPK plots was evaluated.

André Loução Bongo's work is a part of the current research issues related to the improvement of the yield of crops that are the basis of animal and human nutrition. The topics discussed in the dissertation are timely and perfectly match the current needs of African and global agriculture.

Considering the diversity and wide range of research carried out as part of the doctoral dissertation submitted for review, I positively evaluate the study, which is innovative and in my opinion falls within the field of agricultural sciences and is housed in the discipline of agriculture and horticulture.

### **Assessment of work in formal and structural terms**

The thesis presented for review was prepared in the form of a study in a manner typical for doctoral experimental works and includes 140 pages of typescript (112 pages of the main text, 4 pages with lists of tables, figures, maps, schems and photos, and 24 pages with a reference list). The basic/main text is divided into five chapters with subsections systematizing the literature information as introduction, data on the used materials and research methods, a description of the results of the author's own research, discussion and conclusions. The work also contains a list of used acronyms and abbreviations, lists of tables, figures, maps, schems and photos, bibliography and abstracts in Polish and English. At this point, I have only one comment – there is no translation of the title in the Polish abstract.

In terms of the layout, the work has been prepared correctly, it is logical, and the content has been presented in the right order - nevertheless, I believe that the aim of the work is such an important element of the dissertation that it deserves to be clearly distinguished as a chapter, and not only included as part of the “Introductory” chapter - this is, however, only a technical and not a substantive comment.

From a formal point of view, the study does not raise any objections, it is written in a linguistically correct manner - the few editorial errors do not reduce the value of the study. The work is prepared in an interesting and consistent manner, and the collected literature has been cited correctly.

The carefully selected bibliography quoted in the study is impressive as it includes as many as 470 items. The collected publications are mainly the latest articles and older items that were necessary for the proper presentation of the analyzed issue, both from the theoretical and practical side.

The tables 1,2 and 3 show the data concerning main parameters of tested soils, list of primers used for the determination of the presence of specific genes using PCR methods and sequencing, and meteorological data during the field experiment (tables 1-3). In the methodological section is also included map with geographic distribution of sampling sites of

soils in Angola and two schemes of the layout of the field experiment (however, in my opinion, scheme number 1 should be entitled as table) .

The results are presented on pages 52 to 102 and discussed on the 10 following pages (103-111). Tables 4 to 34 show the results of each stage of research. The part documenting the results of the work also includes numerous figures (11 in total) - including dendrograms and classical charts/diagrams, which allowed the author to correctly interpret the results.

The method of compiling the research effects and the statistical analysis of the results prove that Mr. André Loução Bongo is able to maturely and accurately verify scientific information. Presenting some of the results in the form of graphs and dendrograms was a good choice for the author, as it allows the reader to analyze the results faster and easier, which is sometimes difficult in the case of tabular summaries.

The work is also significantly enriched by the selection of photographs documenting the stages of study and obtained results.

Due to the function of the reviewer, I have some minor comments about the work:

- in explanation of the tables 8, 9, 12, 20 (pages respectively: 57, 58, 61, 77) – should be “columns” not “colons”;
- it is a bit confusing when different strain name abbreviations are used in the tables and text and different in the graphs (fig. 1, 2, 3, 4, 5) – for example HLE131, HC4 – on figures LE131, C4 - please avoid it in the future;
- in the aim of the study we can find hypothesis (that there are indigenous rhizobia species in the soils of Huambo, that are able to establish an effective symbiotic relationship with *Phaseolus vulgaris* and improve common bean yield and that the use of two different trapping plants will be more effective for the isolation of rhizobia) - therefore I expected a direct reference to it in the conclusions (or at least in discussion) - the more so because the hypothesis was correct.

Nevertheless, these are no remarks that have any negative impact on the value of the work.

### **Assessment of work in terms of methodology**

In my opinion, the research carried out as part of the doctoral dissertation was prepared correctly, analyzes were cross-sectional and carefully thought out from the methodological point of view. The number of samples taken and the appropriate number of repetitions of tests allows us to believe that the obtained results are reliable and representative for the tested environment.

The methods used in the research are described in details on 16 pages of the paper.

As part of the experimental work submitted for review of the thesis, the PhD student performed a number of analyzes (physico-chemical and microbiological) of soil samples collected from natural forest, fallow, and cultivated fields at different regions of Huambo province, and one from the desert of Namibe province in Angola.

The isolation of indigenous rhizobia was done by traditional plate method (straight from the soil) as well as by trapping method using two promiscuous species (common bean and adzuki bean) - which allowed obtainment of more symbiotically active strains from nodules.

Strains of putative rhizobia were selected, purified and characterized – at first phenotypically and later genotypically.

The pot experiment was set up as primary screening of the ability of the bacteria pure cultures to nodulate common bean cv. Basta - which was the right decision as it allowed to eliminate inactive or weak strains.

For genetic characterization of selected previously isolates the PCR method was used. The research allowed to detect the genes *nod* (*nodC*) - essential for nodulation in all rhizobial species and determinant of host range and *nif* (*nifH*) responsible for nitrogen fixation. The author not only isolated and identified PGPB based on morphological features, but more importantly, he performed molecular analyzes (PCR), and after obtaining the sequences of bacterial isolates, he compared them with known sequences from the NCBI Database, which allowed not only to determine their systematic affiliation and create the phylogenetic tree, but it also reveals the position of species that have probably not been identified so far (two strains, HEC1 and HC4 related to genus *Paraburkholderia*).

The most valuable part of analyzes with selected, endemic, high active strains of PGPB were field experiments conducted in Angola on the on the Experimental field at Gongoinga of University José Eduardo dos Santos from August to December 2019. The field - plot experiment was planned properly as randomized complete block design (RCBD) in triplicate, each main plot had nine subplots as experimental units (details are shown in schemes 1 and 2). In pot and field experiments a double - positive and negative controls were planned, which guarantees reliable results.

The methodology – in my opinion - has been correctly described and supplemented with links to source lists. Logically planned experiments and extensive laboratory and field tests required a lot of work, effects in action, systematic and accuracy from the PhD student;

moreover this type of analysis are very time-consuming, absorbing and burden the researcher very much.

I particularly appreciate the work done on the statistical analyzes, which required not only the author's knowledge of appropriate statistical tools, but also knowledge of relevant software. The collected results of the research have been statistically processed with the use of correct methods [software R i386 (3.6.3 version for Windows)]. The data were subjected to analysis of variance (ANOVA) and means were compared using Tukey's HSD test at  $p = 0.05$ . The correlation analysis was used to determine the relationship and magnitude of the association between the studied parameters.

### **Substantive assessment of the work**

The effective use of nitrogen fixing rhizobia can be very useful as one of the tools to face the current and future challenges of agriculture productivity and minimizing adverse effects on the environment. Improvement of common bean yield through symbiotic bacteria in Angola will contribute, in the short term, to poverty reduction, enhance food security and generate income for smallholder farmers as well as in medium- and long-term agriculture sustainability. The characterization of rhizobia diversity in soils of central Angola will help in selecting more effective rhizobia strains capable of improving common bean yield. Effective strains of native rhizobia with higher nodulation and symbiotic nitrogen fixation efficiencies can be used to produce inoculant as bio-fertilizer that might be available to smallholder farmers in Angola. The use of endemic rhizobia inoculants will also possibly reduce the reliance on nitrogen fertilizers.

Therefore, the author of the work set himself an ambitious goal of evaluation of the usefulness of different methods of isolation of BNF rhizobia and characterization of the diversity of indigenous rhizobia isolated from different soils in the Huambo province of Angola; determination of efficiency of different indigenous rhizobia in nodulating and nitrogen-fixing in symbiosis with the common bean and finally selection of effective rhizobia suitable for inoculation of common bean in African condition.

When preparing the research, author hypothetically assumed that there are indigenous rhizobia species in the soils of Huambo, that are able to establish an effective symbiotic relationship with *Phaseolus vulgaris* and improve common bean yield through the BNF process and that the use of two different promiscuous species as trapping plants will be more effective for the isolation of a greater variety of BNF rhizobia. For this purpose, he decided to

compare the isolation effectiveness of BNF rhizobia directly from the soil on semi-selected medium to trapping technique based on promiscuous common bean which has a long-lasting history of cultivation in Angola and to trapping technique based on adzuki bean which is not yet cultivated in central Angola.

The theoretical foundations related to the research goals were presented by André Loução Bongo in the introduction in which he discussed the issues related to the role of *Fabaceae* in food production, environmental conditions of agricultural production in Africa and Angola, characteristic of *Phaseolus vulgaris* L. – its origin, domestication, evolution, classification, morphology, conditions of growth, cultivars and potential yield of common bean. In this chapter, the reader will also find comprehensive information about biological nitrogen fixation by symbiotic rhizobia, taxonomy of rhizobia nodulating common bean in Sub-Saharan Africa and their interactions, commercial inoculants and factors affecting biological nitrogen fixation and description of methods of rhizobial diversity determination. The author paid special attention to the fact that there are no studies on biological nitrogen fixation in Angola's soils and potential role of BNF for bean yield.

The intended research aim was achieved by the author by conducting a series of analyzes carried out in several stages of laboratory, pot and field (plot) experiments – the last step can even be defined as implementation.

In the first stage of laboratory tests - the assessment of the presence of rhizobia in soils, isolation and their diagnostics, genes determining the ability to establish symbiosis, nodulation and active nitrogen fixation were determined.

The second step was pot experiments - in which the actual ability to symbiosis of the strains was assessed and the impact on plant growth and development was examined.

The field plot experiment was the third and, in my opinion, the most important stage of the research – as it was conducted in Angola and the influence of selected PGPRs on the yield of common bean was assessed in African environmental conditions and soil.

The results were discussed on the background of the available literature.

The title of the dissertation presented for review corresponds to the scope of research carried out during its implementation. Considering the data included in the study, I believe that the aim of the study was achieved and the obtained results allowed for the formulation of relevant conclusions.

Research has shown that the use of common bean and adzuki bean as the trap plants was more efficient to access the rhizobia diversity than isolated directly from the soil by semi selectivemedium; the biodiversity of the BNF rhizobia nodulating the common bean in the studied regions of Huambo is low; the use of one cultivar for authentication is a faithful method to identify the effectiveness and efficiency of the rhizobia; several isolates from the nodules of common bean and adzuki bean were Plant Growth Promoting Bacteria for common bean – and were found in the phylogenetic tree closely related to *Burkholderia diffusa*, *Beijerinckia fluminensis*, *Herbaspirillum huttiense*, *Enterobacter ludwigii*, *Enterobacter wuhouensis* , and *Rhizobium puesense*; the native rhizobia nodulating common bean that dominate in the regions of Huambo were closely related to *R. miluonense* or *R. aegyptiacum*/*R. bangladeshense*/*R. binae*/*R. miluonense* group; the strains isolated from the nodule of adzuki bean were closely related to *Paraburkholderia kirstenboschensis* , *Paraburkholderia caribiensis*; two isolated strains of the genus *Parabulkholderia* were not phylogenetically similar to any recognized species.

But the most important results arising from the work are information that seeds treated with *R. miluonense* strain HBA15a, *R. aegyptiacum* strains HCC321 and HLo8 alone improved common bean in the range 407 - 755%, in comparison to control plants; that inoculation with strains HBA15a, HCC321, and HLo8 in combination with NPKfertilization with additional urea, increase yield in the range 14.3 - 57.2% higher in comparison to plants with the same level of NPK and urea fertilization and that the most effective rhizobia can be a potential candidate for the production of inoculants for common bean in Angola.

The work is an important contribution of André Loução Bongo to the literature on the subject concerning the yielding of legume plants. I believe that such valuable research results should be published in a journal of international importance as soon as possible because the literature lacks such data.

### **Final conclusion**

The doctoral dissertation submitted for review, entitled “Biodiversity of symbiotic nitrogen fixing rhizobia isolated from arable and virgin soils from Huambo province, Angola” prepared by André Loução Bongo is an original solution to a scientific problem, it is innovative and indicates that the author has the necessary theoretical knowledge and great practical skills in the field of agricultural sciences, is able to plan experiments, has mastered laboratory techniques and the ability to independently conduct research in the field of



agricultural sciences in the discipline of agriculture and horticulture, thus meets the requirements for doctoral dissertations specified in the Regulation of the Minister of Science and Higher Education of 19 January 2018. On detailed procedure and conditions for carrying out activities in the doctoral, habilitation proceedings and proceedings for granting the title of professor ((Dz.U. 2018 poz. 261) and the current Act of 3 July 2018 - Regulations introducing the Act - Law on higher education and science (Dz.U. 2018 poz. 1669).

Particularly noteworthy is the application aspect of the research conducted as part of this work. In my opinion, the obtained results are of great importance not only for agriculture in Angola, but probably also for neighboring countries - because the isolation of endemic strains of nodule bacteria from the soils of Africa with a high potential for nitrogen fixing under difficult environmental conditions and their use in the form of a biopreparation (bio-fertilizer) in cultivated soils may significantly affect the size and quality of the obtained crops of plants from the *Fabaceae* family which in many countries are an important, essential and even the basic source of protein in the human diet.

Therefore, I am asking the members of the Discipline Council of the University of Life Sciences in Wrocław to admit André Loução Bongo to further stages of the doctoral procedure.

Considering the applicational value and the possibly significant impact on Angolan agriculture, as well as the future in food production, I am applying for the work to be distinguished.

Dr hab. inż. Maria J. Chmiel, prof. UR

  
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