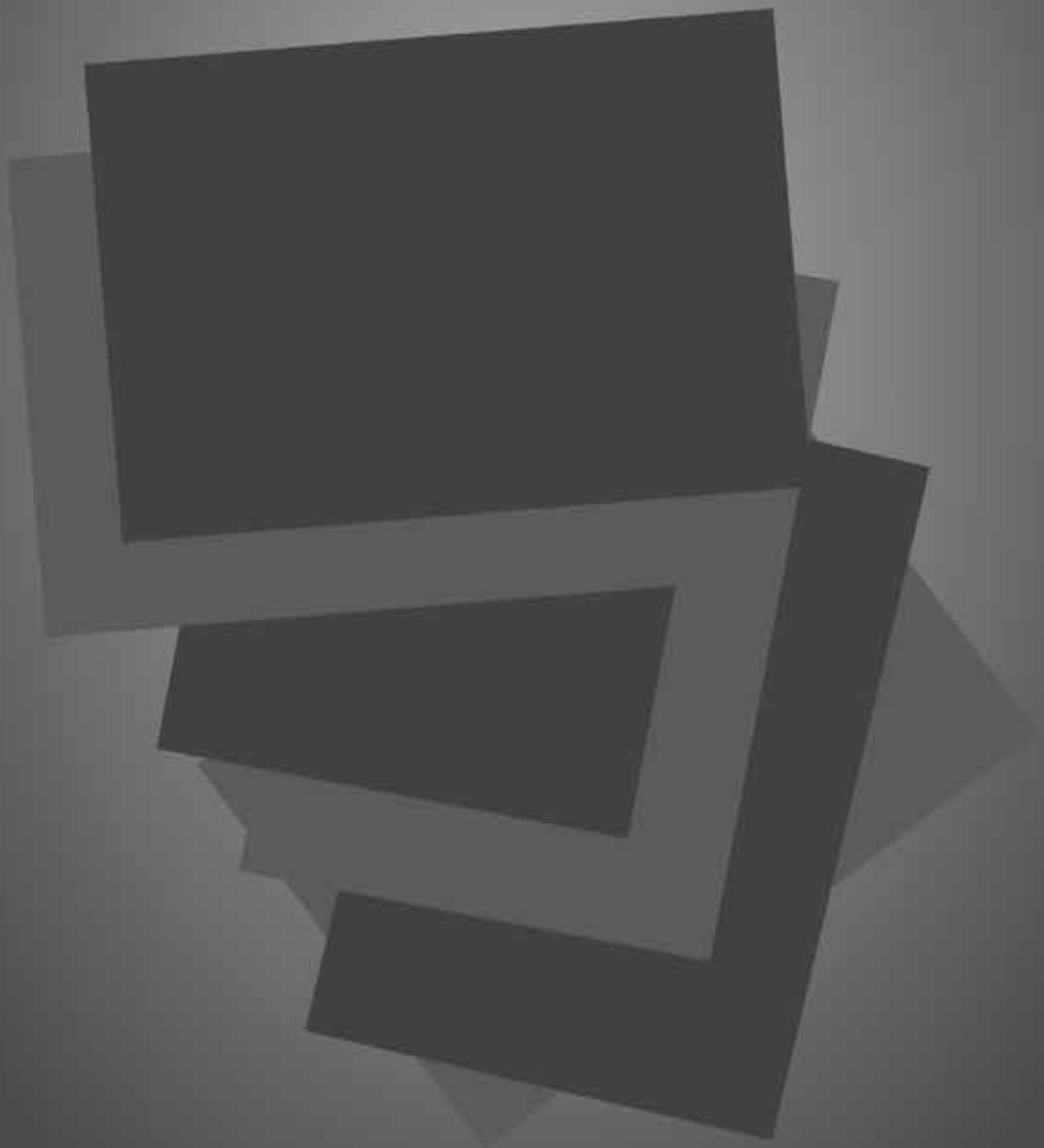
International Journal of PLANT SCIENCES



ELSEVIER

THE UNIVERSITY OF CHICAGO

International Journal of Plant Sciences

Number 9 (2), November/December 2018

VOLUME 179

THE UNIVERSITY OF CHICAGO PRESS

2018

International Journal of Plant Sciences, No.9 (2), (November/December). Volume 179.

The University of Chicago Press, 2018. - Pages 950-1078.

Proceedings of the Journal are located in the **Databases Scopus and Web of Science.**

Source Normalized Impact per Paper (SNIP): 0.937
SCImago Journal Rank (SJR): 0.885

Ranked #82 out of 211 in Plant Sciences

Impact factor: 1.748

2016 Journal Citation Reports®, Thomson Reuters

ISSN: 1058-5893

E-ISSN: 1537-5315

CONTENTS

TNAU PDB - Tamil Nadu Agricultural University Proteome DataBase - Black Gram Proteome

S. Swathi, N. Senthil, V. Vinod Kumar,
N. Jagadeesh Selvam
and M. Raveendran,
Department of Plant Molecular Biology & Bioinformatics,
Tamil Nadu Agricultural University, Coimbatore,
Tamil Nadu, India,
S. Sathish,
Department of Seed Science and Technology,
Seed Centre,Tamil Nadu Agricultural University,
Coimbatore, Tamil Nadu, India

Abstract: Tamil Nadu Agricultural University Proteome DataBase (TNAU PDB) — Black gram proteome is an open accessible database that focuses on proteome of Black gram (*Vigna mungo* L.). Currently, the database contains reference maps of Two-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE) of proteins obtained from artificially aged black gram seeds of variety TNAU blackgram CO 6, which is compared to that of fresh seeds. The database provides information about experimentally identified properties, such as molecular weight, pI value, of the differentially expressed protein due to accelerated ageing and protein sequences obtained using MALDI-TOF mass spectrometry. This database runs on WAMP server with HTML as the front end and MySQL as the backend using PHP as interface and it is hosted in TNAU genomics domain. The basic intention of this database is to provide the detailed information about proteome of black gram. This will help us to understand adaptive and general protective mechanism related to seed aging and effect of ageing on germination.

Keywords: accelerated ageing; black gram; database; proteomics; proteome database; TNAU PDB.

1. INTRODUCTION

Black gram (*Vigna mungo* L) is a protein rich food, containing about 26 percent protein, which is almost three times that of cereals. It ranks fourth among the major pulses cultivated in India. Black gram supplies a major share of protein requirement of vegetarian population of the country. It is consumed in the form of split pulse as well as whole pulse, which is an essential supplement of cereal based diet. In India, black gram occupies 12.7 percent of total area under pulses and contribute 8.4 per-cent of total pulses production. However, area and production of black gram has declined from 3.01 million ha and 1.30 million tonnes in 2000-01 to 2.97 million ha and 1.23 million tonnes, respectively in 2009-10 [1]. In the current scenario of flourishing omics segment that provides deep insight into the mechanism of crop physiology and assist in further crop improvement, proteomics of blackgram is not yet completely exploited as that of cereals like paddy [2]. Since analysis of the proteome provides a direct link of genome sequence with biological activity, data on proteomes are slowly mounting [3]. Currently, in our lab, proteome study on blackgram crop were set in motion. Since seed is the prime source of crop production [4] and storage is inevitable for carryover seeds [5], knowledge on molecular basis of seed ageing is warranted for effective maintenance of seed quality during storage. Accelerated ageing has been widely used to study the pattern of seed deterioration in various crops [6-9]. As a initial phase of proteome study in blackgram, proteome of seed ageing was analysed through comparative study of accelerated aged and fresh seeds and differentially expressed proteins due to ageing were identified. This growing data on proteome of blackgram and unavailability of no separate database concentrating on proteomes of black gram had encouraged us to develope a database to integrate the essential data derived in our lab which would help the researchers to understand the biological process and thereby assists in further research. The main objective of this database is to promote quantitative and qualitative proteome research in black gram that helps in understanding the mechanism of various biotic and abiotic stress related proteins which in turn helps in annotating their function. Currently detailed proteome information about the blackgram variety TNAU blackgram CO 6 seeds are made available in the database.

2. METHODOLOGIES

2.1 Database Content and Source

The black gram proteome database consists of proteome information about pods, leaves, stem and root. Currently, only proteome data of pods alone is made available. Possible changes in proteins due to artificial ageing on dry seeds of blackgram variety TNAU blackgram CO 6 were analyzed and the quantitative and qualitative proteome changes were recorded. Artificial ageing was done for 6 days by packing seeds in perforated butter paper bags and placed in an ageing jar containing 100 ml of distilled water to maintain 98 ± 2% relative humidity and incubated at a temperature of 40 ± 1°C [10]. In the first dimension, 150 µg of protein was loaded on a 17 cm IPG strip with a linear gradient of pH 4-7. In the second dimension, 12% SOS-PAGE gels were used with molecular eight standards. Proteins were visualized by silver staining as suggested by Blum et al. [11]. Their properties such as molecular weight, pI values and their expression were calculated using image analyzer software. Significant spots were cut, digested in trypsin and analysed in MALDI-TOF for sequencing [12]. The generated data were uploaded in the database.

2.2 Languages and Software's Used

The front end of the web application is developed on HTML 5.0 (Hyper Text Markup Language) and the validations are done using javascript. The server side scripting was done on PHP 5.3.0 (Hypertext Pre Processor) and the application was connected to the database using MySQL 5.0.8. Web application was created by using wamp server 2.2 and each spot in the gel image was linked to the corresponding protein information with the help of Macromedia dreamweaver.

2.3 Database Architecture

The application is built on a three tier architecture model consisting of presentation layer, logic layer and the database layer. The presentation layer is the front end of the application created using HTML with which the user interacts. The middle layer is the application server or logical layer created using PHP, which serves the application with data and accepts the requests from the user. The data layer contains the relational database which contains the data to be fetched by the application.

3. RESULTS AND DISCUSSION

3.1 Flow of Data

Proteome database of blackgram has been added as a sub link under TNAU genomics web page which has home page under the link http://www.tnaugenomics.com. From the home page the blackgram database can be reached through the drop down link named TNAU PDB under the tab databases. The flow of data and the procedure to access the database were meticulously depicted in Fig. 1. Similar flow of data can also be seen in a database developed for maize [13].

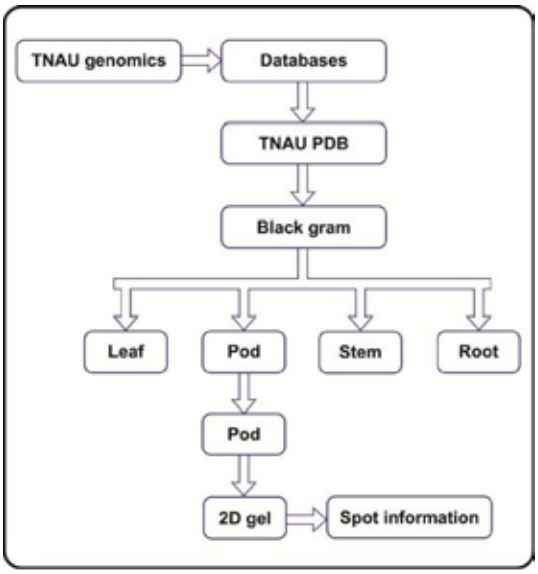


Fig. 1. Flow of data

3.2 Database Schema and Its Features

The data is classified based on the tissue such as pods, leaf, stem, root, etc. from which it is identified. Proteomes obtained using the 20 PAGE and MALDI-TOF methods are displayed using image analyzer software. The description of each spot is stored in the database and they are mapped to the corresponding spots. Based upon the request from the user, the application queries the database and fetches the spot information.

Currently, database contains the proteome information obtained from pods of black gram. The reference 20-PAGE gel shows the position of each identified protein in that crop. The entire list of protein can also be obtained by selecting the crop name displayed inside the table. By selecting the spot in the gel image the entire information about that protein can be obtained. The results are displayed in such a manner

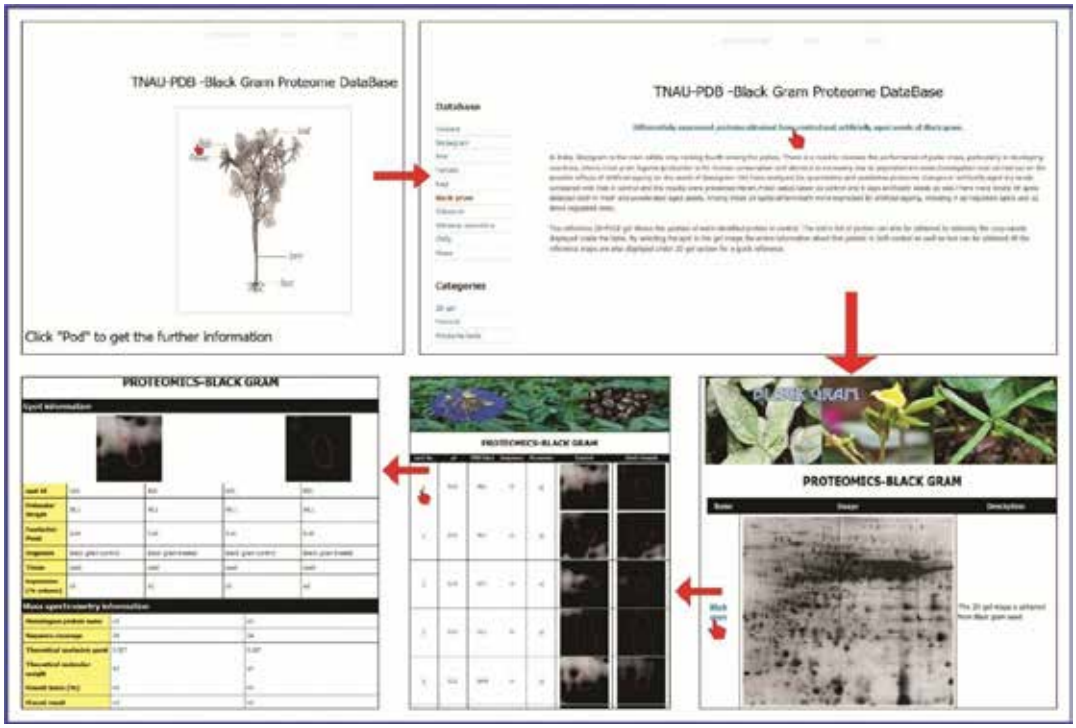


Fig. 2. Procedure to access differentially expressed proteins due to artificial ageing in blackgram seeds

that they are also compared with their control (Fig. 2). All the reference maps are also displayed under 20 gel section for a quick reference. The experiment protocols are listed under the protocol section. Some of the major proteomics tools like Mascot, Compute pl/Mw tool in ExPASy and ExPASy Proteomics tools are displayed under proteome tools section. Similar advanced proteome databases were developed for Arabidopsis and maize by Cornell university [14] and Oyna Prot 20 for dynamic online access to proteomes and two-dimensional electrophoresis gels [15].

3.3 Utility

The primary users of this database will be plant breeders, seed technologist, students and other researchers concentrating on blackgram improvement. It shows the proteome changes that could take place at the dry state of aged seeds. Determination of the expression patterns in response to stress, and an understanding of their functions in stress adaptation will provide the researchers with the basis for effective genetic engineering strategies for improving the tolerance of crops to various stresses.

3.4 Future Developments

The database content will be updated routinely. In the near future proteome data for whole plant parts such as root nodules, stem, leaves and expression of protein under different environmental stress conditions will be made available. With the availability of ample proteome data, architecture of the web page might be modified to suit to the excess flow of information.

4. CONCLUSION

Tamil Nadu Agricultural University Proteome DataBase (TNAU PDB) Black gram proteome contains 2D reference map of black gram seeds developed through comparative proteome analysis of artificially-aged and fresh seeds and shows the identified differentially expressed proteins due to seed ageing. The database content will be updated routinely. It will be useful for plant breeders, seed scientist and other researchers working in black gram.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

References:

1. ASSOCHAM. Study paper on Emerging pulses scenerio in 2015 - productivity, awareness and affordability to drive pulses economy in India. The Associated Chambers of Commerce and Industry of India, New Delhi, India. 2012;1- 18.
2. Setsuko Komatsu, Keiichi Kojima, Kouji Suzuki, Kazuo Ozaki, Kenichi Higo. Rice Proteome Database based on two-dimensional polyacrylamide gel electrophoresis: its status in 2003. Nucleic Acids Research. 2004;32(Database issue): D388 - D392.
3. Montpetit B. Proteomics in the post-genomics era: weighing in the data. Bio Teach Journal. 2003;1:9- 12.
4. Ajeigbe HA, Abdoulaye T, Chikoye D. Legume and cereal seed production for improved crop yields in Nigeria. Proceedings of the Training Workshop on Production of Legume and Cereal Seeds, 2008. Accessed 4 June 2013. Available: http://www.iita.org/c/document_library/get_file?uuid=e2d9972d-41f1-4e25-a38a- fb0309716105&groupId=25357.

5. Justice OL, Bass LN. Principles and practices of seed storage. 1st ed. Science and Education Administration's Federal Research; 1978.
6. Jatoi SA, Afzal M, Nasim S, Anwar R. Seed deterioration study in pea, using accelerated ageing techniques. Pakistan Journal of Biological Sciences. 2001; 4(12):1490-1494.
7. Scialabba A, Bellani LM, Dell Aquila A. Effects of ageing on peroxidase activity and localization in radish (*Raphanus sativus* L.) seeds. Eur. J. Histochem. 2002;46:351-358.
8. Rajjou L, Lovigny Y, Groot SPC, Belghazi M, Job C, Job D. Proteome-wide characterization of seed aging in arabidopsis: a comparison between artificial and natural aging protocols. Plant Physiology. 2008;148:620-641.
9. Xin X, Lina XH, Zhoub YC, Chena XL, Liua X, Lua XX. Proteome analysis of maize seeds: the effect of artificial ageing. Physiologia Plantarum. 2011;143:126-138.
10. Delouche JC, Baskin CC. Accelerated ageing techniques for predicting the relative storability of seed lots. Seed Sci. & Technol. 1973;1:427-452.
11. Blum H, Beier H, Gross HJ. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis. 1987;8:93-99.
12. Shevchenko A, Wilm M, Vorm O, Mann M. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal. Chem. 1996;68:850-858.
13. Murukarthick J, Senthil N, Raveendran M, Prabhakaran P, Sreedevi G, Sumanth Kumar M, Shobhana VG, Dhanya S, Khushboo R, Arumugasamy S, Ravikesavan R, Jagadish SVK. Biogen Base An Interactive Maize Database for Phenomics Platform. International Journal of Computer Application. 2011;2:56-61.
14. Qi Sun, Zybailov B, Majeran W, Frisco G, Olinares PDB, Wijk KJV. PPDB, the Plant Proteomics Database at Cornell. Nucleic Acids Research Database issue. 2009;37:D969-D974.
15. Drews O, Gorg A. Dyna Prot 2D: an advanced proteomic database for dynamic online access to proteomes and two-dimensional electrophoresis gels. Nucleic Acids Research Database issue. 2005;33:D583-D587.

A Metrological Contribution to the Diagnosis of Bovine Tuberculosis

R. A. Valdes, H. L. Costa, M. D. Nascimento,
Faculty of Mechanical Engineering, Federal University of Uberlandia, (UFU), Av. Joso Naves de Avila, Campus Santa Monica, Uberlandia, MG, Brazil,
A. Piratelli-Filho,
Department of Mechanical Engineering, Faculty of Technology, University of Brasilia, Campus Universitario Darcy Ribeiro, Asa Norte, Brasflia, DF, Brazi,
A. M. C. L. Ribeiro, P. M. Soares,
Federal University of Uberlandia (UFU), Faculty of Veterinary Medicine, Av. Para, Campus Umuarama, Bloco 2T, Uberlandia, MG, Brazil

Abstract: The present paper aims to evaluate the actual relevance of the application of metrological criteria for the diagnosis of bovine tuberculosis using Comparative Cervical Tuberculin (CCT) inoculation tests. The present work involves the following steps: identification of the instruments used to measure skin thickness in tuberculin inoculation tests; calibration of the measurement instruments (callipers) using gauge blocks; identification of the variables that can affect the calibration results and the measurement results from inoculation tests; development of a methodology to evaluate the uncertainties associated with both the calliper calibration and with the measurements carried out during diagnosis; mathematical modelling of calliper calibration process and measurement process with the calliper; CCT tests performed in a total of 40 cattle comprising Nellore breed and mixed-breed dairy animals. To determine the effects of uncertainty on the test diagnosis, callipers with resolutions of 0.1 mm and 0.01 mm were compared. The results obtained showed that measurement uncertainty influences the final diagnosis. Therefore, the application of metrological criteria can increase scientific rigor and quality of the results obtained with CCT tests, and consequently, the reliability of the final diagnosis.

Keywords: bovine tuberculosis; tuberculin inoculation; calibration; measurement uncertainty.

ABBREVIATIONS

CCT: *Comparative Cervical Tuberculin,* ***CF:*** *Caudal Foldal Test,* ***GUM:*** *Guide to the Expression of Uncertainty in Measurement,* ***MAPA****: Brazilian Ministry for Agriculture and Livestock,* ***PNCEBT:*** *Brazilian National Program for Control and Eradication of Animal Brucellosis and Tuberculosis,* ***PPD:*** *Purified Protein Derivative,* ***SCT:*** *Single Cervical Tuberculin,* ***TB:*** *Tuberculosis,* Δ***B:*** *Increase in skin fold thickness,* A_0***:*** *Thickness of the skin fold measured before inoculation with avian,* A_{72}***:*** *Thickness of the skin fold measured after inoculation with avian,* B_0***:*** *Thickness before injection with bovine PPD tuberculin,* B_{72}***:*** *Skin thickness 72 hours after inoculation,* c_i***:*** *Sensitivity coefficient of the input variable i,* ***k****: Coverage factor,* ***L:*** *Calliper indication,* L_{Oi}***:*** *Mean indicated value at the point i,* ***M:*** *Variation in the skin fold thickness between the two inoculation tests,* ***n:*** *Number of readings,* ***P:*** *Probability of the variable assuming a standard value higher than the calculated z-score,* ***R:*** *Calliper resolution,* ***RP:*** *Reproducibility of the calliper,* ***s(L):*** *Variability of the value indicated by the calliper at each point,* ***u****: Standard uncertainty,* u_C*: Combined standard uncertainty,* ***U:*** *Expanded uncertainty,* UC_{GB}***:*** *Uncertainty associated with the gauge block calibration,* UC_C***:*** *Uncertainty associated with the calliper calibration,* UC_{Ci}***:*** *Un-certainty of the value obtained with the calliper at the point i during calibration,* ***CV:*** *Conventional value,* ***X:*** *Upper legislation limit,* ***z:*** *Score,* ***s:*** *Standard deviation,* X_i***:*** *Measur and,* x_i***:*** *Estimation of measure and,* $_0$***T:*** *Temperature variation during calibration,* $\Delta\alpha$***:*** *Differential expansion between the materials of the calliper and of the gauge blocks,* Δ***A:*** *Thickness variation before and after inoculation with avian PPD tuberculin,* Δ***B:*** *Thickness variation before and after inoculation with bovine PPD tuberculin,* $\Delta s(L)_i$***:*** *Correction associated with the variability of the value indicated by the calliper at the point i,* Δ***R:*** *Correction associated with the calliper resolution,* ΔIC_{GBi}***:*** *Correction associated with the gauge block calibration,* Δ***T:*** *Difference between the calibration temperature and the reference temperature of 20gC,* Δ***Rp:*** *Correction associated with the calliper reproducibility,* ΔUC_c***:*** *Correction due to the uncertainty associated with the calliper calibration,* v_{ef}***:*** *Effective degree of freedom.*

1. INTRODUCTION

Bovine tuberculosis (TB) caused by Mycobacterium bovis was first described in 14 A.D, but only with the discovery of the tubercle bacillus in 1882 by Robert Koch it started to be properly researched [1].

Bovine tuberculosis still poses serious risks to human health, since cattle to man infection is possible via milk and unpasteurized dairy products and via the respiratory route [2].

Even though the impact on human health is a strong determinant for initiating programs for the control of bovine tuberculosis, economic losses have also been recognized [3]. Bovine tuberculosis has significant consequences for farming economies throughout the world [4].

The economic costs of this zoonosis associated to farming include direct losses due to death, reduction in weight gain, reduction in milk production, premature slaughtering for control of the disease, loss of cattle with high zootechnical value, condemnation of carcasses during slaughtering, etc. [5].

When suitable control measures are not taken, the effects on economy and health evolve slowly and steadily, and sometimes the consequences can be dramatic [6]. They can include direct life losses, mainly due to miscarriages, low reproduction levels, increase of the interval between births, death of calves, and interruption of genetic lineages. The commercial value of infected rural properties and of their animals decreases. The regions and properties where the disease is endemic are in disadvantage when disputing new markets. Indirect losses include human contamination. If it is not treated in due time, the chronic development of the disease in humans leads to economic losses resulting from diagnosis and treatment costs, besides the costs associated with the time away from work during treatment [5].

In industrialized countries, programs for control and eradication of bovine tuberculosis, together with pasteurizing techniques and vaccination [7], have drastically reduced the incidence of infection by *Mycobacterium bovis* both in cattle and in humans. North America, Europe [8,9], Australia and New Zealand [10] have been more successful in controlling and eradicating bovine tuberculosis than Latin American countries [3,11] and other developing countries [12,13]. However, bovine tuberculosis remains a problem for countries both with and without control programs [14-16].

The diagnosis of bovine tuberculosis can be carried out using both direct and indirect methods. The direct methods involve the detection and identification of the infecting agent in biological samples [17,18], whereas the indirect methods investigate immune responses of individuals to the infecting agent. An example of an indirect method is the tuberculin inoculation test, which involves a cellular immune response against *Mycobacterium bovis* manifested as a delayed hypersensitisation reaction [2].

Diagnosis using tuberculin inoculation is fast, safe and relatively cheap [5]. The tuberculin tests are the internationally accepted standard and the most robust tool currently available for the diagnosis of infection by *Mycobacterium bovis* [19].

The use of tuberculin inoculation tests has drastically reduced bovine tuberculosis [14]. However, the infection of feral animals in preservation areas around farms makes the eradication of this disease from cattle herds difficult even in countries with successful tuberculosis control [20-22]. Cattle-to-cattle transmission has also lead to a slight increase of bovine tuberculosis in some developed countries [16, 23].

Diagnosis using results of inoculation tests involve measurements of skin thickness before and after tuberculin inoculation using callipers. However, the majority of the documents with norms and specifications for using tuberculin inoculation tests in eradication programs do not mention either the technical characteristics of the calliper or the qualification of the staff involved in the measurements. For example, the national program for bovine tuberculosis eradication in Spain [24] only states that the callipers must be in good condition, whereas the use of callipers which are specific for tuberculin inoculation tests are the sole recommendation by the Brazilian national program for bovine tuberculosis eradication [5].

In 2006, a report was a produced for the Defra (UK) and the Welsh Assembly Government reviewing risks involved in bovine TB tests [19]. The report emphasizes the need for a methodical and well-defined test procedure in order to guarantee a reliable result for each animal. In particular, this report revealed that equipment used during TB screening tests, including callipers for skin thickness measurements, can incur in deviations of the final results. This probably occurs because this equipment has not been improved for decades. They suggest that some fresh ideas and professional considerations should be given to help manufacturers improve the design

of equipment used in TB tests, including skin measurement. Also, although manuals generally specify all the procedures to be followed during tuberculin inoculation tests, it is not uncommon that personnel involved in the tests do not follow strictly all the recommendations. This behaviour was associated to various reasons: the use of difficult language in manuals, many cross-references and a general failure to consider the level of knowledge of the users when designing and writing the procedures may jeopardize the understanding of the procedures; rules are broken, because they are felt to be irrelevant or because people no longer appreciate the dangers, creating a culture that tolerates violations; lack of local resources; and insufficient procedural guidance or inexperienced staff.

In order to obtain valid results from skin thickness measurements for tuberculosis diagnosis, the measurement instrument (calliper) must be adequate in terms of accuracy and precision and must be traceable in terms of the international length standard (metre). Traceability includes the declaration of the uncertainty at all levels of the traceability chain, including for the measurement results [25]. According to ISO TAG 4/WG 3 [26], popularly known as GUM (Guide to the Expression of Uncertainty in Measurement), any measurement result must declare the reliability associated with the measurement, denominated measurement uncertainty.

Therefore, improvements in the design of the equipment and conformity with procedure regulations would not suffice to reduce deviations that occur in the results from tuberculin inoculation tests. Manuals must be improved to include recommendations related to: the need for calibration of all the equipment involved, aiming the traceability of the results and the reduction of errors; the calculation of measurement uncertainty; the consideration of measurement uncertainty to interpret the results; and the technical specification of the metrological parameters of the equipment, such as accuracy, precision and resolution.

The present paper aims to evaluate the actual relevance of the application of metrological criteria for the diagnosis of bovine tuberculosis using Comparative Cervical Tuberculin (CCT) inoculation tests. The criteria investigated in this study are: calibration of the calliper using gauge blocks; development of a methodology to evaluate the uncertainties associated with both the calliper calibration and with the measurements carried out during diagnosis; discussion of the effects of uncertainty

on the test diagnosis; and comparison of results obtained using callipers with resolutions of 0.1 mm and 0.01 mm.

2. THEORETICAL BACKGROUND

A simple methodology to diagnose bovine tuberculosis involves the intradermal injection of tuberculin and assessment of the test site. In most cattle infected with *Mycobacterium bovis*, this will cause the immune system of the animal to react to the tuberculin and cause a localised allergic reaction (swelling) of the skin a few days after the injection. The presence of induration or swelling, or the measurement of these reactions in millimetres, is carried out at 72 (±6) hours following the injection. A variety of test methods have been used over the years, but they are classically described as a delayed-type hypersensitivity response, relying on the individual response in vivo of the animal to the injection. Estimates of the sensitivity of tuberculin tests range from 68% to 95% while specificity is estimated to be between 96% to 99% [27].

Although tuberculin was first produced by Robert Koch in 1890, Purified Protein Derivative (PPD) tuberculin was developed in 1934 by Seibert. PPD tuberculins, despite being commonly described as "pure", are complex mixtures of proteins, lipids, sugars and nucleic acids including a great variety of antigens, many of which are common to several mycobacterial species [27]. In Brazil, bovine PPD tuberculin is produced from *Mycobacterium bovis* AN5, containing 1 mg of protein per ml (32.500 IU) and avian PPD tuberculin is produced from *Mycobacterium avium* D4, containing 0.5 mg of protein per ml (25.000 IU) [5].

The Brazilian National Program for Control and Eradication of Animal Brucellosis and Tuberculosis (PNCEBT) presents three test methods that involve tuberculin inoculation: i) the caudal fold test; ii) the single cervical test, and iii) the comparative cervical test [5].

The Caudal Foldal (CF) Test is mainly used in North America, Australia and New Zealand [27]. In this test, a 0.1 ml dose of bovine tuberculin PPD is injected intradermally at the centre of the caudal fold approximately 6 cm to 10 cm distal to the base of the tail.

Reading of the test is by palpation of the injection site at 72 hours post injection. Cattle are classified as negative when there is no detectable response at the

injection site. Any increase in the thickness of the caudal fold at the injection site result in an animal being classified as either "suspect" or "reactor".

The Single Cervical Tuberculin (SCT) test is carried out in the skin of the neck using bovine tuberculin. It is the main screening test used in most countries of the European Union [27] and is also largely used in Brazil [5].

During SCT tests, intradermal injection of 0.1 ml of approved bovine tuberculin is made at the junction of the anterior and middle thirds of the neck. The interpretation of reactions is based on clinical observations and records of the increase in skin fold thickness at the site of injection 72 hours later.

(Table 1) summarizes reference values used to interpret clinical observations and thickness measurements and therefore to diagnose the animal [5].

Table 1. Reference values for the interpretation of results obtained with SCT tests, ΔB is the increase in skin fold thickness at the injection site [5]

ΔB (mm)	Pain sensitivity	Consistency	Other interpretations	Diagnosis
0 to 1.9	—	—	—	negative
2.0 to 3.9	Some pain	endured	delimited	inconclusive
2.0 to 3.9	Intense pain	soft	exudation, necrosis	positive
4.0	—	—	—	positive

Therefore, the test involves two measurements of the skin fold thickness at the inoculation site. The thickness measured immediately before injection with bovine PPD tuberculin (B_O) and a second measurement of the skin fold thickness, carried out 72 hours after inoculation (B_{72}). The increase in skin fold thickness at the injection site (ΔB) is calculated using Eq. (1) as the difference in thickness due to PPD tuberculin inoculation.

$$\Delta B = B_{12} - B_0 . \quad (1)$$

Cattle are sometimes infected with other types of mycobacteria which may cause the animal to react to the test. In order to distinguish between animals infected with *Mycobacterium bovis* and those infected by other mycobacteria, another test called Comparative Cervical Tuberculin (CCT) also involves the injection with tuberculin produced from *Mycobacterium avium*, an organism that can cause tuberculosis in birds. The size and nature of the reactions to both tuberculins (avian

and bovine) is compared to determine whether the test result is considered positive, negative or inconclusive.

The CCT test is a confirmatory test to be used in animals that reacted in either CF tests or in SCT tests. The thickness of the skin fold is measured using callipers before (A_O and B_O) and after inoculation with avian (A_{72}) and bovine PPD tuberculin (B_{72}). The increase in skin fold thickness due to avian (ΔA) tuberculin inoculation is then calculated as:

$$\Delta A = A_{12} - A_0 . \quad (2)$$

A comparison of the values obtained with Eq. (1) and Eq. (2) is then carried out with reference values Table 2, in order to obtain a final diagnosis.

Table 2. Reference data for tuberculosis diagnosis using comparative cervical tests, ΔB is the increase in skin fold thickness due to bovine inoculation and ΔA is the increase in skin fold thickness due to avian inoculation [5]

	ΔB- ΔA (mm)	Diagnosis
ΔB<2.0	—	Negative
ΔB<ΔA	<0	Negative
$\Delta B \Delta A$	0.0 to 1.9	Negative
ΔB>ΔA	2.0 to 3.9	Inconclusive
ΔB>ΔA	4.0	Positive

The results of diagnosis carried out using SCT and CCT tests depend on the values obtained with the calliper. Therefore, the scientific rigor of the diagnosis depends on the quality of the measurements. Some uncertainty will always exist in relation to how correctly the measurement result represents the value being measured, *i.e.*, the measurement result is only an approximation or estimative of measure and value. Many factors can influence the measurement quality, so that when measurement results are presented, some quantitative indication of the measurement quality must always be provided. This allows users of such results to evaluate their reliability. Measurement results cannot be compared without some indication of the measuement quality, either between themselves or with a reference value [26].

Measurement uncertainty is defined as a non-negative value that characterizes the dispersion of the values that can be attributed to the measure and, based on the used information. The methodology proposed by ISO TAG 4/ WG 3 [26] can be used to evaluate measurement uncertainty. However, this methodology does not substitute

critical thinking, intellectual honesty and professional ability. The evaluation of measurement uncertainty is neither a routine task nor a purely mathematical task. It depends on a detailed knowledge about both the measure and nature and the measurement. The quality and usefulness of the uncertainty indicated for a result depend on knowledge, critical thinking and honesty of those involved in finding the uncertainty value.

The evaluation of the measurement uncertainty is particularly useful for decision making [28]. When maximum or minimum tolerance limits exist for the measure and, dictated, for example, by some legislation, uncertainty becomes essential for a correct interpretation of the measurement result. Weckenmann et al. [29] have graphically represented how measurement uncertainty can affect the established limits, reducing the conformance zone.

The authors show that all zones are affected by the expanded uncertainty value associated with the measurement. The expanded uncertainty is distributed around the limit values, generating ranges where no analysis can be obtained without risk.

The probability of the measure and value being above the maximum value allowed by specification (legislation) can be evaluated taking into account the uncertainty measurement. For that, first the variable is transformed into a *z*-score:

$$z = \frac{(X - x_i)}{u_c(y)}, \quad (3)$$

where X is the upper legislation limit, x_i is the measurement result and $u_c(y)$ is the value of the combined standard uncertainty, which is equivalent to a dispersion measurement of a standard deviation, obtained by U/k, where U is the expanded uncertainty and k is the coverage factor.

In sequence, the probability of the variable assuming a standard value higher than the calculated *z*-score is defined:

$$P(X > z) = 1 - P(X\ z). \quad (4)$$

This type of information allows users to evaluate and define an acceptable risk during decision making. When a user of the measurement decides to approve a sample, he or she will know the risk of making the wrong decision, i.e., approving a sample that should be rejected. This concept of risk evaluation, which requires the knowledge of the measurement uncertainty, can be extended to various situations.

Therefore, when uncertainty is not evaluated and expressed properly, the interpretation of the results can be jeopardized, leading to errors.

3. MATERIALS AND METHODS

Comparative Cervical Tuberculin (CCT) tests were carried out in a total of 40 cattle comprising Nellore breed and mixed-breed dairy animals. The tested animals were from the Glory Experimental Farm of Federal University of Uberlandia, located in Uberlandia, MG. All animals, male and female sex, with age equal or superior to six week were tested. The tests were carried out in the morning, at environment temperature ranging from 22℃ a 28℃. In this farm, the animals are kept in pasture continuous stocking with approximately 1 hectare (ha) and the number of animals ranges from 30 to 40 animals, according to the accessibility of the trough and size of the animals. Therefore, it is estimated an area of 250 m^2 for each animal. The nutrition of the animals is performed by providing feed in the trough once a day approximately 1 kg for animal. During the dry season, silage is added in their food. The source of water comes from artesian post in shaded and cooler near the trough.

First, hair was shaved around the two injection sites located on the same side of the cervical area of each animal (Fig. 1a). A skin foldat both sites was measured with callipers (Fig. 1b). Readings using the analogic calliper combine a fixed scale and a moving scale. A trigger and combined with a spring system ensure the application of a constant measuring force. The spring system is responsible for returning the moving measuring surface, which makes manipulation by users easy and comfortable. A screw in the upper region of the instrument support allows to fix the moving measuring base in the correct position. A dial system facilitates readings during the tests.

Small amounts (0.1 ml) of bovine PPD tuberculin and of avian PPD tuberculin were injected at room temperature into the shaved skin using 22 G x 3 mm multi-dose syringes at two different sites separated by a distance between 15 mm and 20 mm.

Fig. 1. CCT test: (a) Inoculation of the bovine PPD tuberculin; (b) measurement of the skin fold with callipers, 72 hours after inoculation [5]

The PPD tuberculins were used according to the regulations by the Brazilian Ministry for Agriculture and Livestock (MAPA). They were stored at temperatures between 2℃ and 8℃, protected from direct sunlight and, after opening, bottles were completely used within 24 hours.

After 72 hours, the animal identity was checked, the skin folds at both sites were measured with the same calliper and the thickness of the skin fold was recorded.

For the measurements, an analog calliper (Fig. 2), manufacturer SUPRIVET, located in Divinopolis, MG, Brazil (http://www.suprivet.com.br), with a resolution of 0.1 and a nominal range of 40 mm, was used.

3.1 Calibration of the Calliper

Initially, the calliper was calibrated using a box of steel gauge blocks (Fig. 2), model Starrett, with calibration certificate n.1505/11 issued in July 2011 by LAROY S. STARRETT Metrology Laboratory (LAROYLAB), located in Itu, SP, Brazil, (http://www.inmetro.gov.br/laboratorios/rbc/detalhe_laboratorio.asp?num_certificado-B7&are a-DIMENSIONAL).

Fig. 2. Analog calliper during the calibration process

Calibration was carried out in a metrology laboratory at a controlled temperature of (20±1)°C, according to recommendations by NM-ISO 1 (Standard Reference Temperature for Industrial Length Measurements) [30]. During calibration, temperature was monitored using a digital thermo-hygrometer with a resolution of 0.1°C and a nominal range of -20 to 60°C. Calibration in discrete points within the

measurement range used gauge blo ks with the following lengths: 5.1 mm, 15.0 mm, 22.B mm, 25.0 mm, and 35.3 mm. The zero point was also calibrated. Five measurement cycles allowed the estimation of the arithmetic mean and of the standard deviation for each point, in order to obtain the error curve for the calliper.

3.2 Evaluation of the Uncertainty Associated with the Calibration of the Calliper using Gauge Blocks

The uncertainty was evaluated according to recommendations from ISO TAG 4 WG/3 (Guide to the Expression of Uncertainty in Measurement) [26]. Initially, the variables that could affect the calibration result were identified: *i)* variability of the value indicated by the calliper at each point $s(L)_i$; *ii)* calliper resolution (R); *iii)* uncertainty associated with the gauge block calibration (UC_{GB}); *iv)* difference between the measurement temperature and the reference temperature of 20℃ (LT); and *v)* temperature variation during the measurements ($0T$).

A mathematical model was proposed to assess the uncertainty associated with each evaluated point, which results from the algebraic sum of the corrections associated with the identified variables:

$$C_C = \Delta s(L)_i + \Delta R + \Delta UC_{GBi} + L_{0i}\Delta\alpha\Delta T + L_{0i}\Delta\alpha 0T, \quad (5)$$

where:

C_c - Value obtained with the calliper at the point *i* during calibration;

$ss(L)_I$ - correcti on associated with the variability of the value indicated by the calliper at the point *i*;

s_R - correction associated with the calliper resolution;

$s_{UC_{GBi}}$ - correction associated with the gauge block calibration;

sT - Difference between the calibration temperature and the reference temperature of 20℃;

$0T$ -Temperature variation during calibration;

L_{0I} - Mean indicated value at the point *i*;

$\Delta\alpha$ - Differential expansion between the materials of the calliper and of the gauge blocks.

3.3 Evaluation Associated With the Measurement of the Skin Fold

The mathematical model for the estimation of the uncertainty associated with the measurement of the skin fold is given by:

$$M = \Delta B - \Delta A, \quad (6)$$

where *M* represents the variation in the skin fold thickness between the two inoculation tests; *sA* is the thickness variation before and after inoculation with avian PPD tuberculin; and *sB* is the thickness variation before and after inoculation with bovine PPD tuberculin.

The variation of the measurement of the skin fold thickness due to bovine PPD inoculation is given by Eq. (1), as the difference between the measurement of the skin fold thickness 72 hours after inoculation (B_{72}) and the measurement of the skin fold thickness before inoculation (B_O). Similarly, the variation of the measurement of the skin fold thickness due to avian PPD inoculation is given by Eq. (2) as the difference between the measurement of the skin fold thickness 72 hours after inoculation (A_{72}) and the measurement of the skin fold thickness before inoculation (A_O).

In this case, the uncertainty associated with the variation of the skin fold thickness between the tests with bovine and avian inoculation depends on the uncertainties associated with the measurements of A_O, A_{72}, B_O and B_{72}. Since those variables were obtained using the same measurement system, they can be considered correlated variables. Therefore, the mathematical model to evaluate uncertainty is given by Eq. (7).

$$M = \Delta B - \Delta A = (B_{72} - B_0) - (A_{72} - A_0), \quad (7)$$

The variables that can contribute to the uncertainty during the measurements of A_O, A_{72}, B_O and B_{72} were identified as: *i)* reproducibility of the calliper (*Rp*), ii) resolution of the calliper (*R*), and (iii) uncertainty associated with the calliper calibration (UC_C). In this study reproducibility condition of measurement is a set of conditions that includes different locations, operators and replicate measurements on the same objects.

The variables that contribute to the uncertainty to determine A_O are shown in Eq. (8), where *sRp* is the correction associated with the calliper reproducibility,

sR represents the correction due to the calliper resolution, and sUC_C is the correction due to the uncertainty associated with the calliper calibration.

$$A_0 = \Delta Rp + \Delta R + \Delta UC_{C.} \quad (8)$$

The mathematical model presented in Eq. (8) can also be used to evaluate the uncertainty associated with the measurement of A_{72}, B_O and B_{72}. It must be pointed out that for the determination of the numerical value of measurement uncertainty, the factors that influence the measurements are the same.

Most measurement processes involve various readings of the same measure and under similar conditions in order to allow statistical treatment of the data, detection of possible gross errors, and evaluation of the uncertainty measurement. However, in the case of the tuberculin inoculation tests, repetition of the readings is almost impossible, since the inoculated site generally becomes sore.

In this case, to calculate the standard uncertainty associated with the variability of the readings, 30 measurements were carried out under reproducibility conditions. So, uncertainty can be evaluated with a Type A evaluation using a normal distribution and *n-1* degrees of freedom, as shown in Eq. (9).

$$u(\Delta Rp) = \frac{Rp}{\sqrt{n}}, \quad (9)$$

where *n* is the number of readings.

In relation to the calliper resolution, a Type B evaluation can be applied using a rectangular distribution and an infinite number of degrees of freedom, Eq. (10).

$$u(\Delta R) = \frac{\text{Resolution.}}{\sqrt{3}} \quad (10)$$

The standard uncertainty associated with the calliper calibration ($u(sIC)$) can be obtained by dividing the extended uncertainty (U) declared in the calibration certificate by the coverage factor (k), Eq. (11).

$$u(\Delta IC) = \frac{U(\text{Calibration}).}{k} \quad (11)$$

In this case, a Type B evaluation is applied using a normal probability distribution. The number of degrees of freedom can be determined using a *t*-student

distribution table for the coverage factor *(k)* and the coverage probability, declared in the calibration certificate.

After the calculation of all standard uncertainties, the combined standard uncertainty (u_c) can be estimated. For that, the law of propagation of uncertainty is applied to the initial mathematical model, as shown in Eq. (12). In this equation, all the partial derivatives (sensitivity coefficients) assume unitary values.

$$u_c^2(A_0) = \left(\frac{aA_0}{adRp}\right)^2 \cdot u^2(dRp) + \left(\frac{a\,A_0}{adR}\right)^2 \cdot u^2(dR) + \left(\frac{a\,A_0}{adUC}\right)^2 \cdot u^2(dUC). \quad (12)$$

Equation (12) also allows the evaluation of the combined standard uncertainty associated with the measurements of A_{72}, B_0 and B_{72}.

To calculate the expanded uncertainty *U*, the combined standard uncertainty was multiplied by a coverage factor *k,* obtained from the *t*-student table according to the measurement effective degree of freedom v_{et}, in order to increase the coverage probability to 95%, as shown in Eq. (13). The measurement effective degree of freedom v_{et} is obtained from the Welch-Satterwaite expression, Eq. (14), where c_i is the sensitivity coefficient of the input variable *i*.

$$U = k.u_{c,} \quad (13)$$

$$v_{ef} = \frac{u_c^4(y)}{\sum_{i=1}^{N} \frac{u^4(y_i)}{v_i}} = \frac{u_c^4(y)}{\sum_{i=1}^{N} \frac{(u(x_i)\cdot c_i)^4}{v_i}}. \quad (14)$$

4. RESULTS AND DISCUSSION

4.1 Calibration of the Calliper

(Table 3) shows the values obtained during calibration of the calliper, where *CV* represents the length of the gauge block; *L1* to *LS* represent the readings and *s* is the experimental standard deviation. The table also presents arithmetic mean and bias (error).

The bias values are positive within the whole calliper nominal range, reaching 0.2 mm for the points 5.1 mm and 15.0 mm. Therefore, the measurement instrument tends to provide values higher than the measure and.

The uncertainty associated with the calliper calibration was then evaluated. From the calibration certificate for the gauge blocks, the expanded uncertainty associated with their calibration is 0.09 µm for *k* = 2.00 and a coverage probability

of 95%. The values of expanded uncertainty for each point evaluated during calibration are shown in (Table 4), which evidences identical values of 0.2 mm for *k* = 2.00 and a coverage probability of 95%, for all the points evaluated during calibration.

Table 3. Results of the calliper calibration (mm)

CV	*L 1*	*L 2*	*L 3*	*L 4*	*L S*	Mean	*s*	Bias
0.000	0.0	0.0	0.0	0.0	0.0	0.0	0.00	0
5.100	5.3	5.3	5.3	5.3	5.3	5.3	0.02	0.2
15.000	15.2	15.2	15.2	15.2	15.3	15.2	0.02	0.2
22.800	22.9	22.9	23.0	23.0	23.0	22.9	0.03	0.1
35.300	35.5	35.4	35.4	35.4	35.4	35.4	0.02	0.1

Table 4. Combined standard uncertainty (U_c) and expanded uncertainty (U) for the points evaluated during calibration

	0 mm	5.1 mm	15.0 mm	22.8 mm	25.0 mm	35.3 mm
u_c(mm)	0.1	0.1	0.1	0.1	0.1	0.1
v_{ef}	125	124	125	125	125	124
k	2	2	2	2	2	2
U(mm)	0.2	0.2	0.2	0.2	0.2	0.2

4.2 Skin Fold Thickness Measurements

The measurement results of the skin fold thickness after inoculation tests are summarized in (Table 5), where: A_O is the skin fold thickness before inoculation with avian PPD; A_{72} is the skin fold thickness 72 hours after inoculation with avian PPD; *sA* is the thickness difference before and after inoculation with avian PPD; B_O is the skin fold thickness before inoculation with bovine PPD; B_{72} is the skin fold thickness 72 hours after inoculation with bovine PPD; and *sB* is the thickness difference before and after inoculation with bovine PPD. In the last column, the difference between the results with each inoculation is presented.

Comparing the values in (Table 5), which do not consider measurement uncertainty, with the reference values shown in (Table 2), the CCT tests carried out for the 40 cattle identified 39 animals with skin fold thickness variation (*sB*-*sA*) below 2 mm, indicating negative diagnosis. One animal (animal 33) showed positive diagnosis, which requires measurements to be taken according to regulations [5].

4.3 Measurement Uncertainty

(Table 6) exemplifies the calculation of measurement uncertainty (coverage probability = 95%) associated with A_O for Animal 1. Similar procedures can be extended for the calculation of measurement uncertainties associated with A_{72}, B_O and B_{72}.

(Table 6) shows that for this animal, the expanded uncertainty for k = 2 and coverage probability of 95% associated with A_O was 0.2 mm. This uncertainty value can be extended to the values of A_O, A_{72}, B_O and B_{72} for all the animals, since the variables that influence each value are the same and assume the same values. If a larger value of coverage probability is desired, for example, 99%, the coverage factor is 3.36 and therefore the extended uncertainty becomes 0.3 mm.

Table 5. Results of the measurements of the skin fold thickness (mm) for inoculation tests using bovine (ΔB) and avian PPD tuberculin (ΔA)

Animal	A_0	A_{72}	A_{72}-A_0=ΔA	B_0	B_{72}	B_{72}-B_0=ΔB	ΔB-ΔA
1	6.1	9.9	3.8	4.6	8.5	3.9	0.1
2	7.6	11.5	3.9	8.9	11.6	2.7	-1.2
3	6.4	7.0	0.6	8.2	8.6	0.4	-0.2
4	6.5	8.0	1.5	8.0	8.7	0.7	-0.8
5	5.3	6.6	1.3	5.3	5.9	0.6	-0.7
6	6.2	6.4	0.2	6.2	6.4	0.2	0.0
7	9.5	9.1	-0.4	9.9	10.0	0.1	0.5
8	6.4	6.5	0.1	6.5	6.8	0.3	0.2
9	5.0	5.5	0.5	5.2	5.7	0.5	0.0
10	7.6	7.6	0.0	8.7	9.8	1.1	1.1
11	6.7	7.2	0.5	7.7	9.7	2.0	1.5
12	7.4	7.5	0.1	8.3	8.3	0.0	-0.1
13	6.3	6.4	0.1	8.9	9.0	0.1	0.0
14	10.0	10.0	0.0	9.0	9.5	0.5	0.5
15	6.4	10.2	3.8	7.8	10.0	2.2	-1.6
16	7.6	9.0	1.4	6.8	7.8	1.0	-0.4
17	8.0	10.6	2.6	7.3	8.6	1.3	-1.3
18	8.5	10.1	1.6	8.7	9.5	0.8	-0.8
19	8.7	9.0	0.3	8.3	10.5	2.2	1.9
20	8.2	12.3	4.1	8.2	9.9	1.7	-2.4
21	7.3	8.5	1.2	7.1	8.0	0.9	-0.3
22	7.6	10.6	3.0	6.1	7.5	1.4	-1.6
23	8.1	9.5	1.4	6.4	7.5	1.1	-0.3
24	7.6	8.2	0.6	7.6	8.0	0.4	-0.2
25	7.4	7.5	0.1	7.0	7.4	0.4	0.3
26	8.6	9.5	0.9	7.3	9.4	2.1	1.2
27	7.6	7.6	0.0	7.3	7.7	0.4	0.4
28	7.6	7.8	0.2	7.1	8.2	1.1	0.9
29	6.0	8.5	2.5	6.5	8.5	2.0	-0.5
30	7.3	7.6	0.3	7.0	8.0	1.0	0.7
31	7.1	10.0	2.9	7.1	8.5	1.4	-1.5
32	7.0	7.5	0.5	7.9	8.7	0.8	0.3
33	7.1	7.9	0.8	6.7	15.5	8.8	8.0

34	8.5	9.6	1.1	7.8	10.6	2.8	1.7
35	9.7	9.4	-0.3	7.3	8.7	1.4	1.7
36	7.2	7.6	0.4	7.6	8.1	0.5	0.1
37	7.0	7.2	0.2	6.5	7.4	0.9	0.7
38	8.1	8.8	0.7	8.5	10.2	1.7	1.0
39	8.0	8.5	0.5	8.2	10.0	1.8	1.3
40	7.6	8.2	0.6	6.3	7.4	1.1	0.5

The uncertainty associated with the calliper calibration is the variable with the strongest influence on the combined standard uncertainty and therefore on the expanded uncertainty. (Tables 7 and 8) present as exemplified, for Animal 1, the uncertainties associated with the variation in the skin foldthickness due to both avian PPD inoculation *sA* and to bovine PPD inoculation *sB* were calculated.

Table 6. Parameters for the calculation of measurement uncertainty associated with the variation in the skin fold thickness due to avian PPD inoculation for a coverage probability of 95%

Measur and (X_i)	Estimation (X_i)	Probability distribution	Sensitivity coefficient	Degrees of freedom	Standard uncertainty
Rp	0.058 mm	Normal	1	29	0.0106 mm
R	0.1 mm	Restangular	1		0.0577 mm
IC	0.19 mm	Normal	1	100	0.0850 mm
Combined standard uncertainty (u_c), in mm					0.1033
Effective degree of freedom (v_{ef})					218
Coverage factor (95%)					k = 2.00
Expanded uncertainty (U), in mm					0.2

Table 7. Uncertainty associated with the variation in the skin fold thickness due to avian PPD inoculation for a coverage probability of 95%

Measur and (X_i)	Estimation (X_i)	Probability distribution	Sensitivity coefficient	Degrees of freedom	Standard uncertainty
A_0	9.6 mm	Normal	1	218	0.1033 mm
A_{72}	12.2 mm	Normal	1	218	0.1033 mm
Combined standard uncertainty (u_c), in mm					0.1461
Effective degree of freedom (v_{ef})					436
Coverage factor (95%)					k = 2.00
Expanded uncertainty (U), in mm					0.3

Table 8. Uncertainty associated with the variation in the skin fold thickness due to bovine PPD inoculation for a coverage probability of 95%

Measur and (X_i)	Estimation (X_i)	Probability distribution	Sensitivity coefficient	Degrees of freedom	Standard uncertainty
B_0	8.6 mm	Normal	1	218	0.1033 mm
B_{72}	10.1 mm	Normal	1	218	0.1033 mm
Combined standard uncertainty (u_c), in mm					0.1461
Effective degree of freedom (v_{ef})					436
Coverage factor (95%)					k = 2.00
Expanded uncertainty (U), in mm					0.3

Finally, the uncertainty associated with the difference in skin fold thickness variation between the two inoculations *(sB-sA*) was calculated for a coverage probability of 95%, which is exemplified in (Table 9) for animal 1.

For *sA* and *sB* the expanded uncertainty was 0.3 mm, whereas the difference (*sB-sA)* presented an uncertainty of 0.4 mm, for k = 2.00 and coverage probability of 95%. These values can be extended to all tested animals.

Table 9. Uncertainty associated with the difference (∆*B* - ∆*A*)

Measur and (X_i)	Estimation (X_i)	Probability distribution	Sensitivity coefficient	Degrees of freedom	Standard uncertainty
A	2 mm	Normal	1	436	0.1461 mm
B	1.5 mm	Normal	1	436	0.1461 mm
Combined standard uncertainty (u_c), in					0.2066
Effective degree of freedom (v_{ef})					436
Coverage factor (95%)					k = 2.00
Expanded uncertainty (U), in mm					0.4

The values from (Table 5) can be compared again with the reference values in (Table 2), but now taking into account measurement uncertainty. Animals 34 and 35 presented values of (*sB* - *sA*) = 1.7 mm and for animal 19 this value was 1.9 mm. Without taking measurement uncertainty into account, these animals had been diagnosed as negatives. Considering the expanded uncertainty of 0.4 for k = 2.00 and coverage probability of 95%, they fall into the uncertainty zone. Using Eq. (3), it is possible to calculate that deciding for a negative diagnosis for animals 34 and 35 implies in a risk of 7% of taking the wrong decision, when in fact the result is inconclusive. For animal 19, the chance of surpassing the maximum limit allowed for a negative diagnosis is significantly higher, around 31%.

A value of expanded uncertainty associated with (*sB* - *sA*) of 0.4 mm can be considered excessively high, since it reduces the maximum limit allowed for a negative diagnosis in around 20%. Therefore, it is recommended the use of a calliper with a better resolution in order to reduce the uncertainty associated with the measurements.

As a comparative example, uncertainty values were obtained for a digital calliper with a resolution of 0.01 mm and a nominal range of 30 mm, manufacturer Agrozootec (Brazil), with calibration certificate n.1300/11 issued in February 2011 by QUALIMETRO metrology laboratory. The manufacturer is located in Itu, SP, Brazil (http://www.agrozootec.com.br/contatti.asp). The uncertainty associated with the calibration of the digital calliper was evaluated using the mathematical model given by Eq. (5). The only difference is that for the evaluation of the uncertainty associated with calibration and/or measurement using digital instruments or measuring systems, the resolution must be divided by two, since this is the maximum error expected during readings.

(Table 10) compares the results obtained using the methodology proposed in Eqs. (8-14), where Calliper A is the analog calliper, model SUPRIVET, with a resolution of 0.1 mm and Calliper B is the digital calliper, model Agrozootec, with a resolution of 0.01 mm.

The use of a calliper with better resolution reduced the expanded uncertainty associated with the result from 0.41 mm (Calliper A) to 0.32 mm (Calliper B), which represents a reduction of 22%. The standard uncertainty associated with the resolution reduced from 0.0577 mm (Calliper A) to 0.0190 mm (Calliper B). Reproducibility varied from 0.0106 mm (Calliper A) to 0.0015 mm (Calliper B). (Fig. 3) summarizes the effect of expanded uncertainty on the values established for the final diagnosis for both callipers.

(Fig. 3) evidences that a calliper with better resolution (0.01 mm) must have a better precision and therefore the uncertainty zone for diagnosis is reduced. Despite the availability in the market of callipers of a variety of models and resolutions generally varying from 0.1 mm to 0.01 mm, this work recommends the use of calibrated and traceable callipers with a resolution of 0.01 mm for tuberculin inoculation tests.

Table 10. Comparison of the uncertainty for both callipers

	Calliper A (mm)	Calliper B (mm)
u(Rp)	0.0106	0.0015
u(R)	0.0577	0.0029
u(C)	0.0850	0.0800
$u_c(A_0)$	0.2066	0.0801
$u_c(A_{72})$	0.2066	0.0801
$u_c(B_0)$	0.2066	0.0801
$u_c(B_{72})$	0.2066	0.0801
$u_c(sA)$	0.2922	0.1133
$u_c(sB)$	0.2922	0.1133
U(sB-sA)	0.4132	0.3205

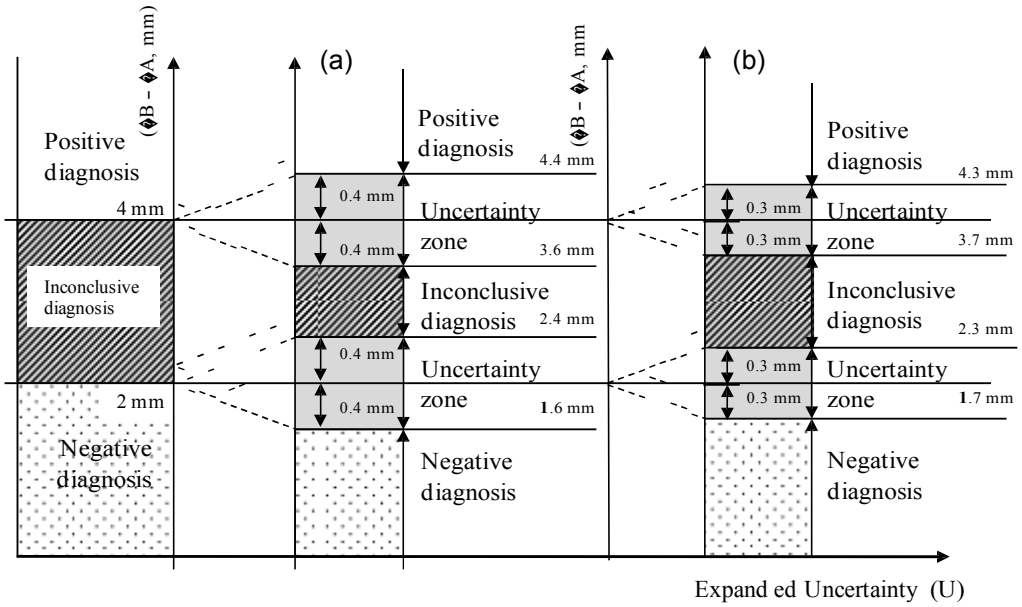


Fig. 3. Effect of the uncertainty on limit values for diagnosis: (a) Calliper A; (b) Calliper B

5. CONCLUSION

This work investigated metrological aspects associated with the diagnosis of bovine tuberculosis using tuberculin inoculation tests.

A methodology was applied to evaluate uncertainty of the measurements carried out during diagnosis in order to increase scientific rigor and reliability of the measurements, and therefore the quality of diagnosis obtained from tuberculin inoculation tests.

It was observed that when measurement uncertainty is used to interpret the results, the final diagnosis can change, so that animals that could be diagnosed as negatives should in fact have an inconclusive diagnosis.

The expanded uncertainty associated with the final result was 0.4 mm for an analog calliper with a resolution of 0.1 mm, but it was reduced to 0.32 mm (22%) when a digital calliper with a resolution of 0.01 mm was used.

ACKNOWLEDGEMENTS

The authors are grateful to the program PIAIC/UFU and to FAPEMIG and CAPES for financial support.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

References:

1. Wood P, Mogaghan M, Rothel J. Preface. Veterinary microbiology. 1994;40:7. DOI: 10.1016/0378-1135(94)90040-x.
2. Grange JM, Yates MD. Zoonotic aspects of *Mycobacterium bovis* infection. Veterinary Microbiology. 1994;40:137-151. DOI: 10.1016/0378-1135(94)90052-3.
3. De Kantor IN, Ritacco V. Bovine tuberculosis in Latin America and the Caribbean: current status, control and eridication programs. Veterinary Microbiology. 1994;40:5-14. DOI: 10.1016/0378-1135(94)90042-6.
4. Neill SD, Pollock JM, Bryson DB, Hanna J. Pathogenesis of *Mycobacterium bovis* infection in cattle. Veterinary Microbiology. 1994;40:41-52. DOI: 10.1016/0378-1135(94)90045-0.
5. Lage AP, Roxo E, Muller EE. Programa Nacional de Controle e Erradicacao da Brucelose e da Tuberculose Animal (PNCEBT). Brasflia. 2006;188. Portuguese.
6. Roberts T. A retrospective assessment of human health protection benefits from removal of tuberculous beef. J Food Protect. 1986;49:293-298.
7. Thom ML, McAulay M, Vordermeier HM, Clifford D, Hewinson RG, Villarreal-Ramos B, Hope JC. Duration of Immunity against *Mycobacterium bovis* following neonatal vaccination with Bacillus Calmette-Guerin Danish: Significant protection against infection at 12, but Not 24, Months. Clinical and Vaccine Immunology. 2012;19:1254-1260. DOI: 10.1128/cvi.00301-12.
8. Caffrey JP. Status of bovine tuberculosis eradication programmes in Europe. Veterinary Microbiology. 1994;40:1-4. DOI: 10.1016/0378-1135 (94) 90041-8.

9. Welby S, Govaerts M, Vanholme L, Hooyberghs J, Mennens K, Maes L, Van der Stede Y. Bovine tuberculosis surveillance alternatives in Belgium. Preventive Veterinary Medicine. 2012;106:152-161. DOI: 10.1016/j.prevetmed.2012.02.010.
10. Tweddle NE, Livingstone P. Bovine tuberculosis control and eradication programs in Australia and New Zealand. Veterinary Microbiology. 1994;40:23-39. DOI: 10.1016/0378-1135(94)90044-2.
11. Furlanetto LV, Figueiredo EES, Conte CA, Silva FGS, Duarte RS, Silva JT, Lilenbaum W, Paschoalin VMF. Prevalence of bovine tuberculosis in herds and animals slaughtered in 2009 in the State of Mato Grosso, Brazil. Arquivo Brasileiro De Medicina Veterinaria E Zootecnia. 2012;64:274-280. Portuguese.
12. Cosivi O, Grange JM, Daborn CJ, Raviglione MC, Fujikura T, Cousins D, Robinson RA, Huchzermeyer HF, de Kantor I, Meslin FX. Zoonotic tuberculosis due to *Mycobacterium bovis* in developing countries. Emerg Infect Dis. 1998;4:59-70.
13. Hernandez C, Cetner AS, Jordan JE, Puangsuvan SN, Robinson JK. Tuberculosis in the age of biologic therapy. J Am Acad Dermatol. 2008;59:363-380.
14. Thom A, Morgan JH, Hope JC, Villarreal-Ramos B, Martin A, Howard CJ. The effect of repeated tuberculin skin testing of cattle on immune responses and disease following experimental infection with *Mycobacterium bovis*. Vet Immunol Immunop. 2004;102:399-412.
15. Thom ML, Hope JC, McAulay M, Villarreal-Ramos B, Coffey TJ, Stephens S, Vordermeier HM, Howard CJ. The effect of tuberculin testing on the development of cell-mediated immune responses during *Mycobacterium bovis* infection. Vet Immunol Immunop. 2006;114:25-36.
16. Green LE, Carrique-Mas JJ, Mason SA, Medley GF. Patterns of delayed detection and persistence of bovine tuberculosis in confirmed and unconfirmed herd breakdowns in cattle and cattle herds in Great Britain. Preventive Veterinary Medicine. 2012;106:266-274. DOI: 10.1016/j.prevetmed. 2012.04.008.
17. Jia K, Yu M, Zhang GH, Zhang J, Lin ZX, Luo CB, Yu HQ, Li SJ. Detection and identification of mycobacterium tuberculosis and *Mycobacterium bovis* from clinical species using DNA microarrays. Journal of Veterinary Diagnostic Investigation. 2012;24:156-160. DOI: 10.1177/1040638711417141.

18. Tipu MY, Chaudhary ZI, Younus M, Rabbani M. A Cross Sectional Study of *Mycobacterium bovis* in dairy cattle in and Around Lahore City, Pakistan. Pak J Zool. 2012;44:393-398.
19. Archer R, Comer P. Review of TB testing procedures. Defra and the Welsh Assembly Government; 2006.
20. Clifton-Hadley RS, Wilesmith JW, Richards MS, Upton P, Johnston S. The occurrence *Mycobacterium bovis*. Infection in cattle in and an around area subject to extensive badger (Meles meles) control. Epidemiol Infect. 1995;114:179-193.
21. Coleman JD, Cooke MM. *Mycobacterium bovis* infection in wildlife in New Zealand. Tuberculosis. 2001;81:191-202.
22. Payeur JB, Church S, Mosher L, Robinson-Dunn B, Schmitt S, Whipple D. Bovine tuberculosis in Michigan wildlife. Ann Ny Acad Sci. 2002;969:259-261.
23. Goodchild AV, Clifton-Hadley RS. Cattle-to-cattle transmission of *Mycobacterium bovis*. Tuberculosis. 2001;81:23-41.
24. PNTB. National Program of Bovine Tuberculosis Eradication Presented by Spain for 2012. Ministerio de Medio Ambiente y Medio Rural y Marino. 2012;69. Spanish.
25. ISO. General requirements for the competence of testing and calibration laboratories. 2005;36.
26. ISO. Guide to the expression of uncertainty in measurement. Geneva, Switzerland, 2008;131.
27. Monaghan ML, Doherty ML, Collins JD, Kazda JF, Quinn PJ. The tuberculin test. Veterinary Microbiology. 1994;40:111-124. DOI: 10.1016/0378-1135(94)90050-7.
28. RS RM. Measurement uncertainty: practical guide evaluator laboratories. Procedimento do sistema de gestao da qualidade revisao. 2009;32. Portuguese.
29. Weckenmann A, Knauer M, Killmaier T. Uncertainty of coordinate measurements on sheet-metal parts in the automotive industry. J Mater Process Tech. 2001;115:9-13.
30. NM ISO 1. Standard reference temperature for industrial length measurements. 1996;2.

Physiological and Biochemical Responses of Two Cultivars of Phaseolus Vulgaris L. to Application of Organic Fertilizers and Nile Compost in Sandy Soil

Salwa Mohamed Abbas, Hanan Helmy Latif and Nora Magdy,
Department of Biological and Geological Science,
Faculty of Education, Ain Shams
University, Cairo-Egypt

ABSTRACT

Aims: The present work aims to stimulate some physiological changes in the plants using organic fertilizer and compost by enhancing some compounds such as total amino acids and phytohormones in two cultivars of bean.

Study Design: The pots of the (*Phaseolus vulgaris*) L. cv. bronco were divided into 7 sub-groups they will be prepared as in the seven treatments via 1 - control, Nile compost, compost and rice straw, compost and maize stalks, rice straw and maize stalks, rice straw, maize stalks and were replicate times for *Phaseolus vulgaris* L. cv. paulista from T_8-T_{14}. After 45 days (vegetative stage), 90 days for (flowering stage) and 130 days for (fruiting stage) the plants were harvested.

Place and Duration of Study: Department of Biological and Geological Science, Faculty of Education, Ain Shams University, Cairo-Egypt June 2012.

Methodology: Growth, yield, free amino acids and phytohormones of two cultivars of *Phaseolus vulgaris* L. cv. paulista and cv. bronco was investigated.

Results: The results showed that the significant differences in growth in all the stages and percent of free amino acids and phytohormones in shoot in vegetative stage in two cultivars of bean were obtained with mixture of compost or maize stalk and maize stalk decompost.

Conclusion: Generally, the addition of organic fertilizer with compost led to improve the yield of two cultivars as compared to control. Hence, it could be suggested that the treated plants, with these organic residues and Nile compost increased the growth, yield and the above chemical compositions.

Keywords: Phaseolus vulgaris; sandy soil; free amino acids; phytohormones.

1. INTRODUCTION

Fertilizer is any material, organic or inorganic, natural or synthetic, that supplies plants with the necessary nutrients for plant growth and optimum yield. Organic fertilizers are natural materials of either plant or animal origin, including livestock manure, green manures, crop residues, household waste, compost, and woodland litter. Organic manure plays direct role in plant growth as a source of all necessary macro and micronutrients in available forms during mineralization and improving physical and chemical properties of soils [1]. It plays an important role in increasing growth, yield and yield components of many crops. [2] reported that organic manures significantly affected tomato plant height, leaf area and fruit number plant.

Compost has been recognized as a low cost and environmentally sound process for treatment of many organic wastes [3]. It is a plant residue, animal residue or a mixture of both that has been decomposed and recycled as a fertilizer and soil amendment. The application of compost has been shown to positively affect the structure, porosity, water holding capacity, compression strength, nutrient content and organic matter content of the soil all of these improve plant growth, crop yield and crop quality [4]. Organic fertilizer effect on amino acid on plant, that it increased the latter by treating the soil with different organic fertilizer. [5] showed that organic fertilizer leads to new amino acids compared with the amino acid in the control treatment in wheat grain and dry shoots. [6] showed that application of compost significantly increased the level of total free amino acid in the leaves of mustard when compared to control.

Furthermore, organic matter increased plant hormone-like activity [7,8]. The alteration in different aspect of cellular metabolisms including the content of phytohormones could be arising from the different compounds present in the organic fertilizer.

Phaseolus vulgaris L. is one of the most important members of leguminous crops in Egypt grown for either local consumption or exportation, it is known as green bean or snap bean it is an important source of protein and energy for many developing countries. It's Rich in protein, dietary fibers, minerals (Ca, P, Fe, K, Mg & Mn) and vitamins (A, 81, 82 & C) with high amino acids [9].

The present work aims to stimulate some physiological changes in the plants using organic fertilizer and compost by enhancing some compounds such as total amino acids and phytohormones in two cultivars of bean.

2. MATERIALS AND METHODS

Seeds of (*Phaseolus vulgaris*) L. cv. bronco and *Phaseolus vulgaris* L. cv. paulista were obtained from Agriculture Research Center, Ministry of Agriculture, Giza, Egypt.

Seeds of Snap bean were surface sterilized for 1 min in 70% (v/v) ethanol, 20 min in 5% (v/v) sodium hypochlorite and rinsed five times with sterile bidistilled water. A pot experiment was conducted in the greenhouse of Faculty of Education; Cairo, Egypt with a sandy soil collected from Arab Guhaina, Qalyubia, Egypt has pH 7.3, EC 2.56 MHz/cm, organic matter 0.2%, CaCO3 2.3%. All pots will contain equal amounts of sandy soil and kept inside an open-air wire house during plant growth and development. 140 pots (about 30 cm) were divided into two groups consisting 70 pots for each cultivar of *Phaseolus vulgaris.*

The pots of the first group were divided into 7 sub-groups they will be prepared as in the following scheme:

Seven treatments via:

1 - control T_1.

2 - Nile compost T_2. (Composition of Nile compost) Soft lime Super phosphate contains 15 % phosphate 2 or 5 soluble in water.

3 - compost and rice straw T_3.

4 - compost and maize stalks T_4.

5 - rice straw and maize stalks T_5.

6 - rice straw T_6. (Composition of rice straw) the chemical composition of rice straw varies between varieties and growing seasons, with higher nitrogen and cellulose contents in early-season rice compared to others. The composition of 50 rice varieties was measured for a number of factors. Ash and fiber content ranged from 13.4 percent to 20.4 percent and 56.3 percent to 68.9 percent respectively. Lignin. ranged from 3 percent to 4.4 percent and silica ranged from 8.8 percent to 13.3 percent. Nitrogen ranged from 0.19 percent to 0.86 percent; fat ranged from 0.80 percent to 1.13 percent.

7- maize stalks T_7. (Composition of maize stalks) the chemical compositions of corn (maize) stalk. The nutrients composition % of corn stalk on DM basis were DM;

90, OM; 93, CP; 5, CF; 35, EE; 1.3, Ash; 7 while the fiber fraction were CF; 35, NDF; 44, ADF; 70. and for the corn fodder on DM basis were DM; 37, OM; 93, CP; 9, EE; 2.4, Ash; 7 while the fiber fraction were CF; 25, NDF; 29, ADF; 48.

And were replicated times for *Phaseolus vulgaris* L. cv. paulista from T_8-T_{14}.

The pots were watered for the germination of seeds. After 45 days (vegetative stage) 90 days for (flowering stage) and 130 days for (fruiting stage) the plants were harvested and washed with distilled water. The morphology fresh weights of shoots and roots and shoot length were determined in 10 plants. The samples were oven dried at 70°C for 72 h. and the dry weights of shoot and root were determined. The obtained data were analyzing statistically by using t test at 5% and 1%.

2.1 Determination of Phytohormones

Extraction and estimation of phytohormones were carried out as according to the method of [10]. Indol 3-acetic acid (IAA), Gibberellic acid (GA), Abscisic acid (ABA) was analyzed. Five gram fresh weight samples were placed in 100 ml methanol: chloroform: 2 N ammonium hydroxide (12:5:3 v/v/v) and homogenized using a Kinematic Polytron Homogenizer. After addition 1 µg/100 ml Butylated Hydroxy-toluene (BHT), the samples were frozen at -80°C for one week, for further analysis. Then the extracts were transferred into 250 ml conical flasks and added 22.4 ml bi-distilled water. To obtain a homogeneous mixture, the conical flasks were shaken 3 or 4 times. Thus, with the exception of plant growth substances, the other organics in methanol were allowed to pass into the chloroform phase. The extraction, purification and quantitative determination of total IAA, GA3 and ABA were done according to literature methods of [10].

2.2 Quantitative Determination of Total Amino Acids

Total amino acid composition of Snap bean seeds was determined by amino acid analyzer apparatus model "Eppendorf LC3000" using the method of [11].

2.2.1 Acid hydrolysis

0.3 g of Snap bean seeds powder was defeated with soaking in diethylether overnight to be sure that the sample does not contain any fats and remove pigments and impurities in the samples to be clear. A known weight (0.3 g) of defeat plant

material received 10 ml 6 N hydrochloric acid in a sealed tube, and then placed in an oven at 110°C. For 24 hours.

Hydrolyzates were transferred quantitatively into a porcelain dish and the hydrochloric acid was then evaporated to dryness at 50-60°C on a water bath. Distilled water (5 ml) was added to the hydrolyzate and then evaporated to dryness to remove the excess of hydrochloric acid and finally the residue was dissolved in 10 ml distilled water and filtrate through a 0.45 mm filter. The filtrate was dried under vacuum with a rotary evaporator, then 10 ml of distilled water was added and the samples dried a second time. One ml of 0.2 N sodium citrate buffers at pH 2.2 was added and the samples stored frozen in a sealed vial until separation of amino acids by the amino acid analyzer.

Separation of amino acids by amino acid analyzer: Samples of amino acids were injected in amino acid analyzer (Eppendorf LC 3000). Each amino acid is separated at specific pH, and then colored by reagent named Ninhydrin. Ninhydrin (triketohydrindene hydrate) is an oxidating agent which leads to the oxidative deamination of alpha-amino groups. It is very important for the detection and the qualitative analysis of amino acids. Ninhydrin also reacts with primary amines however the formation of carbon dioxide is quite diagnostic for amino acids. Alpha amino acids yield a purple substance that absorbs maximally at 570 NM. Amino acids (Proline) yield a yellow product (absorption maximum 440 NM).

2.2.2 Statistically analyzed

Data were statistically analyzed using F-test and LSD at 5 and 1% levels of probability according to [12].

3. RESULTS AND DISCUSSION

3.1 Effect of Organic Fertilizer on Growth Parameters

The data in (Table 1, 2) revealed that there are significant differences between the studied cultivars in the vegetative stage length of stems, length of roots, number of leaves and leaflets, fresh weight of stems and roots.

These results indicate that significant increases occurred in all growth parameters in two cultivars at groups T_4, T_7, T_{11} and T_{14} in compared to control, at the same time the two cultivars were shown no significant differences in the

number of leaflets at T_2, T_3, T_5, T_6, T_9, T_{10}, T_{12} and T_{13} treatment in both the cultivars. On the other hand no significant difference in the number of leaves for all treatments in both the cultivars.

At the second stage all tested organic fertilizers in combination with Nile compost caused a highly significant increase in the growth parameters (length of stems and roots, number of leaves and leaflets, fresh weights of stems and roots and the number of flowers in *Bronco* cv. (Table 3). On the other hand there were no significant differences in the number of leaves except the treatment at a mixture of compost and maize T_{14}. Similary in case of Paulista cv., (Table 4). The number of leaflets increased significant except in the treatment of T_{12} and T_{13} as compared to control.

The fresh weight of roots showed no significant difference due to adding straw rice only T_{13}. Furthermore, the data revealed that no. of leaves in *Paulista* cv. Showed no significant differences in all treatments, except in T_9, T_{11} and T_{14}.

In fruiting stage, growth parameters of bronco cv. cultivar exhibited highly significant difference at L.S.D. 0.05% and 0.01%, in all treatments, except in leaves and leaflets number of T_2 and in root fresh weight of T_5. The increases were highly significant in length of stem, number, of legume, number of seed legume, weight of pods, fresh weight of stem, and root (Table 5).

The number of leaves showed no significant difference in all treatments except, decomposted maize stalk only T_{14} which showed highly significant difference at 0.05% and 0.01% levels.

The number of leaflets was exhibited (Table 6) no significant difference as a result of organic fertilizer application alone or combined with Nile compost in all groups except T_{11} and T_{14} where a highly significant increase was observed as compared to control. Organic fertilizer plays a direct role in plant growth [13].

[14] observed that similar results were obtained in wheat treated with the application of organic manure and compost. The effects of organic manure on the vegetative growth parameters could be related to the role of released nitrogen, phosphorus and potassium from organic manure. In addition they play a vital role in photosynthesis, carbohydrate transport, protein synthesis, control of ionic balance, regulation of plant stomata functions, water use and activation of plant enzymes and other processes [15,16,17].

[18] observed that carbon compounds like cellulose and hemicellulose contained in plant residues are easily broken down and then can exert a considerable depressing effect on the nitrifying of the low-N materials. This could cause temporary immobilization of N in the soil, which would thus interfere with plant growth. However, the effects of organic fertilizers on plant growth seem to vary, and some studies showed decreased plant growth or yields when using organic fertilizers compared with conventional fertilizers [19].

The increase in growth and development during the following data could be due to the presence of phytohormones in organic fertilizers that stimulate plant growth [20]. Data presented in this investigation show varietal differences between both cv. bronco and cv. paulista in some growth characters at 30, 60 and 120 days after sowing. These differences between bean cultivars may be due to genetical differences between genotypes concerning partition of dry matter. In this regard, [21] found that the addition of organic manure combined with chemical fertilizers improved vegetative growth of sweet pepper plants.

[22] showed that in the early growth stage, there is a obviously lower in the stem length, the stem diameter and the dry weight of the organic manure cultivation in compared to another two stages; the mainly limit factors is the nutrient deficiency in the soil caused by the organic manure has not been fully decomposition. However, the findings of this investigation results of the composted maize stalks and mixed between rice straw and maize stalks treatment were found best than other mixed treatments. The maximum overall growth and yield record from the compost treatment and admixed with FYM were found consistent with the findings of [23]. The maximum biomass obtained may be due to high composition of Nitrogen in organic fertilizers, which supplement to the plant's vegetative phase. The experiment results revealed that the highest productivity by composted maize stalks and mixed between rice straw and maize stalks treatments may be due to the improvement of physico-chemical properties of the soil and can be used as a resource for maximum crop productivity with more financial output in comparison to those chemical fertilizers.

It is clear also from results that application of organic fertilizer increase most growth characters. The highest increases in the characters mentioned before were obtained by rice straw and mixed between rice straw and maize stalks and the least values were observed with compost and rice straw only. This trend could be explained on a basis that maintaining sufficient available nutrients during the growth period could be achieved through organic materials application rather than through

the mineral fertilization. These results are also in agreement with those obtained by [24] who showed that organic manure plus mineral fertilizer increase vegetative growth of broccoli plants.

All organic manures treatments increase the dry matter accumulation in the different plant organs, i.e. roots, shoots and consequently the completely peanut plants. This finding indicates the vital role of organic fertilization in more release of available nutrient elements to be absorbed by plant roots and this in turn increase dry matter content in the different peanut plant organs [25]. On the other hand, the ability of organic compost to produce such effects varies greatly with the type of organic waste used. Application of composted rice straw exhibited higher values in dry weight of different peanut organs as compared to the other organic wastes, while composted water hyacinth along recorded the highest R/S ratio [26].

The fresh weight and number of nodules were increased because of organic wastes application as compared to chemical fertilizer treatment [27]. The favorable influence of Nile compost on growth might be attributed to its effect on supplying the trees with their requirements from various nutrients, reducing soil pH, encouraging of microorganism's activity and producing natural auxins. The compost could serve as a naturally produced, slow release source of plant nutrients and their amendment has been shown to increase plant dry weight [28]. Application of compost in combination with chemical fertilizer resulted in larger leaf area index [29]. A higher leaf area index, plants become photosynthetically more active, which would contribute to improvement in yield, attributes [30].

3.2 Effect of Organic Fertilizer on Yield

All tested organic wastes, caused increases in the yield components compared to control i.e. number and yield of pods, and seed yield. [31] stated that organic manure alone or in combination with synthetic fertilizers significantly increased grain and biological yield against control.

No significant differences in yield of pods and seeds were obtained between different organic wastes alone and NPK treatment. [1] reported that incorporating rice straw into soil has increased grain yield 15–18%. Direct seeded rice (DSR) may also show the effects on grain yield of incorporation of rice straw for a couple of years in the same way as transplanted rice.

Regarding the characteristic of bean as related with the quality of yield, data indicate that, significant differences calculated in number of pods and seeds per 100 g, number of seeds per pod, weight of 100 g pods and seed index between

different composted organic wastes. These results may be due to the higher levels of organic matter and nutrients in composts [32] as well as the positive effect of composting on reduction of the germination capacity of weeds and soil-borne pathogens. The positive action of different N sources on growth and nutritional status could result in enhancing the yield. In coincidence with the present results those obtained by [33].

The addition of rice straw and mix it in the soil led to the improvement of soil properties in the form of soil penetration resistance as well as access to good specifics yield. Thus increasing the productivity of wheat and rice, which were grown with successive seasons during throughout the experiment compared to cultivated crop in the treatment without rice straw.

There was a consistent trend for similar or higher yield with rice straw, with some significant differences. Higher or similar wheat yield under rice straw mulch was also reported in other studies in the same environment [34]. The higher yields with maize stalks in our experiments were probably due to increased soil water availability compared with the control.

3.3 Effect of Organic Fertilizer on Phytohormones

Data in Table 7 revealed that the effect of organic fertilizers stimulated the synthesis of phytohormone in two cultivars. The phytohormone IAA increased in all treatments in two cultivars except by adding straw rice, there was reduced in IAA synthesis in comparison to control. However, using compost, mixture of compost and maize stalk, mixture of maize stalk and straw rice and decompost maize stalk only, increased GA when compared to control in cv. bronco. On the other hand, there were increases in GA synthesis in cv. Paulista due to organic fertilization. ABA differd values in most of tested samples. However there was reduced in ABA when treated with a mixture of compost and maize stalk and decompost maize stalk only. The better efficiency of organic manures might be due to the fact that the organic manures would have provided the micronutrients such as Zn, Cu, Fe, Mn, and Mg in an optimum level. Zinc is involved in the biochemical synthesis of the most important phytohormone, IAA through the pathway of conversion of tryptophan to IAA [35].

Table 1. Effect of compost and organic fertilizers on growth parameters of *Phaseolus vulgaris* bronco cv. (vegetative stage)

Soil treatments	*Quantity (g/pot)*	*Length of stem (cm)*	*Length of root (cm)*	*No. of leaves (/ 10 plants)*	*No. of leaflets (/ 10 plants)*	*Fresh weight of stem g/plant*	*Fresh weight of root g/plant*
T_1	---	11.50	25.60	3.00	5.00	2.67	2.04
T_2	17 gm l pot	13.00**	22.30**	3.00 ns	5.00 ns	2.76*	2.02 ns
T_3	8.5 gm of each component l pot	12.80**	22.00**	3.00 ns	5.00 ns	3.00**	1.85*
T_4	8.5 gm of each component l pot	13.80**	28.00**	3.00 ns	6.00**	3.51**	2.60**
T_5	8.5 gm of each component l pot	13.00**	24.00**	3.00 ns	5.00 ns	3.50**	2.21*
T_6	17 gm l pot	12.00**	21.10**	3.00 ns	5 .00ns	2.50**	1.50**
T_7	17 gm l pot	13.50**	31.00**	3.00 ns	6.00**	3.95**	4.01**
L.S.D. 0.05%		0.14	0.63	0.00	0.09	0.09	0.14
0.01%		0.20	0.91	0.00	0.12	0.13	0.21

*No: Number, CV: Cultivar, LSD: Least significant difference, **: Highly significant difference, ns: no significant difference. (T_1, Control;T_2, composts; T_3, compost + rice straw;T_4, compost + maize stalk; T_5, rice straw + maize stalk;T_6, rice straw; T_7, maize stalk).*

Table 2. Effect of compost and organic fertilizers on growth parameters of *Phaseolus vulgaris* paulista cv. (vegetative stage)

Soil treatments	*Quantity (g/pot)*	*Length of stem (cm)*	*Length of root (cm)*	*No. of leaves (/ 10 plants)*	*No. of leaflets (/ 10 plants)*	*Fresh weight of stem g/plant*	*Fresh weight of root g/plant*
T_8	---	13.70	19.30	3.00	5.00	2.10	0.96
T_9	17 gm I pot	18.20**	26.00**	3.00ns	5.00ns	2.90**	1.20**
T_{10}	8.5 gm of each component I pot	14.50**	19.80ns	3.00ns	5.00ns	2.80**	1.30**
T_{11}	8.5 gm of each component I pot	15.60**	26.20**	4.00ns	6.00**	3.62**	1.80**
T_{12}	8.5 gm of each component I pot	15.00**	26.00**	3.00ns	5.00ns	3.40**	1.72**
T_{13}	17 gm I pot	14.00*	19.00ns	3.00ns	5.00ns	3.08**	1.41**
T_{14}	17 gm I pot	16.00**	29.00**	4.00**	8.00**	3.09**	1.20**
L.S.D. 0.05%		0.27	0.72	0.09	0.20	0.09	0.05
0.01%		0.38	1.04	0.12	0.29	0.12	0.08

*No: Number, CV: Cultivar, LSD: Least significant difference, **: Highly significant difference, ns: no significant difference.*
(T_8, Control;T_9, composts; T_{10}, compost + rice straw;T_{11}, compost + maize stalk; T_{12}, rice straw + maize stalk; T_{13}, rice straw; T_{14}, maize stalk).

Table 3. Effect of compost and organic fertilizers on growth parameters of *Phaseolus vulgaris* bronco cv. (flowering stage)

Soil treatments	Quantity (g/pot)	length of stem (cm)	length of root (cm)	No. of leaves (/ 10 plants)	No. of leaflets (/ 10 plants)	Fresh weight of stem g/plant	Fresh weight of root g/plant	No. of flowers (/plant)
T_1	---	17.00	20.10	4.00	12.00	5.45	1.50	5.00
T_2	17 gm I pot	19.50**	21.50**	5.00**	9.00**	4.97**	1.93**	6.00**
T_3	8.5 gm of each component I pot	18.50*	18.30**	7.00**	13.00**	3.48**	1.73**	8.00**
T_4	8.5 gm of each component I pot	20.90**	27.20**	7.00**	16.00**	5.77**	2.90**	8.00**
T_5	8.5 gm of each component I pot	19.00**	20.20ns	5.00**	17.00**	5.04**	2.08**	6.00**
T_6	17 gm I pot	20.00**	20.90**	5.00**	15.00**	5.10**	3.07**	8.00**
T_7	17 gm I pot	23.80**	22.90**	9.00**	17.00**	7.29**	3.29**	14.00**
L.S.D. 0.05%		0.37	0.50	0.30	0.52	0.20	0.13	0.50
0.01%		0.54	0.72	0.44	0.75	0.29	0.18	0.71

*No: Number, CV: Cultivar, LSD: Least significant difference, **: Highly significant difference, ns: no significant difference. (T_1, Control; T_2, composts; T_3, compost + rice straw; T_4, compost + maize stalk; T_5, rice straw + maize stalk; T_6, rice straw; T_7, maize stalk).*

Table 4. Effect of compost and organic fertilizers on growth parameters of *Phaseolus vulgaris* paulista cv. (flowering stage)

Soil treatments	Quantity (g/pot)	length of stem (cm)	length of root (cm)	No. of leaves (/ 10 plants)	No. of leaflets (/ 10 plants)	Fresh weight of stem g/plant	Fresh weight of root g/plant	No. of flowers (/plant)
T_8	---	18.00	21.00	4.00	10.00	3.88	1.01	5.00
T_9	17 gm l pot	17.20**	23.60**	5.00**	13.00**	5.61**	1.71**	6.00**
T_{10}	8.5 gm of each component l pot	17.40**	23.00**	4.00ns	11.00**	5.52**	1.98**	6.00**
T_{11}	8.5 gm of each component l pot	19.00**	24.30**	6.00**	13.00**	6.55**	2.26**	7.00**
T_{12}	8.5 gm of each component l pot	17.50**	21.40*	4.00ns	10.00ns	4.88**	1.15*	6.00**
T_{13}	17 gm l pot	17.40**	20.70**	4.00ns	10.00ns	4.04ns	1.12ns	6.00**
T_{14}	17 gm l pot	21.00**	25.00**	7.00**	17.00**	6.71**	2.88**	7.00**
L.S.D. 0.05%		0.24	0.30	0.21	0.45	0.20	0.12	0.12
0.01%		0.35	0.43	0.31	0.65	0.28	0.17	0.17

*No: Number, CV: Cultivar, LSD: Least significant difference, **: Highly significant difference, ns: no significant difference. (T_8, Control; T_9, composts; T_{10}, compost + rice straw; T_{11}, compost + maize stalk; T_{12}, rice straw + maize stalk; T_{13}, rice straw; T_{14}, maize stalk).*

Table 5. Effect of compost and organic fertilizers on growth of yield of *Phaseolus vulgaris* bronco cv. (fruiting stage)

Soil Tretments	Quantity (g/pot)	length of stem (Cm)	length of root (Cm)	No. of leaves/ plant	No. of leaflets /plant	No. of legume /plant	No. of seeds /pod	Weight of legume (g)	Fresh weight of stem g/plant	Fresh weight of root g/plant
T_1	---	17.00	21.50	6.00	13.00	2.0	4.0	1.94	2.33	1.91
T_2	17 gm l pot	18.50ns	22.50**	6.00ns	13.00ns	3.00**	6.00**	3.04**	3.22**	1.76*
T_3	8.5 gm of each component l pot	18.00**	23.10**	7.00**	15.00**	4.00**	9.00**	4.13**	3.33**	2.31**
T_4	8.5 gm of each component l pot	20.00**	24.03**	7.00**	15.00**	4.00**	10.00**	4.26**	4.81**	2.54**
T_5	8.5 gm of each component l pot	19.00**	23.40**	7.00**	14.00**	3.00**	8.00**	3.05**	3.03**	1.98ns
T_6	17 gm l pot	18.00*	23.20**	7.00**	14.00**	3.00**	6.00**	3.55**	2.74**	2.09**
T_7	17 gm l pot	22.00**	26.80**	8.00**	17.00**	4.00**	12.00**	6.66**	5.40**	3.71**
L.S.D. 0.05%		0.75	0.29	0.12	0.24	0.13	0.48	0.26	0.20	0.12
0.01%		1.08	0.42	0.17	0.35	0.19	0.69	0.37	0.28	0.17

*No: Number, CV: Cultivar, LSD: Least significant difference, **: Highly significant difference, ns: no significant difference. (T_1,Control;T_2, composts; T_3, compost + rice straw;T_4, compost + maize stalk; T_5, rice straw + maize stalk;T_6, rice straw; T_7, maize stalk).*

Table 6. Effect compost and organic fertelizers on growth of yield of *Phaseolus vulgaris* paulista cv. (fruting stage)

Soil Tretments	Quantity (g/pot)	l. of stem (Cm)	l. of root (Cm)	No. of leaves/ plant	No. of leaflets / plant	No. of legumes/ plant	No. of seeds/ leguems	Weight of pods (g/Plants)	Fresh w. of stem g/plant	Fresh w. of root g/plant
T_8	-	21.20	20.9	6.00	13	2	5	3.32	3.96	1.99
T_9	17 gm / pot	22.30**	25.00**	6.00ns	13.00ns	4.00**	9.00**	3.87**	3.58**	1.75**
T_{10}	8.5 gm of each componet / pot	22.00**	23.60**	6.00ns	13.00ns	4.00**	9.00**	4.78**	3.03**	1.72**
T_{11}	8.5 gm of each component / pot	25.80**	27.20**	6.00ns	14.00**	4.00**	9.00**	5.79**	4.63**	2.16**
T_{12}	8.5 gm of each component / pot	22.00**	23.00**	6.00ns	13.00ns	4.00**	9.00**	4.43**	3.06**	1.67**
T_{13}	17 gm / pot	21.80*	21.00ns	6.00ns	13.00ns	4.00**	9.00**	4.37**	3.44**	1.25**
T_{14}	17 gm / pot	27.90**	29.20**	5.00**	16.00**	4.00**	11.00**	7.91**	5.03**	3.51**
L.S.D. 0.05%		0.44	0.54	0.07	0.20	0.14	0.32	0.27	0.13	0.13
0.01%		0.64	0.78	0.10	0.29	0.20	0.46	0.38	0.19	0.18

*No: Number, CV: Cultivar, LSD: Least significant difference, **: Highly significant difference, ns: no significant difference. (T_8, Control; T_9, composts; T_{10}, compost + rice straw; T_{11}, compost + maize stalk; T_{12}, rice straw + maize stalk; T_{13}, rice straw; T_{14}, maize stalk).*

Table 7. Effect of compost and organic fertilizers on Phytohormones of two cultivars of *Phaseolus vulgaris* (vegetative stage)

No.	Phytohormones mg/1OOg		
	GA3	IAA	ABA
1	16.332	7.233	14.463
2	21.033	8.934	23.721
3	11.968	9.776	20.130
4	55.012	10.291	8.902
5	43.562	10.221	3.492
6	2.888	7.028	10.451
7	64.918	24.714	4.542
8	2.546	8.054	12.611
9	5.993	9.368	16.017
10	4.131	11.122	7.232
11	21.237	16.334	7.249
12	13.344	12.921	11.648
13	4.131	1.111	11.159
14	46.202	22.185	6.967

Indol 3-acetic acid (IAA), Gibberellic acid (GA), Abscisic acid (ABA) Phaseolus vulgaris (cv. bronco.) (T_1, Control; T_2, composts; T_3, compost + rice straw; T_4, compost + maize stalk; T_5, rice straw + maize stalk; T_6, rice straw; T_7, maize stalk).
Phaseolus vulgaris (cv. paulista) (T_8,Control; T_9, composts; T_{10}, compost + rice straw; T_{11}, compost + maize stalk; T_{12}, rice straw + maize stalk; T_{13}, rice straw; T_{14}, maize stalk).

[36] reported that organic manures activate many species of living organisms which release phytohormones and may stimulate the plant growth and absorption of nutrients.

This data was directly related to leaf nitrogen content due to the action of this nutrient on the process of cell multiplication and plant organ development. Furthermore, nitrogen is a factor and considered as the characteristic constituent of functional plasma, an integral part of chlorophyll molecules, proteins, amino acids, nucleic acids, nucleotides, alkaloids, enzymes, coenzymes, hormones and vitamins [37]. It alteration in different aspect of cellular metabolisms including the content of phytohormones could be arising from the different compounds present in the used vermicompost [38].

The gibberellic acid (GA) is involved in many aspect development throughout the life-cycle of higher plants. They also mediate certain environmental effects on plant development and are signaling molecules that regulate and integrate developmental processes during the entire life-cycle of higher plants, including shoot elongation and root development [39]. Gibberellin GA signaling may enable integration of aerial and root development [40]. Result concerning the effect of organic fertilizers on fruit endogenous hormones during the experimental season. This result may be due to the use of plant growth regulators (GA3) which could lead to an increase in fruit set of deciduous trees. In addition, [41] decided the same results on citrus. Treatment maize stalks and rice straw in cv. Paulista recorded the highest yield and hormones value and maize stalks in cv. Bronco. These results are owing to the use of GA3 in plant

and micronutrients, which led to an increase in fruit set, and, GA3 played a major role in enlarging fruit size. In general, these results are in line with those obtained by [42].

3.4 Effect of Organic Fertilizer on Free Amino Acid

The amino acid content in leaves exhibited difference in values in treating samples as response to organic fertilizers (Table 8). Fifteen of amino acid compositions were estimated in the shoot in vegetative stage. However, the application mixture of compost and maize or decomposed maize stalk only increased most of amino acids in two cultivars when compared to control.

The results showed that compost, the mixture of compost and maize and maize stalk decomposing only, shown the highest number of amino acids compared with the amino acid in the rest treatments.

The results agreed with [43] who showed that the increased percentage of crude protein, free amino acids and nitrate in tubers of potato increased with increasing the rate of the fertilizers [5] reported that organic fertilizer lead to new amino acids compared with the control in wheat Also [6] reported that application of compost significantly increased the level of total free amino acid in leaves of mustard when compared to control.

The results in this investigation are in accordance with the conclusions of other researchers who found that the quantity of albumins-globulins is scarcely influenced by N nutrition. As noted previously, protein composition of the wheat grain is influenced by genotype, as well as by cultivation system and environmental conditions [44]. In other words, although increased nitrogen supply correlated significantly to an increase in all protein components, its effect on grain protein also depends on the cultivar sown, due to different uses of available soil N, especially during stem elongation. It seems that organic matter can improve the physical properties of the soil and would have caused increased root development that acted positively in more uptakes of water and nutrients [45]. In addition, our results was harmony with [46] that there were an increase of formation of amino acid and consequently protein formation.

[45] stated that the soluble proteins are increased with better N supply and favorable growth condition by treatment with organic manure.

Our results confirmed an increase of glutamine level in maize stalks and rice straw application. The supply of ammonium increased considerably the concentrations of the primary amino acids, and asparagine was the most predominant acid, followed by glutamine.

Table 8. Effect of compost and organic fertilizers on amino acid contents of two cultivars of *Phaseolus vulgaris* (vegetative stage)

	***Phaseolus vulgaris* (CV. bronco.)**							***Phaseolus vulgaris* (CV. paulista)**						
Total amino acids %	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Asp	**4.262**	2.716	2.845	4.740	**3.418**	**1.895**	5.896	**2.281**	**3.973**	**4.054**	**5.063**	**4.864**	**2.686**	**5.547**
Thr	**0.710**	---	**0.564**	**0.925**	**0.715**	**0.630**	**2.805**	0.394	**0.761**	**0.694**	**0.872**	**0.782**	**0.508**	**1.134**
Ser	**0.520**	0.678	**0.598**	**2.647**	**2.283**	**0.650**	**4.736**	2.925	**4.284**	3.171	4.265	3.140	3.088	**3.614**
Gly	**1.508**	**1.830**	**1.653**	**2.190**	**2.110**	1.425	**2.565**	1.385	**2.198**	1.886	**2.457**	**2.287**	**0.940**	**2.802**
Ala	**1.804**	**1.590**	**1.653**	**2.190**	**1.872**	**1.445**	**2.282**	**1.908**	**2.064**	1.688	**2.324**	**2.265**	**1.178**	**2.387**
Val	**1.130**	**1.332**	**1.460**	**1.573**	**1.312**	**0.602**	**2.120**	0.795	1.489	**1.398**	**1.520**	**1.493**	**1.404**	**1.770**
Lle	**0.756**	**0.496**	**0.864**	**1.067**	**0.867**	**0.605**	**1.215**	**0.311**	**0.652**	**0.572**	**0.847**	**0.667**	**0.542**	**1.018**
Leu	**2.150**	**1.160**	**2.251**	**2.540**	**2.272**	**1.861**	**2.625**	1.198	**2.080**	2.502	**2.685**	**2.598**	**2.209**	**3.460**
Tyr	**0.890**	**0.350**	**0.639**	**0.972**	**0.899**	0.314	1.294	0.618	0.795	**0.876**	1.475	**1.100**	**0.681**	**1.526**
Phe	**0.805**	**0.128**	**0.588**	**1.095**	**0.828**	0.400	1.450	**0.705**	0.719	1.014	**1.312**	**1.125**	**0.980**	1.356
Lys	**0.597**	---	**0.508**	**0.835**	**0.632**	**0.470**	**0.932**	**0.402**	0.456	**0.558**	**0.710**	**0.637**	**0.391**	**1.556**
MET	0.587	0.409	**0.984**	**1.155**	**1.062**	0.096	**1.370**	**0.365**	**0.280**	**0.344**	**1.050**	**0.395**	0.043	**1.306**
Arg	2.217	**1.102**	**4.540**	**6.684**	**6.175**	1.951	**6.802**	**1.149**	**2.028**	**1.605**	**4.605**	**2.223**	1.641	**4.857**
Glu	---	**3.097**	---	⋯	---	---	---	---		---	---	---	---	**1.033**
His	----	·	---	**0.415**	---	---	**0.792**	·	---	---	**1.267**	---	---	---

Abbreviations of amino acids: ALA = alanine, ARG = arginine, ASP = aspartic acid, GLU =glutamic acid, GLY = glycine, HIS = histidine, ILE = isoleucine, LEU = leucine, LYS = lysine, MET = methionine, PHE = phenylalanine, PRO = proline, SER = serine, THR = threonine, TYR = tyrosine, VAL = valine.

cv. bronco (T_1, Control; T_2, composts; T_3, compost + rice straw; T_4, compost + maize stalk; T_5, rice straw + maize stalk; T_6, rice straw; T_7, maize stalk).

cv. paulista (T_8, Control; T_9, composts; T_{10}, compost + rice straw; T_{11}, compost + maize stalk; T_{12}, rice straw + maize stalk; T_{13}, rice straw; T_{14}, maize stalk).

4. CONCLUSION

Application of (mixture of compost and maize stalk and decomposed maize stalk only) were found as the most effective ones to increase of that contents of amino acid and phytohormone of two cultivars. Therefore, it could be suggested that the treated plants, with these organic residues and Nile compost increased the growth, yield and the above chemical compositions.

ACKNOWLEDGEMENTS

To the spirit of our father and our teatcher Dr. Mohamed Abed-Elhamid Hassan as long as science and scientists, we dedicate this modest effort.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

References:

1. Chaterjee B, Ghanti P, Thapa U, Tripathy P. Effect of organic nutrition in spro broccoli (*Brassica aleraceae* var. italica plenck). Vegetable Sci. 2005;33(1):51-54.
2. Ryan J, Harik SN, Shwayri I. A short-term greenhouse evaluation of non-conventional organic wastes. Amer. Univ. of Berirut Lebanon Agric. Wastes. 1985;12(4):241-249.
3. Hoitink HAJ. Proceedings Review: International Symposium on composting research. Compost Science and Utilization. Spring. 1993;37-42.
4. Smith WH. Utilizing composts in land management to recycle organics. In: Bertoldi, Sequi, M.P. Lemmes, B. and T. Papi (Eds.). The science of compo-sting Part 1. 1996;413–422. Blackie, Glasgow, UK.
5. Rajaa FH, Saadi SK. Effect of Gibberellic acid and Organic fertilizer on certain chemical compounds for Wheat plant Triticum aestivum L. Al-Anbar J. Agric. Sci. 2011;9:70-79.
6. Banerjee A, Datta JK, Mondal NK. Biochemical changes in leaves of mustard under the influence of different fertilizers and cycocel. J. Agric. Technol. 2012; 8(4):1397-1411.

7. Atiyeh RM, Lee S, Edwards CA, Arancon NQ, Metzger JD. The influence of humic acids derived from earthworm-processed organic wastes on plant growth. Bioresource Technol. 2002;84:7-14.
8. Bachman GR, Metzger JD. Growth of bedding plants in commercial potting substrate amended with vermicompost. Bioresource Technol. 2008;99:3155–3161.
9. Sehirali S. Yemeklik tane baklagiller Ders Kitabi. (Seed Legumes, Lecture Notes), Publication No. 1089 Faculty of Agriculture, University of Ankara. 1988;314. Turkey.
10. Unyayar S, Topcuoglu SF, Unyayar A. A modified method for extraction and identification of Indole-3-Acetic Acid (IAA), Gibberellic Acid (GA3), Abscisic Acid (ABA) and zeatin produced by *Phanerochaete chrysosporium* ME446. Bulg. J. Plant Physiol. 1996;22:105-110.
11. Steven AC, Michael M, Thomas LT. A Manual of advanced techniques for amino acids analysis. The Pico-Tag method, Millipore Corporation, Printed in USA; 1989.
12. SAS-Programme. SAS user's Guide Statistics SAS Institute, INC, Raleiegh. North Carolina. 1982;584.
13. Mazhar AAM, Nahed G, Shaymaa IS, Sahar MZ. Effect of Nile Compost application on growth and chemical constituents of *Jatropha curcas* grown under different salinity levels of diluted sea water. Aus. J. Basic and App. Sci. 2011;5(9):967-974.
14. Muhammad I, Anwar H, Ehsan EV. Response of wheat growth and yield to various levels of compost and organic manure. Pak. J. Bot. 2008;40(5):2135-2141.
15. El-Ghamry AM. Soil fertility and potato production as affected by conventional and organic farming systems. J. Soil Sci. and Agric. Eng. Mansoura Univ. Egypt. 2011;2(2):141–156.
16. Najm AA, Haj SH, Fazeli MRF, Shamorady MTD. Effect of utilization of organic and inorganic nitrogen source on the potato shoots dry matter, leaf area index and plant height, during middle stage of growth. International J. Agric. and Biol. Sci. 2010;1(1):26-29.
17. Al-Hisnawy EAK. The Effect of Spraying Organic Fertilizer (LIQ Humus) on the Growth and Yield of Three Cultivars of Potato plant *Solanum tubersum* L. M.Sc. Thesis. College of Agric. Univ. Kufa Iraq. (in Arabic); 2011.

18. Rubins EJ, Bear FE. Carbon-nitrogen ratios in organic fertilizer materials in relation the availability of their nitrogen. Soil Sci. 1942;54:411-424.
19. Treadwell DD, Hochmuth GJ, Hochmuth RC, Simonne EH, Davis LL, Olczyk WL, Sprenkel RK, Osborne LS. Nutrient management in organic green house herb production. Hort Technol. 2007;17:461–466.
20. Nogales R, Cifuentes C, Benitez E. Vermicomposting of winery wastes: A laboratory study. J. Environ. Sci. and Health. 2005;1234:659-573.
21. Shams ASA. Response of sweet pepper crop to organic and biofertilizer application. M.Sc Thesis Fac. Agric. Moshtohor, Zagazig Univ., Egypt. 2003;148.
22. Xiangyang L, Guangxi R, Yan S. The effect of organic manure and chemical fertilizer on growth and development of *Stevia rebaudiana* bertoni. Energy Procedia. 2011;5:1200-1204.
23. Yadav H, Vijayakumari B. Influence of vermicompost with organic and inorganic manures on biometric and yield parameters of chilli [*Capsicum annuum* (L.) var. Plri]. Crop Res. 2003;25(2):236-243.
24. Rakesh S, Chaurasia SNS, Singh SN. Response of nutrient sources and spacing on growth and yield of broccoli (*Brassica oleraceae* var. italica Plenck). Vegetable science. 2006;33(2):198-200.
25. Ahiabor BD, Hirata H. Characteristic responses of three tropical legumes to the inoculation of two species of VAM fungi in Andosol soils with different fertilities. Mycorrhiza. 1994;5:63–70.
26. Yanar D, Gebologlu N, Yanar Y, Aydin M, Cakmak P. Effect of different organic fertilizers on yield and fruit quality of indeterminate, tomato (*Lycopersicon esculentum*). Sci. Res. Essay. 2011;6(17):3623-3628.
27. Radwan SMA. The Use of Biofertilizers in Increasing the Uptake of Plant Nutrients in Some Vegetable Crops. Ph.D. Thesis, Fac. Agric. Ain Shams Univ. Cairo. 1990;243-260.
28. Edwards CA. Historical overview of vermicomposting. Biocycle. 1995;36(6):56-58.
29. Jeyabal A, Kuppuswamy G. Recycling of organic wastes for the production of vermicompost and its response in rice-legume cropping system and soil fertility. European J. Agronomy. 2001;15(3):153-170.

30. Sharma AR, Mittra BN. Effect of green manuring and mineral fertilizer on growth and yield of crops in rice-based cropping on acid lateritic soil. Journal of Agricultural Science, Cambridge. 1988;110:605–608.
31. Saeed N, Hussain M, Saleem M. Interactive effect of biological sources and organic amendments on the growth and yield attributes of sunflower (*Helianthus annuus* L.). Pakistan Journal of Agricultural Sciences. 2002;39(2):135-136.
32. Badr El-Din SMS, Attia M, Abo-Sedera SA. Field assessment of composts produced by highly effective cellulolytic microorganisms. Biol. Fertil. Soils. 2000;32:35-40.
33. Mansour AEM, Ahmed FF, Ragab AM, Mohamed GA. Yield and quality of Anna apple trees in response to application of some slow and fast release N fertilizers. Egypt. J. Hort. 1999;26(1):1-6.
34. Chakraborty D, Nagarajan S, Aggarwal P, Gupta V, Tomar R, Garg R, Sahoo R, Sarkar A, Chopra U, Sarma K. Effect of mulching on soil and plant water status, and the growth and yield of wheat (*Triticum aestivum* L.) in a semi-arid environment. Agric. Water Manage. 2008;95(12):1323-1334.
35. Premsekha M, Rajashree V. Influence of Organic Manures on Growth, Yield and Quality of Okra. Am.-Eur. J. Sustainable Agric. 2009;3(1):6-8.
36. Arisha HME, Gad AA, Younes SE. Response of some pepper cultivars to organic and mineral nitrogen fertilizer under sandy soil conditions. Zagazig J. Agr. Res. 2003;30:1875-1899.
37. Castellanos JZ, Uvalle-Bueno JX, Aguilar-Santelises YA. Manual de interpretacion de analisis de suelos, aguas agricolas, plantas y ECP. 28 ed. INIFAP, Chapingo, Mexico; 2000.
38. Moghadam ARL, Ardebili ZO, Saidi F. Vermicompost induced changes in growth and development of *Lilium asiatic* hybrid var. *Navona*. Afr. J. Agric. Res. 2012;7(17):2609-2621.
39. Lange T, Kappler J, Fischer A, Frisse A, Padeffke T, Schmidtke S, Lange M. Gibberellin biosynthesis in developing pumpkin seedlings. Plant Physiol. 2005;139:213–223.
40. Gou J, Strauss SH, Tsai CJ, Fang K, Chen Y, Jiang X, Busov VB. Gibberellins regulate lateral root formation in populus through interactions with auxin and other hormones. Plant Cell. 2010;22:623–639.

41. El-Sayed Ahalam A. Effect of foliar application of liquid organic fertilizer and/or GA3 in fruiting and leaf mineral composition of Washington Navel orange trees. J. Agric. Res., Zagazig Univ. 2005;32(4):763-775.
42. El-Seginy AM, Khalil BM. Effect of spraying some nutrients and gibberellic acid on leaf mineral content, fruit characters and yield of Le-Conte pear trees. J. Agric. Sci., Mansoura Univ. 2000;25(6):3529-3539.
43. Bohm H, Dewes T. Effects of increasing rates of farmyard manure on yield, quality and postharvest quality of selected potato cultivars. Schriftenreihe institute fur organischen. Landbau. 1997;4:368-374.
44. Pedersen L, Jorgensen JR. Variation in rheological properties of gluten from three biscuit wheat cultivars in relation to nitrogen fertilization. J Cereal Sci. 2007; 46:132–138.
45. Brady NC, Weil RR. The nature and properties of soil 13 rd edn. Macmillan Publishing Co, New York; 2005.
46. Agamy RA, Mohamed GF, Rady MM. Influence of the Application of Fertilizer Type on Growth, Yield, Anatomical Structure and Some Chemical Components of Wheat (*Triticum aestivum* L.) Grown in Newly Reclaimed Soil. Australian Journal of Basic and Applied Sciences. 2012;6(3):561-570.

Yield Selection Within Coffee Arabica cv. Ruiru 11

B. M. Gichimu, E. K. Gichuru,
Coffee Research Foundation, Ruiru, Kenya,
G. E. Mamati and A. B. Nyende,
Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya

ABSTRACT

Aims: This study was aimed at identifying high yielding Ruiru 11 sibs in varying growing conditions. The study also intended to measure the extent to which cherry yields of Ruiru 11 are affected by the environment.

Study Design: Randomized Complete Block Design with three replications.

Place and Duration of Study: The study was conducted in three different agro-ecological zones in Kenya namely Mariene in Meru County, Kisii near Kisii town in Kisii county and Koru in Kericho County between November 2008 and September 2011.

Methodology: Thirty four (34) Ruiru 11 sibs, all of which are resistant to Coffee Berry Disease and Coffee Leaf Rust, were evaluated in this study alongside two entries of SL28, a cultivar susceptible to the two diseases. One entry of SL28 was sprayed with copper fungicides to control, while the other SL28 entry was not sprayed with any fungicides. Planted at a spacing of 2 m by 2 m, each entry had 12 trees per plot per rep, giving a total of 1296 plants per experiment per site. Cherry yield recording was done during the peak harvesting period of May to July at Mariene and July to September at Koru and Kisii. The data was subjected to Analysis of Variance (ANOVA) using XLSTAT version 2012 statistical software and effects declared significant at 5% level.

Results: Significant (P = .05) yield differences among Ruiru 11 sibs were obtained in all years of evaluation at Koru but only in 2011 at Kisii and Mariene. There was a greater discrimination between sibs at Koru, followed by Kisii and then Mariene. Year effect was highly significant (P < .001) and equally distinguished in all sites but year x sib interactions were significant (P = .05) only at Kisii. Combined analysis for all environmental combinations showed highly significant (P < .001) differences between sibs, environments and their interaction. Environments made a greater contribution (42.6%) to the variation compared to sibs (7%). The interaction

term also made a significant contribution (18.7%). The best sibs per site and those adapted to contrasting environments were identified.

Conclusion: The expression of high yield variation among Ruiru 11 sibs is a sign of high potential of intra-selection within the cultivar for yield improvement. Identified sibs can be recommended to farmers and also exploited in future breeding programmes for improvement of Ruiru 11 productivity and agronomic adaptability. The occurrence of significant sib by environment (G x E) interactions was an indication that the best improvement strategy should be a multi-site selection.

Keywords: coffee; Ruiru 11; cherry yields; Kenya.

1. INTRODUCTION

Behind oil, coffee is the second most traded commodity in the world. Its cultivation is mainly by smallholder farmers who hardly break even mainly due to low yields, high production cost and low world market prices. Increasing productivity, while reducing the cost of production is a main breeding objective of most producing countries [1]. New arabica cultivars with higher yield potential and resistance to Coffee Leaf Rust (CLR) and/or Coffee Berry Disease (CBD) have started to replace traditional varieties on a large scale in several countries [2]. The cultivar Ruiru 11 is a composite of about 60 F1 hybrid sibs each derived from a cross between a specific female and male population [3]. The cultivar was developed at the Coffee Research Station, Ruiru, Kenya, and released to growers in 1985. It combines resistance to CBD and CLR with high yield, fine quality and compact growth amenable to high density planting [3].

The economic value of Arabica coffee *Coffea arabica* L. is determined both by the yield potential and the bean quality [4]. Yields of 5 tons ha^{-1} and higher have been obtained in some close-spaced and unshaded Arabica coffee blocks e.g. in Brazil, Colombia and Kenya [5]. However, most smallholder Arabica coffee farms with no access to external inputs often produce less than 300 kg ha^{-1} $year^{-1}$ green coffee beans, while intensively managed plantations at conventional spacing may yield an average of 2 tons ha^{-1} annually [6]. Data from field trials at Coffee Research Foundation, in Kenya shows that Ruiru 11 cultivar planted at a density 3300 trees/ha produces between 2.5 and 3.0 tons ha^{-1} $year^{-1}$ [6]. Depending on conditions, coffee yields fluctuate from year to year and from location to location [7;8].

Success of a new variety depends to a great extent on its adaptability to a wide range of climatic and soil conditions [8]. Coffee can be cultivated on a wide range of soil types, provided these are at least 2 m deep, free-draining loams with a good water-holding capacity and a pH of 5–6, fertile and contain at least 2% organic matter. High-quality, acidic Arabica coffees are mostly produced on soils of volcanic origin [6]. Arabica coffee is grown in altitude ranges between 1400 and 1800 m above sea level [9]. The optimum mean annual temperature range for Arabica coffee is 18-21°C [10]. Rainfall requirements depend on the retention properties of the soil, atmospheric humidity and cloud cover, as well as cultivation practices. The optimum annual rainfall range is 1200-1800 mm for Arabica coffee [10] with a maximum of 2500 mm [6]. Coffee plants grow and yield better if exposed to alternate cycles of wet and dry seasons [6]. Abundant rainfall throughout the year often results in scattered harvest and low yields [10]. The distribution of sunshine also has a strong influence on flowering, bean expansion and ripening. Shade decreases coffee tree productivity by about 20%, but reduces the alternate bearing pattern [11].

Knowledge of the effects of environment and genotype by environment (G x E) interaction is important to breeders in making decisions regarding the development, evaluation and release of new cultivars [7,8]. Identifying high yielding coffee genotypes is often time consuming and difficult to achieve due to the perennial nature of the crop, biennial bearing, and the large environmental component of variance for yield [1]. This study aimed at identifying high yielding Ruiru 11 sibs in varying growing conditions. The study also intended to measure the extent to which cherry yields of Ruiru 11 are affected by the environment.

2. MATERIALS AND METHODS

2.1 Description of Study Sites

The study was conducted in three different agro-ecological zones in Kenya namely Mariene in Meru County, Kisii near Kisii town in Kisii county and Koru in Kericho County. Mariene is located at 0^{0}N, 37° 35'E, at an elevation of 1524 m above sea level. The soils are ando-humic acrisols, friable clays, strongly acidic, very low in bases and moderate in organic matter. Koru is located at 0° 07'S, 35° 16'E and has an elevation of 1554 m above sea level.

The soils are eutric nitosols, friable clays, and weakly acidic to neutral, rich in bases, available phosphorous and moderate inorganic matter. Kisii is located at 0° 41'S, 34°47'E at 1700 m above sea level. The soils are molic nitosols, friable clays with acidic pH, low to moderate bases and are high in organic matter. The experimental plots in Koru and Kisii were established in April 1990 while Meru plot was established in April 1991. All the plots have undergone change of cycle twice and were therefore almost of the same status. Other agronomic practices were carried out as recommended. All the sites were laid out in a Randomized Complete Block Design (RCBD) with three replications.

2.2 Plant Materials and Field Layout

Thirty four (34) Ruiru 11 sibs (Table 1) were evaluated in this study alongside two entries of SL28 used as checks. One entry of SL28 was sprayed with copper fungicides to control CBD and CLR, while the other SL28 entry was not sprayed with any fungicides. All the sites were laid out in a Randomized Complete Block Design (R CBD) with three replications. Planted at a spacing of 2 m by 2 m, each entry had 12 trees per plot per rep, giving a total of 1296 plants per experiment per site. Cherry yield recording was done during the peak harvesting period of May to July at Mariene and July to September at Koru and Kisii. Rainfall was recorded in all the three sites for the three production seasons (years) at various berry development stages (Table 2).

2.3 Data Analysis

The data was subjected to Analysis of Variance (ANOVA) using XLSTAT version 2012 statistical software and effects declared significant at 5% level. Separate as well as combined analysis of variance was performed on data from all locations over the three production years. Least Significance Difference (LSD) was used to separate the means.

Table 1. The pedigree of the 34 Ruiru 11 sibs evaluated

	Female parent						
Male parent	**Cat.86**	**Cat.88**	**Cat.90**	**Cat.124**	**Cat.127**	**Cat.128**	**Cat.134**
SL34 x [(SL34 x RS) HT]	-	-	-	135	-	137	-
SL28 x [(SL28 x RS) (B x HT)]	1,11,41	22,42	3,23	5	6	7	50
SL28 x [(N39 x HT) (SL4 x RS)]	71	72	-	-	-	-	80
SL28 x [(K7 x RS) (SL34 x HT)]	-	52	-	-	-	-	-
SL28 x [(SL34 x RS) HT]	91,111, 121,131	112,142	93,103, 123,143	105,115,125	106	107,117	100

Key: RS = Rume sudan, HT = Hibrido de Timor, B = Bourbon, Cat. = Catimor,
The numbers in the table are Ruiru 11 sibs e.g. 1 = Ruiru 11 sib 1 and so on

Table 2. Rainfall in mm received at the three locations at different berry development stages

Kisii												
Stages	**Flowering**	**Pinhead**	**Berry expansion**				**Filling**		**Ripening**			**Total**
Month	**Nov**	**Dec**	**Jan**	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Jul**	**Aug**	**Sept**	**rainfall**
2008/09	153.4	82.6	111.2	661.3	188.3	231	297.4	152.3	63.2	197.2	160.3	2298.2
2009/10	151.7	305.5	49.8	99.6	203.4	233.7	406.8	202.4	79.6	204.3	292.1	2228.9
2010/11	109.1	188.5	97.5	42.5	138.5	237.2	267.8	91.6	100.5	233.6	225.3	1732.1
Koru												
Stages	**Flowering**	**Pinhead**	**Berry expansion**				**Filling**		**Ripening**			**Total**
Month	**Nov**	**Dec**	**Jan**	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Jul**	**Aug**	**Sept**	**rainfall**
2008/09	92.1	28.5	122.6	87.8	59.9	267.7	177.6	102.6	113.8	83.1	176.6	1312.3
2009/10	106.2	343	102.8	215.5	211.8	163.4	258.9	140.6	132	118.4	89	1881.6
2010/11	80	163.3	67.7	88	177.5	60.3	198.5	138.4	77.4	205.9	211.6	1468.6
Mariene												
Stages	**Flowering**	**Pinhead**	**Berry expansion**				**Filling**		**Ripening**			**Total**
Month	**Nov**	**Dec**	**Jan**	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Jul**	**Aug**	**Sept**	**rainfall**
2008/09	37.5	3.5	19	181.4	138.3	0.6	147	15.6	156.5	221.6	96	1017.0
2009/10	12.6	16.8	3	303.8	420.5	194.7	192.9	118.7	348.4	504.2	121.1	2236.7
2010/11	21.3	21	1.4	181.8	370.5	30.6	49	22.8	52.8	252.5	148.4	1152.1

3. RESULTS

Cherry yield data was obtained from two locations (Koru and Mariene) over three years and two years at Kisii making a total of 8 environmental combinations. The Kisii site was omitted in 2009 as it recorded very low yields as the trees were recovering from hailstorm damage. Analysis of variance (ANOVA) obtained significant (P = .05) yield differences among Ruiru 11 sibs in all the years at Koru but only in 2011 at Kisii and Mariene. This was an indication of some genetic variation between the sibs which are considered to be closely related. Examination of the F values at each location showed that there was a greater discrimination between sibs at Koru, followed by Kisii and then Mariene. The year effect was highly significant (P < .001) and equally distinguished in all sites but year x sib interactions were significant (P = .05) only at Kisii (Table 3).

Table 3. Sib variations for cherry yield at the three sites over three years

	Sib variations			Combined variations		
	2009	2010	2011	Year	Sibs	Year x Sib
Kisii	-	0.0941^{ns}	0.0038^{**}	0.0001^{***}	0.0027^{**}	0.0358^{*}
Koru	0.0387^{*}	0.0181^{*}	0.0062^{**}	0.0001^{***}	0.0001^{***}	0.9392^{ns}
Mariene	0.1554^{ns}	0.5341^{ns}	0.0149^{*}	0.0001^{***}	0.0003^{***}	0.8501^{ns}

Analysis of variance of the individual years with the locations combined revealed that the site effect was significant (*P* = .05) in all the years. All the sites recorded their best yields in 2010. Mariene trial consistently recorded the lowest yields in all the years that were evaluated while Koru trial recorded moderate yields. Kisii and Koru recorded similar yields in 2010 but the former yielded highest in 2011 (Table 4).

Table 4. Site variations in average (Av.) cherry yields (in grams) over the three years

	2009		2010		2011	
	Av. yield	Variation	Av. yield	Variation	Av. yield	Variation
Kisii	-	-	11825.29	A	10018.58	A
Koru	8785.29	A	11091.30	A	7515.34	B
Mariene	4419.74	B	5033.40	B	4188.93	C
LSD	713.97		790.22		851.07	

NB: Means sharing the same letter along the column are not significantly different (P = .05)

Multi-site analysis for the 8 environmental combinations recorded significant differences between sibs, environments and their interaction (Table 5). Further scrutiny of their contribution to total sum of squares indicated that environments made a greater contribution (42.6%) to the variation compared to sibs (7%). The interaction term also made a significant contribution (18.7%).

Table 5. Multi-site analysis of variance for cherry

Source	DF	SS	MS	F	p
Blocks	2	25401139.7	12700570	1.40733	0.2456 ns
Environment	7	7030918459	1.00E+09	111.298	0.0000 ***
Sib	35	1152790161	32936862	3.64967	0.0000 ***
Environment x Sib	245	3077380387	12560736	1.39183	0.0008 ***
Error	574	5180123925	9024606.1<-		
Total	863	1.65E+10			

Key: df = degrees of freedom, SS = Sum of Squares, MS = Mean Squares, F = Fishers value, P = level of significance

Overall, Kisii and Koru in 2010 produced the highest yields. They were followed by Koru in 2011, Koru in 2009, Kisii in 2011 and Mariene 2010 in that order, all of which recorded cherry yields that were significantly (P = .05) different from each other. The lowest yields were recorded at Mariene in 2011 and 2009 (Table 6).

Table 6. Environmental effect on cherry yield of Ruiru 11 sibs

Rank	Environment	Yields (g)	Variation
1	Kisii 2010	11825.287	A
2	Koru 2010	11091.305	A
3	Koru 2011	10018.583	B
4	Koru 2009	8785.288	C
5	Kisii 2011	7515.341	D
6	Mariene 2010	5033.398	E
7	Mariene 2009	4419.741	EF
8	Mariene 2011	4188.927	F
	LSD	802.938	

NB: Means sharing the same letter along the column are not significantly different (P = .05)

Significant yield differences were observed among the sibs in all the locations. Evaluated sibs were found to produce average yields between 3 – 16 kgs (Table 7). The high yielding but susceptible SL28 cultivar was used as a check. In all the three sites, SL28 sprayed with fungicide recorded slightly higher yields than the unsprayed SL28 in absolute terms but statistically similar. Therefore, spraying SL28 against fungal diseases had no significant effect on yield. At Kisii site, the yields of SL28 (both sprayed and unsprayed) were highly comparable to those of most Ruiru 11 sibs. The yields of sprayed SL28 were not significantly different from those of the first 30

Ruiru 11 sibs (except 143, 107, 106 and 112) while the yields of unsprayed SL28 were statistically similar to those of all Ruiru 11 sibs except R11-112. At Koru, all Ruiru 11 sibs produced better yield than SL28 in absolute terms with 17 sibs recording significantly (P = .05) higher yields than SL28. At Mariene, 8 Ruiru 11 sibs recorded significantly (P = .05) higher yields than SL28 (Table 7).

The best performing sibs per location are shown in Table 8. The most suited sibs for Kisii site which recorded high yields in both seasons were found to be R11-131, R11-52, R11-7, R11-117, R11-6, R11-142, R11-1 and R11-41. The Koru site was found to be favourable for most of the sibs but best performing were R11-107, R11-91, R11-80, R11-117, R11-142, R11-52, R11-137, R11-11, R11-100 and R11-135.

The above mentioned sibs for both Kisii and Koru sites consistently recorded high yields in varying environmental conditions. For Mariene, the best performing sibs were R11-1, R11-135, R11-11 and R11-52. The four were the only ones that yielded consistently better under all conditions and were regarded to be highly stable in terms of yields. The sibs were best discriminated at Mariene and the site was considered the best for yield selection followed by Kisii.

The most widely adapted sibs which performed better in varying climatic conditions are shown in Table 8. R11-52 and R11-117 were the best sibs overall, consistently recording high yields in all the environments. Other sibs that consistently recorded high yields in varying environments are R11-131, R11-11, R11-105, R11-142, R11-7, R11-100 and R11-121. In addition, R11-80, R11-135, R11-22, R11-72, R11-137, R11-115, R11-6 and R11-91 consistently recorded high yields in more than one environment (Table 9).

4. DISCUSSION

Although some studies have been carried out to assess variation of Ruiru 11 sibs in quality [4;12;13;14] and disease resistance [3], there is little information about their variation in yield. Ruiru 11 sibs evaluated were found to differ significantly in yields. This was an indication of high genetic variation between Ruiru 11 sibs. Similar results were obtained by Wamatu *et al*. [8] when evaluating related coffee clones some of which have been utilized as Ruiru 11 male parents. In Brazil, carvalho *et al*. [15] observed large variability in cherry yields among F1 generation plants obtained by crossing selected coffee trees and among bourbon coffee progenies that have been harvested for 12 to 15 consecutive years. When assessing cup quality of Ruiru 11, Ojijo [12], Agwanda *et al*. [4], Omondi [13], Kathurima *et al*. [14], also reported significant variability within the cultivar.

Table 7. Average performance of each sib per location

Kisii				Koru				Mariene			
Rank	Sib	Mean yield	Variation	Rank	Sib	Mean yield	Variation	Rank	Sib	Mean yield	Variation
1.	R11-131	14115.97	a	1	R11-80	15995.37	a	1	R11-52	5976.15	a
2.	R11-52	12981.67	ab	2	R11-107	14223.80	ab	2	R11-1	5914.93	a
3.	R11-137	12671.94	abc	3	R11-137	13115.73	abc	3	R11-11	5890.04	ab
4.	R11-117	12437.08	abcd	4	R11-117	13038.19	abcd	4	R11-135	5640.22	abc
5.	R11-6	11747.50	abcde	5	R11-91	12886.57	abcde	5	R11-3	5580.67	abcd
6.	R11-7	11587.22	abcdef	6	R11-142	12423.47	bcdef	6	R11-22	5323.28	abcde
7.	R11-1	11545.28	abcdef	7	R11-52	12032.70	bcdefg	7	R11-117	5270.78	abcde
8.	SL28(S)	11208.06	abcdefg	8	R11-100	12016.00	bcdefg	8	R11-121	5173.85	abcdef
9.	R11-111	11015.00	abcdefg	9	R11-131	11765.36	bcdefgh	9	R11-7	4987.00	abcdefg
10.	R11-42	10817.64	abcdefgh	10	R11-11	11620.89	bcdefghi	10	R11-100	4827.81	abcdefgh
11.	R11-41	10655.86	abcdefghi	11	R11-135	11494.28	bcdefghij	11	R11-131	4823.04	abcdefgh
12.	R11-121	10604.86	abcdefghi	12	R11-115	11407.59	bcdefghij	12	R11-115	4822.93	abcdefgh
13.	R11-50	10587.08	abcdefghi	13	R11-125	10888.70	bcdefghijk	13	R11-143	4767.89	abcdefgh
14.	SL28(NS)	10317.94	abcdefghij	14	R11-105	10351.90	cdefghijk	14	R11-106	4663.63	abcdefgh
15.	R11-142	10238.75	abcdefghij	15	R11-123	10295.81	cdefghijk	15	R11-6	4654.26	abcdefgh
16.	R11-22	10142.78	bcdefghij	16	R11-7	10269.14	cdefghijk	16	R11-123	4551.48	bcdefghi
17.	R11-105	9948.75	bcdefghij	17	R11-121	9951.22	cdefghijk	17	R11-137	4531.41	cdefghi
18.	R11-72	9867.50	bcdefghij	18	R11-72	9677.07	cdefghijkl	18	R11-105	4525.30	cdefghi
19.	R11-5	9686.25	bcdefghij	19	R11-22	9569.67	defghijkl	19	R11-72	4518.41	cdefghi
20.	R11-23	9507.08	bcdefghij	20	R11-6	9384.40	efghijkl	20	R11-142	4484.33	cdefghi
21.	R11-91	9202.64	bcdefghijk	21	R11-42	9350.75	fghijkl	21	R11-80	4482.04	cdefghi
22.	R11-125	9198.75	bcdefghijk	22	R11-23	9125.94	fghijkl	22	R11-125	4410.22	cdefghi
23.	R11-115	8988.75	cdefghijk	23	R11-93	9040.70	fghijkl	23	R11-112	4335.44	cdefghi
24.	R11-11	8825.42	cdefghijk	24	R11-41	8880.03	ghijkl	24	R11-23	4270.07	defghi
25.	R11-103	8661.94	defghijk	25	R11-103	8731.01	ghijkl	25	R11-93	4187.93	efghi
26.	R11-100	8387.36	efghijk	26	R11-50	8370.11	hijkl	26	R11-5	4036.63	efghi
27.	R11-123	8261.67	efghijk	27	R11-143	8122.30	ijklm	27	R11-91	4000.74	efghi
28.	R11-80	8058.47	efghijk	28	R11-112	8033.38	jklm	28	R11-111	3891.11	fghi
29.	R11-135	7995.14	efghijk	29	R11-1	7865.91	klm	29	R11-42	3823.26	ghi
30.	R11-3	7841.11	fghijk	30	R11-111	7800.23	klm	30	R11-71	3788.44	ghi

31.	R11-71	7588.33	ghijk	31	R11-3	7624.22	klm	31	R11-41	3773.70	ghi
32.	R11-93	7503.33	ghijk	32	R11-106	7574.63	klm	32	R11-107	3711.15	ghi
33.	R11-143	7012.78	hijk	33	R11-5	7456.94	klm	33	SL28(S)	3652.52	ghi
34.	R11-107	6886.67	ijk	34	R11-71	7451.14	klm	34	SL28(NS)	3585.78	hi
35.	R11-106	6461.39	jk	35	SL28(S)	6169.75	lm	35	R11-50	3583.89	hi
36.	R11-112	5573.33	k	36	SL28(NS)	4737.24	m	36	R11-103	3244.48	i

Table 8. The best 15 Ruiru 11 sibs for the Koru, Kisii and Mariene sites

Kisii				Koru						Mariene					
2010		2011		2009		2010		2011		2009		2010		2011	
Sib	Mean yields (g/tree)	Sib	Mean yields (g/tree)	Sib	Mean yields (g/tree)	Sib	Mean yields (g/tree)	Sib	Mean yields (g/tree)	Sib	Mean yields (g/tree)	Sib	Mean yields (g/tree)	Sib	Mean yields (g/tree)
R11-131	15937.8	R11-137	14248.3	R11-107	16241.7	R11-80	18608.3	R11-80	16933.3	R11-1	6443.7	R11-3	6906.3	R11-106	6122.2
R11-52	14820.0	R11-131	12294.2	R11-91	14348.6	R11-117	15797.9	R11-137	14975.0	R11-135	6223.0	R11-22	6636.7	R11-52	5596.1
R11-50	14000.0	R11-117	11584.2	R11-80	12444.4	R11-131	14638.0	R11-115	13350.0	R11-11	5909.3	R11-52	6489.0	R11-11	5539.4
R11-72	13546.7	R11-111	11443.3	R11-117	11758.3	R11-100	14445.2	R11-107	13250.0	R11-52	5843.3	R11-121	6314.3	R11-1	5477.8
R11-11	13455.6	R11-52	11143.3	R11-142	11394.4	R11-142	14242.6	R11-91	12634.0	R11-121	5793.3	R11-11	6221.3	R11-7	5388.3
R11-7	13381.1	R11-105	11093.1	R11-52	11361.1	R11-121	14187.0	R11-131	12433.3	R11-117	5671.7	R11-123	5949.3	R11-135	5271.7
R11-117	13290.0	R11-42	10867.5	R11-137	10584.7	R11-137	13787.5	R11-11	12075.0	R11-3	5617.3	R11-117	5865.7	R11-42	5062.8
R11-6	12826.7	R11-6	10668.3	R11-11	10440.3	R11-107	13179.7	R11-135	11925.0	R11-22	5374.0	R11-1	5823.3	R11-5	4867.2
R11-142	12746.7	R11-1	10585.0	R11-125	10175.0	R11-52	13095.3	R11-100	11666.7	R11-6	5211.7	R11-100	5600.0	R11-93	4804.4
R11-22	12737.8	R11-121	9856.4	R11-115	9991.7	R11-135	12874.5	R11-52	11641.7	R11-100	5187.3	R11-6	5470.0	R11-105	4643.9
R11-1	12505.6	R11-7	9793.3	R11-100	9936.1	R11-125	12866.1	R11-142	11633.3	R11-80	4928.0	R11-115	5432.0	R11-41	4569.4
R11-5	12290.0	R11-41	9163.6	R11-135	9683.3	R11-11	12347.4	R11-123	11616.7	R11-123	4857.3	R11-135	5426.0	R11-91	4555.6
R11-93	12286.7	R11-125	8541.9	R11-121	9058.3	R11-72	11988.2	R11-117	11558.3	R11-131	4844.0	R11-131	5360.7	R11-115	4514.4
R11-41	12148.1	R11-115	8384.2	R11-42	8987.5	R11-7	11910.2	R11-6	11250.0	R11-7	4722.0	R11-143	5312.0	R11-137	4415.6
R11-3	11922.2	R11-142	7730.8	R11-105	8970.8	R11-91	11677.1	R11-22	11175.0	R11-142	4720.7	R11-112	5062.7	R11-143	4400.0

Table 9. Most widely adapted sibs

	2009		2010		2011	
No.	**Sib**	**Most adapted at**	**Sib**	**Most adapted at**	**Sibs**	**Most adapted at**
1	R11-117	Koru and Mariene	R11-131	All Sites	R11-137	All Sites
2	R11-80	Koru and Mariene	R11-117	All Sites	R11-131	All Sites
3	R11-52	Koru and Mariene	R11-52	All Sites	R11-52	All Sites
4	R11-11	Koru and Mariene	R11-11	All Sites	R11-117	All Sites
5	R11-142	Koru and Mariene	R11-121	Koru and Mariene	R11-105	All Sites
6	R11-135	Koru and Mariene	R11-100	Koru and Mariene	R11-115	All Sites
7	R11-100	Koru and Mariene	R11-142	Koru and Kisii	R11-41	All Sites
8	R11-121	Koru and Mariene	R11-22	Kisii and Mariene	R11-7	Kisii and Mariene
9	R11-105	Koru and Mariene	R11-72	Koru and Kisii	R11-6	Kisii and Koru
10	R11-7	Koru and Mariene	R11-7	Koru and Kisii	R11-91	Koru and Mariene

The observed site differences indicated that the environment has a strong effect on the expression of yield potential. We attribute differences in yield to the particular edaphic and climatic conditions of each site. Wamatu *et al*. [8] and Anim-Kwapong and Adomako [1] also reported large environmental component of variance for yield in coffee. The three sites partly fulfilled the conditions of good selection and testing environment which include high genetic variances, high mean performance and high heritability [4]. On the basis of average performance, Koru could be the best selection site followed by Kisii as they consistently recorded the highest means which better portrayed the potential of the sibs. There was also greater discrimination between sibs at Koru, followed by Kisii and then Mariene. However, Mariene could also be a good selection site to discriminate the sibs under less favourable conditions while Koru could be the best selection site based on high genetic variances.

In our study, rainfall was taken as the first most important limiting factor and thus used to explain the observed site differences. A similar approach was also applied by Agwanda *et al*. [4] when selecting for cup quality. The observed seasonal (year) effects can be partly explained by varying quantity and distribution of rainfall and partly by the biennial bearing nature of coffee. All the sites recorded their best yields in 2010. This was because the sites received adequate rainfall which was well distributed in 2009/2010 production year thus resulting in high yields. In 2008/2009 production year, the Koru trial experienced reduced rainfall especially in the early stages of berry development which resulted in reduced yields. A similar effect was observed in 2008/2009 production year at Mariene and also in 2010/2011 production year at both Mariene and Kisii. Seasonal (year) x sib interactions were not significant except at Kisii and this effect was attributed to biennial bearing.

Genotype by Environment (G x E) interactions is a measure of stability and adaptability of genotypes in varying environments. In this study, significant G x E interactions was observed indicating that different Ruiru 11 sibs responded differently to different environments. When evaluating related coffee clones some of which have been utilized as Ruiru 11 male parents, Wamatu *et al*. [8] also observed significant G x E interactions. Apart from yields, significant G x E interactions has also been reported on other desirable traits in Ruiru 11 and other types of Arabica coffee. For example, on coffee quality of Ruiru 11, Agwanda *et al*. [4], Omondi [13] and Kathurima *et al.* [14] reported G x E interactions of significant magnitude. Mawardi and Hulip [16] and Agwanda *et al*. [4] observed highly significant G x E interactions in bean

characteristics of Arabica coffee. High G x E interactions for desirable traits have been reported as a major setback in achieving faster progress in selection [4]. These significant interactions might be to a large extent attributable to the low precision in balancing the growing conditions in the multi-site trials and may also be partly explained by trial characteristics.

The study further identified several sibs that are best suited for each of the three locations. These sibs should be recommended to farmers in these agronomic locations for production of high quality Ruiru 11 coffee. Besides, the study identified the most widely adapted Ruiru 11 sibs with a high yielding potential in varying climatic conditions. These included R11-52, R11-117, R11-131, R11-11, R11-105, R11-142, R11-7, R11-100 and R11-121. These consistently recorded high yields in highly varying environments. Others that consistently recorded high yields in more than one environment include R11-80, R11-135, R11-22, R11-72, R11-137, R11-115, R11-6 and R11-91. Some of these sibs including R11-52, R11-117, R11-131, R11-107, R11-121, R11-11, R11-137 and R11-22 have also been found to have high bean and cup quality with good climatic stability [17]. Kathurima *et al*. [14] also recorded high cup quality from R11-41, R11-11, R11-91 and R11-131 in a multi-site study involving ten Ruiru 11 sibs. Such sibs can be recommended to farmers and also be exploited in future breeding programmes for improvement of Ruiru 11 yield agronomic adaptability.

5. CONCLUSION

The study demonstrated the existence of a high yield variation among Ruiru 11 sibs. There is therefore high potential of intra-selection within the cultivar for yield improvement. The most widely adapted Ruiru 11 sibs as well as the best sibs for the studied coffee growing areas on the basis of cherry yield were identified. These will be recommended to farmers and also be exploited in future breeding programmes for improvement of Ruiru 11 yield agronomic adaptability. The growing environment was found to have a strong effect on the expression of yield potential as portrayed by high site variations. The occurrence of significant G x E interactions was an indication that the best improvement strategy should be a multi-site selection. Future studies should therefore include many locations with more variable climatic conditions ranging from marginal to suitable coffee growing areas. Rainfall intensity and distribution especially during the early stages of berry development was also found to be critical as the highest yields were where rainfall was adequate and well distributed.

ACKNOWLEDGEMENTS

This work was co-financed by coffee Research Foundation (cRF) and the common Fund for commodities (cFc) through the coffee Leaf Rust Project (cFc/IcO/40) supervised by International coffee Organization (IcO). Additional financial support was provided by the European Union through the Quality coffee Production and commercialization Programme (QcPcP). Thanks are due to the technical staff of cRF Breeding section who participated in this study. This work is published with the permission of the Director of Research, cRF, Kenya.

COMPETING INTERESTS

Authors declared that no competing interests exist.

References:

1. Anim-Kwapong E, Adomako B. Genetic and environmental correlations between bean yield and agronomic traits in *Coffea canephora*. J. Plant. Breed. Crop Sci. 2010;2(4):064-072.
2. Van der Vossen HAM. Coffee Breeding and Selection: Review of Achievements and Challenges. Proceedings, 19th ASIC International conference on Coffee Science, Trieste, Italy, 14-18 May 2001.
3. Omondi CO, Ayiecho PO, Mwang'ombe AW, Hindorf H. Resistance of *Coffea arabica* cv. Ruiru 11 tested with different isolates of *Colletotrichum kahawae*, the causal agent of Coffee Berry Disease. Euphytica. 2001;121:19 - 24.
4. Agwanda CO, Baradat P, Eskes A, Cilas C, Charrier A. Selection for bean and liquor qualities within related hybrids of Arabica coffee in multilocal field trials. Euphytica. 2003; 131(1):1-14.
5. S¨ondahl MR. Van der Vossen HAM, Piccin AM and Anzueto F. The Plant. In: Viani R., Editor. Espresso Coffee: the Chemistry of Quality. 2nd ed. Academic Press Ltd, London; 2005.
6. Van der Vossen HAM. The Cup Quality of Disease-Resistant cultivars of Arabica Coffee (*Coffea arabica*). Experimental Agriculture. 2009;45:323-332.
7. Gichimu BM, Omondi CO. Early performance of five newly developed lines of Arabica coffee under varying environment and spacing in Kenya. Agric. Biol. J. N. Am. 2010; 1(1):32-39.

8. Wamatu JN, Thomas E, Piepho HP. Responses of different arabica Coffee (*Coffea arabica* L.) clones to varied environmental conditions. Euphytica. 2003;129: 175-182.
9. Hindorf H, Omondi CO. A review of three major fungal diseases of *Coffea arabica* L. in the rainforests of Ethiopia and progress in breeding for resistance in Kenya. J. Adv. Res. 2010;2(2):109-120.
10. DaMatta FM, Ramalho JDC. Impacts of drought and temperature stress on coffee physiology and production: A review. Brazilian J. Plant. Physiol. 2006;18:55-81.
11. Vaast P, Van Kanten R, Siles P, Dzib B, Franck N, Harmand JM, Genard M. Shade: a key factor for Coffee sustainability and quality. Proceedings, 20th ASIC International Conference on Coffee Science, Bangalore, India, 2004.
12. Ojijo NKO. Comparative evaluation of cup quality of Kenya Arabica cultivars. CRF Ann. Rep., 1993.
13. Omondi CO. Coffee quality assessment: the case of two Kenyan cultivars, Ruiru 11 and SL28. Proceedings, 22nd ASIC International conference on coffee Science, campinas, Brasil, Lausanne, Switzerland, 2008.
14. Kathurima CW, Kenji GM, Muhoho SM, Boulanger R, Davrieux F. Discrimination of *Coffea arabica* Hybrids of the Composite Cultivar Ruiru 11 by Sensorial Evaluation and Biochemical characterization. Adv. J. Food Sci. Technol. 2010; 2(3):148-154.
15. Carvalho A, Monaco LC, Antunes FH. Coffee Breeding XV: Variability Observed in coffee Progenies. Bragantia. 1959;18:373-386.
16. Mawardi S, Hulip R. Genotype by Environment Interaction of Bean Characteristics in Arabica Coffee. Proceedings, 16th ASIc International Conference on coffee Science, Kyoto, Japan, 1995.
17. Gichimu BM, Gichuru EK, Mamati GE, Nyende AB. Selection within *Coffee arabica* Cv. Ruiru 11 for high cup quality. African Journal of Food Science. 2012;6(18):456-464.

Microrganisms in Plant Protection

Zlata Holečková, Martin Kulhánek, Jiří Balík,
Department of Agro-Environmental Chemistry and Plant Nutrition,
Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Kamýcká 129, Prague 165 21, Czech Republic

Abstract: Biocontrol agents (BA) are products which contain live microorganisms or their spores as the active substances. Their application could be one possible way that should: i) improve resistance to diseases and pathogens, ii) growth of roots and aboveground biomass and iii) nutrient uptake by plants. The agent's function is based on many different mechanisms. Experiments with BA were carried out under different conditions (fields, pots, greenhouses), with different varieties of tested plants as well as using different application strategies (seed incrustation, application on the leaves and others). Therefore, many different results were published in scientific journals. The aim of this study is to review published results focused on the usage of BA within a plant protection. It might be useful mainly for the ecological farming and healthy food production. This review summarizes the most recent knowledge in this scientific field.

Keywords: biocontrol agents, microoragnisms, plant protection, organic farming.

1. Introduction

The enormous growth of Earth's population requires to provide adequate food resources and to find out alternative strategies for a sufficient crop production. One of the crop production's crucial factors is the achievement of effective plant protection. However the plant protection agents can often expose to danger the environment and human health by food chain's pollution with different chemical compounds. The nowadays problem is limited areas of productive agricultural land and an increasing occurrence of plant diseases and pests within the crop production also. It is also necessary to look for other approaches and strategies (Neumann 2012; Hogenhout *et al*. 2009). The use of pesticides is a traditional method but it causes negative side impacts on the environment by progressive resistance of the pathogens to active

substance. This proccess incites a futher research to find out more alternative strategies that would eliminate pathogens. Recently, there has been an effort made towards development of harmless products that are based on microorganisms and their influences (bacteria, fungi) and active natural substances (extracts from soil, compost or seaweeds, microbial residues, plant extracts). The proposal and design of new strategies requires better public discourse about the consequences of the farming impacts on the environment resulting in a better understanding of the soil-plant relationship. It is expected that the development of new strategies will have a significant economical and environmental impact, particularly for future generations (Roy 2017; Withers *et al*. 2014).

2. Microorganisms in plant protection

This chapter lists a selection of microorganisms that are used in the plant protection and crop production. In current time the methods of biological control with the use of microorganisms attract attention of research as a promising alternative to chemical control. Biological protection with the use of antagonistic microorganisms has proved to be a viable alternative. Development of the Biocontrol agents (BA) increases due to the potential use of these substances in organic farming (El-Gremi *et al*. 2017). The current focus on a plant disease management has been shifted from chemical pesticides to more ecofriendly biopesticides in order to reduce an environmental pollution and to minimize the risk of development of pesticide-resistant strains of plant pathogens. Many bacteria have the potential to reduce crop losses through biocontrol mechanism (Vallabhaneni 2016). BAs are divided into two main groups, according to which type of microorganism contain: fungal strains (*Trichoderma*, *Penicillium* and *Sebacinales)* and bacterial strains (*Bacillus* and *Pseudomonas)* (Neumann 2012). BA are highlighted here with more evidences through field or pot experiments and greenhouse studies. The experiments and studies include a broad spectrum of crops such as corn, rice, soybeans, tomatoes, cotton, energy cane, oil palms, millets, oilseeds, banana, coconut, lime, coffee, tea, rubber, flower, spices, herbs, lawns, ornaments, trees, biofuel and forage grass (Janarthanam 2013), sugar beet, tobacco, cucumber, watermelon, muskmelon, cucumber, tropical crops (Choudhary & Johri 2009).

2.1. Fungal BioControl

As mentioned before BA can be divided into two main groups - fungal and bacterial. Several fungal representatives have been selected and described further in this section. At the end of this section (2.1.) in Table 1. There are selected bacteria and their impact on plant protection.

2.1.1. *Trichoderma* ssp.

Strains of the genus *Trichoderma* spp. are wild filamentous fungi occurring in the most of soils and different habitats. *Trichoderma* is a fungal genus that includes species that are currently being used as BA or as biofertilizer (Dominguez *et al*. 2016; Hermosa *et al*. 2012). *Trichoderma* is known for producing several enzymes and antibiotics. The varietes of physiological, antifungal and insecticidal effects are attributed to this species. It operates against a broad spectrum of plant pathogens. These fungi increase the growth of plants' above ground biomass as well as the development of the root system (El-Gremi *et al*. 2017; Galletti *et al*. 2015; Ferrigo *et al*. 2014; Do Vale *et al*. 2012; Raja 2007). It has also been observed that selected *Trichoderma* strains can improve plant nutrient uptake (Yedidia *et al*. 2001) which has indirect influence on the plant health as well. The above mentioned increase of growth occurs due to its strong anti-pathogenic activity, biosynthesis of hormones, improving nutrient uptake from the soil, root development, or increasing the rate of carbohydrates metabolism and photosynthesis as well (El-Gremi *et al*. 2017). The main hydrolytic enzymes secreted by the *Trichoderma* strains are proteases, chitinases and endochitinases. The glycoside hydrolase family, including chitinases, and other enzymes are representing 51% of the total secretome (totality of secreted organic molecules and inorganic elements by biological cells, tissues, organs, and organisms). Few representatives are classified in the protease family (8.9%), others (17.6%) are mostly intracellular proteins. The endochitinases are proteins involved in chitin degradation. The mechanism of chitinases action can be divided into two major groups: endochitinases and exochitinases. In general, endochitinases belongs into chitinases that cleave chitin randomly inside the chain. Exochitinases are subclassified into chitobiosidases and chitobiases. All of these enzymes act in a mutual, synergistic on chitin and on cell wall degradation (Do Vale *et al*. 2012; Duo-Chuan 2006). Chitinases are produced e.g. by bacteria, algae, fungi, plants, insects, nematodes, molluscides, vertebrates, including human and also certain viruses (Gooday 1999). *Trichoderma* is also the main component in several commercially

produced biofungicides. The biofungicide is intended to apply in a foliar application, seed protection and into a soil. The soil application is used for the treatment and suppression of various diseases caused by pathogens such as *Botrytis*, *Fusarium*, and *Penicillium* spp. This group of fungicides is used against pests also. It improves a plants' health and environmental monitoring (Gomes *et al*. 2015; Samuels *et al*. 2014). This filamentous fungus increases the resistance of plants against biotic and abiotic stresses and therefore indirectly increases e.g. nitrogen use efficiency. The plants' deep and developed roots allow to withstand drought that was confirmed at e.g. for maize and ornamentals. The above mentioned characteristics are applied as a seed treatment against various pathogens and mycotoxins (Galletti *et al*. 2015; Ferrigo *et al*. 2014; Raja 2007).

2.1.2. *Trichoderma harzianum*

Trichoderma harzianum is wild filamentous fungus; it occurs in soil. *Trichoderma* belongs to the fungi that includes species which are currently used as biological control agents (Dominguez *et al*. 2016; Hermosa *et al*. 2012). Mycoparasitic fungi, such as *T. harzianum*, produce an arsenal of chitin-degrading enzymes to hydrolyze the host cell wall and can also generate high contents of cellulases under appropriate culture conditions (Do Vale *et al*. 2012). Strain T22 was also reported as one enabling to improve the efficiency of photosynthesis and growth of tomatoes (El-Gremi *et al*. 2017). As a notable BA, *Trichoderma harzianum* can antagonize a diverse array of phytopathogenic fungi, including *Botrytis cinerea*, *Rhizoctonia solani* and *Fusarium oxysporum*. Elucidating the biocontrol mechanism of *T. harzianum* in response to the pathogens enables to be exploited in the control of plant diseases (Yang *et al*. 2009). Vitti *et al*. (2016) researched the influence of *T. harzianum* (strain T-22) application under laboratory conditions on the occurrence of *Cucumber mosaic virus* in tomato. And the results prove that early inoculation of this strain is able to induce a defense response. The reduction of mosaic occurrence affects enzyme (dismutase and catalase) and phytohormones (ethylene, abscisic acid, salicylic acid, and jasmonic acid) production. As well Kerroum *et al*. (2015) carried out a study with tomatoes. This study involved pot experiments and confirmed the antagonistic effect of *T. harzianum* against *F. oxysporum* f. sp. radicis-lycopersici that causes root crown rot of tomatoes. Altinok & Erdogan (2015) conducted laboratory and pot trials with *T. harzianum*, strains T16 and T23. These strains

significantly inhibited growth of the pathogenic fungus *Fusarium oxysporum*. Ahmad *et al*. (2015) realized a pot trial with *Brassica juncea* testing the influence of soil salinity on brassica after application of *T. harzianum*. Soil salinity stress caused that the plants were smaller with slower growth, changes of plants' physical and biochemical properties and decrease in the biomass yield was found out. Results showed that the seedling plants treated with *T. harzianum* were significantly more resistant to stress conditions caused by salinity in comparison with untreated plants.

2.1.3. *Pythium oligandrum*

The biocontrol agent *Pythium oligandrum*, a soil-inhabiting oomycete, colonizes the rhizosphere of many crop species and it is responsible for the reduction of diseases caused by a number of soil-borne fungal pathogens (Al-Rawahi & Hancock, 1997). *P. oligandrum* promotes plant growth, as a result of interactions' complex, which includes an indirect effect through control of pathogens in the rhizosphere and/or a direct one mediated by plant-induced resistence. The increased plant growth is caused by the interaction between *P. oligandrum* and roots. It is proved that during this interaction the fungus produces auxin compound - tryptamine (Le Floch *et al*. 2003). This fungus produces an elicitor that activates plant defence reactions (Takenaka *et al*. 2003). Therefore, it is postulated that *P. oligandrum* is able to reduce disease through a plant-mediated resistance mechanism i.e. referred as induced resistance. Hase *et al*. (2008) proved that treatment of tomato roots (*Solanum lycopersicum*) with *P. oligandrum* induces an increased amount of ethylene, reducing the severity of bacterial disease caused by *Ralstonia solanacearum*. Hase *et al*. (2008), Glazebrook (2005) next published that plant growth regulators play important role in the plant defence responses to pathogens i.e. jasmonic acid and salicylic acid. Therefore Hase *et al*. (2008) conducted study and laboratory experiment with the involvement of jasmonic acid and salicylic acid. These acids are dependent on signal transduction pathways in resistance to *R. solanacearum.* The experiments were carried out with tomato roots treated with *P. oligandrum* at two tomato cultivars. The first used tomato cultivar was Micro-Tom, i.e. wild-type and the second one was Moneymaker, the type that does not accumulate a salicylic acid. The occurrence of *R. solanacearum* was suppressed in the both tomatoes cultivars after application of *P. oligandrum*. The enhanced resistance was induced at 5 days after treatment. It seems be proved that *P. oligandrum* generally induces resistance to *R. solanacearum* in tomatoes. Takenaka *et al*. (2003) published

conclusions that the application of *P. oligandrum* enhances resistance to root-rot-causing agents *Aphanomyces cochlioides* and *Rhizoctonia solani* in sugar beet. Holmes *et al*. (1998) conducted study and pot experiments, where sugar beet seeds were treated with *P. oligandrum* against damping-off of sugar beet. The results indicated that used of *P. oligandrum* significantly reduced a disease caused by *P. ultimum* but at pH values between 7.0 and 7.5 only.

Table 1. Plant protection promoting fungi as BA against various plant diseases.

Fungi	Experimental conditions	Disease	References
Trichoderma ssp.	Laboratory	Diseases caused by pathogens such as *Botrytis*, *Fusarium* or *Penicillium* spp	Samuels *et al*. (2014)
Trichoderma harzianum	Laboratory	*Cucumber mosaic virus* in tomato	Vitti *et al*. (2016)
	Laboratory and pot experiments	*Fusarium oxysporum*	Altinok & Erdogan (2015)
	Laboratory	*Botrytis cinerea*, *Rhizoctonia solani*, *Fusarium oxysporum*	Yang *et al*. (2009)
Pythium oligandrum	Laboratory	*Ralstonia solanacearum* in tomato	Hase *et al*. (2008)
	Laboratory	Root-rot caused by *Rhizoctonia solani* and *Aphanomyces cochlioides* in sugar beet	Takenaka *et al*. (2003)
	Laboratory	Damping-off caused by *P. ultimum* in sugar beet	Holmes *et al*. (1998)

2.2. Bacterial BioControl

Several promising bacterial representatives have been selected and described further in this section. And also at the end of this section (2.2.) in Table 2. There are selected fungi and their impact on plant protection.

2.2.1. *Pseudomonas* spp.

Pseudomonas sp. is ubiquitous microorganism in agricultural soils, well adapted to grow in the rhizosphere. *Pseudomonas* is well suited as biocontrol and growth-promoting agents (Vallabhaneni 2016). They are often used as BA because they display a broad range of mechanisms to control diseases (Arseneault *et al*. 2016). The inoculation of seeds or roots with fluorescent *Pseudomonas* has been a widely used in practice to increase plant vigor and productivity in tobacco. The *Pseudomonas* has a beneficial effect against a wide range of root phytopathogens, e.g. *Rhizoctonia solani*, *Pythium aphanidermatum* and *Fusarium oxysporum* belong to them. The mechanisms suggested to achieve such inhibition include: production of antibiotics, iron-chelating compounds, hydrolytic enzymes and biosurfactants, competition for favourable nutritional sites or as mycorrhiza helping bacteria (Vallabhaneni 2016). Proteins produced by certain species of *Pseudomonas* increase resistance to *Xanthomonas oryzae var. oryzae* in rice and to Tobacco *Mosaic Virus*. These proteins cause hypersensitivity reactions, higher expression levels of genes related to defense against pathogens and promoting of growth. Therefore they have a potential for development as protein-type BA. When they are applied to tobacco or rice plants then proteins derived from harpin are able to induce resistance to Tobacco *Mosaic Virus* and to *leaf blight disease* in rice with varying degrees. The functional peptide fragments, which were identified there, may result in the effective control of diseases as well as increase a productivity of crops. The condition is that they are developed into a form of microbial pesticides for agricultural applications. This could be an environmentally friendly alternative to some of the chemical pesticides currently in use (Wu *et al*. 2017). The appearence of fluorescent *Pseudomonas* in the rhizosphere microflora depends on characteristics such as soil texture, rhizosphere pH, soil matrix potential, soil water flow, temperature, plant species (Vallabhaneni 2016). Mikicinski *et al*. (2016) used the isolate of *Pseudomonas graminis* (strain 49M) under laboratory and greenhouse conditions but in an orchard also. The aim was to protect apple blossoms and apple terminal shoots. This study identified

Pseudomonas graminis, strain 49M's ability to suppress the fire blight in an immature pear and apple flower and its fitness on flowers in an orchard. The strain 49M is highly protective against fire blight on different plant tissues (up to 73.3% on flowers and 86.2% on terminal shoots, compared to the controls) during the entire bloom period in an orchard. This is the first report showing that *Pseudomonas graminis* strain 49M is a prospective candidate for a future development as the biopesticide that will be used against the fire blight. Vallabhaneni (2016) conducted a study and his results suggest that the *Pseudomonas fluorescens* utilization to control *Rhizoctonia solani* is the promising strategy of disease management. This statement is supported by the fact that all tested *P. fluorescens* isolates reduced the disease severity in tobacco seed beds. Such reduction was evident due to the decrease of affected seedlings number, decrease in the number of sclerotium formation and symptoms' disappearance of severe disease on seed beds. Nine isolates of *P. fluorescens* were selected and evaluated in terms of their antagonistic activity against *R. solani* under vitro conditions. Knot *et al*. (2013) reported that the *Pseudomonas* increases germination of *Poa pratensis* seeds under laboratory conditions, especially of 2-4 years old seeds. Yusran *et al*. (2009) reported the application of *Pseudomonas* and *Bacillus amyloliquefaciens* (individually or in a combination) into soil caused that the state of tomato roots improved in a pot trial. They were healthier and significantly higher colonized by arbuscular mycorrhizal fungi.

2.2.2. *Bacillus amyloliquefaciens*

Bacillus amyloliquefaciens is gram-positive, aerobic and endospore-forming bacteria. They are often used as is commercial chemicals in industry (Zhang *et al*. 2016; Chowdhury *et al*. 2015; Kröber *et al*. 2014). They are one of the beneficial agents used for the plant growth promotion and the suppression of soil-borne diseases in agriculture as well. *B. amyloliquefaciens* produces many metabolites such as are e.g. enzymes (chitinase, peroxidases and proteases), casein, elastin, gelatin, starch, nitrites, esculin and arbutin, phosphatases, adenine, cellulose, guanine, hypoxanthine, pectin, testosterone, tyrosine, many types of antibiotics (e.g. bacillomycins, fengycin, difficidin) and other substances (El-Gremi *et al*. 2017; Chowdhury *et al*. 2015; Lagerlöf *et al*. 2015; He *et al*. 2013; Priest *et al*. 1987). Production of antibiotic that inhibite a growth of antifungal pathogens El-Gremi *et al*. (2017), as well as antibacterial and antinematocidal effects for plants and also the ability to produce a wide variety of secondary metabolites, which aims to suppress

competing bacteria, fungi, viruses or nematodes in the rhizosphere of plants. Lagerlöf *et al*. (2015), Kröber *et al*. (2014), He *et al*. (2013), Chen *et al*. (2009) and Koumoutsi *et al*. (2004) declare that the bacteria reduce the influence of plant abiotic stress conditions such as drought, salinity or lack of nutrients. Proteins secreted by *Bacillus amyloliquefaciens* FZB42 protect plants against disease by eliciting innate immunity (Kierul *et al*. 2015). He *et al*. (2013) reported that *Bacillus amyloliquefaciens* belongs to beneficial soil microorganisms, which colonize the plant roots and stimulate the growth of its host. The use of these bacteria offers great potential to increase the yield and reduce the plant disease caused by numerous microorganisms. Kim *et al*. (2015) reported that these bacteria attract attention by their increasing importance in the last time, particularly by their fungicidal effect. PT14 strain proved its property to be a broad spectrum of antifungal activity against *Fusarium solani* and *Fusarium oxysporum*. Nevertheless this strain was not active against bacterial strains. Furthermore Lagerlöf *et al*. (2015), Talboys *et al*. (2014), Fan *et al*. (2012), Burkett-Cadena *et al*. (2008) reported that *B. amyloliquefaciens* promotes a plant growth that is based primarily on the production of secondary metabolites suppressing competing microbial pathogens and diseases occurring in the rhizosphere of plants. It encourages a root development and improves seed germination as well. Some plants, e.g. maize (Baudoin *et al*. 2003), soybean (Yang *et al*. 2012), lupin (Egle *et al*. 2003), rice (Aulakh *et al*. 2001) produce a lactic acid in root exudates. This acid with the other root exudates becomes a energy source for *B. amyloliquefaciens*. Chowdhury *et al*. (2015) carried out experiments which demonstrated that FZB42 strain is able to reduce the disease severity of bottom root caused by soil-borne pathogen *Rhizoctonia solani* on lettuce. Kröber *et al*. (2014) reported results of their pot and field experiments which demonstrated that the strain FZB42 is able to effectively colonize the rhizosphere of lettuce (*Lactuca sativa*) and promotes a significant suppression of bottom rot disease caused by *Rhizoctonia solani*.

2.2.3. *Bacillus subtilis*

Bacillus subtilis is a ubiquitous gram-positive bacteria commonly found in water, soil, air and decomposition of plant residues. However, the primary presence of these bacteria was found in soil (Tam *et al*. 2006; Kunst *et al*. 1997). The bacteria produce endospores that allow it to endure and overcome some extreme temperatures and dry periods. *B. subtilis* produce a series of proteases and other enzymes. This bacterium is considered a benign organism, as it has not properties

that cause disease and is not pathogenic or toxic for humans, animals or plants (Kunst *et al*. 1997). Many years ago Korzybski *et al*. (1978) and Katz and Demain (1977), published that the *B. subtilis* produces a wide spectrum of antibacterial and antifungal compounds and furthermore also antibiotics such as difficidin and oxydifficidin that are effective against the broad range of aerobic and anaerobic bacteria.

These bacteria are widely used in agriculture to promote plant growth. They may be taken into account as a promising approach how to protect plants against diseases (Ma *et al*. 2015). Orio *et al*. (2016) reported that the application of *B. subtilis* had a strong effect against fungal pathogen that causes pink disease of roots (*Setophoma terrestris*) at onions.

Table 2. Plant protection promoting bacteria as BA against various plant diseases.

Bacteria	Experimental sites	Disease	References
Pseudomonas spp.	Laboratory condition	*Xanthomonas oryzae* in rice and *Tobacco Mosaic Virus* in tobacco	Wu et al. (2017)
	Greenhouse condition, pot experiment	Fire blight of pear and apple	Mikicinski *et al*. (2016)
	Laboratory condition	Diseases caused by *Rhizoctonia solani* in tobacco	Vallabhaneni (2016)
	Laboratory condition, pot experiment	Meloidogyne javanica in tomato	Siddiqui & Shaukat (2004)
	Laboratory condition	Disease of bottom root caused by *Rhizoctonia solani* on lettuce	Chowdhury *et al*. (2015)
Bacillus amyloliquefaciens	Greenhouse and field conditions	Rot disease caused by *Rhizoctonia solani* on lettuce	Kröber *et al*. (2014)
	Laboratory condition	*Erwinia carotovora* in *Arabidopsis*	Ryu et al. (2004)
	Field condition	Tomato mottle virus	Murphy et al. (2000)

Bacillus subtilis	Laboratory condition	Pink disease of roots at onions	Orio et al. (2016)
	Laboratory condition	*Erwinia carotovora* in *Arabidopsis*	Ryu et al. (2004)
	Greenhouse and field conditions	Downy mildew in pearl millet	Raj et al. (2003)

3. Conclusions

The current research is focused on the partial replacement of chemicals used in agriculture to protect plants against pests and diseases. Within this context it is examined an usage of BAs, where e.g. the interactions between organisms leads to damage to other plant pathogen organism.

Many studies reported positive influence of fungal as well as bacterial BAs on plant health and growth, respectively. These studies were mostly realized in laboratory conditions, where many negative factors can be excluded. Therefore, before transferring these technologies in agronomic practice, pot and especially field trials are strongly needed to confirm the laboratory results in field conditions. Nowadays, there are only several studies that confirmed the positive influence of BAs in the pots or fields. Generally, BAs presents the promising way in plant protection, which required further testing.

Acknowledgements

This research was financially supported by the Resource Preservation by Application of bioeffectors in European Crop Production nr. 7. RP 312117.

This manuscript was proof readed by: Proof-Reading-Service.com.

References:

1. Ahmad P., Hashem A., Abd-Allah E.F., Alqarawi A.A., John R., Egamberdieva D., Gucel S. (2015): Role of Trichoderma harzianum in mitigating NaCl stress in Indian mustard (Brassica juncea L) through antioxidative defense system. Frontiers in Plant Science. 6: 868. DOI: 10.3389/fpls.2015.00868.
2. Altinok H.H., Erdogan O. (2015): Determination of the In vitro Effect of Trichoderma harzianum on Phytopathogenic Strains of Fusarium oxysporum.

Notulae Botanicae Horti Agrobotanici Cluj-Napoca. 43 (2). 494-500. DOI:10. 15835/nbha4329788.

3. Al-Rawahi A.K., Hancock J.G. (1997): Rhizosphere competence of Pythium oligandrum. Phytopathology. 87 (9): 951-959. DOI: 10.1094/PHYTO. 1997. 87.9.951.
4. Arseneault T., Goyer C., Filion M. (2016): Biocontrol of Potato Common Scab is Associated with High Pseudomonas fluorescens LBUM223 Populations and Phenazine-1-Carboxylic Acid Biosynthetic Transcript Accumulation in the Potato Geocaulosphere. PHYTOPATHOLOGY. 106 (9): 963-970. DOI: 10.1094/PHYTO -01-16-0019-R.
5. Aulakh M.S., Wassmann R., Bueno C., Kreuzwieser J., Rennenberg H. (2001): Characterization of root exudates at different growth stages of ten rice (Oryza sativa L.) cultivars. PLANT BIOLOGY. 3 (2): 139-148. DOI: 10.1055/s-2001-12905.
6. Baudoin E., Benizri E., Guckert A. (2003): Impact of artificial root exudates on the bacterial community structure in bulk soil and maize rhizosphere. SOIL BIOLOGY & BIOCHEMISTRY. 35 (9): 1183-1192. https://doi.org/10.1016/S0038-0717(03) 00179-2.
7. Burkett-Cadena M., Kokalis-Burelle N., Lawrence K.S., van Santen E., Kloepper J.W. (2008): Suppressiveness of root-knot nematodes mediated by rhizobacteria. Biological Control. 47 (1): 55-59. DOI: 10.1016/j.biocontrol.2008.07.008.
8. Chen X.H., Borriss R., Scholz R., Schneider K., Vater J., Süssmuth R., Piel J., Koumoutsi A. (2009): Genome analysis of Bacillus amyloliquefaciens FZB42 reveals its potential for biocontrol of plant pathogens. Journal of Biotechnology. 140 (1-2). 27-37. DOI: 10.1016/j.jbiotec.2008.10.011.
9. Choudhary D.K., Johri, B.N. (2009): Interactions of Bacillus spp. and plants - With special reference to induced systemic resistance (ISR). Microbiological Research. 164 (5): 493-513. DOI: 10.1016/j.micres.2008.08.007.
10. Chowdhury S.P., Hartmann A., Gao X.W., Borriss R. (2015): Biocontrol mechanism by root-associated Bacillus amyloliquefaciens FZB42. FRONTIERS IN MICROBIOLOGY. 6. 780. DOI: 10.3389/fmicb.2015.00780.
11. Do Vale L.H.F., Gómez-Mendoza D.P., Kim M.S., Pandey A., Ricart C.A.O., Filho E.X. F., Sousa M.V. (2012): Secretome analysis of the fungus Trichoderma

harzianum grown on cellulose. Proteomics. 12 (17): 2716-2728. DOI: 10.1002/pmic.201200063.

12. Dominguez S., Rubio M.B., Cardoza R.E., Gutierrez S., Nicolas C., Bettiol W., Hermosa R., Monte E. (2016): Nitrogen Metabolism and Growth Enhancement in Tomato Plants Challenged with Trichoderma harzianum Expressing the Aspergillus nidulans Acetamidase amdS Gene. Frontiers in Microbiology. 7:1182. DOI: 10.3389/fmicb.2016.01182.
13. Duo-Chuan L. (2006): Review of fungal chitinases. MYCOPATHOLOGIA. 161 (6): 345-360. DOI: 10.1007/s11046-006-0024-y.
14. Egle K., Romer W., Keller H. (2003): Exudation of low molecular weight organic acids by Lupinus albus L., Lupinus angustifolius L. and Lupinus luteus L. as affected by phosphorus supply. Agronomie. 23 (5-6): 511-518. DOI: 10.1051/agro:2003025.
15. El-Gremi S.M., Draz I.S., Youssef W.A.E. (2017): Biological control of pathogens associated with kernel black point disease of wheat. Crop Protection. 91: 13-19. DOI: 10.1016/j.cropro.2016.08.034.
16. Fan B., Carvalhais C.L., Becker A., Fedoseyenko D., von Wirén N., Borriss R. (2012): Transcriptomic profiling of Bacillus amyloliquefaciens FZB42 in response to maize root exudates. BMC Microbilogy. 12: 116. DOI: 10.1186/1471-2180-12-116.
17. Ferrigo D., Raiola A., Rasera R., Causin R. (2014): Trichoderma harzianum seed treatment controls Fusarium verticillioides colonization and fumonisin contamination in maize under field conditions. Crop Protection. 65: 51-56. DOI: 10.1016/j.cropro.2014.06.018.
18. Galletti S., Fornasier F., Cianchetta S., Lazzeri L. (2015): Soil incorporation of brassica materials and seed treatment with Trichoderma harzianum: Effects on melon growth and soil microbial activity. Industrial Crops and Products. 75 Part A: 73-78. DOI: 10.1016/j.indcrop.2015.04.030.
19. Glazebrook J. (2005): Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annual Review of Phytopathology. 43: 205-27. DOI: 10.1146/annurev.phyto.43.040204.135923.
20. Gooday G.W. (1999): Aggresive and defensive roles for chitinases. In: Jollès P., Muzzarelli R.A.A. (eds.). Chitin and Chitinases. Birkhäuser Verlag. Basel. 157-165. DOI: 10.1007/978-3-0348-8757-1_11.

21. Gomes E.V., Costa M.D., de Paula R.G., de Azevedo R.R., da Silva F.L., Noronha E.F., Ulhoa C.J., Monteiro V.N., Cardoza R.E., Gutierrez S. (2015): The Cerato-Platanin protein Epl-1 from Trichoderma harzianum is involved in mycoparasitism, plant resistance induction and self cell wall protection. Scientific Reports. 5: 1-13. DOI: 10.1038/srep17998.
22. Hase S., Takahashi S., Takenaka S., Nakaho K., Arie T., Seo S., Ohashi Y., Takahashi H. (2008): Involvement of jasmonic acid signalling in bacterial wilt disease resistance induced by biocontrol agent Pythium oligandrum in tomato. PLANT PATHOLOGY. 57 (5): 870-876. DOI: 10.1111/j.1365-3059.2008.01858.x.
23. He P., Hao K., Blom J., Rückert Ch., Vater J., Mao Z.C., Wu Y.X., Hou M.S., He P.B., He Y.Q. et al. (2013): Genome sequence of the plant growth promoting strain Bacillus amyloliquefaciens subsp. plantarum B9601-Y2 and expression of mersacidin and other secondary metabolites. Journal of Biotechnology. 164: 281-291. DOI: 10.1016/j.jbiotec.2012.12.014.
24. Hermosa R., Viterbo A., Chet I., Monte E. (2012): Plant-beneficial effects of Trichoderma and of its genes. Microbiology. 158: 17-25. DOI: 10.1099/mic.0.052274-0.
25. Hogenhout S.A., Van der Hoorn R.A.L., Terauchi R., Kamoun, S. (2009): Emerging Concepts in Effector Biology of Plant-Associated Organisms. Molecular Plant-Microbe Interaction. 22 (2): 115-122. DOI: 10.1094/MPMI-22-2-0115.
26. Holmes K.A., Nayagam S.D., Craig G.D. (1998): Factors affecting the control of Pythium ultimum damping-off of sugar beet by Pythium oligandrum. Plant Pathology. 47: 516-522. DOI: 10.1046/j.1365-3059.1998.00253.x.
27. Janarthanam L. (2013): Bioprotectant with multifunctional microorganisms: A new dimension in plant protection. Journal of Biopesticides. 6 (2): 219-204.
28. Katz E., Demain A.C. (1977): Peptide antibiotics of Bacillus: Chemistry, biogenesis and possible functions. Bacteriological Reviews. 41 (2): 449-474.
29. Kerroum F., Noureddine K., Eddine H.J., Mebrouk K. (2015): Biological Control of Fusarium Crown and Root Rot Disease of Tomato by Trichoderma Harzianum in the West of Algeria. International Journal of Science and Nature. 6 (2): 141-146.
30. Kierul K., Voigt B., Albrecht D., Chen X.H., Carvalhais L.C., Borriss R. (2015): Influence of root exudates on the extracellular proteome of the plant growth-promoting bacterium Bacillus amyloliquefaciens FZB42. MICROBIOLOGY-SGM. 161: 131-147. DOI: 10.1099/mic.0.083576-0.

31. Kim Y.G., Kang H.K., Kwon K.D., Seo C.H., Lee H.B., Park Y. (2015): Antagonistic Activities of Novel Peptides from Bacillus amyloliquefaciens PT14 against Fusarium solani and Fusarium oxysporum. JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY. 63 (48): 10380-10387. DOI: 10.1021/acs.jafc.5b04068.
32. Knot P., Pancikova J., Raus J., Sochorec M. (2013): The Effect of Proradix and Headstart Treatment Methods on Germination of Poa pratensis Caryopsis. In: Book of Abstracts Seed and Seedlings XI. Scientific and Technical Seminar. Czech University Life Sciences Prague. February 7, 2013.
33. Korzybski T., Kowszyk-Gindifer Z., Kurylowicz W. (1978): Antibiotics isolated from the genus Bacillus (Bacillaceae) In: Antibiotics - Origin. Nature and Properties. Vol. III. American Society of Microbiolog. Washington. DC. 1529-1661.
34. Koumoutsi A., Chen X.H., Henne A., Liesegang H., Hitzeroth G., Franke P., Vater J., Borriss R. (2004): Structural and Functional Characterization of Gene Clusters Directing Nonribosomal Synthesis of Bioactive Cyclic Lipopeptides in Bacillus amyloliquefaciens Strain FZB42. Journal of Bacteriology. 186 (4). 1084-1096. DOI: 10.1128/JB.186.4.1084-1096.2004.
35. Kröber M., Wibberg D., Grosch R., Eikmeyer F., Verwaaijen B., Chowdhury S.P., Hartmann A., Puhler A., Schluter A. (2014): Effect of the strain Bacillus amyloliquefaciens FZB42 on the microbial community in the rhizosphere of lettuce under field conditions analyzed by whole metagenome sequencing. Front. Microbiol. 5:252. DOI: 10.3389/fmicb.2014.00252.
36. Kunst F., Ogasawara N., Moszer I., Albertini A.M., Alloni G., Azevedo V., Bertero M.G., Bessieres P., Bolotin A., Borchert S. et al. (1997): The complete genome sequence of the Gram-positive bacterium Bacillus subtilis. Nature. 390 (6657): 249-256. DOI: 10.1038/36786.
37. Lagerlöf J., Ayuke F., Bejai S., Jorge G., Lagerqvist E., Meijer J., JohnMuturi J., Söderlund S. (2015): Potential side effects of biocontrol and plant-growth promoting Bacillus amyloliquefaciens bacteria on earthworms. Applied Soil Ecology. 96: 159-164. DOI: 10.1016/j.apsoil.2015.08.014.
38. Le Floch G., Rey P., Benizri E., Benhamou N., Tirilly Y. (2003): Impact of auxin-compounds produced by the antagonistic fungus Pythium oligandrum or the

minor pathogen Pythium group F on plant growth. PLANT AND SOIL. 257 (2): 459-470. DOI: 10.1023/A:1027330024834.

39. Ma X., Wang X., Cheng J., Nie X., Yu X., Zhao Y., Wang W. (2015): Microencapsulation of Bacillus subtilis B99-2 and its biocontrol efficiency against Rhizoctonia solani in tomato. Biological Control. 90: 34-41. DOI: 10.1016/j.biocontrol.2015.05.013.
40. Mikicinski A., Sobiczewski P., Pulawska J., Maciorowski R. (2016): Control of fire blight (Erwinia amylovora) by a novel strain 49M of Pseudomonas graminis from the phyllosphere of apple (Malus spp.). EUROPEAN JOURNAL OF PLANT PATHOLOGY. 145 (2): 265-276. DOI: 10.1007/s10658-015-0837-y.
41. Murphy J.F., Zehnder G.W., Schuster D.J., Sikora E.J., Polston J.E., Kloepper J.W. (2000): Plant growth-promoting rhizobacterial mediated protection in tomato against tomato mottle virus. Plant Disease. 84 (7): 779-784. DOI: 10.1094/PDIS.2000.84.7.779.
42. Neumann G. (2012): EU-funded research collaboration on use of bio-effectors in agriculture launched. Press Release. Germany: University of Hohenheim. Available at http://www.biofector.info/about-biofector.html (accessed on Sep 15, 2014).
43. Orio A.G.A., Brucher E., Ducasse D.A. (2016): A strain of Bacillus subtilis subsp subtilis shows a specific antagonistic activity against the soil-borne pathogen of onion Setophoma terrestris. European Journal of Plant Pathology. 144 (1): 217-223. DOI: 10.1007/s10658-015-0762-0.
44. Priest F.G., Goodfellow M., Shute L.A., Berkeley R.C.W. (1987): Bacillus amyloliquefaciens sp. nov., nom. rev. International Journal of Systematic and Evolutionary Microbiology. 37 (1): 69-71.
45. Raj S.N., Deepak S.A., Basavaraju P., Shetty H.S., Reddy M.S., Kloepper J.W. (2003): Comparative performance of formulations of plant plant promoting rhizobacteria in growth promotion and suppression of downy mildew in perl millet. Crop Protection. 22 (4): 579-588. https://doi.org/10.1016/S0261-2194(02)00222-3.
46. Raja U. (2007): Trichoderma Harzianum. Greenmax Agro Tech. India. Available at http://www.greenmaxagrotech.com/enquiry.html (accessed on Nov 25, 2014).

47. Roy E. (2017): Phosphorus recovery and recycling with ecological engineering: A review. Ecological Engineering. 98: 213-227. DOI: 10.1016/j.ecoleng. 2016.10.076.
48. Ryu C.M., Farag M.A., Hu C.H., Reddy M.S., Kloepper J.W., Pare P.W. (2004): Bacterial volatiles induce systemic resistance in Arabidopsis. Plant Physiology. 134 (3): 1017-1026. DOI: 10.1104/pp.103.026583.
49. Samuels G.J., Chaverri P., Farr D.F., McCray E.B. (2014): Trichoderma online. Systematic Mycology and Microbiology Laboratory. ARS. USDA. The Regents of the University of California. Available at http://genome.jgi.doe.gov/Triha1/ Triha1.home.html (retrieved/accessed on Nov 26, 2016).
50. Siddiqui I.A., Shaukat S.S. (2004): Systemic resistance in tomato induced by biocontrol bacteria against the root-knot nematode, Meloidogyne javanica is independent of salicylic acid production. JOURNAL OF PHYTOPATHOLOGY. 152 (1): 48-54. DOI: 10.1046/j.1439-0434.2003.00800.x.
51. Takenaka S., Nishio Z., Nakamura Y. (2003): Induction of defense reactions in sugar beet and wheat by treatment with cell wall protein fractions from the mycoparasite Pythium oligandrum. Phytopathology. 93 (10): 1228-32. DOI: 10.1094/PHYTO.2003.93.10.1228.
52. Tam N.K.M., Uyen N.Q., Hong H.A., Duc L.H., Hoa T.T., Serra C.R., Henriques A.O., Cutting S.M. (2006): The Intestinal Life Cycle of Bacillus subtilis and Close Relatives. Journal of Bacteriology. 188 (7): 2692-2700. DOI: 10.1128/JB.188.7.2692-2700.2006.
53. Talboys P.J., Owen D.W., Healey J.R., Withers P.J.A., Jones D.L. (2014): Auxin secretion by Bacillus amyloliquefaciens FZB42 both stimulates root exudation and limits phosphorus uptake in Triticum aestivum. BMC PLANT BIOLOGY. 14: 51. DOI: 10.1186/1471-2229-14-51.
54. Vallabhaneni S.D. (2016): Biocontrol of Rhizoctonia solani in Tobacco (Nicotiana tabacum) Seed Beds Using Pseudomonas fluorescens. AGRICULTURAL RESEARCH. 5 (2): 137-144. DOI: 10.1007/s40003-016-0207-9.
55. Vitti A., Pellegrini E., Nali C., Lovelli S., Sofo A., Valerio M., Scopa A., Nuzzaci M. (2016): Trichoderma harzianum T-22 Induces Systemic Resistance in Tomato Infected by Cucumber mosaic virus. FRONTIERS IN PLANT SCIENCE. 7: 1520. DOI: 10.3389/fpls.2016.01520.

56. Withers P.J., Sylvester-Bradley R., Jones D.L., Healey J.R., Talboys P.J. (2014): Feed the crop not the soil: rethinking phosphorus management in the food chain. Environmental Science & Technology. 48 (12): 6523-6530.
57. Wu H.J., Zhang Y., Zhang H.Y., Gu Q., Gao X.W. (2017): Identification of functional regions of the HrpZ (Psg) protein from Pseudomonas savastanoi pv. glycinea that induce disease resistance and enhance growth in plants. EUROPEAN JOURNAL OF PLANT PATHOLOGY. 147 (1): 55-71. DOI: 10.1007/s10658-016-0979-6.
58. Yang T.Y., Liu G.L., Li Y.C., Zhu S.M., Zou A.L., Qi J.L., Yang Y.H. (2012): Rhizosphere microbial communities and organic acids secreted by aluminum-tolerant and aluminum-sensitive soybean in acid soil. BIOLOGY AND FERTILITY OF SOILS. 48 (1): 97-108. DOI: 10.1007/s00374-011-0608-7.
59. Yang H.H., Yang S.L., Peng K.C., Lo C.T., Liu S.Y. (2009): Induced proteome of Trichoderma harzianum by Botrytis cinerea. MYCOLOGICAL RESEARCH. 113 (9). 924-932. DOI: 10.1016/j.mycres.2009.04.004.
60. Yedidia I., Srivastva A.K., Kapulnik Y., Chet I. (2001): Effect of Trichoderma harzianum on microelement concentrations and increased growth of cucumber plants. Plant Soil. 235: 235-242. DOI: 10.1023/A:1011990013955.
61. Yusran Y., Weinmann M., Neumann G., Römheld V., Müller T. (2009). Effects of Pseudomonas sp. ”Proradix” and Bacillus amyloliquefaciens FZB42 on the Establishment of AMF Infection, Nutrient Acquisition and Growth of Tomato Affected by Fusarium oxysporum Schlecht f.sp. radicis-lycopersici Jarvis and Shoemaker. 26-30 August 2009. The Proceedings of the International Plant Nutrition Colloquium XVI. University of California. UC Davis: Department of Plant Sciences. Available at http://eprints.cdlib.org/uc/item/22h2v2h7 (accessed on Nov 22, 2014).
62. Zhang N., Yang D.Q., Kendall J.R.A., Borriss R., Druzhinina I.S., Kubicek C.P., Shen Q. R., Zhang R.F. (2016): Comparative Genomic Analysis of Bacillus amyloliquefaciens and Bacillus subtilis Reveals Evolutional Traits for Adaptation to Plant-Associated Habitats. FRONTIERS IN MICROBIOLOGY. 7: 2039. DOI: 10.3389/fmicb.2016.02039.

Influence of Bioeffectors Application on Maize Growth, Yields and Nutrient Uptake

Zlata Holečková, Martin Kulhánek, Jiří Balík,

Department of Agro-Environmental Chemistry and Plant Nutrition, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Kamýcká 129, Prague 165 21, Czech Republic

Abstract: Application of bioeffectors should improve the mobilisation of nutrients (especially phosphorus) from less available forms in soil, improve plant growth and contribute to mycorrhiza development. Bioeffectors should also increase resistance to diseases and pathogens. Consequently, bioeffectors should lead to a higher yields. The aim of this research is to estimate the influence of bioeffector application on plant growth and nutrient uptake of maize (*Zea mays, L*. var. Colisee). Three bioeffectors in combination with two phosphorus fertilisers were tested in a pot experiment with cambisol Humpolec. The bioeffectors used were: Trianum (*Trichoderma harzianum*), Proradix (*Pseudomonas sp.*) and RhizoVital (*Bacillus amyloliquefaciens*) in combination with triple superphosphate and rock phosphate. The use of bioeffectors did not positively influence nutrient uptake, dry matter or plant growth. Results of the pot experiments did not show significant positive effects of bioeffector application on plant growth, dry mass or availability of nutrients from less soluble forms in the soil.

Keywords: bioeffector, maize, nutrients, phosphorus, soil.

Introduction

Phosphorus in soil is an irreplaceable macro-element necessary for plant growth and development. Despite its necessity in plant metabolism is its content in the soil is relatively low (Mengel 1991; Blume et al. 2010). In the majority of soil types a higher phosphorus content is found in the close-to-surface layers due to increased biological activity, which results in the accumulation of organic material. Application of organic and mineral fertilisers can often influence soil phosphorus amount. The content of phosphorus in soil can vary depending on the nature of parent material, texture and other farming factors (the ratio and type of supplied phosphorus and

method of soil cultivation) (Ivanič et al. 1984; Sharpley 1995). Phosphorus in soil can be divided in two basic groups: inorganic phosphorus and organic phosphorus (Sharpley et al. 1987). The amount of phosphorus bound in the soil fractions depends mainly on the timing of fertilizers application, including the impact of earlier interventions (McGehan and Lewis 2002). The degree of availability for plants depends on chemical, physical-chemical and physical properties of the particular soil type, seasonal dynamics of water, air and temperature regimes, biological activity of soil, the plant species, etc. (Sharpley 1995; Nash et al. 2014). Today's society relies on inorganic phosphorus compounds (fertilisers, feed or food additives) to exploit the limited natural resources of phosphates. For these reasons, there is an overall need to develop more sustainable mechanisms to maintain phosphorus availability for crops and livestock but using a smaller amount of supplied mineral phosphorus, which will lead to improvement of soil functions. Creation of a new strategy requires better public awareness about the consequences of farming approaches on the environment, a better understanding of phosphorus dynamics in the soil-plant relationship, the creation of new innovative technologies to reduce the dependance of the population on mined phosphate and increase the efficiency of phosphorus fertilisation. The development of new strategies is expected to have a significant economical and environmental impact, particularly for future generations (Withers et al. 2014). Due to a growing world population it is expected that demand for food and feed will increase. Limited availability of productive agricultural land and increasing dependance on mineral fertilisers make it necessary to develop alternative strategies for plant nutrition (Hogenhout et al. 2009; Neumann 2012). In 2012 a project was introduced that includes the use of so-called bioeffectors in crop production. This project should contribute to the reduction of mineral fertilisers used in agriculture and to proper and efficient land use and involves testing under real conditions at different geographic locations (Smalla et al. 2012). It is an integrated project focused on the development of new approaches based on activity of live microorganisms and active natural substances (Hogenhout et al. 2009). Bioeffectors can contribute, depending on soil and climate conditions, to overcome limitations in the availability of nutrients. These compounds contain microorganisms (bacteria, fungi) and active natural substances, such as extracts from soil or compost, microbial residues, plant extracts or products of biological processes. These products are developed for a wide variety of crops (e.g. maize, wheat, tomatoes, rape, spinach, grass, ornamentals). Their

effective use should cause the mobilisation of nutrients from less bioavailable forms in soil (Neumann 2012; Smalla et al. 2012) and further support root growth (Ferrigo et al. 2014; Galletti et al. 2015) and mycorrhiza development (Yusran et al. 2009). The aim of this study is to evaluate the effect of bioeffectors on maize plant growth and selected nutrient uptake by the above ground mass of a plant, particularly focusing on phosphorus management.

Materials and methods

Pot experiments were established in a vegetation hall on the 30th of April 2014. Five maize seeds (*Zea mays*, variety Colisée) were sown into the pots (volume 5 L). On the 28th of May 2014, plants were selected on the final count of three per pot.

The tested soil was obtained from experimental stations of the Crop Research Institute (Humpolec site). Further site characteristics are mentioned in Table 1.

Table 1. Characteristics of experimental fields.

Site	Humpolec
Latitude	49°33'15" N
Longitude	15°21'02" E
Altitude (m above sea level)	525
Mean yearly temperature (°C)	7.0
Mean yearly rainfall (mm)	665
Soil type	cambisol
Soil sort	sandy loam
pH[1)]	5.1
P (mg/kg)[2)]	77 (± 10) B[3)]

1) Estimated in air-dried soil, 0.01 mol/l $CaCl_2$, 1:10 w/v

2) Average basic data estimated using Mehlich 3 method

3) Category B = low content

The substrate was composed of soil and quartz sand at a 2:1 ratio. In this experiment three bioeffectors in combination with two fertilisers were tested with the same dose of nitrogen and potassium (Table 2).

Table 2. Scheme of pot experiments.

Treatment No.	Treatment	Treatment No.	Treatment
1	BE0 + NK	7	BE2 + RP + NK
2	BE1 + NK	8	BE3 + RP + NK
3	BE2 + NK	9	BE0 + TSP + NK
4	BE3 + NK	10	BE1 + TSP + NK
5	BE0 + RP + NK	11	BE2 + TSP + NK
6	BE1 + RP + NK	12	BE3 + TSP + NK

Nitrogen was supplied in the form of calcium nitrate (0.50 g N pot^{-1}) and potassium in the form of K-fertilizer Patentkali (0.85 g K pot^{-1}). The effectiveness of bioeffectors was tested using rock phosphate (RP) and triple superphosphate (TSP), which were applied at the same dose of phosphorus (0.26 g P pot^{-1}). All treatments were compared with a control to which was applied only an inactive bioeffector (demineralized water). The experimental plants were harvested on the 13th of August 2014.

Bioeffectors used in the pot experiment, together with the active substance (in parentheses) were: (i) BE 0: Control (water only); (ii) BE 1: Trianum (*Trichoderma harzianum*, strain T-22, 10^9 spores g^{-1}, Koppert Biological Systems), 0.1175 g pot^{-1}; (iii) BE 2: Proradix (*Pseudomonas sp.,* strain DSMZ 13134, $6.6x10^{10}$ colony forming units (cfu g^{-1}, Sourcon Padena GmbH & Co.KG), 0.1375 g pot^{-1}; (iv) BE 3: RhizoVital (*Bacillus amyloliquefaciens*, strain FZB42, $2.5x10^{10}$ cfu g^{-1}, ABiTEP GmbH), 0.35 ml pot^{-1}. All bioeffectors were applied locally to the seeds in the form stock solution at a dosage of 25 ml per pot (5 ml of stock solution to each seed).

Plant height was measured four times during the experiment (5th of June 2014, 18th of June 2014, 3rd of July 2014, 13th of August 2014). After harvesting the pot experiments, the above ground biomass weight, % of dry mass, the content of macro- and selected micro-nutrients in above ground biomass and their uptake, were measured. For the estimation of nutrients, fine milled above ground dry biomass was analysed via dry decomposition at 500°C. Thereafter, samples were transferred to a solution of 1.5% nitric acid (provided by Mader et al. 1998). The extracts were measured by inductively coupled plasma optical emission spectrometry (ICP-OES) (Varian VistaPro, Australia). All results were statistically analysed (tests for the normality of distribution, One way ANOVA, Scheffes test at significance level 0.05) using the statistical software application STATISTICA (StatSoft 2016).

Results

The pot experiment was based on the hypothesis that the application of bioeffectors would increase the amount of available phosphorus and other important nutrients for plants. This would result in better phosphorus and other nutrient uptake, greater plant growth and higher yields (Table 3). Table 3 shows plant height measured during the experiment. It is obvious that in the initial growth stages (recorded on 5th and 18th of June), plant height was influenced mainly by TSP use. On the 18th of June a significant positive effect of rock phosphate application on plant height was recorded as well. In later stages, nonsignificant differences between the studied variants occured. This was probably due to competition among the plants in the pots. In terms of plant height variant BE0 showed the smallest plant heights on the 5th and 18th of June and the 3rd of July; however, the impact of the application of various bioeffectors was not statistically verified.

Table 3. Height of maize plants (cm) on specific dates (three plants).

Treatment	Height 5th June	Height 18th June	Height 3rd July	Height 13th August
1	24.2^{a}	47.8^{a}	88.8^{a}	157^{a}
2	27.2^{a}	56.1^{a}	91.6^{a}	147^{a}
3	26.9^{a}	57.1^{a}	94.6^{a}	152^{a}
4	28.1^{a}	56.5^{a}	95.9^{a}	149^{a}
5	29.3^{a}	65.3^{b}	103^{a}	136^{a}
6	31.3^{a}	66.1^{b}	99.5^{a}	140^{a}
7	32.3^{a}	70.5^{b}	103^{a}	137^{a}
8	31.7^{a}	67.4^{b}	98.0^{a}	134^{a}
9	45.7^{b}	82.1^{c}	102^{a}	135^{a}
10	43.1^{b}	84.4^{c}	105^{a}	126^{b}
11	51.4^{b}	82.4^{c}	102^{a}	128^{a}
12	46.4^{b}	78.3^{c}	99.5^{a}	132^{a}
F-test	42.5	38.3	3.57	4.71
p ≤ *	0.01	0.01	n.s.	0.05

* p = significance level

Figure 1 indicates the average above ground dry biomass yield for each variant (Fig. 1). The highest dry mass weight was recorded for treatment BE1 + TSP. This was probably caused by application of TSP. The lowest weight of dry matter was recorded for treatment 4 (BE3 + NK) and treatment 7 (BE2 + RP + NK). From Figure 1 it is obvious that the application of selected bioeffectors had no statistically significant effect on the dry matter yield.

Figure 1. Dry matter weight of the above ground biomass of maize (g per three plants).

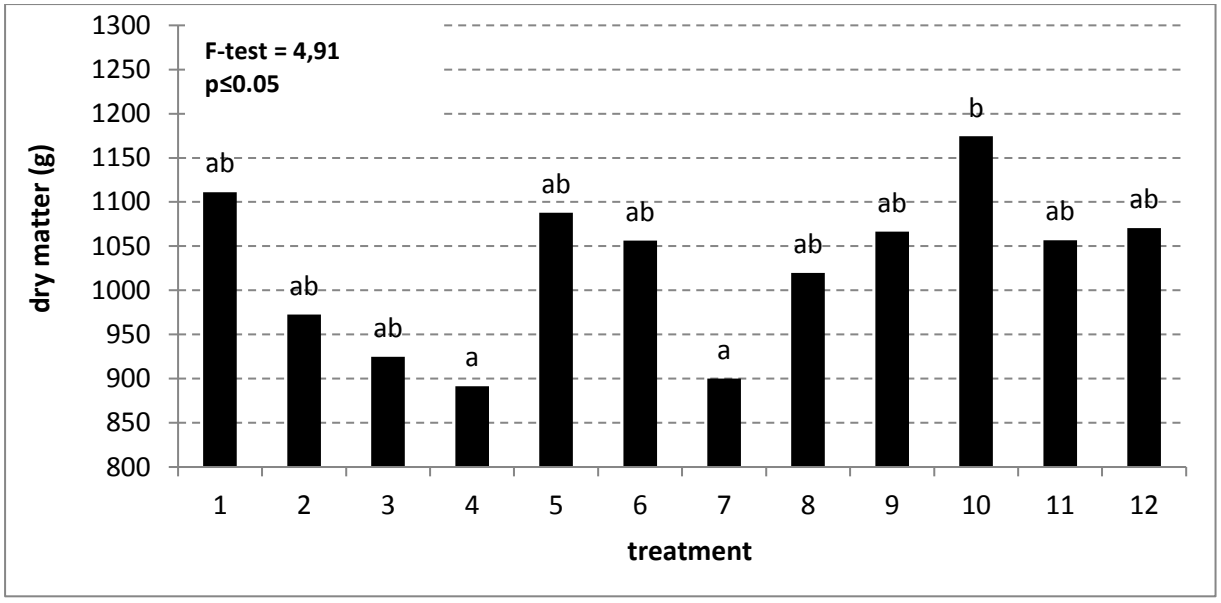


Table 4 lists the nutrients content in the above ground biomass of maize (Table 4). Significant differences between treatments were obtained only for nitrogen and calcium, whereby the nitrogen content under treatment 3 was higher in comparison to treatments 6, 9, 10 and 11. Under treatment 3 the bioeffector BE2 + NK was applied. On the other hand under treatment 6 (BE1 + RP + NK) was applied, under treatment 9 (BE2 + RP + NK) was applied, under treatment 10 (BE1 + TSP + NK) was applied and under treatment 11 (BE2 + TSP + NK) was applied. Calcium content under treatment 1 was significantly higher in comparison to that under treatments 7 and 10. Under treatment 1 (BE0 + NK) was applied, while under treatments 7 and 10, BE2 + RP + NK and BE1 + TSP + NK were applied, respectively. The rest of the results were not statistically significant but the highest contents of analysed elements were found in the following treatments: phosphorus = var. 11; potassium = var. 4; magnesium, sulphur, iron, copper and zinc = var. 1 and

manganese = var. 5. The lowest contents of analysed elements were found in the following variants: phosphorus = var. 6; potassium = var. 9; magnesium = 6; sulphur = 6 and 10; iron = var. 2; copper = var. 9 and zinc and manganese = var. 10.

Table 4. Average content of nutrients in plants (mg/kg).

Var.	N	P	K	Ca	Mg	S	Fe	Cu	Zn	Mn
1	5296^{ab}	1031^{a}	12729^{a}	2219^{b}	1259^{a}	529^{a}	57.38^{a}	1.44^{a}	23.49^{a}	29.17^{a}
2	5164^{ab}	1033^{a}	12339^{a}	1446^{ab}	1043^{a}	470^{a}	21.42^{a}	1.22^{a}	11.16^{a}	24.85^{a}
3	6678^{b}	1260^{a}	12127^{a}	1676^{ab}	1215^{a}	497^{a}	32.74^{a}	1.37^{a}	12.49^{a}	32.32^{a}
4	5115^{ab}	1038^{a}	13815^{a}	1438^{ab}	1032^{a}	458^{a}	54.52^{a}	1.24^{a}	11.66^{a}	29.33^{a}
5	4894^{ab}	1033^{a}	11485^{a}	1840^{ab}	1197^{a}	457^{a}	50.76^{a}	1.32^{a}	10.27^{a}	32.78^{a}
6	4010^{a}	1011^{a}	11344^{a}	1406^{ab}	930^{a}	405^{a}	30.56^{a}	0.95^{a}	9.24^{a}	24.66^{a}
7	5404^{ab}	1257^{a}	11862^{a}	1201^{a}	944^{a}	424^{a}	39.83^{a}	0.96^{a}	10.58^{a}	26.46^{a}
8	4836^{ab}	1072^{a}	10781^{a}	1534^{ab}	1052^{a}	469^{a}	54.85^{a}	1.18^{a}	10.05^{a}	26.78^{a}
9	3583^{a}	1277^{a}	10258^{a}	1293^{ab}	913^{a}	444^{a}	39.48^{a}	0.84^{a}	8.74^{a}	28.79^{a}
10	4388^{a}	1270^{a}	10811^{a}	1062^{a}	822^{a}	405^{a}	21.71^{a}	0.92^{a}	7.93^{a}	23.76^{a}
11	4418^{a}	1293^{a}	10478^{a}	1331^{ab}	951^{a}	471^{a}	26.16^{a}	0.97^{a}	8.96^{a}	29.38^{a}
12	4808^{ab}	1247a	12536^{a}	1392^{ab}	988^{a}	413^{a}	32.55^{a}	1.01^{a}	9.23^{a}	32.08^{a}
F-test	6.81	1.73	2.73	4.81	3.28	2.16	1.26	3.68	1.70	1.41
p ≤ *	0.01	n.s.	n.s.	0.01	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

* p = significance level

Table 5 shows the average amount of element uptake by plants for each treatment. This research focused especially on phosphorus because phosphorus sources are limited and bioeffectors are developed specifically to increase phosphorus availability (Table 5). Significant differences between treatments ($p \leq 0.01$) were obtained only for nitrogen, calcium and sulphur, whereby the nitrogen uptake under treatment 3 was higher in comparison to treatment 9. Under treatments 3 and 9, BE2 + NK and BE0 + TSP + NK were applied, respectively. Statistically verified differences in calcium uptake were identified under treatment 1 and treatments 2, 4, 7, 9, 10 and 11. Under treatment 1 the highest uptake of calcium was recorded and BE0 + NK was applied. Under treatments 2, 4, 7, 9, 10 and 11 (BE1 + NK, BE3 + NK, BE2 + RP + NK, BE0 + TSP + NK, BE1 + TSP + NK and BE2 + TSP + NK) were applied, respectively. The final statistically verified difference was

of sulphur uptake, whereby the sulfur content under treatment 1 was higher in comparison to treatment 7. Under treatment 1 (BE0 + NK) was applied and under treatment 7 (BE2 + RP + NK) was applied. The rest of results were not statistically significant but the highest uptakes of analysed elements were under the following treatments: phosphorus = var. 10; potassium, magnesium, iron, copper and zinc = var. 1 and manganese = var. 5. The lowest uptakes of analysed elements were under following variants: phosphorus = var. 4; potassium, magnesium, copper and manganese = var. 7; iron - var. 2 and zinc = var. 10.

Table 5. Average uptake of nutrients by plants (mg per three plants).

Var.	N	P	K	Ca	Mg	S	Fe	Cu	Zn	Mn
1	5853^{ab}	1139^{a}	14114^{a}	2458^{a}	1393^{a}	585^{b}	64.79^{a}	1.61^{a}	26.68^{a}	32.59^{a}
2	5023^{ab}	1005^{a}	12013^{a}	1407^{b}	1015^{a}	458^{ab}	20.83^{a}	1.19^{a}	10.87^{a}	24.20^{a}
3	6167^{a}	1146^{a}	11116^{a}	1539^{ab}	1113^{a}	455^{ab}	29.85^{a}	1.25^{a}	11.42^{a}	29.77^{a}
4	4546^{ab}	922^{a}	12310^{a}	1285^{b}	921^{a}	408^{ab}	48.80^{a}	1.11^{a}	10.36^{a}	26.19^{a}
5	5323^{ab}	1124^{a}	12508^{a}	1998^{ab}	1301^{a}	497^{ab}	55.00^{a}	1.44^{a}	11.16^{a}	35.54^{a}
6	4211^{ab}	1062^{a}	12030^{a}	1494^{ab}	982^{a}	428^{ab}	32.58^{a}	1.00^{a}	9.67^{a}	25.98^{a}
7	4837^{ab}	1139^{a}	10657^{a}	1083^{b}	858^{a}	384^{a}	35.93^{a}	0.86^{a}	9.54^{a}	23.22^{a}
8	4942^{ab}	1093^{a}	10977^{a}	1557^{ab}	1070^{a}	477^{ab}	54.95^{a}	1.20^{a}	10.24^{a}	27.20^{a}
9	3829^{b}	1357^{a}	10922^{a}	1380^{b}	973^{a}	472^{ab}	42.25^{a}	0.89^{a}	9.30^{a}	30.88^{a}
10	5115^{ab}	1489^{a}	12739^{a}	1244^{b}	960^{a}	473^{ab}	25.59^{a}	1.07^{a}	9.21^{a}	27.60^{a}
11	4676^{ab}	1375^{a}	11087^{a}	1417^{b}	1011^{a}	500^{ab}	27.88^{a}	1.04^{a}	9.53^{a}	31.25^{a}
12	5130^{ab}	1326^{a}	13345^{a}	1487^{ab}	1054^{a}	440^{ab}	34.88^{a}	1.07^{a}	9.88^{a}	34.70^{a}
F-test	4.16	2.78	2.00	5.89	3.57	3.31	1.31	3.44	1.675	1.87
p ≤ *	0.01	n.s.	n.s.	0.01	n.s.	0.01	n.s.	n.s.	n.s.	n.s.

* p = significance level

Discussion

To date several studies have evaluated the different effects of bioeffectors on plants and included various different parameters. For example, Yusran et al. (2009) reported that after Proradix and RhizoVital application (individually or in combination) to soil in a pot experiment, a significant improvement in the state of tomato plant roots occurred. The roots were healthier and showed significantly higher colonisation by arbuscular mycorrhizal fungi. In our experiments these parameters are not rated

but the health of the plants should improve plant growth and yield. However, we did not confirm a significant positive effect of bioeffector application on maize yield. Kumar et al. (2015) conducted pot experiments to support Pigeon Pea (*Cajanus cajan* L.) plant growth after inoculation with bacteria *Pseudomonas fluorescens*. For the study 75 fluorescent Pseudomonas strains from different agro-ecosystems in India were isolated. The isolated strain P17 showed considerable support for growth in terms of root length, dry matter, chlorophyll, carbohydrates, nitrogen, calcium, iron and manganese. *Pseudomonas sp*. strain P17 was identified as a potential rhizobacteria to support plant growth and increase nutrient uptake. In our experiments we tested *Pseudomonas sp.*, strain DSMZ 13137 and found that it did not have a positive influence on plant growth or nutrient uptake. Further, Chiarini et al. (1998) conducted a pot experiment in greenhouse conditions with *Sorghum bicolor* and inoculation with microorganisms *Burkholderia cepacia*, *Pseudomonas fluorescens* and *Enterobacter sp*. The results showed that all three microorganisms have the ability to colonise the root system of *Sorghum*, but only the *B. cepacia* and *P. fluorescens* supported plant growth via inoculation with one microorganism only. Dual inoculation had no further effect on plant growth. Our results did not show a positive influence of *Pseudomonas sp.* strain DSMZ 113134 on increasing plant growth or uptake of nutrients. Dual inoculation was not evaluated in our experiments.

In this research was not influence of bioeffectors was confirmed on plant height nor on yield or dry matter weight. Higher values were probably caused by the addition of TSP. Similar plant heights in the later stages could be caused by the correlative stimulating effects of the roots and subsequent growth of the above ground plant parts, or the production of fytohormones (gibberellins, cytokinins, auxins) (Šebánek et al. 1991). Statistically significant differences between treatments on nutrients content in the above ground were obtained only for nitrogen and calcium. And statistically significant differences on uptake of nutrients by plants were obtained only for nitrogen, calcium and sulfur.

Conclusions

Although some positive results in other studies, bioeffectors did not positively influenced maize yields, as well as the macro- and selected microelements content in above ground biomass of plants in our experiments. Results from the pot experiments showed only that the TSP application increased the plant height during the initial growth stages as well as P uptake with above ground biomass of harvested

plants. Therefore it is clear that bioeffectors works only in specific conditions and should be further tested.

Acknowledgements

This research was financially supported by the Resource Preservation by Application of bioeffectors in European Crop Production nr. 7. RP 312117.

This manuscript was proof readed by: Proof-Reading-Service.com.

References:

1. Blume H.P., Brümmer G.W., Horn R., Kandeler E., Kögel-Knabner I., Kretzschmar R., Stahr K., Wilke B.M. (2010). Textbook of soil science (Lehrbuch der Bodenkunde) – Scheffer/Schachtschabel. 15. Edition. Berlin: Spektrum Akademischer Verlag GmbH. Heidelberg. 593 p.
2. Chiarini L., Bevivino A., Tabacchioni S., Dalmastri C. (1998). Inoculation of Burkholderia cepacia, Pseudomonas fluorescens and Enterobacter sp. on Sorghum bicolor: Root colonization and plant growth promotion of dual strain inocula. Soil Biology and Biochemistry. 30 (1): 81-87. DOI: 10.1016/S0038-0717(97)00096-5.
3. Ferrigo D., Raiola A., Rasera R., Causin R. (2014). Trichoderma harzianum seed treatment controls Fusarium verticillioides colonization and fumonisin contamination in maize under field conditions. Crop Protection. 65: 51-56. DOI: 10.1016/j.cropro.2014.06.018.
4. Galletti S., Fornasier F., Cianchetta S., Lazzeri L. (2015). Soil incorporation of brassica materials and seed treatment with Trichoderma harzianum: Effects on melon growth and soil microbial activity. Industrial Crops and Products. 75 Part A: 73-78. DOI: 10.1016/j.indcrop.2015.04.030.
5. Hogenhout S.A., Van der Hoorn R.A.L., Terauchi R., Kamoun S. (2009). Emerging Concepts in Effector Biology of Plant-Associated Organisms. Molecular Plant-Microbe Interaction. 22 (2): 115-122. DOI: 10.1094/MPMI-22-2-0115.
6. Ivanič J., Havelka B., Knop K. (1984). Nutrition and fertilization of plants (In Slovak: Výživa a hnojenie rastlín). Nature Bratislava - SZN Praha. 482 p.
7. Kumar G.P., Desai S., Reddy G., Amalraj E.L.D., Rasul A., Ahmed S.K.M.H. (2015). Seed bacterization with Fluorescent Pseudomonas spp. enhances nutrient uptake and growth of Cajanus cajan L. Communications in Soil Science and Plant Analysis. 46 (5): 652-665. DOI: 10.1080/00103624.2015.1005219.

8. Mader P., Száková J., Miholová D. (1998). Classical dry ashing of biological and agricultural materials, Part II. Losses of analytes due to their retention in an insoluble residue. Analusis. 26 (3): 121-129. DOI: 10.1051/analusis:1998121.
9. McGechan M.B., Lewis D.R. (2002). Sorption of phosphorus by soil, Part 1: Principles, equations and models. Biosystems Engineering. 82 (1): 1-24. DOI: 10.1006/bioe.2002.0054.
10. Mengel K. (1991). Plant nutrition and metabolism (Ernährung und Stoffwechsel der Pflanze). 7. Edition. Gustav Fischer Verlag Jena. 466 p.
11. Nash D.M., Haygarth P.M., Turne B.L., Condron L.M., McDowell R.W., Richardson A.E., Watkins M., Heaven M.W. (2014). Using organic phosphorus to sustain pasture productivity: A perspective. Geoderma. 221: 11-19. DOI: 10.1016/j.geoderma.2013.12.004.
12. Neumann G. (2012): EU-funded research collaboration on use of bio-effectors in agriculture launched. Press Release. Germany: University of Hohenheim. Available at http://www.biofector.info/about-biofector.html (accessed on Sep 15, 2014).
13. Sharpley A.N. (1995). Soil phosphorus dynamics: agronomic and enviromental impacts. Ecological Engineering. 5 (2-3): 261-279. DOI: 10.1016/0925-8574(95)00027-5.
14. Smalla K. (2012). EU-funded research collaboration on use of bio-effectors in agriculture launched. First kick-off meeting at University of Hohenheim. Germany: Julius Kühn-Institut. Available at http://www.jki.bund.de/en/startseite/presse/english-press/eu-funded-research-collaboration-on-use-of-bio-effectors-in-agriculture-launched.html (accessed on Nov 17, 2014).
15. Sharpley A.N., Tiessen H., Cole C.V. (1987). Soil phosphorus forms extracted by soil tests as a function of pedogenesis. Soil Science Society of America Journal. 51: 362-365. doi:10.2136/sssaj1987.03615995005100020019x.
16. Šebánek J., Sladký Z., Procházka S., Kutáček M. (1991). Experimental Morphogenesis and Integration of Plants. Amsterdam: Elsevier Science Publishers. 417 p.
17. Withers P.J., Sylvester-Bradley R., Jones D.L., Healey J.R., Talboys P.J. (2014). Feed the crop not the soil: rethinking phosphorus management in the food chain. Environmental Science & Technology. 48 (12): 6523-6530. DOI: 10.1021/es501670j.

18. Yusran Y., Weinmann M., Neumann G., Römheld V., Müller T. (2009). Effects of Pseudomonas sp. ”Proradix” and Bacillus amyloliquefaciens FZB42 on the Establishment of AMF Infection, Nutrient Acquisition and Growth of Tomato Affected by Fusarium oxysporum Schlecht f.sp. radicis-lycopersici Jarvis and Shoemaker. 26-30 August 2009. The Proceedings of the International Plant Nutrition Colloquium XVI. University of California. UC Davis: Department of Plant Sciences. Available at http://eprints.cdlib.org/uc/item/22h2v2h7 (accessed on Nov 22, 2014).

Formation of the Seed Layer From the Organomineral Mixture During the Seed Pelleting of Coniferous Tree Species

Ostroshenko V.Yu.,
Federal State Budgetary Education Institution of Higher Education
Primorskaya State Agricultural Academy,
Prospect Blucher 44, Ussuriysk, Russia,
Federal Scientific Center of the East Asia Terrestrial Biodiversity,
Far East Branch of Russian Academy of Sciences,
Prospect of the 100th anniversary of Vladivostok 159, Vladivostok, Russia,
Akimov R.Yu., Gamaeva S.V., Inshakov S.V.,
Ostroshenko V.V., Ostroshenko L.Yu., Zamyshlyaev S.V.,
Federal State Budgetary Education Institution of Higher Education
Primorskaya State Agricultural Academy,
Prospect Blucher 44, Ussuriysk, Russia

Abstract: The seed pelleting is one of the most perspective way of the presowing processing of seeds. It covers seeds with a shell of organomineral materials, polymer binders and target additives and helps to smooth their surface, provide seedlings in the early phases of development with the necessary elements of mineral nutrition, protecting them from pests and diseases. At present, this technique is used in agriculture for seeding small seeds. The studies presented in this article are aimed at optimizing the technological process of seed pelleting of coniferous tree species, namely, Scots pine (*Pinus silvestris* L.) and Korean pine cedar (*Pinus koraiensis* Siebold et Zucc.). Experimental studies were performed using an electromechanical seed drazhirator. The organomineral mixture was used in a ratio of 0.48 kg per 1 kg of seeds. At the end of the process, the bonding strength of the filler to the seeds was determined. The dynamics of formation of the layer thickness was determined on the cross sections of the treated seeds using an optical microscope. As a result, the dependence of the thickness of the layer on the time and repetition of the seed pelleting was established. The article presents diagrams of experimental information with its subsequent equalization by a theoretical law having a high agreement on a given time interval. The thickness of the first layer formed around the seeds of Scotch

pine was 0,3 mm - 0,4 mm and Korean pine cedar - 0,5 - 0,7 mm. The seeder does not destroy this shell. When increasing the coating layer for 1 stage, the optimum thickness is reached within 7-8 minutes. When it is planned to increase the thickness of the layer, the draining process is expediently divided into 2 stages. The time of pelleting at stage 1 is 2 minutes, on the second - 6 minutes. When the thickness of the limiting layer is reached and the pelleting is continued, it is possible to reduce time.

Keywords: seed pelleting, covering (pelleting) layer, seeds, Scotch pine, Korean pine cedar.

Introduction. In recent decades, in the forestry of Russia and abroad, experimental work has been activated to grow planting material using growth stimulators (regulators) during reforestation. Preseeding seed treatment is of great positive importance. Seed pelleting is one of the persperctive methods of such processing. It is a coating of seeds in a shell of organomineral materials, polymer binders and targeted additives, which leads to the smoothing of their surface, providing sprouts in the early phases of development with the necessary elements of mineral nutrition, protecting them from pests and diseases [1-7, 9,10].

As a result of the seed pelleting, granules (pellets) are formed in diameter, depending on the size of the seeds, suitable for storage, transportation and spot-sowing. At the time of germination of the seed in the soil, the layer of the granule dissolves (decomposes), providing the sprout with nutrients and protecting it from aggressive soil flora [6,7].

Processing seeds of forest species and crops have obtained the most widespread technology of pelleting[4-7, 11-20]. At present, this effective method of presowing seed preparation is used on an industrial scale in Russia's agriculture in the sowing of small-seed crops (vegetable, sugar beet, cotton) [6,7].

In the forestry of Russia, the employees of LenNIILKh (St. Petersburg) [6] started the first experiments on the pelting and sowing of small seeds of coniferous tree species (Pinus sylvestris, Ayaan spruce) in the 1980s. In the Far East, the efficiency of seed pelleting is studied by foresters of the Primorskaya State Academy of Agriculture and the “GTS” – a branch of Federal Scientific Center of the East Asia Terrestrial Biodiversity. Possibilities of mechanized coating of seeds of Korean cider pine (*Pinus koraiensis* Siebold et Zucc.), Scots pine (*Pinus silvestris* L.) and Amur larch (*Larix amurensis)* are studied. In addition, the components of the coating mixture have

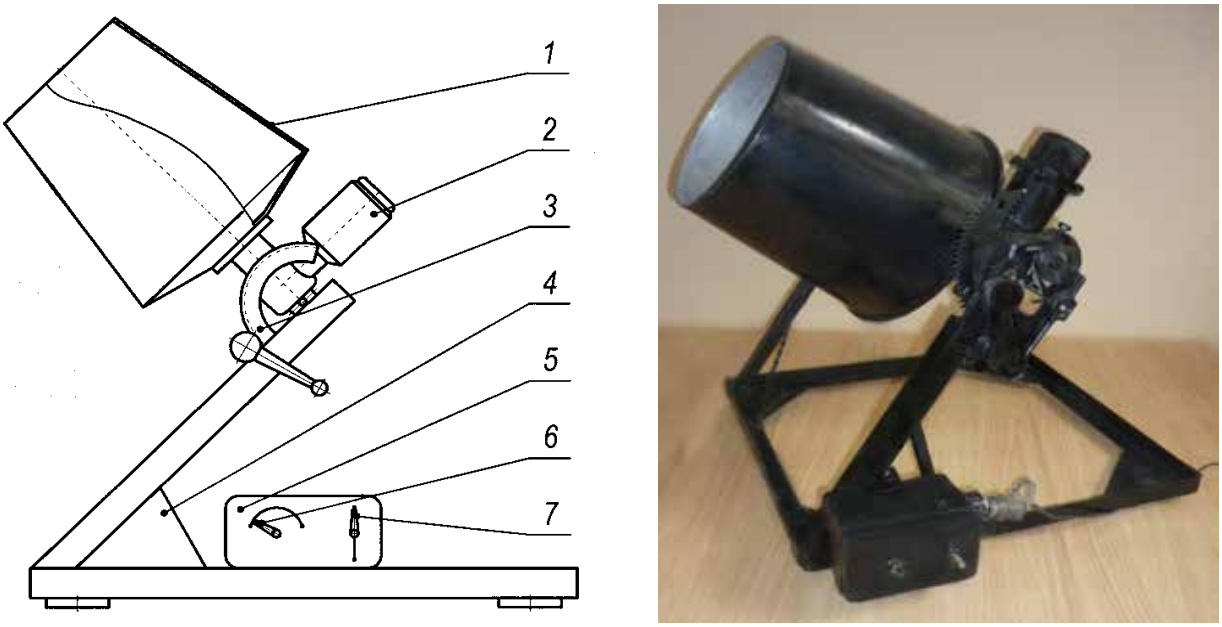
been developed [1,2]. Adhesive has a huge role. It is the filler and the main component and provides the formation of a dragee and nutrient layer on the seeds. Now, the possibility of improving the technological process of seed pelleting is being studied.

The purpose of the research is to analyze the conditions for the formation of a seed layer from a coating mixture when treating seeds of Scotch pine (*Pinus silvestris* L.) and Korean pine cedar (*Pinus koraiensis* Siebold et Zucc.). It is necessary to solve the following tasks to achieve the goal:

1. To carry out the pelleting of seeds of Scotch pine and Korean pine cedar with the help of a mechanical device;

2. To determine the dependence of the layer thickness on the time and repetition of the pelleting.

Materials and methods. Seed pelleting was carried out at the Forestry Department and in the laboratory of the Primorskaya State Academy of Agriculture using an electromechanical drazhirator (Figure 1).



1 - a drum;
2 - drive motor-reducer;
3 - mechanism for changing the angle of inclination of the drum axis;
4 - frame;
5 - the electric control system;
6 - the regulator of frequency of the drum rotation;
7 - the switch of direction of the drum rotation.

Figure 1 - Scheme and photo of the experimental drazhirator

The experienced drazhirator is made based on the motor-reducer MU-100 AGU with the power of 120 W, with the speed of its rotation - 150-300 rpm. The angle of inclination of the tank axis is 40˚ ± 5 °, the volume of the drazhirator tank is 8 l.

The seeds for 40-60 minutes were treated with $KMnO_4$ solution before pelleting. Then seeds were placed in a solution of the growth stimulator Epin-Extra with a concentration of 0.5 ml / 2 l of water for 30 minutes.

The technological process of seed pelleting includes six methods: weighing and filling seeds in a drazhirator; addition to the seeds of the binder (PVA glue); mixing seeds with glue; addition of filler (wood ash); grinding of the lumps of the coating mixture; completion of the pelleting process - extraction of granules from the working capacity of the drazhirator. The most laborious and responsible, in terms of the quality of the work performed, is the mixing of seeds with glue. The execution of this method determines the level of execution of the entire technological operation [2,9,10]. Identification of the amount of adhesive for the production process was produced by the search way. Seeds were mixed with the coating mixture at the rate of 250 g of seeds, 60 g of adhesive (PVA adhesive) and 60 g of wood ash, the production of which in the conditions of high forest cover of the Far Eastern region is not connected with difficulties [1, 2 ,10].

The seed pelleting was carried out by the method of layering the covering mixture with periodic moistening of the seeds. The production rate for the eight-hour shift was 11.4 kg of pelleted seeds. pelleted seeds were dried for 4-6 hours at room temperature (Figure 2).

At the end of drying, the strength of the adherence of the filler to the seeds was determined. Putting the pelleted seeds in water, the time of the beginning of decomposition of the coating layer was revealed.

a) b)

Figure 2 - Pelleted seeds: a) Scots pine, b) Korean cedar pine

The dynamics of formation of the thickness of the layer was determined on the cross sections of the treated seeds. The thickness of the coating weight was measured using an optical microscope with a digital eyepiece and a binocular magnifier. In this case, the plane of the seed cut from the conditional center was divided into eight equiangular sectors, and thickness values X1 X2 ... X8 along the lines coinciding with the sides of the corners of the mentioned sectors were determined (Figure 3).

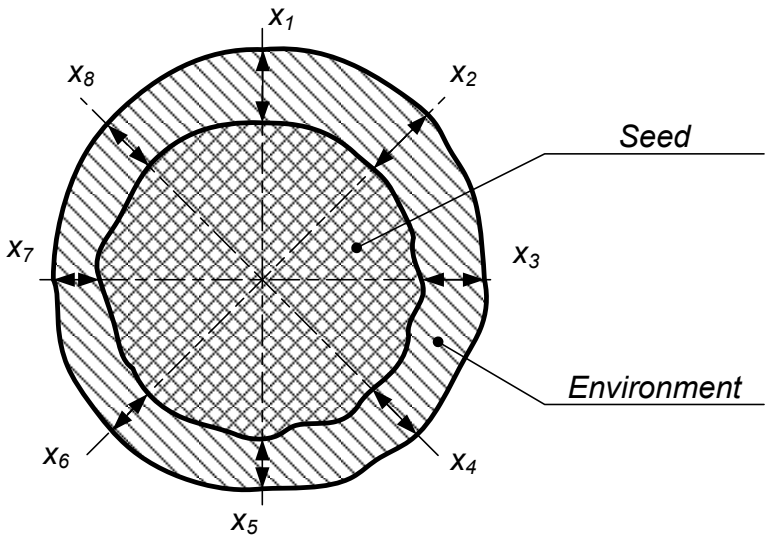


Figure 3 - Scheme for determining the average thickness of the layer

Subsequently, the average thickness of the layer of each seed was determined as the arithmetic mean of the measured values by the formula:

$$\bar{x} = \frac{1}{8}\sum_{i=1}^{i=8} x_i \quad (1)$$

The pelleted seeds were sown on garden bed. Non-pelleted seeds were used for control. Seeds were sown in four replicates; 100 units were sown in each replication. The soil germination was determined.

Results. At the end of the pelleting, the maximum thickness of the first layer (shell) formed around the seeds of Scots pine was observed with a pelletizing time of 7-8 minutes and amounted to 0,3 mm - 0,4 mm (Figure 4). The thickness of the first layer of Korean cedar pine was 0.5 - 0.7 mm (Figure 5). The seeding machine does not destroy this shell. The average diameter of the pelleted seeds was: for Scots pine - 3,2-3,8 mm, for Korean cedar pine - 12-14 mm.

Figure 4 - Thickness of the layer of Scots pine seed (Pinus silvestris L.) at the first stage of pelletizing (time - 7 minutes).

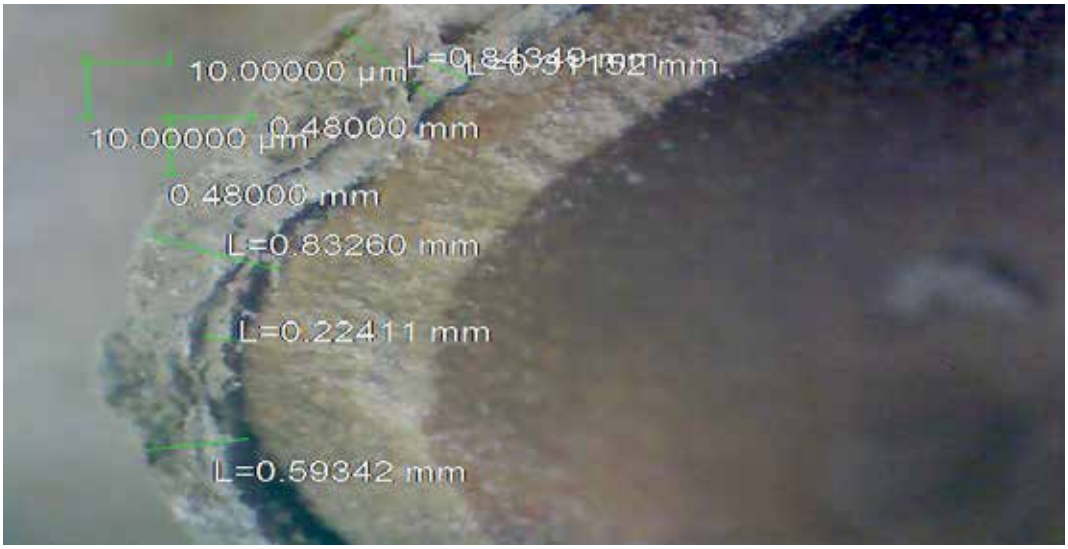


Figure 5 - Thickness of the layer of Korean cedar pine (*Pinus koraiensis* Siebold et Zucc.) at the first stage of pelletizing (time - 8 minutes).

At the second stage of seed pelleting, a higher thickness of the layer of Scots pine seeds was 0.6 mm (Figures 6, 8). It was observed with the duration of pelletizing for 2 + 6 - 8 + 6 minutes. Higher thickness of the layer of Korean cedar pine seeds was 1.1-1.3 mm with a seed pelletizing time of 2+6-8+4 minutes (Figures 7, 9).

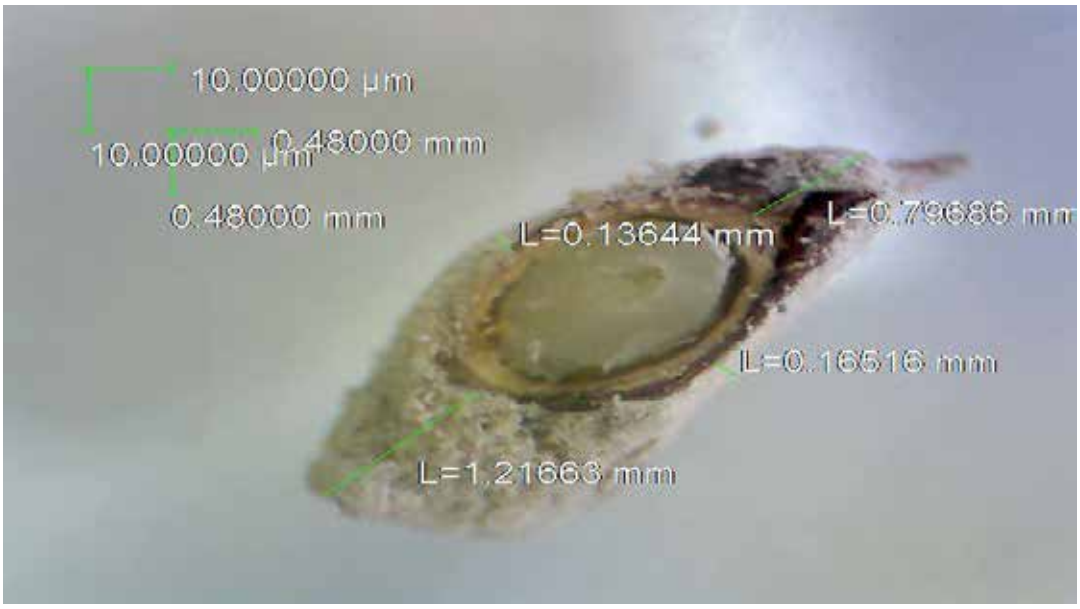


Figure 6 - Thickness of the layer of Scots pine seed (*Pinus silvestris* L.) at the second stage of pelletizing (time - 2 + 6 min.)

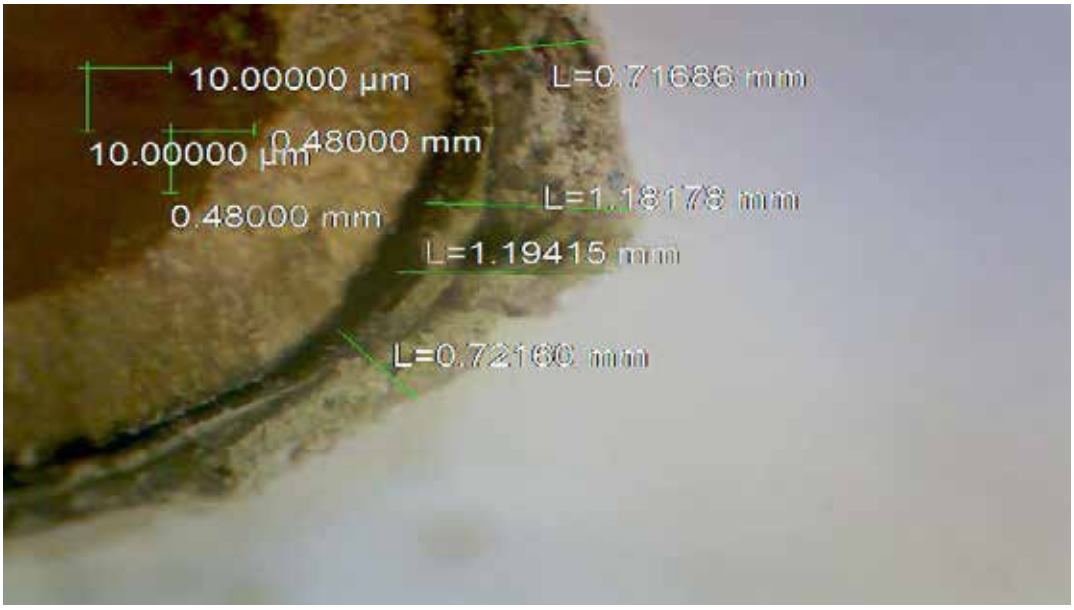


Figure 7 - Thickness of the layer of Korean cedar pine seed (*Pinus koraiensis* Siebold et Zucc.) at the second stage of pelletizing (time - 2 + 6 min.)

The pelleeted seeds placed in water were characterized by a high degree of strength on first day of the experiment. In the following days, the seeds swelled and a decrease in the strength of the attachment of the layer to the seed was noted.

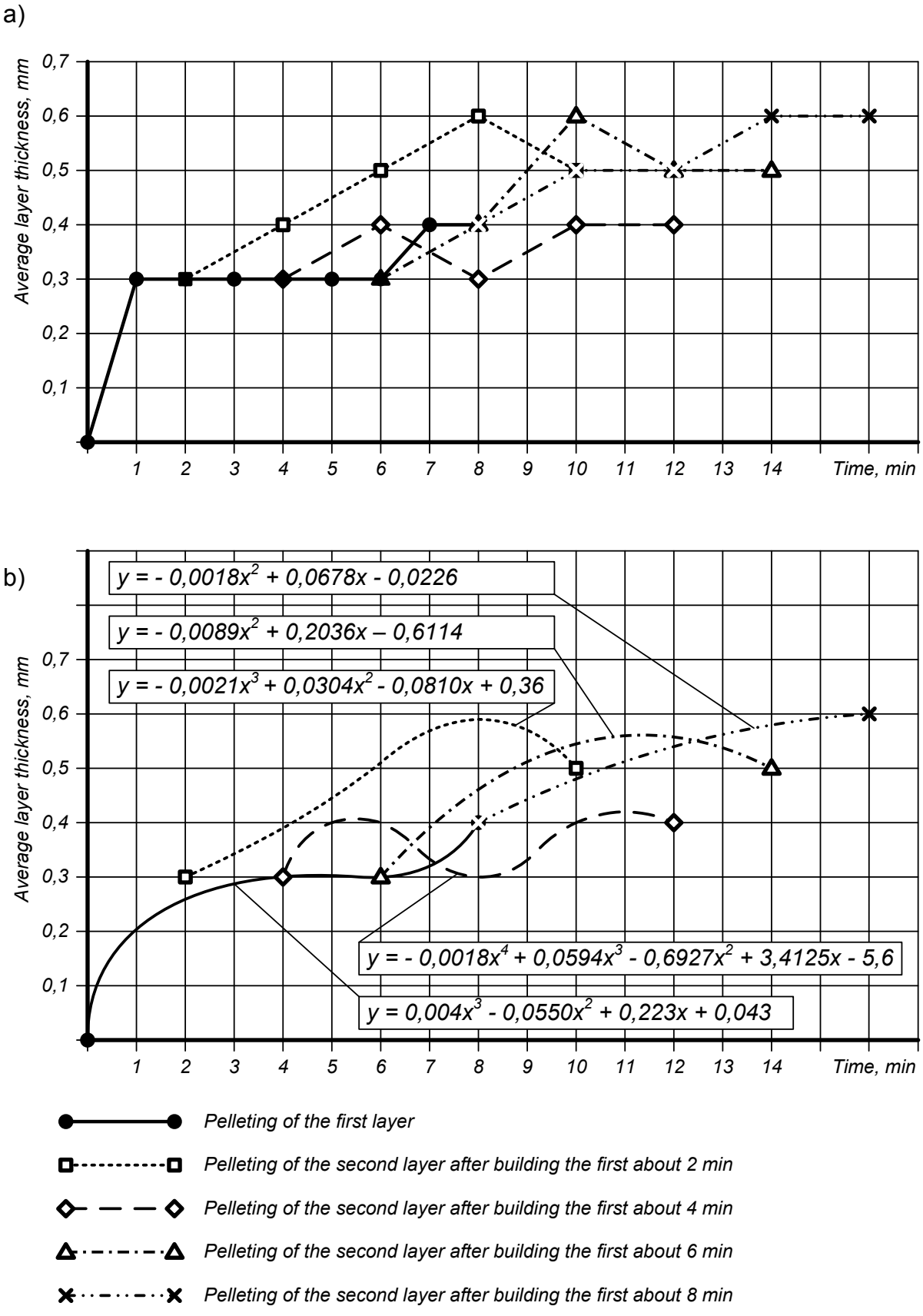


Figure 8 – The dynamics of layer building on seeds of Scots pine (a – an experimental information; b – leveling by the theoretical law)

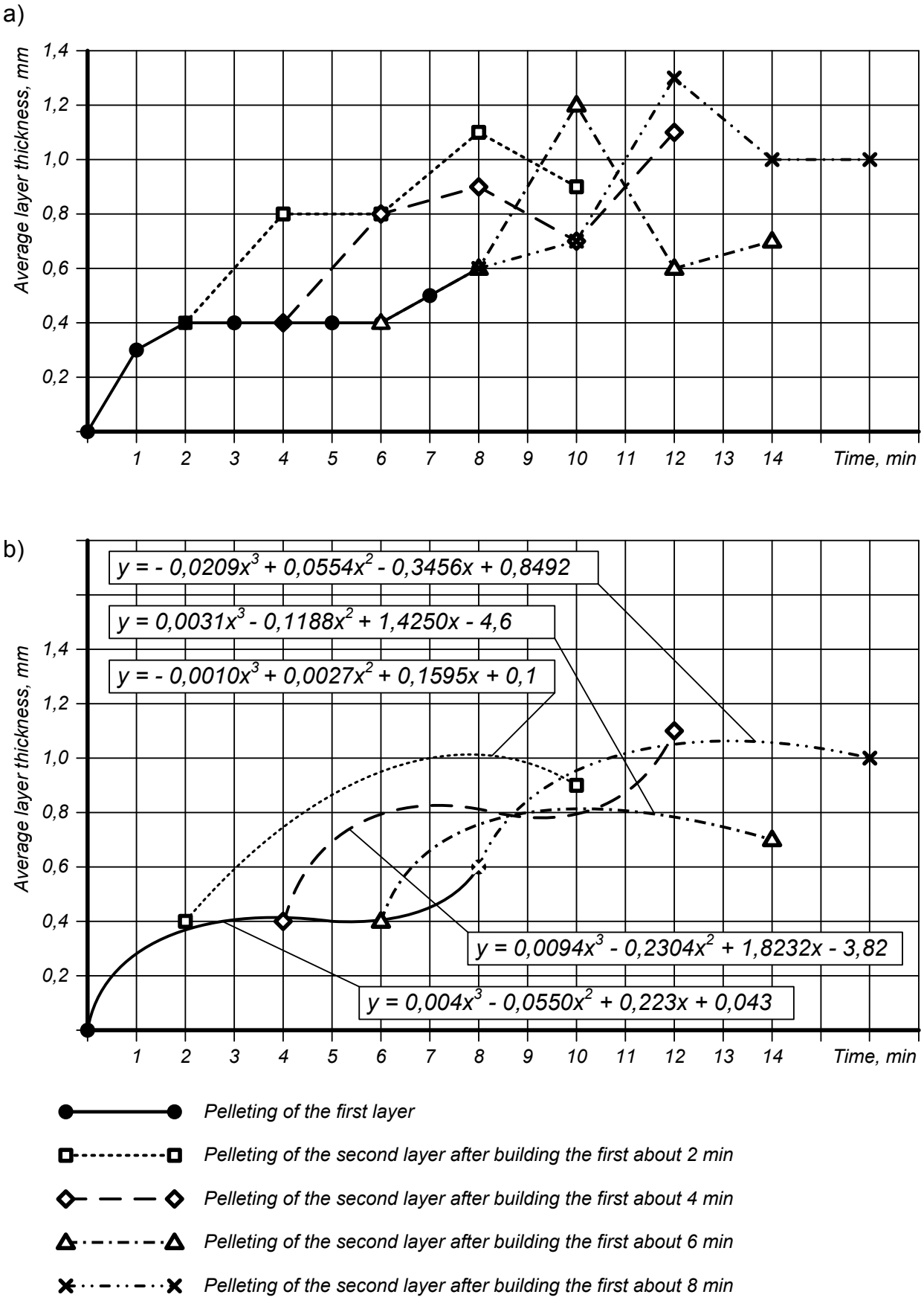


Figure 9 – The dynamics of layer building on seeds of Korean pine (a – an experimental information; b – leveling by the theoretical law)

Conclusions. The developed electromechanical drazhirator allows to carry out seed pelleting of coniferous seed of small (pine - *Pinus* L., larch - *Larix* Mill.) and large (Korean cedar pine - (*Pinus koraiensis* Siebold et Zucc.) sizes.

The pelleting mixture is tightly attached to the seeds, providing seedlings with nutrients. Further studies on the effectiveness of growing planting material from drained seeds are necessary. Optimal layer on seeds of Scots pine and Korean pine is achieved in 1 stage, with the build-up time of 7-8 minutes. If it is intended to build up a larger layer, it would be more appropriate to break up the coating process into 2 stages. So, the pelleted seeds must be unloaded, dried and begin to pellet again. The time for pelleting at 1 stage is 2 minutes, on the second stage is 6 minutes. It should be noted that when the thickness of the limiting layer is reached, it is possible to reduce the layer with further pelleting.

References:

1. Akimov R.Yu., Ostroshenko V.V., Pak A.V. Identification of components when pelleting seeds of coniferous trees. [Lesa i lesnoe khozyaistvo v sovremennykh usloviyakh]. Khabarovsk, FGU DalNIILH, 2011, p. 79-81. (In Russian).
2. Akimov R.Yu., Ostroshenko V.V. The seed pelleting pine cedar pine seeds (*Pinus koraiensis* Siebold et Zucc.) and Amur larch (*Larix amurensis)*. Agrarnyy vestnik Primor'ya [Agrarian Bulletin of Primorye], 2016, No. 4, P. 39 – 4 (In Russian).
3. Ostashevskiy I.Ya., Sushko I.I., Boyko V.P., Prigodskiy A.I., Budko V.S., Rostovtseva T.F., Vasilev N.I., Kolotnover B.Z., Polyakov A.A., Ba-rysh E.A., Petryaev V.P., Dronyuk S.A., Kovalchuk R.V. Apparat dlya drazhirovaniya semyan [Apparatus for seed pelleting]. Patent USSR, no. 3823693, 1997, 3 p.
4. S.V. Zamyshlyaev, V.Yu. Ostroshenko, S.V. Inshakov, V.V. O.S. Overview of devices for coating seeds of agricultural and forest crops. Agrarnyy vestnik Primor'ya [Agrarian Bulletin of Primorye], 2017, no. 2 (6), p. 17-20. (In Russian).
5. Kopytkov V. V. The use of composite polymer preparations for pelleting seeds of coniferous species. Doklady Natsional'noy akademii nauk Belarusi [Reports of the National Academy of Sciences of Belarus]. Minsk, 2013, vol. 57, no. 2, p. 119-123. (In Russian).
6. Maslakov E. L., Lebedenko E. L., Albert V.D. Develop a system of measures and establish standards for the collection, processing, storage and preparation of

pine and spruce seeds for sowing, providing 90% germination when growing seedlings with closed roots in greenhouse conditions. Zaklyuchitel'nyy otchet [Final Report]. Leningrad, 1985, 105 p. (In Russian).

7. Mukhin VD. Drazhirovanie semyan sel'skokhozyaystvennykh kul'tur [Farming of seeds of agricultural crops]. Moscow, Kolos. 1971, 93 p. (In Russian).
8. Ostroshenko V.V., Ostroshenko L.Yu., Akimov R.Yu. Rationing of works on pelleting seeds of coniferous trees. Vestnik KrasGAU [Bulletin of KGAU], 2013, no. 3, p. 139-142. (In Russian).
9. Usov V.N., Ostroshenko V.V., Akimov R. Yu. Investigation of the technological process of pelleting seeds of Korean pine (*Pinus koraiensis* Siebold et Zucc.) on an electromechanical dragee. Vestnik KrasGAU [Bulletin of KGAU], 2015, no.9, p. 145 – 149. (In Russian).
10. Brockwell, J. Studies on seed pelleting as an aid to legume seed inoculation. Coating materials, adhesives, and methods of inoculation. Australian journal of agricultural research. 1962, Vol. 13 (4), P. 638-649.
11. Devi, J. R., Selvaraj J. A. Seed pelleting and soil types on germination and vigor of seeds in ash gourd and ribbed gourd. Madras Agricultural Journal. – 1995, Vol.82, No. 2, P.75-77.
12. Moënne-Loccoz Y., Naughton M., Higgins P.. Effect of inoculum preparation and formulation on survival and biocontrol efficacy of *Pseudomonas fluorescens* F113. Journal of applied microbiology. 1999, Vol. 86, No. 1, P.108-116.
13. Srimathi P., Mariappan N., Sundaramoorthy L., Paramathma M. Effect of organic seed pelleting on seed storability and quality seedling production in biofuel tree species. Journal of Horticulture and Forestry. 2013, Vol. 5 (5), P. 68-73.
14. Ch. Min Ryu, J. Kim, O. Choi. Improvement of biological control capacity of *Paenibacillus polymyxa* E681 by seed pelleting on sesame. Biological Control. 2006, Vol. 39, No. 3, P. 282-289.
15. Rudolf, P.O. A test of pelleted Jack Pine seed. Journal of Forestry. 1950, Vol.48, No. 10, P.703-704.
16. Taylor A.G., Allen P. S., Bennett M. A., Bradford K.J. Seed enhancements. Journal of new seeds. 1998, Vol. 8, No. 2, P. 245-256.
17. Taylor A. G., Eckenrode C.J., Straub R.W. Seed coating technologies and treatments for onion: challenges and progress. Hort Science. 2001, Vol. 36, No. 2, P. 199-205.

18. Fraser J. W., Adams M. J. The effect of pelleting and encapsulation on germination of some conifer seeds native to Ontario. Canadian Forestry Service, Great Lakes Forest Research Centre, Sault Ste. Marie, Ontario. Information Report. No. O-X-319. 1980. 17 p.
19. Yadav M. S., Sharma S. K., Rajora M. P. Effect of pelleting material on seed germination and grassland productivity. Range Management and Agroforestry. 2000, Vol.21, No.2, P.121-127.

Bioindication Assessment of Environmental Quality in Vicinity of Underground Nuclear Explosion Sites on the Territory of the West Yakutia Using the Level of Fluctuating Asymmetry in Plants and Animals

E.G. Shadrina,
Institute for Biological Problems of Cryolithozone SB RAS,
Ya.L. Vol'pert,
677890, Russia, Yakutsk, Lenin av., 41,
Research Institute of Applied Ecology,
North-Eastern Federal University; 677000, Russia, Yakutsk, Lenin av., 1

Abstract: The study was performed in vicinity of two sites of underground nuclear explosion (UNE) that had been conducted in the West Yakutia in 1974 and 1978 and were followed by unplanned radioactivity releases. In contaminated sites γ-background level varied between 15 and 190 μR/h; the main contaminants being Cs-137 and Co-60. For the control we used a site 2 km from the UNE *Crystall* with γ-background of 4-6 μR/h.

For environmental quality assessment we used the level of fluctuating asymmetry (FA), which is small nondirectional deviations from the ideal symmetrical state that are caused by random errors in the course of ontogeny. The objects of research were the dwarf birch (*Betula exilis*) and red vole (*Clethrionomys rutilus*). Asymmetry level in the dwarf birch laminae was assessed by four venation features. In the red vole we used 10 phenes concerning cranial foramina. A total of 1044 leaves and 34 skulls have been examined.

FA level in the control site was 0.040. In vicinity of UNE *Kraton-3* within different distances from the explosion site FA levels in all examined sites were higher than in the control biotope and varied within 0.041-0.050. The most significant increase in FA (to 0.050) was registered on patches with γ-background level within 160-190 μR/h, difference with the control being statistically significant ($p < 0.05$ by Student's t-test). In vicinity of UNE *Crystall* the background radiation generally did not

exceed 5-15 μR/h, with separate patches reaching 50. FA level there was 0.037-0.042, but an increase in phenodeviant occurrence (deviations in lamina venation and uneven basal edge of lamina) was registered. Occurrence of FA manifestations in the red vole in vicinity of both UNEs was 0.42-0.43, while in the site located 250 m from the edge of the "dead forest" it was 0.37 and at a distance of 1-2 km it was 0.36.

An increase in FA level in plants and animals in vicinity of UNEs *Kraton-3* and *Crystall* indicates that occurrence of developmental stability disturbances in the area is increased, which reflects deterioration of habitats. On the whole, positive dependence of FA level on background radiation was registered, with the effect being most pronounced when the background is higher than 150 μR/h.

Keywords: permanently increased background radiation, bioindication, developmental stability, fluctuating asymmetry, dwarf birch, red vole.

INTRODUCTION

One of the most tangible forms of technogenic influence is global and local radionuclide contamination of environment. Negative impact of ionizing radiation on organisms is widely known. Given the capacity of radionuclides for bioaccumulation, even low levels of radiation pollution are dangerous with repeated exposure. Permanently increased background radiation (PIBR) was registered on the territory of many regions of the Russian Federation. It can originate from natural sources as well as from technogenic disasters and accidents, but generally it is mainly caused by the anthropogenic factor.

In applied environmental studies one of the most expedient bioindicational approaches is the method of estimating the environmental quality by the magnitude of deviations from organism developmental stability. The method is based on the assessment of intraindividual variability in morphological structures, such as the level of fluctuating asymmetry (Zakharov, 1987). Fluctuating asymmetry (FA) is small nondirectional deviations from ideal symmetrical state that have no adaptive value of their own and are caused by random errors in the course of ontogeny; primarily, prenatal ontogeny (Zakharov, 1987; Leary, Allendorf, 1989). In normal conditions such deviations are at their minimum but their level rises under any stressful influence, which leads to increasing asymmetry (Zakharov, 1987; Parsons, 1990, 1992; Palmer, Strobeck, 1992). Because resulting phenotypic differences are not true abnormalities and do not noticeably affect the individual's viability, this destabilization

of development turns out to be a highly sensitive indicator that enables a researcher to detect even slightest ontogenetic variations in response to small changes in environment. These changes in environment cannot affect developing structures directly and are mediated by the mother organism, which evidences that the asymmetry results not from different influence on the left and right sides of organs but expresses overall disturbance in the homeostasis of ontogeny (Zakharov, 1987; Palmer, Strobeck, 1992). Owing to that, FA level can be used to estimate the negative impact on the organism by the environment, e.g. social stress in animals or influence of pests, diseases, or climatic factors on plants (Zakharov, 1987; Moller, 1995; Martel *et al.*, 1999; Zakharov *et al.*, 2001; Shadrina & Vol'pert, 2014). Lately FA level has been widely used for assessment of environmental quality in cities and on territories affected by chemical and radiation pollution (Posledstviya …, 1996; Kryazheva *et al.*, 1996; Zakharov *et al.*, 2000 a; Shadrina *et al.*, 2003, 2008, 2009, 2012 a, b; Soldatova & Shadrina, 2007).

METHOD

Objects of our study were shrubs and small mammals. We collected 10 laminae from each bush of the dwarf birch in similar illuminance conditions. FA level was estimated by four lamina structure and venation features (Shadrina *et al*., 2008; Fig. 1); integral FA value was calculated as the absolute value of the mean relative difference per trait (Fig. 1). 1044 birch leaves have been examined.

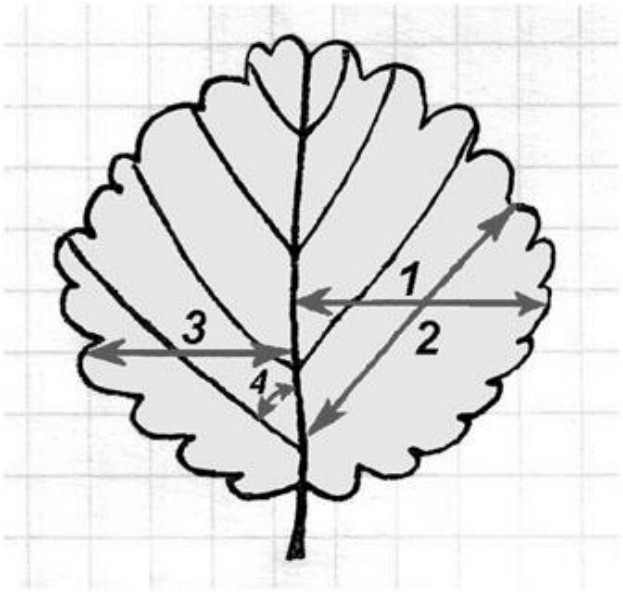


Figure 1 - Leaf measurements used for assessment of FA value

1, width of ½ of the leaf at its middle; 2, length of the first (counting from the leaf base) second-order vein; 3, width of 1/2 of the leaf at its bottom third; 4, angle between the mid-vein and the first from the leaf base second-order vein.

$$FA = ABS\left(\frac{L-R}{L+R}\right) \quad (1)$$

where: FA, fluctuating asymmetry value; ABS, absolute value; L and R, measurements of the left and right halves of the leaf.

In the red vole we counted the amount of cranial foramina and their arrangement; 10 phenes were chosen for the analysis; FA level, occurrence of fluctuating asymmetry manifestations (OFAM) was calculated as the proportion of asymmetrical traits to the total number of accounted traits (Zakharov et al., 2000 b). 34 red vole skulls have been examined.

The statistical treatment of the results was performed using standard methods, correlation analysis was carried out using Spearman's r, statistical significance of differences between samples was assessed using Student's t-test (Zaitsev, 1991).

DESCRIPTION OF THE STUDIED REGION

Two areas characterized by PIBR were selected for the research: territories of the West Yakutia in vicinity of underground nuclear explosions that were carried out in the second half of the 20th century and were followed by unplanned radioactivity releases.

From 1974 to 1987 on the territory of Yakutia 12 non-military underground nuclear explosions (UNE) took place; in two cases they caused the contamination of the environment by fission products.

In 1974, 2.5 km north of the Udachny city, underground nuclear explosion *Crystall* was conducted, it was 1.7 kiloton. The total of 8 such explosions was scheduled in order to create a dam for a tailings pond for the local ore-processing enterprise, but due to the unplanned release of the fission products the plan was abandoned. Eighteen years later the pit was filled up and covered with a sarcophagus (mound) up to 20 meters high (fig. 2).

Figure 2 – The UNE Crystall sarcophagus

In 1978 in the same region another nuclear explosion (*Kraton-3*) was conducted, it took place in the valley of the Markha River (a tributary of the Vilyui River), 577 meters underground. The explosion was 19 kiloton powerful and the objective was to study the Earth core by seismic sounding. Due to the errors committed when organizing the explosion, a release of fission products occurred. The cloud drifting downwind covered the drilling site, remote operating center, and the camp with 80 staff members. Radioactivity level during the passing of the cloud was more than 200 R/h, near-field hazardous zone (0.5 R) stretched to approximately 30 km. The area was severely contaminated; larch forest perished on the territory of more than 100 ha.

Both studied sites are situated in the subzone of larch sparse taiga (fig. 3). Stretches of “dead forest” created by the unplanned releases are clearly visible in vicinity of both shafts even today. At the present time, in vicinity of the UNE *Crystall* γ-background level is 5-15 μR/h, only on certain patches it reaches as high as 50 μR/h. The main contaminants are Cs-137 and Co-60. In vicinity of *Kraton-3* γ-background level varied between 40 and 120 μR/h within the radius of approximately 500 m from the shaft, there were small patches with the background of 160-190

μR/h, increased content of radionuclides was registered not only in soil but in plants and bottom sediments as well.

Figure 3 – The studied region

RESULTS AND DISCUSSION

For assessment of deviations in developmental stability we chose species widely distributed in the region: the dwarf birch (*Betula exilis*) and red vole (*Clethrionomys rutilus*). For the control we used a site 2 km from the UNE *Crystall* with γ-background of 4-6 μR/h.

In vicinity of UNE *Kraton-3* samples were collected at different distances from the shaft. FA values of the dwarf birch in all sites were higher than in the control and varied within 0.041-0.050 (Table, Fig. 4). It is worth noting that almost no difference

in FA level was found between the plants from the sites with γ-background from 20 to 120 μR/h. The background of 160-190 μR/h was recorded on small patches within the zone with general background of 80-130 μR/h. On these patches FA Value was 0.050, difference with the control being statistically significant ($p < 0.05$ by Student's t-test). The differences between the sites with the background of 20-120 μR/h were also noticeable but did not reach statistically significant levels. Correlation analysis showed pretty high level of correlation between developmental stability and radiation pollution; correlation coefficient for FA value was $\rho=0.86$ and for phenodeviant occurrence it was $\rho=0.75$, the main contributor to this dependence being the increased occurrence of anomalies in lamina venation (phenodeviant 1).

In vicinity of the UNE *Crystall* γ-background level was generally not higher than 5-15 μR/h, on certain patches reaching 50 μR/h. The level of deviations in developmental stability of the dwarf birch here was lower than in vicinity of the UNE *Kraton-3* and varied within 0.037-0.042.

Table. FA values of the dwarf birch in vicinity of the UNEs followed by unplanned releases in the Western Yakutia

No.	Sample collection sites	FA value			phenodeviants, %	
		n	M	m	Ph_1	Ph_2
Control						
1	Larch forest, 2 km from UNE *Crystall*	99	0.040	0.002	4.0	5.1
Kraton-3						
2	Larch forest, 500 m from the shaft, 20 μR/h	100	0.043	0.002	9.0	4.0
3	Dead forest, 450 m from the shaft	101	0.041	0.002	11.9	8.9
4	Dead forest, 250 m from the shaft	100	0.041	0.002	10.0	8.0
5	Dead forest, 150 m from the shaft	298	0.043	0.001	6.4	13.1
Crystall						
7	Left bank of the Bysyttakh stream, 260-270 m from the sarcophagus	96	0.038	0.002	7.3	5.2
8	Right bank of the Bysyttakh stream, 250 m from the sarcophagus	102	0.037	0.002	6.9	9.8
9	Dead forest, 5 m from the sarcophagus	148	0.042	0.002	12.2	15.5

Note: n, the amount of lamina; M, arithmetic mean; m, error; Ph_1, occurrence of venation deviations; Ph_2, occurrence of uneven basal edge of lamina.

Note that at the distance of 250-270 m from the sarcophagus FA value already is 0.037-0.038, which is even lower than in the control. In the plants that grow almost under the sarcophagus the value of this parameter is somewhat higher (0.042) but the difference with the control is not statistically significant. At the same time, phenodeviant occurrence at the distance of 250-270 m from the sarcophagus was somewhat higher than in the control but comparable, while in the plants growing near the sarcophagus phenodeviants were found significantly more often (Table).

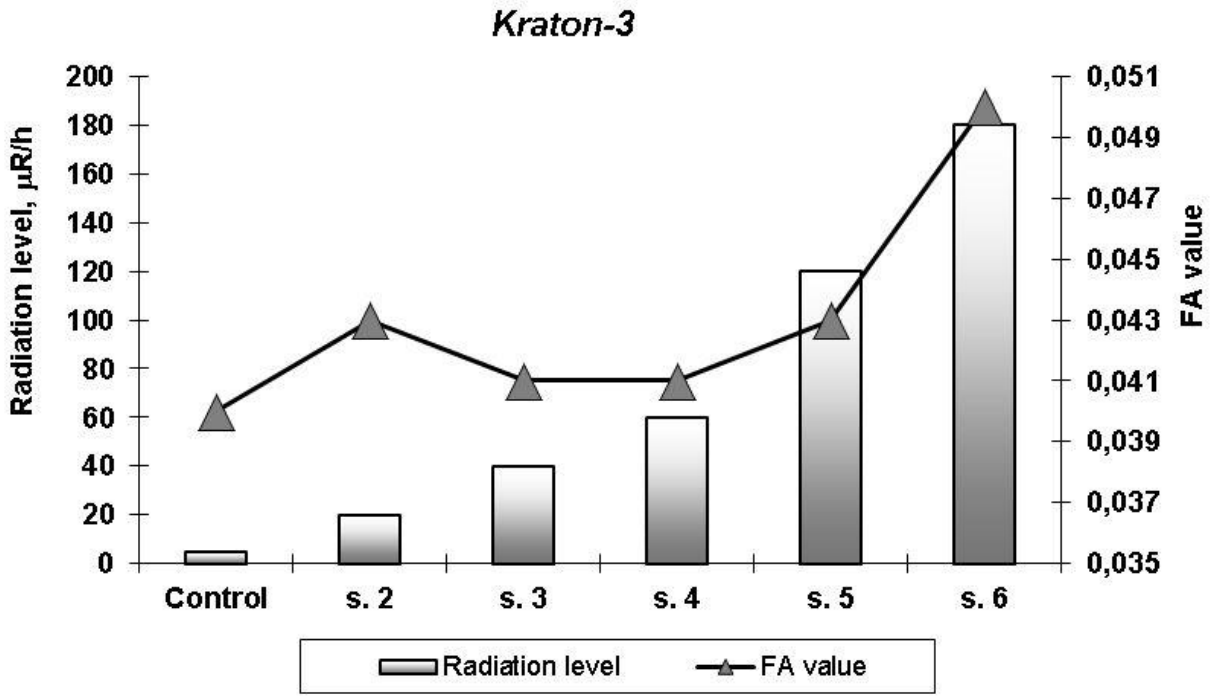


Figure 4 - Dependence of FA value in the dwarf birch on γ-background level

In the site 9 we collected leaves of 15 plants that grew along the perimeter of the sarcophagus. The analysis of variability of FA level within the sample showed significant spread of values: from 0.024 to 0.060. We found that somewhat higher values were exhibited by the plants situated close to the road leading to the sarcophagus (for those two plants average FA value was 0.046) and two plants on the northwestern side of the sarcophagus (0.058 and 0.060). On the whole, even among plants growing close to each other, the spread was very high. It is worth noting that in this area we had already recorded previously a similarly high spread of FA values in another species (*Salix viminalis*) (Shadrina, Shadrin, 2004). This can

serve as a circumstantial evidence of unfavorable conditions for the population, which would cause individual differences in resistance to be displayed more sharply.

Occurrence of fluctuating asymmetry manifestations (OFAM) in the red vole within the taiga zone normally is 0.25-0.35, but in the subzone of sparse taiga this parameter is higher, which appears to be connected with the pressure of unfavorable abiotic factors (Shadrina, Vol'pert, 2014). In vicinity of the studied UNEs, FA value in the red vole was 0.42-0.43, while at the site located 250 m from the edge of the "dead forest" zone it was 0.37, and at the distance of 1-2 km it was 0.36 (Fig. 5). However, it is worth noting that the differences between the control and contaminated territories did not reach statistically significant levels. It is probably explained by the small size of the sample due to the low abundance of small mammals on the studied territory (for contaminated territories n=14, for undisturbed ones n=20).

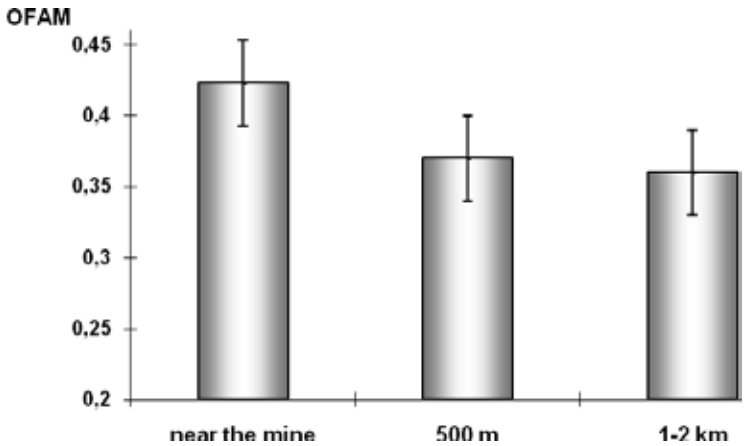


Figure 5 - Occurrence of fluctuating asymmetry manifestations in the red vole in the area of the UNEs followed by unplanned releases

On the whole, our results correspond to the data obtained by other authors. Deviations in developmental stability and rise in occurrence of fluctuating asymmetry manifestations and phenodeviants were registered on the territory of the Bryansk oblast in the area affected by the Chernobyl disaster (Posledstviya …, 1996).

Thus, increase in FA value in plants and animals indicates that developmental stability in conditions of PIBR is subject to disturbances even when the radiation level is low. On the whole, positive dependence of FA value on γ-background level was noted, most significant rise in it being observed when the γ-level is higher than 150 μR/h.

Acknowledgments. We thank Vitalii Danilov, Denis Shadrin, and Victoria Soldatova for their participation in collecting and processing the materials. This work was supported by the state commission No. 5.8169.2017/8.9 of the Ministry of Education and Science of the Russian Federation and the base project AAAA-A17-117020110058-4 of the Russian Academy of Sciences. This article was written at the conclusion of the project "Assessment and major tendencies in the changes of environmental, social and economic state, and human potential of the West economic zone of Sakha (Yakutia) Republic", Program for integrated scientific research in Sakha (Yakutia) Republic aimed at development of its productive forces and social service in 2016-2020".

References:

1. R.F. Leary and F.W. Allendorf. “Fluctuating asymmetry as an indicator stress. Chance use in protect nature”, Acta Zool. Fenica, 1989, vol. 4, pp. 214-217.
2. Palmer, R.A. and Strobeck, C., Fluctuating asymmetry: a measurement, analysis, patterns, Ann. Rev. Ecol. Syst., 1986, vol. 17, pp. 391–421.
3. Parsons P.A. Fluctuating asymmetry: an epigenetic measure of stress. - Biol. Rev. - 1990, N 65. - P. 131-145.
4. Parsons P.A. Fluctuating asymmetry: a biological monitor of environmental and genomic stress. – Heridity. – 1992, N 68. - P. 361-364.
5. Zakharov V.M. (1987). Asymmetry in animals. Moscow, Science: p. 216.
6. Kryazheva N.G., Chistyakova E.K. & Zakharov V.M. (1996). Analysis of developmental stability of the Japanese white birch under conditions of chemical pollution. Russian Journal of Ecology, 6: pp. 441-444.
7. Zakharov V.M., Chubinishvili A.T., Dmitriev S.G., Baranov A.S., Borisov V.I., *et al.* (2000 a). Environmental fitness: estimation practice. Moscow, Center for the environmental policy of Russia: pp. 318.
8. Posledstviya Chernobyl’skoi katastrofy: Zdorov’e sredy (Consequences of the Chernobyl Disaster: Environmental Health), Zakharov, V.M. and Krysanov, E.Yu., Eds., Moscow, 1996.
9. Shadrina E.G., Vol'pert Ya.L., Danilov V.A. & Shadrin D.Ya. (2003). Bioindication of mining industry impact on land ecosystems of the North (morphogenetic approach). Novosibirsk: Nauka. Siberian branch, pp: 110.

10. Shadrina E.G., Vol'pert Ya.L. & Danilov V.A. (2009). Values of developmental stability abnormalities in plants and animals as criteria of environmental quality in the area affected by mining industry facilities on the territory of Yakutia. Problems of Regional Ecology, 3: pp. 43-48.
11. Shadrina E.G., Vol'pert Ya.L., Alekseeva N.N., Danilov V.A. & Pudova T.M. (2012 a). Bioindicational assessment of changes in environmental quality caused by diamond mining facilities. Eurasian Mining, 2: pp. 84-87.
12. Shadrina E.G., Pudova T.M. & Soldatova V.Yu. (2012 b). Bioindication of environmental quality during exploration of raw hydrocarbon deposits (by the example of the South-Western Yakutia). Fundamental investigation, 4: pp. 206-211.
13. Soldatova V.Yu. & Shadrina E.G. (2007). Fluctuating asymmetry of the Japanese white birch (*Betula platyphylla* Sukacz.) as an indicator of environmental quality in a city. Regional Environmental Issues, 5: pp. 70-74.
14. Zakharov V.M., Baranov A.S., Borisov V.I., Valetsky A.V., Kryazheva N.G. *et al.* 2000 b. Environmental fitness: methods of estimation. Moscow: pp. 68.
15. Zaitsev G.N. (1991). Mathematical analysis of biological data. Moscow: Nauka: pp. 184.
16. Shadrina E.G., Soldatova V.Yu., Shadrin D.Ya., Kapitonova N.N. Evaluation of Environmental Quality by the Indices of Disturbance of Stable Development in Plants and Animals in the Conditions of Permanently Increased Radiation Background over the Yakutia Territory. Regional Environmental Issues, 2008, 2: pp. 91-95.
17. Moller A.P. 1995. Leaf-mining Insects and fluctuating asymmetry in elm *Ulmus glabra* leaves. Journal of Animal Ecology, 64: pp. 697- 707.
18. Martel J., Lempa K., Haukioja E. 1999. Effect of stress and rapid growth on fluctuating asymmetry and insect damage in birch leaves. OIKOS, 86: pp. 208-216.
19. Shadrina E., Vol'pert Ya., Soldatova V., Alekseeva N., Pudova T. Evaluation of Environmental Conditions in Two Cities of East Siberia Using Bio-indication Methods (Fluctuating Asymmetry Value and Mutagenic Activity of Soils) // International Journal of Biology; Vol. 7, No. 1; 2014. Pp. 20-32.
20. Shadrina E.G., Vol'pert Ya.L. Developmental Instability of the Organism as a Result of Pessimization of Environment under Anthropogenic Transformation of

Natural Landscapes // Russian Journal of Developmental Biology, 2014, Vol. 45, No. 3, pp. 117–126.

21. Zakharov V.M., Zhdanova N.P., Kirik E.F. & Shkil' F.N. Ontogenesis and population: evaluation of developmental stability in natural populations. Russian Journal of Developmental Biology, 2001, 32: pp. 336-351.
22. Shadrina E.G., Shadrin D.Ya. Assessment of Environmental Quality on Underground Nuclear Explosion Sites Using Measures of Developmental Instability in Plants and Animals. Radiation Safety of the Sakha (Yakutia) Republic / Second Conference of the Republic for Theory and Practice, 2003, Yakutsk, pp.

International Journal of Plant Sciences

Number 9 (2), November/December 2018

VOLUME 179