AGRICULTURAL RESEARCH UPDATES

VOLUME 24

Prathamesh Gorawala
Srushti Mandhatri
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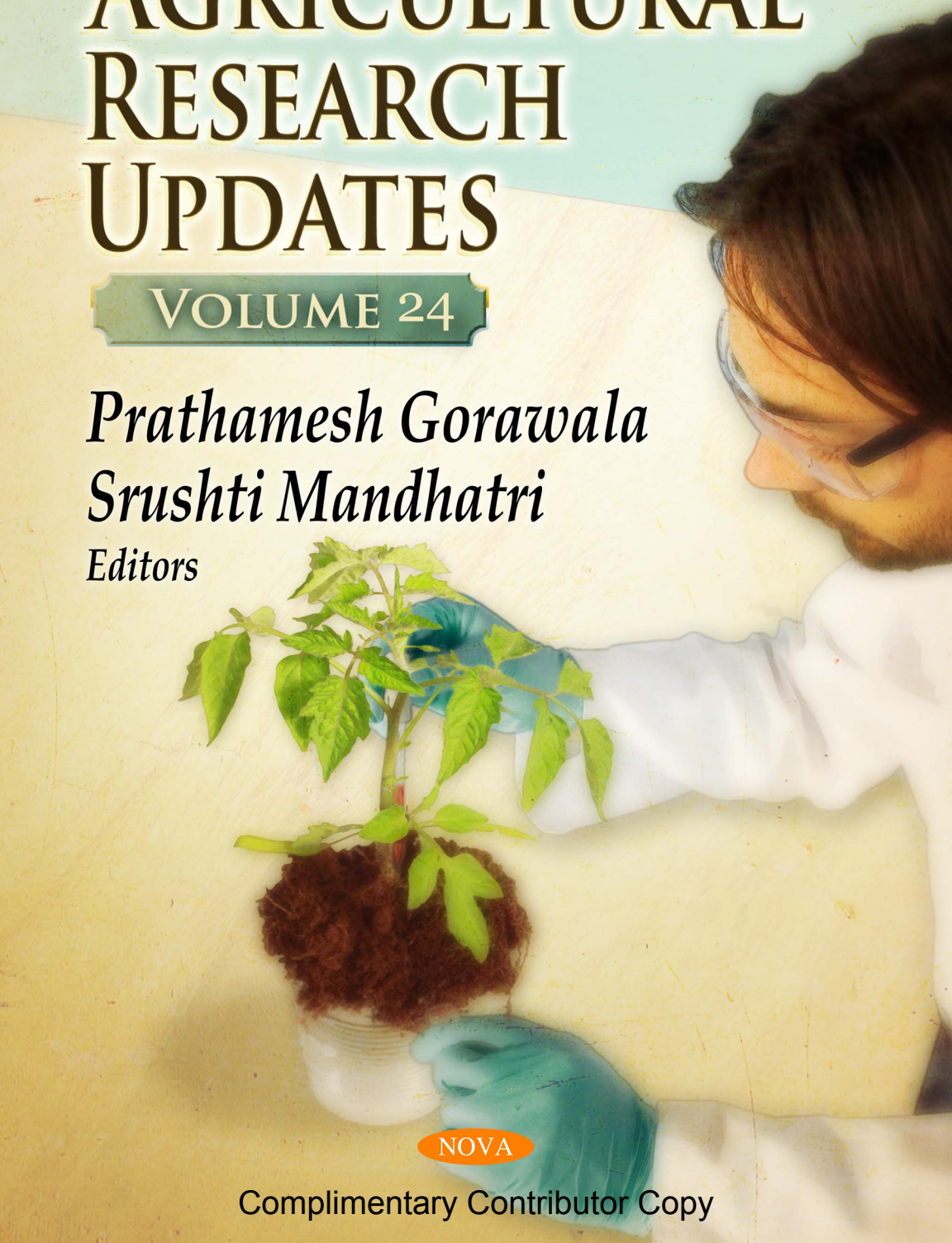

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PRATHAMESH GORAWALA
AND
SRUSHTI MANDHATRI
EDITORS

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CONTENTS

PREFACE

Agricultural Research Updates. Volume 24 begins with a mention of various features of maize chromosomes such as cytological mapping of meiotic chromosomes, heterochromatic knobs, centromeres, neocentromeres and DNA content in the maize genome. The authors report on the variability of tropical maize chromosomes and on chromosomal alterations in maize karyotype that occurred in callus cultures.

Next, the authors present an overview of reports addressing the successful development of rhizobial strains that are symbiotically more efficient by overexpression of single or multiple genes, directly or indirectly associated to the symbiotic process, such as nodulation, nitrogen fixation, stress response and phytohormone production genes among others.

The main objective is of the following chapter is to evaluate the performance of 42 olive varieties in low water conditions and to infer the relationships between the leaf parameters and other variables. The findings of this study provide supplementary information about the performance of olive varieties cultivated intensively under low water conditions and what leaf indices can be used to evaluate their responses.

A chained set of trials was conducted and described with the aim of studying whether corn fertilized by Arbuscular Mycorrhiza could influence crop yields, affect mycotoxins and modify poultry and pig meat

production, in the short term, or after a long period of storage. Two experiments conducted in corn fields treated with a commercial bio-fertilizer have shown that the yield can be improved and that the resistance to fungal attacks had significantly increased.

Additionally, the authors aim to develop and evaluate the cosmetic performance of emulsions containing oils and vegetable butter from Brazilian organic agriculture, verifying the influence of these emollients on the physical-chemical and sensory stability of cosmetic formulations. This is the first study investigating these raw materials from Brazilian organic agriculture in emulgel-type cosmetic formulations.

The next chapter describes a new no-contact method of determining bird mass that does not have the deficiencies of old-fashioned automatic weighing platforms in the poultry industry. The method is based on computer processing of digital top-down images of the birds automatically taken with a camera mounted on the ceiling of a poultry house.

Salacca edulis is known to possess flavonoids, flavanol, tannin, anthocyanin, ascorbic acid, alkaloid and antioxidants which may make it important in disease prevention. The authors aim to assess the long-term impact of *S. edulis* intake on specific populations and their functionality claims.

In the closing study, four native *Bradyrhizobium* isolates (NRI1, NRI2, NRI3, and NRI4) obtained from field trap cultures set in acidic soils of Embu and Tharaka Nithi County, Kenya and a commercial inoculant (USDA 110), were examined for acid tolerance on yeast extract mannitol agar at pH levels of 3, 5, 7 and 9. Moreover, the isolates were examined for nitrogen fixation potential in greenhouse bioassays using limed and non-limed field soil.

Chapter 1 - In this review, the authors mention various features of maize chromosomes such as cytological mapping of meiotic chromosomes, heterochromatic knobs, centromeres, neocentromeres and DNA content in the maize genome. The authors report studies on the variability of tropical maize chromosomes and on chromosomal alterations in maize karyotype that occurred in callus cultures.

Chapter 2 - Some rhizobia are able to establish effective symbiosis with legume species that have high agronomic value, such as soybean, chickpea or common bean, amongst others. This symbiotic relationship is a powerful alternative to the nitrogen fertilizers applied in cultivated soils, which besides being expensive, are often responsible for environmental problems, such as groundwater contamination. Therefore, rhizobial strains that are simultaneously highly efficient in nitrogen fixation, able to compete with native soil populations, persistent on the soil as well as adapted to endure different stress conditions in the field, have been selected over the years to be used as inoculants for legumes plants, aiming to improve their yields. Studies on the genetic manipulation of rhizobia, with the purpose to improve their symbiotic performance, are building up important knowledge that can be used to optimize these symbioses. Enhancing the rhizobia symbiotic performance by the overexpression of specific genes to improve their symbiotic effectiveness, nodulation efficiency, competitiveness and/or stress tolerance might represent a strategy to accelerate the development of more efficient and resilient inoculants. This chapter will present an overview of reports addressing the successful development of rhizobial strains symbiotically more efficient by overexpression of single or multiple genes, directly or indirectly associated to the symbiotic process, such as nodulation, nitrogen fixation, stress response and phytohormone production genes among others.

Chapter 3 - This work was made on 42 olive (*olea europaea*) varieties cultivated in northern Tunisia. The main objective is to evaluate their performance in low water conditions (LWC) and to infer the relationships between the leaf traits and the leaf and soil water variables. In line with these objectives, measurements and estimates of 15 leaf variables were made over two successive years (2015-2016), including the relative water content (RWC, %), the foliar tissue density (D, g/kg), the succulence (S, mg H_2O/cm^2), the sclerophylly (S_c, g/m^2), the water content at saturation (WCS, g H_2O/g DW), the specific leaf area (SLA, cm^2/g) and the water saturated deficit (WSD,%). Data (15 initial variables) were subject to the *Principal Component Analysis* (with the MATLAB program) to select the best indices. The variables S_c, WSD, WCS, RWC, S and D are found to be

suitable for most varieties to describe accurately their response to LWC. Comparative results showed that olive varieties use different means to cope with LWC. The cultivars Gerboui, Coratina and Souri are the most performant; they have high S_c, RWC and S values and low WSD and WCS records, which makes them highly performant. Manzanilla has high D, medium S_c but low RWC. The cultivars Chamchali, Chemlali and Ascolana showed low performance in this area, suggesting that the means used to withstand the lack of water are less effective. Arbequina provided low S_c and D records, which makes it less protected against water loss, but it has low leaf area, which contributes to balancing out the other negative features. On the other hand, multiple relationships were observed between the leaf indices and the water variables, which could be used in the future to identify the spatial variability of responses of olive to LWC. Relationships established between the leaf indices and the other water variables (leaf or soil) showed that most varieties follow the general trends observed between RWC and D, LA and leaf weights and D and S, with some exceptions (Verdal, Coratina...). These varieties behave differently under LWC. Results also showed that the volumetric soil moisture (H_v, %) is poorly correlated to RWC and S_c but it interacts well with SLA, which can accurately traduce the soil water status. To conclude, the findings of this study provide supplementary information about the performance of olive varieties cultivated intensively under LWC and what leaf indices are the best to evaluate their response to the lack of water. The authors consider that further research is needed to see if these performant varieties are also competitive at a productive level.

Chapter 4 - A chained set of symbiotic trials has been conducted with the aim of studying whether and how corn fertilised by Arbuscular Mycorrhiza (AM) and microbial consortia could influence crop yields, affect mycotoxins, and modify poultry and pig meat production, in the short term or after a long period of storage. Two experiments conducted in corn fields treated with a commercial bio-fertiliser have shown that the yield can be improved by +4 to +30% and that the resistance to fungal attacks had significantly increased. The secondary metabolites, fatty acid composition, NIRS properties, and electronic nose profiles were also

modified, with a substantial reduction in the oxidant power of -47% in the grain flour and -19% in the feed. The productive performances, as well as the slaughter and meat colour, were not modified after a fresh corn utilization by broiler poultry and heavy pigs, but the blood biochemical parameters in the poultry revealed a clear amelioration of the physiological functions and of the serum antioxidant capacity. After a twenty-one-month delayed utilization, substantial nutritive differences, related to shelf life and palatability, emerged, since the broilers fed the control diet showed a reduced intake of -26.7%, with a final body weight reduction of -27.7%. Therefore, a symbiotic farming provided by AM and microbial fertilisation may be considered a strategic tool for agronomic sustainability, resilience and for the nutritive usefulness of maize crops.

Chapter 5 - In recent years, the cosmetics industry has been focusing on the development of natural and organic cosmetics, aiming to manufacture products with greater quality and effectiveness, and to fulfil the diverse requirements of cosmetics regulations (Regulation of the European Commission, 2009). Certifiers like the IBD and ECOCERT, produce guidelines for these products. Here in Brazil however, the authors have no institution equal to the Ministry of Industry and Commerce in France, which is respected in more than 80 countries. From 2008 to 2012, Brazil grew 7.4% without the organic and natural cosmetics segment. Brazilian biodiversity products are undergoing a-n expansion process, following a worldwide trend of substituting synthetic products for agricultural raw materials. By organic cosmetics, the authors understand products composed of only natural ingredients, with a minimum of 95% raw materials, produced in accordance with the precepts of organic agriculture. There are also no preservatives, synthetic fragrances or petroleum derivatives used, as well as other components of non-natural origin. In this context, the objective was to develop and evaluate the cosmetic performance of emulsions containing oils and vegetable butter from Brazilian organic agriculture, verifying the influence of these emollients on the physical-chemical and sensory stability of cosmetic formulations. Emulgels were formulated containing *Syagrus coronata* (Licuri), *Dipteryx alata* (Baru) and *Pachira aquatica* (Munguba) oils, from

the Brazilian biomes Caatinga, Cerrado and Amazonia respectively, combined with *Virola surinamensis* (Ucuuba) vegetable butter, the thickener used for natural polymer xanthan gum,. These emulsion systems were submitted to accelerated stability tests (centrifugation, vibration and thermal stress), according to the cosmetic stability guide of the Brazilian National Health Surveillance Agency (ANVISA). The sensory potential (instrumental texture and *in vitro* scattering) of the emulgels in the post-preparation and post-aging accelerated periods was verified. pH and organoleptic characteristics were analyzed at the end of 90 days. All formulations developed were stable, with no presence of cream, flocculation or phase separation. The pH presented over the 90-day period was compatible with the body pH of the skin. Regarding sensory characteristics, the emulgels containing Licuri oil combined with Ucuuba butter showed greater spreadability as well as lower hardness and less adhesiveness. These results reflect a mild and pleasant sensory quality that is conferred by the greater low-level fatty acids in this emollient. Given the results observed, the authors can see that raw materials from Brazilian agriculture generate stable cosmetic products, which can be sensorially optimized, with characteristics that are inherent to the uniqueness of the grease composition. Additionally, this is the first study investigating these raw materials from Brazilian organic agriculture in emulgel-type cosmetic formulations.

Chapter 6 - Old-fashion automatic weighing platforms in the poultry industry require frequent maintenance and re-calibration and are intrinsically inaccurate because bigger birds occasionally avoid them. This chapter describes a new no-contact method of determining bird mass that is free of these deficiencies. The method is based on computer processing of digital top-view bird images automatically taken with image sensors mounted on the ceiling of a poultry house. Experimental data presented in this chapter prove that the average area of such images can be used to predict with good accuracy the average mass of birds. The chapter discusses: (1) hardware setting; (2) algorithm of computer processing of bird images and determining the average image area; (3) lab testing with test objects imitating birds; (4) field testing in two production houses at a

commercial poultry farm; and (5) future directions of research and development. The information provided in the chapter can be useful for agriculture professionals who are interested in: (a) inexpensive and maintenance free tools for continuous monitoring bird mass for timely adjustment of feeding process to keep it on target and thus maximize output and minimize feed conversion ratio and (b) paradigm change in bird's mass monitoring technology that reduces grower's maintenance burden and makes mass monitoring a daily routine in every poultry farm.

Chapter 7 - *Salacca edulis* is known to possess flavonoids, flavanol, tannin, anthocyanin, ascorbic acid, alkaloid and antioxidants. This may make it important in disease prevention. The fruit extracts have high levels of phenolic compounds with anti-proliferative and antioxidant properties, including chlorogenic acid, (-)-epicatechin, singly-linked pro-anthocyanidins which exist as dimers through hexamers of catechin or epicatechin. General inspection of the literature suggests that *S. edulis* has the ability to diminish the generation of fibrinogen which could subsequently reduce the risk of coronary atherosclerosis. The fruit has also been accredited with the ability to inhibit proliferation and induce selective cytotoxicity and apoptosis in cancer cells. In another study *S. edulis* has been shown to possess strong anti-hyperuricemic capacity. However, the long-term impact of *S. edulis* intake on specific populations and their functionality claims has not been fully evaluated. Although several anti-proliferative effects which are based on epidemiological studies have been explained, the mechanism of their actions is not fully understood.

Chapter 8 - Within the rhizospheric soil, rhizobia frequently encounter various abiotic stresses that affect their growth, initial steps of symbiosis with legumes, and their capacity of nitrogen fixation. In sub-Saharan Africa (SSA), acidic soils that characterize many agroecosystems are a major impediment to biological nitrogen fixation. Moreover, this is exacerbated by poor soil health management practices and lack of soil testing services by resource limited smallholder farmers. Consequently, this has led to unabated poor crop production including soybean, which is an important cheap source of proteins to both man and livestock. Therefore, identification of most symbiotically effective and acid tolerant

native bradyrhizobia isolates would lead to development of more efficient and low-cost inocula that are widely adopted by smallholder farmers in SSA. To realize this purpose, four native *Bradyrhizobium* isolates (NRI1, NRI2, NRI3, and NRI4) obtained from field trap cultures set in acidic soils of Embu and Tharaka Nithi County, Kenya and a commercial inoculant (USDA 110), were examined for acid tolerance on yeast extract mannitol agar at pH levels of 3, 5, 7 and 9. Moreover, the isolates were further examined for nitrogen fixation potential in greenhouse bioassays using limed and non-limed field soil. The experiment was set in a complete randomized design with liming as the main factor, inoculated soybean as the sub factor and replicated three times. After 28 days, the crop was harvested and assessed for nodulation, shoot and root dry weight. Interestingly, most native isolates tolerated pH of 5 while the commercial inoculant tolerated pH level of 9. All the isolates were sensitive to pH of 3. Soybean inoculated with the commercial bradyrhizobia showed significantly higher ($p<0.001$) shoot dry weight, nodule number and dry weight, compared to those inoculated with the native bradyrhizobia. Besides, liming of soil enhanced soybean nodulation and growth across all the bradyrhizobia inoculants. This study forms an important step towards the use of most effective rhizobial inoculants which are well adapted to different local agro-climatic conditions.

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Chapter 1

KARYOTYPE VARIABILITY IN MAIZE

Margarida L. R. Aguiar-Perecin[1,*], PhD
Janay A. Santos-Serejo[1,2], PhD
and Mateus Mondin[1], PhD
[1]Department of Genetics, Luiz de Queiroz College of Agriculture,
University of São Paulo, Piracicaba, SP, Brazil
[2]Embrapa Cassava and Fruits,
Brazilian Agricultural Corporation,
Cruz das Almas, BA, Brazil

ABSTRACT

In this review, we mention various features of maize chromosomes such as cytological mapping of meiotic chromosomes, heterochromatic knobs, centromeres, neocentromeres and DNA content in the maize genome. We report studies on the variability of tropical maize chromosomes and on chromosomal alterations in maize karyotype that occurred in callus cultures.

[*] Corresponding Author Email: mlrapere@usp.br.

Karyotype Variability in Tropical Maize Inbred Lines and Hybrids

The identification of chromosomal features and the characterization of the maize karyotype and genome structure have progressed extensively since the development of procedures to identify maize meiotic chromosomes in the 20th century. Classical cytogenetics was carried out by the observation of pachytene chromosomes. The somatic chromosomes have been identified by the C-banding procedure. The characterization of maize somatic and pachytene chromosomes was improved with the development of fluorescence *in situ* hybridization (FISH) using tandemly repeated satellite and single-copy sequences as probes. Chromosome structures such as centromere, knobs, neocentromeres and B chromosomes have been extensively investigated. Here, we report a study of somatic chromosomes of sister inbred lines, derived from a tropical maize population (Jac Duro [JD]) and hybrids between them that were investigated by FISH mapping of satellite DNAs. Heterochromatic knobs visible at pachytene were coincident with 180-bp FISH signals and C-bands on somatic chromosomes. Their number was variable among lines. Small FISH signals of the 180-bp knob repeat on the tip of chromosomes and the signal of subtelomeric 4-12-1 clone were invariant and useful for the identification of specific chromosomes. The centromere position of chromosomes 2 and 4 differed from the patterns reported for standard maize lines. Somatic chromosomes of a JD line and the commonly used KYS line were compared in a hybrid of these lines. The pachytene chromosomes with FISH signals that identified chromosomes 2 and 4 were fully synapsed. The results raise questions on the meiotic pairing of homologous chromosomes possibly differing in their content of repetitive DNA and contribute to the knowledge of maize global diversity.

Involvement of Knobs in Chromosome Breakage in Maize Callus Cultures

The size and number of heterochromatic knobs are variable among races and they may be present in each of the 10 chromosomes of the complement at fixed locations on the chromosome arms in modern maize. Breakpoints resulting in chromosome alterations associated with heterochromatic knobs have been detected in maize plants regenerated from callus culture. Evidence of mitotic instability in callus culture was demonstrated in studies showing that sister chromatids were held together at knob sites, forming bridges in anaphases. After breakage, a breakage-

fusion-bridge (BFB) cycle was initiated and after some mitoses, the chromosomes healed. FISH signals of telomeric sequences on the broken chromosome arms of regenerated plants provided evidence of *de novo* telomere formation. A study of the stability of chromosomes 7 and 9 in C-banded metaphases from subcultures of a callus culture, collected during a period of 30-42 months, is reported here. Chromosome alterations involving knobs were observed. The aberrant chromosomes were stable in the subcultures, thus providing evidence of broken chromosome healing. The results are of interest to investigations on mechanisms that alter the chromosomes during evolution.

Keywords: maize, karyotype, heterochromatic knobs, neocentromeres, genome, DNA content, callus culture, breakage-fusion-bridge cycle

INTRODUCTION

The identification of maize chromosomes and characterization of the karyotype have progressed since the pioneering work of McClintock (1929). The number of chromosomes is 20 and during decades, the maize karyotype analysis was based on observations of the pachytene chromosomes obtained from pollen mother cells. The early cytological maps were constructed based on the identification of relative chromosome length, arm ratio, chromomere and heterochromatin patterns (Longley, 1939; Rhoades, 1950; Dempsey, 1994 Neuffer et al., 1997). Rhoades (1978) described the following structures containing heterochromatin: chromosomal knobs, centric heterochromatin (heterochromatin adjacent to the centromeres), B chromosomes, abnormal chromosome 10 and heterochromatic nucleolus organizing region localized on chromosome 6.

Maize cytogenetics has been extensively reviewed (Carlson, 1988; Birchler *et al*., 2004; Birchler and Bass, 2009; Dawe, 2009). In this review, we focus on characteristics of the maize karyotype and report as illustration, studies on the karyotype of tropical maize lines and chromosomal alterations in callus cultures.

MEIOTIC CHROMOSOMES

The analysis of pachytene chromosomes has produced a description of a general meiotic karyotype (Rhoades, 1950; Dempsey, 1994; Neuffer et al., 1997). This contribution allowed the genetic linkage groups to be associated with the respective chromosomes and led to many findings ranging from the demonstration of the cytological basis of the crossing over (Creighton and McClintock, 1931) to the organization of collections of trisomics, monosomics, reciprocal translocations (A-A translocations), inversions and deficiencies. The cytological position of translocation breakpoints along the pachytene chromosomes was mapped (Longley, 1961; Beckett, 1978). These stocks are available from the Maize Genetics Cooperation Stock Center at the University of Illinois (http://maizecoop.cropsci.uiuc.edu/) and have been important cytogenetic tools. The B-A translocations are another important tool. They involve interchanges between the supernumerary B chromosome and various arms of the A set. The B chromosome is found in some maize lines and contains no known genes. The number of B chromosomes can vary because they suffer non disjunction at the second pollen mitosis. All these collections have been important as genetic markers and to the possibility to make inferences regarding the positions of gene loci in the physical chromosomes (for details see McClintock, 1930, 1950; Coe, 1994; Freeling and Walbot, 1994; Birchler *et al*., 2004; Birchler and Bass, 2009).

Recently, maps based on fluorescence *in situ* hybridization (FISH) data localizing repetitive DNA sequences have been developed (Chen *et al*., 2000). Also, single-copy sequences were mapped (Sadder et al., 2000) and using marker-selected sorghum (*S. propinquum*) BAC clones (Koumbaris and Bass, 2003; Amarillo and Bass, 2007). Fifteen probes were visualized on chromosome 9. They were produced as pooled polymerase chain reaction products based on sequences of genetic markers or repeat-free portions of mapped bacterial artificial chromosome (BAC) clones. The cytological positions of most sequences correspond on the pachytene, somatic and finger print contig (FPC) maps (Danilova and Birchler, 2008). The integration of restriction fragment length polymorphism (RFLP) and

FISH maps was achieved using Core Bin Marker *loci*. Because the RFLP probes are below the FISH detection limit, the authors used BACs from sorghum, a small relative of maize, thus with a lower content of repetitive DNA, as surrogate clones for FISH mapping (Figueroa *et al*., 2011).

Besides maps of DNA sequences visualized by FISH, maps of recombination nodules (RN) have been developed. The cytological map units have been referred as centiMcClintock (cMC), i.e., 1 cMC refers to 1% of the length of the chromosome arm upon which a given locus resides (Lawrence et al., 2006). Recombination nodule distribution data was based on the observation that RNs are closely correlated with crossing over, and because they are observed by electron microscopy of synaptonemal complexes (SCs) in extended pachytene chromosomes. A SC karyotype was prepared in which each SC was identified by relative length and arm ratio and related to the proper linkage group using inversion heterozygotes (Anderson *et al*., 2003). Cytological maps of crossing over based on RNs were used to predict the physical position of genetic markers on each of the 10 chromosome of maize. Predictions for chromosome 9 were tested using genetically mapped, single copy markers that were independently mapped on pachytene chromosomes using *in situ* hybridization. The correlation between predicted and observed location was very strong, indicating a virtual 1:1 correspondence (Anderson *et al*., 2004). Pachytene FISH maps developed for six maize chromosomes, using fragment length polymorphism marker-selected *Sorghum propinquum* bacterial artificial chromosomes (BACs), were merged into one composite karyotype for direct comparative analysis with recombination nodule-predicted cytogenetic, genetic linkage and B 73 genomic physical map using the relative marker positions of the *loci* on all the maps. The cytogenetic pachytene FISH map positions resembled the nodule-based predictions. The cytogenetic and linkage comparisons agreed with previous studies showing a decrease in marker spacing in the pericentromeric heterochromatic region on the genetic linkage maps. On the other hand, considerable variation was observed between the relative arm positions of *loci* when comparing the cytogenetic FISH map to the B 73 genomic physical maps (Figueroa and Bass, 2012).

THE MAIZE GENOME

The maize genome was sequenced using the B73 line as reference genome version 1 (B73 RefGen_v1) (Schnable et al., 2009). The full complement of maize transposable elements (TEs) was identified from B73 RefGen_v1, which includes class II DNA TEs and an abundance of class I RNA TEs (SanMiguel and Bennetzen, 1998). Almost 85% of the B73 RefGen_v1 consist of TEs. The size of the maize genome has expanded dramatically (to 2.3 gigabases) via the proliferation of long terminal repeat retrotransposons (SanMiguel and Bennetzen, 1998).

The maize genome size evaluated by the analysis of DNA content through image cytometry is variable. The genus *Zea* shows both inter and intra-specific variations in DNA amount (Laurie and Bennett, 1985; Poggio et al., 1998; Bennett and Leitch, 2005; Diez *et al*., 2013.). It has been proposed that this variation is due to differences in heterochromatin amount, mainly located in the knobs, as well to the presence of B chromosomes (Laurie and Bennett, 1985; Rayburn et al., 1985; Poggio et al., 1998; Rosato et al., 1998). Recently, Realini *et al*. (2015) did not found a significant correlation between genome size and percentage of heterochromatin in maize populations from Northeastern Argentina. This would be due to other non-coding repetitive DNA sequences that are contributing to the genome size variation, like retrotransposons, which in maize make up over 70% of the nuclear genome (SanMiguel and Bennetzen, 1998). In addition, Jian *et al*. (2017) observed a moderate positive correlation between genome size and the 180-bp knob abundance determined by high-throughput sequencing in tropical and temperate maize inbred lines.

Silva *et al*. (2018) identified the individual mitotic chromosomes measuring the chromosomal DNA amount of a maize variety. Chromosomes displaying knobs had higher DNA amount. The chromosomal DNA amount of the variety analyzed was higher in all chromosomes compared to sequencing data of the B 73 reference genome. This result may reflect an excessive DNA amount in the variety studied or an underestimation of the bp number in sequencing, for the genome of *Z.*

mays has a high amount of repetitive DNA which is often not considered in the sequencing-based assembly.

CENTROMERES

Centromeres are chromosome structures that bind to kinetochores, which bind to spindles and move the chromosomes during anaphase in mitosis and meiosis. Most centromeres in plants and animals are characterized by a simple repeat array. In maize the major tandem repeat is a 156 bp sequence known as CentC. (Ananiev *et al*., 1998c). The same repeat is found at each chromosome, but centromeres of related species evolved very quickly and show variation among them. For instance, this variation is found among the grasses. In maize, the extension of the CentC arrays varies among the chromosomes from about < 100 kb to several thousand kb. These values were evaluated in analyses of stretched DNA fibers (Jin *et al*., 2004). Although repeat arrays are found at centromeres in plants and animals, evidences have shown that functional kinetochores can be formed in regions that have no sequence similarity to centromeres. But it has been considered that the repetitive structure of centromeric DNA have a strong impact on centromeric stability over evolutionary time (Dawe and Henikoff, 2006; see Dawe, 2009 for details). In addition, a chromosome 4-specific repeat was found in a procedure that showed homology between this repeat and B chromosome centromere sequences (Page *et al*., 2001).

Grass centromeres contains a class of retroelements known as Centromeric Retroelements (Jiang *et al*., 2003) that have been designated as CR elements. Maize CR elements have been called CRM. CR elements have been shown to be portions of Ty3/Gypsy retroelements (Presting et al., 1996). CR elements are present at centromere and pericentromeric regions, which are different at the cytological chromatin level (see Dawe, 2009). Nagaki *et al*. (2003) sequenced two maize bacterial artificial chromosome (BAC) clones, which consisted of defined centromeric DNA, composed of satellite sequences and retrotransposons that can be classified

as centromere specific and noncentromere specific. The CR elements inserted randomly into either CentC repeats or other retrotransposons.

The nature of centromere specification is epigenetic. A protein known as CENH3 is the fundamental protein of the kinetochore. CENH3 is similar to H3 in the core domain that binds to other histones, but differs in the N-terminal domain. (Henikoff et al., 2000). To determine if the major centromeric DNAs CentC and CRM elements are part of the functional centromere/kinetochore complex, an antiserum to CENH3 was generated. With specific antisera, chromatin immunoprecipitation (ChIP) was employed to investigate the DNAs that interact with the kinetochore. By this method, CentC and CRM elements were shown to interact specifically with CENH3 (Zhong et al., 2002). CENH3 is thought to recruit many of the centromeric protein required to chromosome movement.

Considering that the DNA sequence of the centromere has little or no role in perpetuating kinetochores, this aspect is a striking example of genetic information being transmitted in a manner that is independent of DNA sequence (epigenetically) (see Dawe, 2009). It has been shown that RNA transcribed from centromere remains bound within the kinetochore region, and this population of RNA is thought to be part of the epigenetic marking system. A genetic and biochemical study of CENPC, a key inner protein, showed that the DNA-binding reaction is sensitive to single-stranded RNA. Long single-stranded nucleic acids strongly promoted the binding of CENPC to DNA. The types of RNAs that stabilize DNA binding matched in size and characters to RNAs present on kinetochores *in vivo*. The data suggested that centromeric RNA helps to recruit CENPC to the inner kinetochore (Du *et al*., 2010).

Another interesting question concerning centromeres is their size variability across species. Zhang and Dawe (2012) estimated centromere sizes in ten species of the grass family including rice, maize and wheat. Measurements were made using an antibody to rice CENH3. A clear linear relationship between total centromere size and genome size was found.

Centromere inactivation has been observed in plants containing B-A translocation chromosomes. Dicentric minichromosomes, derived from the chromosome type of breakage-fusion-bridge (BFB) cycle described by

McClintock (1939; 1941), contained two regions with DNA sequences typical of centromeres. Immunolabeling of CENH3 revealed only one functional centromere per chromosome (Han et al., 2006). One of the mechanisms involved in this transition between activated and inactivated state was observed in plants containing the supernumerary B chromosome. Using a fiber-based technique that can be used to assess the level of cytosine methylation associated with repetitive DNA sequences, Koo *et al*. (2011) revealed that the DNA sequences in the normal B centromere exhibited hypomethylation. This methylation pattern was slightly changed when the B chromosomes was transferred to oat as an addition chromosome. An inactive version of this same chromosome showed hypermethylation, indicating that the inactive centromere was modified into a different epigenetic state at the DNA level.

The study of the centromere sequences provided evidence of frequent recombination events resulting in the formation of partial retrotransposons, deletion within retrotransposons, chimeric retrotransposons, segmental duplications including higher orders of CentC repeats, a deleted CentC monomer, centromere-proximal inversions and insertion of mitochondrial sequences. These observations resulted from a study involving nearly complete assembly of the 1.85 Mb maize centromere 10 from inbred B73, using PacBio technology and BACs from the reference genome project. (Wolfgruber *et al*., 2016).

Maize (Zea mays mays) is an attractive model for studying centromere position and sequences. In the genomes of *Z mays mays* and wild relatives (Z. *parviglumis*, *Z. mays mexicana* and *Z. huehuetenangensis*), the major satellite sequence CentC has a tenfold range in abundance, and sequences of individual CentC copies are highly polymorphic. The distributions of mutations in CentC copies are strikingly similar among species and subspecies. Polymorphic CentC copies are strongly enriched in CENH3 ChIP samples despite their dissimilarity to the consensus sequence (Gent *et al*., 2017).

KNOBS AND NEOCENTROMERES

Knobs are darkly stained structures observed in pachytene chromosomes and mitotic prophases. The number of knobs is variable and they may be present in each of the ten chromosomes of the complement at fixed locations on the chromosome arms, in modern maize and its relatives, including species of *Zea* (teosintes) and *Tripsacum*. Knobs are found at several positions near the ends or in interstitial sites of the maize chromosome arms, suggesting the occurrence of strong selection pressure at these locations. They have been used as chromosome markers and are useful for chromosome identification. They are highly variable among the maize races. Some lines have no visible knobs and others can have many knobs (McClintock, 1978; McClintock *et al.*, 1981). In their survey of maize races, McClintock (1978) observed characteristic chromosomal patterns that suggested the existence of karyotype groups in some geographic regions. For example, in the so-called "Andean complex," most of the examined race collections that were made at highlands in South America had the same or nearly the same karyotype characteristics, including small knobs on the long arm of chromosome 6 in position 3 (K6L3) and on the long arm of chromosome 7 (K7L). Knobs can also vary in their molecular structure among chromosomes. They are composed primarily of 180-bp repeats (Peacock et al., 1981). Recently another knob repeat called TR-1 element was identified (Ananiev *et al*., 1998a). This repeat is present in some knobs but in a lower proportion in comparison with the 180-bp unit. Some knobs contain a mixture of both sequences (Ananiev *et al*., 1998a; Kato *et al*., 2004, Albert *et al*., 2010; Ghaffari *et al*., 2013). Knobs also contain retrotransposons (Ananiev *et al*., 1998b; Mroczek and Dawe, 2003).

Knobs have been located on cytogenetic maps based on pachytene analysis (Longley, 1939 Rhoades, 1950; Dempsey, 1994; Neuffer *et al*., 1997). Recently they have been placed on the genetic map (Lawrence *et al*., 2006), and mapped relative to the maize reference genome assembly (Ghaffari *et al*., 2013). The authors used FISH to map visible knobs in recombinant inbred lines, and three knobs from the B73 inbred were

accurately placed on the B73 reference genome. These data demonstrated that knobs lie in gene-dense regions, generally high recombination areas. It was also revealed that knobs in heterozygous condition can reduce local recombination (Ghaffari *et al*., 2013).

The genetic effects of knobs have been discussed in many reviews (Rhoades, 1978; Aguiar-Perecin *et al*., 2000 and Dawe, 2009). One interesting characteristic of the knobs is their activity as neocentromeres resulting in meiotic drive. This meiotic event is a mechanism by which small regions of the genome are preferentially transmitted to the progeny. Meiotic drive occurs in plants that have an uncommon form of chromosome 10, the abnormal chromosome 10 (Ab 10). This chromosome has a large knob near the tip of the large arm and three conspicuous chromomeres on a proximal region (Longley, 1938). In the presence of this chromosome, the knobs are converted in neocentromeres and move forward and arrive at spindle poles in advance of the centromeres during anaphase I and II of female meiosis (Rhoades and Vilkomerson, 1942). The Ab10 is also preferentially segregated to progeny when crossed as a female (Rhoades, 1942). The preferential segregation or meiotic drive occurs only in plants that are heterozygous for a knob. Preferential segregation proceeds because recombination between the knob and the centromere occurs, resulting in chromatids known as heteromorphic dyads, which are oriented towards the pole, as neocentromere activity commences at meiosis I. In meiosis II, the chromosomes are oriented with the knobs facing towards the outside of what will become the linear tetrad. Then, the upper three megaspores die, leaving only the bottom cell containing the Ab10 or knobbed chromosomes to become the egg. In this connection, it is interesting to note that the origin of knob polymorphism in maize and its wild teosinte progenitors, including number and size, has been discussed in several reports. Buckler *et al*. (1999) proposed that meiotic drive was responsible for the evolution of maize knobs.

It has been suggested that the neocentromere movement on the spindle is different from the kinetochore mediated movement. Neocentromeres lack the three key kinetochore proteins CENH3, CENP-C and MAD2 (Dawe *et al*., 1999; Yu, 2000; Dawe and Hiatt, 2004). This suggests that

Ab 10 encodes one or more novel trans-acting factors that act specifically on knobs. Neocentromeres move along the side of microtubules, whereas the kinetochores interact with micro tubules in an end-on fashion (Yu et al., 1997). While the centromeres/kinetochores are moving slowly towards the poles, neocentromeres rapidly slide along microtubules sidewalls.

The Ab structure has been extensively investigated and the long arm can be subdivided into four major domains: the distal tip, the large knob, the central euchromatin and the differential segment. The knob contains almost entirely the 180-bp knob repeats, while the differential segment contains three chromomeres composed of TR1 (see Dawe 2009).

The functional portion of the Ab 10 is referred as Ab 10 haplotype. The neocentromere activity has been investigated and a mutant screen has been developed to identify genes that control meiotic drive. This screen resulted in the finding of mutants and deletions, as well as a set of terminal deficiencies (Hiatt and Dawe, 2003). Data also show that the central euchromatin or "shared region" contains inversions of genes located on chromosome 10. Recombination between Ab10 and N10 are blocked due to these nested inversions that include genes from N10. Information on this issue can be found in Dawe (2009).

Additionally, Kanizay *et al*. (2013) described the behavior of a large knob on chromosome 10 called K10L2, composed entirely of the TR-1 repeat and linked to a strong activator of TR-1 neocentromere activity. K10L2 shows weak meiotic drive when paired with the normal chromosome 10 (N10), and reduces the meiotic drive exhibited by Ab10 in Ab10/K10L2 heterozygotes. These data suggested that both the TR-1 and knob 180-bp repeats exhibit meiotic drive and that the two repeats can operate in competition with each other.

Recently Dawe *et al*. (2018) described the structure of the Ab 10 chromosome, showing that the breakpoint of the deficiencies (Df [L]) is located on the tip of the chromosome. The authors identified a cluster of eight genes on Ab 10, called Kinesin driver (*Kindr*) complex that are required for both neocentromeres motility and preferential transmission. This complex is also located on the tip of the chromosome. The genes are

similar in overall structure, but can be divided into four classes by SNP profile, and three of the classes are expressed.

Mutant screens identified two suppressors of meiotic drive mutants (Ab10-smd1 and Ab10-smd12) that lack neocentromere activity but retain a cytologically intact Ab chromosome (Dawe and Cande, 1996). Dawe *et al.* (2018) showed that these mutants cause uniform silencing of all the genes of the *Kindr* complex. This study showed that Ab10-smd1 and Ab10-smd12 are *kindr* epimutants, where the observed decreases in gene expression are related with increase in DNA methylation across the entire gene cluster. As an independent test of the role of *Kindr* in meiotic drive, plants were transformed with an RNAi construct directed against the first and second exon of *Kindr*. Two of five independent RNAi lines showed the expected reduction of meiotic drive in these lines. The data established the importance of *Kindr* in meiotic drive, as genetic screens targeting meiotic drive (Ab10-smd1, Ab10-smd12) implicated *Kindr*, and targeting *Kindr* by RNAi (Ab10-ki1) resulted in a loss of meiotic drive. *Kindr* localizes to neocentromeres and specifically associates with knobs containing 180-bp repeats and not those containing TR1 repeats. Kinesin gliding assays and immunolocalization revealed that *Kindr* is a functional minus-end-directed kinesin that localizes specifically to knobs containing 180-bp repeats. Sequence comparisons suggest that *Kindr* diverged from a Kinesin-14A ancestor ~12 mya and has driven the accumulation of >500Mb of knob repeats and affected the segregation of thousands of genes linked to knobs on all 10 chromosomes. The results (Dawe *et al*., 2018) illustrate the potential of meiotic drive to strongly impact karyotipic evolution. In addition, the data show that the impact of Ab10on maize has been profound adding as much as 500 Mb of new DNA and indirectly affecting the Mendelian segregation of the majority of genes.

SOMATIC MAIZE KARYOTYPE

As mentioned above, the identification of maize chromosomes according to their relative length, centromeric position, knobs and

prominent chromomeres determined from pachytene studies (Rhoades, 1950; Neuffer et al., 1968, McClintock et al., 1981) was well standardized and well accepted. Somatic chromosomes analyzed in mitotic metaphase chromosomes from root tips are condensed and difficult to identify in spreads stained with conventional methods. Chen (1969) and Filion and Walden (1973) reported an identification of maize somatic chromosomes according to their relative length and arm ratio in Feulgen stained metaphases. In fact, the unequivocal chromosome identification in such preparations is that of the satellited chromosome 6, which possess the nucleolar organizer region (NOR).

The examination of C-banded somatic chromosomes stained with Giemsa has allowed the detection of bands corresponding with knobs visualized in pachytene chromosomes (Ward, 1980; Aguiar-Perecin, 1985; Aguiar-Perecin and Vosa, 1985), and was proven to be useful for the detection of homozygous and heterozygous knobs in individuals of a population from which inbred lines were selected (Decico, 1991). However, the unequivocal identification of somatic chromosomes may be difficult due to the degree of chromatin condensation and the presence of large knobs that alters the size of chromosome arms. Thus, the use of C-banding must be supplemented with an analysis of pachytene chromosomes, in which details of chromosome structure can be visualized. The employment of C-banding has been useful in studies involving the detection of changes in knobbed chromosomes in cells of callus culture, in which the knob composition was previously known (Fluminhan *et al*., 1996; Santos-Serejo and Aguiar-Perecin, 2016, Santos-Serejo *et al*., 2018), as mentioned bellow. Also, C-banding was used to determine the number of knobs in maize populations (Rayburn *et al*., 1985). So, the analysis of somatic chromosome has the advantage of the possibility of investigating plant populations in a short time without the need to grow the plants to analyze meiosis.

Improvements in the methods for maize somatic chromosome preparation were important for the identification of mitotic chromosomes. One procedure was the application of pressurized nitrous oxide gas to root tips to yield a very high number of metaphase spreads. Root tips from

young seedlings or from older plants were treated in 10 atmospheres of nitrous oxide (Kato, 1999). In addition, the pretreatment of root tips with a combination of 8-hydroxiquinoline, a mitotic fuse inhibitor, and cycloheximide, a protein synthesis inhibitor, resulted in a high index of metaphase and prometaphase accumulation (Bertão and Aguiar-Perecin, 2002).

Other important method was the use of probes of repeated DNA sequences in fluorescent *in situ* hybridization (Kato *et al*., 2004). Specific arrays at localized regions in the genome were used for the identification of the somatic chromosomes in metaphase spreads. This probe cocktail included the 18S and 5S ribosomal RNA genes, the CentC centromere unit, the Cent4 repeat specific of the chromosome 4 centromere, a TAG microsatellite cluster of variable locations but usually on chromosomes 1, 2, and 4, two types of knob sequence (180-bp and TR1) and two types of subtelomere repeat, that have a variable distribution on the chromosomes. This collection of probes allowed the identification of the ten homologues in maize inbreds, in which the knob composition was variable (Kato et al., 2004). FISH analyses using probes of these satellite DNAs have allowed the study of karyotype diversity in maize inbred lines commonly used in cytogenetic and genetic studies including e.g., B73, KYS and Mo17 among others and in lines of a nested association mapping (NAM) population (Albert *et al*., 2010).

As illustration, we report here a study of sister inbred lines (S6 progenies) derived from a tropical maize population designed Jac Duro (Mondin *et al*., 2014). These lines are referred as JD lines and belong to two families, JD 1-3 and JD 4-4. In addition, hybrids between two lines of family JD 1-3, and between a line of family JD 4-4 and the temperate line KYS were analyzed. A previous analysis of C-banded metaphases and of pachytene cells showed that in these lines the presence of knobs on the long arm of chromosomes 3 and 5 (K3L and K5L) and on the short arms of chromosomes 7 and 9 (K7S and K9S) was variable. In addition, these lines were homozygous for the knobs K6L2, K6L3, K7L, K8L1 and K8L2 (Table 1). K refers to knob, the number refers to the chromosome, and L and S designate the long and short arms respectively. K6L2, K6L3, K8L1

and K8L2 refer to different knob positions on the chromosomes 6 and 8, according to the literature (McClintock *et al*., 1981).

For the FISH procedure, the following probes were used: the primary knob 180-bp repeat and the centromeric repeat CentC. Chromosome-specific probes were used to identify chromosome 2 (5S rDNA), chromosome 4 (Cent4), chromosome 6 (9.1-kb repeating unit of the NOR DNA) and other specific chromosomes (subtelomeric 4-12-1 clone). The origin and details on these probes are described by Mondin *et al.* (2014). The Cent4 and subtelomeric probes were labeled with biotin-14-dATP by nick translation (Bionick Labelling System, Invitrogen, USA) and detected with mouse anti-biotin followed by TRITC-conjugated rabbit anti-mouse (red) and TRITC-conjugated swine anti-rabbit antibodies (DAKO, Denmark), with the exception of the KYS x 441311 hybrid somatic karyotype, for which the CentC probe was detected with FITC-conjugated antibodies (green). The NOR rDNA was detected with a mixture of 50% rabbit anti-mouse FITC and 50% anti-mouse TRITC, resulting in a yellow signal (not shown). The knob 180-bp and 5S rDNA sequences were labeled with digoxigenin 11-dUTP (Roche, Germany) by random priming and detected with FITC- or rhodamine-conjugated sheep anti-digoxigenin (Roche, green or red).

The FISH procedure, the image capture, processing and karyotype analysis were performed as previously described (Mondin *et al*., 2007; 2014). The chromosomes of somatic C-banded metaphases from the lines 441123, 444331 and 441123 x 444331 hybrid were measured, and their relative lengths (expressed as percent of chromosome 10, as reported by Aguiar-Perecin and Vosa, 1985) and arm ratios were estimated (Table 2). An ideogram that was based on these data was presented. Pachytene chromosomes of the hybrid 441123 x 444331 were measured to estimate their arm ratios (Table 2). The values were according to the literature (Rhoades, 1950; Aguiar-Perecin and Vosa, 1985; Dempsey, 1994), except for the arm ratios of chromosomes 2 and 4, higher in chromosome 2 (about 1.44 in somatic chromosomes and 1.71 in pachytene), compared with chromosome 4 (about 1.3 and 1.44 respectively in somatic and pachytene

chromosomes). Data from the literature describe the chromosome 4 with the centromere position more submedian than chromosome 2.

FISH signal locations of the satellite DNA sequences were determined in the 441311 and 133425 lines and in the 133425 x 132331 and 441311 x KYS hybrids (Figure 1). KYS is a maize line commonly used in cytogenetic research and its chromosomes have been well characterized (Chen *et al*., 2000: Kato *et al*., 2004). The locations of signals on the chromosomes of JD lines were as follows: the 180-bp knob repeat was variable according with the knob location observed at pachytene (Table 1); in addition, a small signal of this sequence was observed on the tip of the short arm of chromosomes 1 and 6 in all lines, and a thin 180-bp signal was detected on the knobless chromosome 9; the subtelomeric 4-12-1 sequence was detected on 1S, 2S, 4SL, 5S and 8L in all lines, and in the KYS line it was present also on 5L; the 5S rDNA signal was observed on the submetacentric chromosome 2, in comparison with the metacentric chromosome 4, labeled with the Cent4 signal.; the CentC signals detected in the 441311 x KYS hybrid by three antibodies were strong in most of the chromosome pairs and did not discriminate specific chromosomes. The 180-bp knob sequence signals on the KYS were at 1S, 5L, 6SL, 7L and 9S.

Table 1. JD 1-3 and JD 4-4 line families and their knob composition visualized in pachytene chromosomes. (Mondin *et al*., 2014. Front. Plant Sci. 5:544)

Lines	Knob composition								
	K3L	K5L	K6L2	K6L3	K7S	K7L	K8L1	K8L2	K9S
JD 1-3 Family									
132331	00	00	++	++	++	++	++	++	++
133425	00	00	++	++	++	++	++	++	00
JD 4-4 Family									
441123	++	++	++	++	++	++	++	++	++
441311	++	++	++	++	++	++	++	++	++
442612	00	++	++	++	00	++	++	++	++
444331	00	++	++	++	00	++	++	++	00

K, Knob; numbers refer to chromosomes; L, long arm; S, short arm; L1, L2, L3 refer to different knob positions; +, presence of knob; 0, absence of knob.

Table 2. Relative lengths and arm ratios of somatic chromosomes and arm ratios of pachytene chromosomes of JD lines. Large knobs alter arm length of mitotic chromosomes. K6L2/L3 and K8L1/L2 are detected as a single band in mitotic chromosomes. (Mondin *et al.*, 2014. Front. Plant Sci. 5:544)

Chromosome features	Chromosome rank													
	1	2	3		4	5		6	7		8	9		10
			KL	*		KL	*	KL2/L3	KS/KL	KL	KL1/L2	KS	*	
Metaphase														
RL	178.1	149.3	155.66	146.99	143.00	158.99	135.99	120.3	159.4	137.55	131.3	137.55	106.6.	100
AR	1.24	1.44	1.97	1.85	1.30	1.35	1.02	1.85	1.83	2.83	3.0	0.91	1.64	1.81
Pachytene														
AR	1.28	1.71	2.59		1.44	1.13		3.82	2.69		3.82	1.59		2.68

RL, relative length (expressed as percent of chromosome 10 length); AR, arm ratio.

KL, knob on the long arm; KS, knob on the short arm; KL2/L3, knob positions on chromosome 6; KL1/L2, knob positions on chromosome 8; * without knob.

From these observations, the markers used allowed the unambiguous identification of the somatic chromosomes of the lines analyzed represented in the ideogram showed in (Figure 2).

With the 180-bp probe, the variable knobs were identified, and small signals observed at 1S, 6S and 9S provided evidence that at these positions there would be a detectable copy number of the 180-bp sequence in the chromosomes of several maize varieties as shown in the literature (Kato *et al*., 2004; Albert *et al*., 2010). As above mentioned, knobs have been located on cytogenetic maps based on pachytene analyses (Longley, 1939; McClintock *et al*., 1981). Recently, they have been placed on the genetic map (Lawrence *et al*., 2006), and mapped relative to the maize reference genome assembly (Ghaffari *et al*., 2013).

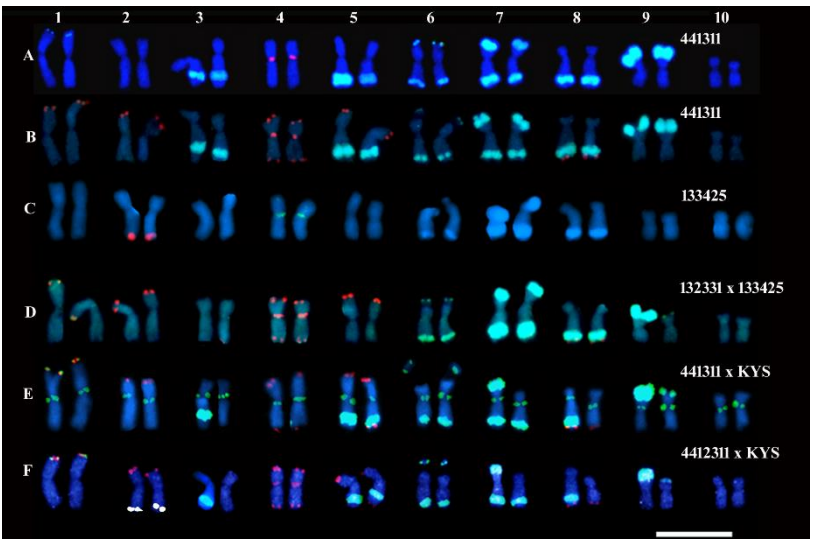

Figure 1. Somatic karyotypes of JD lines **(A-C)** and hybrids **(D-F**), labeled by FISH with probes for the knob 180-bp repeat **(A**, **B**, **D-F**, green**)**, Cent4 **(A**, **B**, **D, F**, red and **C**, green**)**, subtelomeric 4-12-1 **(B**, **D-F**, red**)**, 5S rDNA **(C**, red and **F**, pseudo-colored white**)**, CentC (**E**, green). Note that the large knobs can be detected as DAPI bands in **(C)** and that the knobless chromosome 9 from the parent 133425, in hybrid 132331 x 133425, has a small 180-bp signal on 9S **(D)**. Chromosomes 2, 3, 5, 7, 8 and 9 from the 441311 parent (placed on the left) can be recognized in the 441311 x KYS hybrid **(E, F)**. The chromosomes were counterstained with DAPI. Scale bar = 10µm. (Mondin *et al*., 2014. Front. Plant Sci. 5: 544).

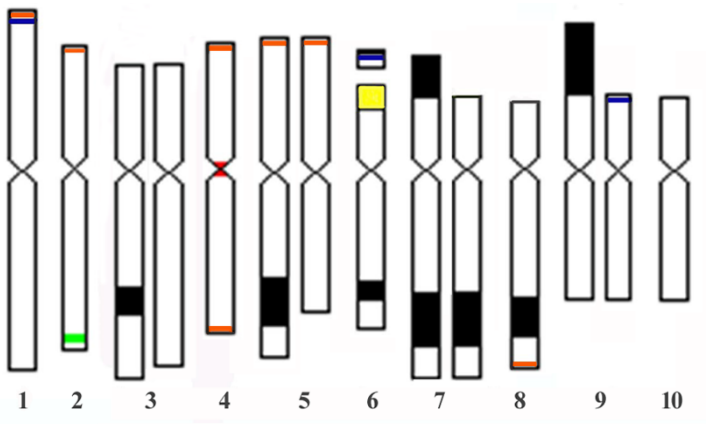

Figure 2. Ideogram representative of the somatic chromosomes of JD lines showing large knobs on chromosomes 3, 5, 6,7,8 and 9, detected by C-banding and FISH probed with the knob 180-bp repeat (black), small 180-bp repeat signals (blue), subtelomeric 4-12-1 clone (orange), Cent4 (red), 5S rDNA (green) and NOR rDNA (yellow). Knobless homologues of chromosomes 3, 5, 7 (without K7S) and 9 are displayed. Mondin et al.(2014).(Mondin *et al*., 2014. Front. Plant Sci. 5: 544).

In the JD lines reported here, the presence of large 180-bp signals corresponding with the positions and size of knobs visible at pachytene stage and C-banded somatic metaphases suggests that these knobs are composed primarily of 180-bp repeats. TR1 elements have been found in knobs on 2L, 4L and 6S in most lines that have been investigated, but they are also detected on 6L 8L 9S and 10L in some lines (Kato *et al*., 2004; Albert *et al*., 2010; Ghaffari *et al*., 2013; Kanizay *et al*., 2013). In the JD lines, knobs on 2L, 4L and 10L were not observed, but further investigation is necessary to assess whether TR-1 elements are present on 6S, 6L2, 6L3, 8L1, 8L2 and 9S.

The presence of the subtelomeric signal on 1S, 2S, 4SL, 5S and 8L in all of the JD lines and in KYS provided evidence that they may probably be detected in these positions in various maize varieties, then being important markers for these chromosomes. They have been observed in maize lines but varying among materials (Kato *et al*., 2004; Albert *et al*., 2010) including KYS, in which we observed subtelomeric signals at 5S and 5L.

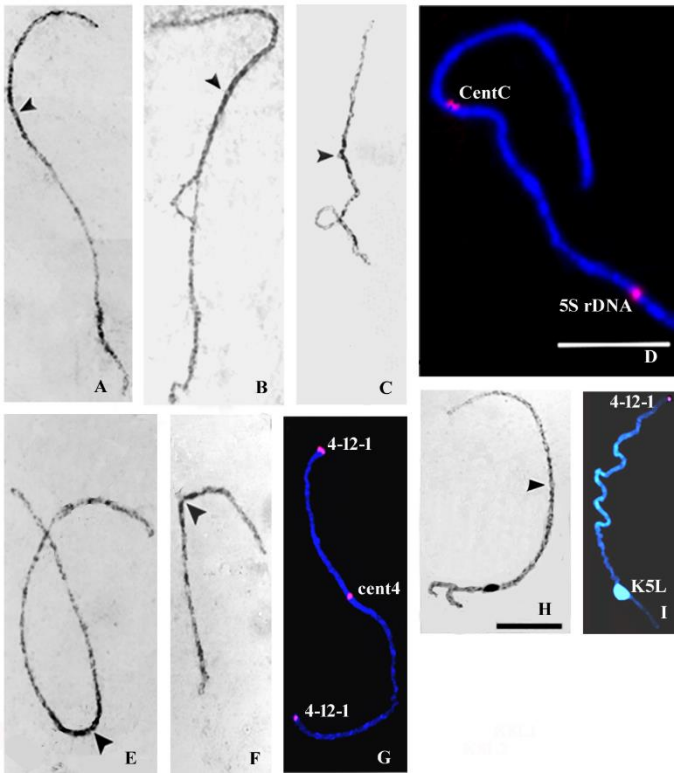

Figure 3. Chromosomes 2 **(A-D)**, 4 **(E-G)** and 5 **(H-I)** at pachytene stage from the 441311 x KYS hybrid **(A**, **B**, **D**, **E**, **G, H)** and KYS **(C, F)** and 444331 **(I)** lines. The homologues are completely synapsed in the hybrid, with exception of one cell in which a loop possibly resulting from a pairing failure occurred in a segment of the bivalent 2 **(B)**. The arrowheads indicate the centromeres in carmine-stained chromosomes **(A-C**, **E**, **F, H**). FISH signals (red) of CentC and 5S rDNA probes on chromosome 2 **(D)**, Cent4 and subtelomeric 4-12-1 clone (red) on chromosome 4 **(G)** and 4-12-1 (red) on chromosome 5 **(I)** are displayed. Scale bars for carmine stained chromosomes and FISH images = 10µm. (Mondin et al., 2014. Front. Plant Sci. 5: 544).

The pachytene chromosomes of the 441311 x KYS were measured to investigate the pairing behavior of chromosome 2 and 4. Chromosome 5 was also included in this analysis. (Table 3). Carmine-stained chromosomes 2 and 4 from the 441311 and KYS lines were analyzed to compare their arm ratios (Figures 3A-C, E, F). Chromosomes of the 444331 line were also analyzed The arm ratios of the chromosome 2 of 441311, 444331 and 441311 x KYS were very similar (about 1.70), while

in KYS (Figure 3C) it was 1.36, corresponding to data in the literature (see review of KYS data in Anderson *et al*., 2003). The homologous chromosomes of the bivalent 2 were completely synapsed (Figure. 3A) and in only one microsporocyte, a loop was detected, suggesting the occurrence of a pairing failure in a chromosomal segment. The chromosome 4 arm ratios in 441311, 44431 and 441311 x KYS were not similar (about 1.37 in the lines and 1.47 in the hybrid). The centromeric position of the bivalent 4 appeared to be more variable among cells of the hybrid, but the pairing between homologues was complete. The chromosome arm ratio of KYS was 1.63, which is also consistent with data in the literature. The data gave evidence of differences in the centromere position of the chromosomes 2 and 4 between the JD and KYS lines. The centromere position of the chromosome 2 in the 441311 x KYS hybrid was similar to that observed in the 441311 line, while the arm ratio of chromosome 4 was intermediate between values estimated for JD and KYS lines. The arm ratio of the metacentric chromosome 5 was about 1.10 in the lines and hybrid, with no difference among them.

Fluorescence *in situ* hybridization using chromosome-specific probes to identify chromosomes 2 and 4 showed features of the chromosome pairing in the 441311 x KYS hybrid. The bivalent 2 labeled with the Cent C and 5S rDNA probes (Figure. 3D) and the bivalent 4 labeled with Cent4 and 4-12-1 subtelomeric clone probes (Figure 3G) showed normal synapsis at the repetitive DNA sites analyzed. Other chromosomes presented normal pairing and chromosome 5 was included here as example. The pair 5, which possessed knobs with different sizes on 5L on the homologues, showed normal synapsis at this region (Figure 3H). As in KYS the knob is described at 5L, the analysis of this hybrid was also important to give evidence that the knob on the metacentric chromosome 5 is also located on 5L in the JD lines. This can be seen in chromosome 5 of the 444331 line with the large knob on 5L and the 4-12-1 subtelomeric signal on 5S (Figure 3I). The arm ratios for the JD and KYS lines and the hybrid were consistent with the median position of the centromere in chromosome 5. (Table 3).

Table 3. Arm ratios of chromosomes 2, 4 and 5 at pachytene stage from lines 441311, 444331, KYS and the hybrids 441311 x KYS compared with KYS data from various studies. (Mondin *et al*., 2014. Front. Plant Sci. 5:544)

Materials	Chromosomes		
	2	4	5
441311	1.69 (1.63; 1.75) a	1.37 (1.33; 1.41) c	1.09 (1.00; 1.18) a
444331	1.70 (1.61; 1.79) a	1.36 (1.30; 1.41) c	1.12 (1.06; 1.19) a
KYS*	1.37 (1.27; 1.44) b	1.63 (1.54; 1.71) a	1.04 (0.97; 1.11) a
441311 x KYS	1.69 (1.62; 1.77) a	1.47 (1.42; 1.52) b	1.09 (1.02; 1.16) a
Rhoades (1950)#	1.26	1.59	1.20
McClintock et al. (1981)#	1.20	1.57	1.16
Dempsey (1994)#	1.25	1.6	1.10

*Present study; #KYS data from other studies. Chromosome arm ratio for each line and hybrid were tested through an analysis of variance. Values with the same letter are equal based on their confidence intervals in brackets.

It is beyond the scope of this study to discuss the mechanism involved in the pairing of homologues differing in centromere positions, which was observed in the 441311 x KYS hybrid, but this finding raises some questions that may be considered in further investigations. The axial contraction along the fibers during meiotic mid-prophase has been shown to be uniform, using BAC FISH mapping of selected *loci* on maize chromosome addition lines of oats (Figueroa and Bass, 2012). In their study, the authors found that the relative *loci* positions along pachytene chromosomes did not change as a function of total arm length at early and late pachynema. However, they observed considerable variation between the relative arm positions of *loci* when comparing the cytogenetic FISH map to the B73 genomic physical map. The authors mention that this could occur in some cases in which the cytogenetic FISH map and genomic physical map are of different genotypes. Differences in DNA content among maize lines are well known (Laurie and Bennett, 1985; see Figueroa and Bas, 2012). According with Figueroa and Bass (2012), variation in relative map positions could result from genotype-specific

variation in DNA packing along the pachytene chromosome axis of individual chromosome arms. Alternatively, the authors argued that genome sizes could be similar, but repetitive DNA sequences may have accumulated in different regions of the chromosome arms. Satellite DNA and TEs were identified in inbred line B73, and it was estimated that ~85% of the B73 RefGen v1 were comprised of TEs (Schnable *et al*., 2009). In addition, the distribution of some retrotransposon families on the chromosome of maize lines was shown to be non-random, with distinct patterns revealed by FISH (Lamb *et al*., 2007b).

From these considerations, we could infer that the KYS and JD lines have different contents of repetitive DNA along the arms of the knobless chromosomes 2 and 4, resulting in differences in relative arm lengths. The complete synapsis observed at pachytene stage may also involve non-homologous pairing at some chromosome regions. Synapsis of non-homologous parts of chromosomes at pachynema has been detected in maize (McClintock, 1933; Lamb *et al*., 2007a).

In conclusion, the results of our study highlight problems concerning meiotic chromosome pairing and levels of chromatin contraction along chromosomes with different distributions of repetitive DNA, besides contributing for the knowledge of global maize chromosome variability.

INVOLVEMENT OF KNOBS IN CHROMOSOME BREAKAGE IN MAIZE CALLUS CULTURES

Alterations in chromosome structure have been observed in plants regenerated from tissue culture. Chromosomal breakage and its consequences (deficiencies, duplications, translocations and inversions) are events that occur in callus cultures. Breakpoints involved in alterations associated with heterochromatic knobs have been detected in maize regenerated plants (reviewed by Peschke and Phillips, 1992; Aguiar-Perecin *et al*., 2000). In these plants, most breakpoints were located between the centromere and knobs. One hypothesis that was proposed to

explain the role of heterochromatin in inducing chromosome breakage was that normally late-replicating heterochromatic knobs may replicate later in culture, thus leading to the formation of a bridge, as a result of the delayed separation of chromatids at knob sites (Lee and Phillips, 1987). In studies in cells of maize embryogenic calli, bridges resulting from delayed separation of chromatids at knob sites were observed. Typical bridges, i.e., bridges resulting from dicentric chromatids were also observed. The examination of C-banded anaphases showed that sister chromatids were held together at C-band sites (corresponding to knobs) (Fluminhan *et al*., 1996). Typical bridges with and without knobs were observed. These events were interpreted as evidence of the occurrence of the chromatid type breakage-fusion-bridge (BFB) cycle initiated by chromatids that were broken during the primary event (Fluminhan *et al*., 1996; Santos-Serejo and Aguiar-Perecin, 2016).

The first studies on the chromatid BFB cycle showed that a BFB cycle initiated by a chromosome broken at meiosis occurs in gametophyte mitoses and in the endosperm and that healing of broken chromosome ends occurs in the zygote (McClintock, 1939, 1941). The healing of broken chromosomes, i.e., the addition of telomere sequences at broken ends, has been reported for diverse organisms. In plants, it has been investigated by FISH using probes of telomere repetitive sequences of *Arabidopsis thaliana*. In wheat, hybridization signals were observed at the broken ends of deleted chromosomes and at the centromeric region of telomeric chromosomes (Werner et al., 1992; Tsujimoto, 1993).

To illustrate a case of chromosome healing, we report here a study involving a cytogenetical analysis of plants regenerated from a maize callus culture (Santos-Serejo and Aguiar-Perecin, 2016), in which a deficiency-duplication (Df-Dp) on the chromosome 7 short arm was interpreted as being derived from a chromatid type BFB cycle and healing of the broken arm (Fluminhan *et al.*, 1996; Santos-Serejo and Aguiar-Perecin, 2016). The stability of this chromosome was investigated in mitosis and meiosis of regenerated plants. R_1 and R_2 generations of plants regenerated from a 12-month-old callus designated 3-57 were used. This embryogenic callus was derived from a F_2 hybrid 1315 x 132331 whose

parents were JD lines belonging to the family 1-3 mentioned above (Mondin et al., 2014). The knob composition of these lines was: K6L2, K6L3, K7S, K7L, K8L1, K8L2 and K9S. Then, the chromosome 7 had a terminal knob on the short arm (K7S) and an interstitial knob on the long arm. In several cells of the 3-57 culture, one altered chromosome 7 with two knobs on the short arm and a terminal euchromatic segment in this arm was observed. According to a previous interpretation (Fluminhan *et al.*, 1996; Santo-Serejo and Aguiar-Perecin, 2016), the two knobs on the short arm would bear a deficiency in the terminal region. These knobs and the regions designated as b segments would be reverse tandem duplications (RTD) resulting from a delayed separation of sister chromatids at K7S and formation of a bridge in anaphase, followed by chromatid breakage, a chromatid type BFB cycle and healing of the broken chromosome end (Figure 4).

The plants regenerated (R_0) from the 3-57 culture were expected to be heterozygous for the altered chromosome 7. C-banded metaphases of root tips from 24 R1 and 52 R2 plants obtained by self-pollination were investigated. Plants homozygous for the normal chromosome 7 and heterozygous for the altered chromosome were recovered (Figures 5A, 5B), but homozygotes for the altered chromosome were not observed. The Df-Dp chromosome 7 was stable and transmitted to 45.83% and 11.76% of the R1 and R2 plants examined, respectively. Probably due to inviability of pollen grains or of homozygous seeds, the genotype ratios observed were not the expected according to Mendelian segregation (Santos-Serejo and Aguiar-Perecin, 2016).

The structure of the aberrant chromosome 7 was stable and similar to that observed in the original callus culture. The FISH procedure carried out using the telomeric probe $(TTTAGGG)_6$ showed signals at the terminus of the short arm in mitotic metaphases of the R_1 plants (Figures 5 C-E). Thus, the results provided evidence that de novo telomere formation occurred at the broken chromosome end, presumably due to telomerase activity in the original 3-57 callus (Santos-Serejo and Aguiar-Perecin, 2016).

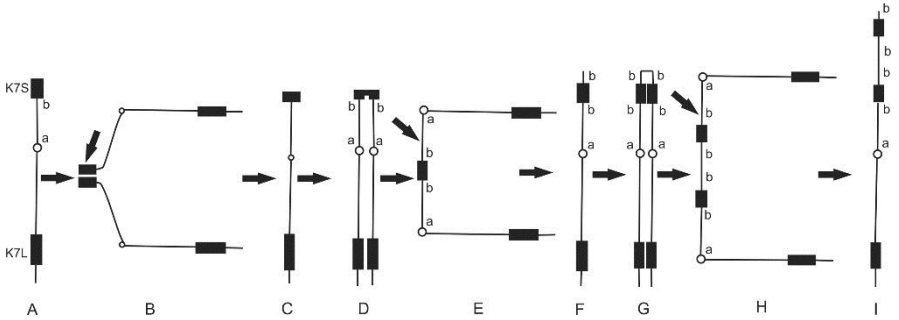

Figure 4. BFB cycle that would give rise to a chromosome 7 bearing a deficiency and duplication (Df-Dp) showing: normal chromosome **(A)**, anaphase with delayed separating chromatids and breakage at K7S **(B)**, chromatid with a deficient K7S **(C),** fused after replication **(D)**, breakage at anaphase **(E)**, chromatid with duplication of the b segment **(F)** fused at broken ends after replication **(G)**, anaphase bridge and breakage **(H),** and the resulting chromatid with two knobs and tandem reverse duplications (RTD) of the b segment **(I)**. Arrows at the anaphases indicate breakpoints. (Santos-Serejo and Aguiar-Perecin, 2016. Genome 59, 367-378).

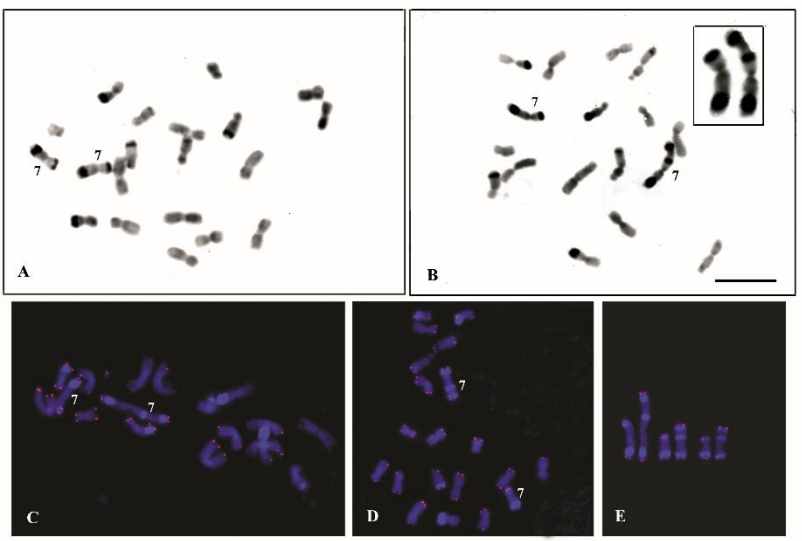

Figure 5. Somatic chromosomes of R1 plants. C-banded mitotic metaphases of homozygote for normal chromosome 7 showing knobs (C-bands) on the long and short arms **(A)**, heterozygote for the Df-Dp chromosome 7 with two knobs on the short arm (an amplification of pair 7 is shown in the square) **(B)**, telomeric FISH signals on early and full metaphases **(C, D)**, and homologous pairs of chromosome 7 **(E)**. Note the FISH signal on the terminus of the Df-Dp chromosome 7 short arm. Scale bar = 10 µm. (Santos-Serejo and Aguiar-Perecin, 2016. Genome 59, 367-378).

The expression of telomerase has been reported for some plant tissues, such as the meristematic tissue of cauliflower and undifferentiated cells from *Arapdopsis*, soybean and carrot suspension cultures, but low or undetectable in differentiated tissues (Fitzgerald et al., 1996). In our study, we observed the addition of a new telomere at a euchromatic region, which would be non-telomeric, then suggesting telomerase expression in maize callus culture. Evidence of healing of broken chromosomes was observed at different sites of chromosomes 7 and 9 in long-term cultures as shown below.

The meiotic behavior of the Df-Dp chromosome 7 was normal. In the heterozygous plants the homologues showed normal pairing at pachytene. In the diplotene and diakinesis stages, a heteromorphic pair corresponding to chromosome 7 was observed, as expected for the heterozygotes (Santos-Serejo and Aguiar-Perecin, 2016).

New evidences of the healing of broken chromosomes were obtained by the analysis of C-banded metaphases of long-term cultures. Samples of the 3-57 culture after 18 months of culture initiation were maintained in the original MS medium, supplemented as described by Santos-Serejo and Aguiar-Perecin (2016) and designated as subculture MS2, supplemented with 2mg/L 2,4-dichlorophenoxyacetic acid (2,4D). Samples were also transferred to MS medium supplemented with 1 mg/L 2, 4D (subculture MS1) and to N6 medium supplemented with 1.5 mg/L 2,4D (subculture N6). Two subcultures in each medium were maintained and were designated as cell lines 1-MS2, 2-MS2, 1-MS1, 2-MS1, 1-N6 and 2-N6 (Santos-Serejo and Aguiar-Perecin, 2016).

The analysis of C-banded metaphases of the cell lines detected new alterations in the chromosomes 7 and 9, and the occurrence of delayed chromatid separation and bridges in anaphases provided evidence of new BFB cycle events in these long-term cultures. In the original 18-month-old callus culture 3-57, two types of altered chromosome 7 were detected (Figure 6). One of the chromosomes was the Df-Dp chromosome described above. The other type of aberrant chromosome 7 possessed K7S on an interstitial position of a duplicated short arm, and was previously interpreted (Fluminhan *et al.*, 1996) as being derived from the same type of

BFB cycle shown in Figure 4. In the investigated cell lines the following types of chromosome 7 were detected (Figure 7A): 7A, normal type; 7B, with a duplicated short arm and a subterminal K7S; 7C, with two knobs on the short arm and a terminal euchromatic segment; 7D, similar to the 7C but without the terminal euchromatic segment; 7E, similar to 7D with a smaller terminal K7S; and 7F, with a larger short arm, a very large interstitial K7S, and without K7L on the large arm. The 7A, 7B and 7C types were present in cells from the 18-month-old original callus culture (Figure. 6). The 7C chromosome was similar to that observed in the regenerated plants (Figures 5B and 7E). Figures 7B and 7C illustrate the 7D and 7B chromosome types respectively, and the 7E type can be seen in Figure 7D. The 7F type can be seen in the pedigree of the 1-MS1 cell line (42-month-old subculture, Figure. 6). In addition, the following types of chromosome 9 were found (Figure 7A): 9A, normal, with a very large K9S; 9B, with a smaller K9S; 9C, with a smaller subterminal K9S; 9D, without the knob; 9E, minichromosome interpreted as derived from chromosome 9, as shown below. Figure 7B illustrates the 9A, 9C and 9E types. The 9B type can be seen in Figures 7D and 7E. The 9D type without knob was detected in the 31-month-old subculture of the 1-MS1 and in the cell lines 2-MS2 and 2-MS1 (Figure 6).

The cell line pedigree analysis revealed karyotype diversity among the cell lines, but homogeneity within some of them was observed in samples harvested at different age transfers (Figure 6). Gross aberrations were not found in the chromosome 6 and 8 which possess knobs, but smaller than those found on chromosomes 7 and 9. The two original altered chromosomes 7 (7B and 7C types) were maintained in the cell lines, except for the 42-month-old subculture of the 2-N6 cell line, in which a chromosome 7 with a smaller distal K7S (7E type) was detected. In addition, in the 1-MS1 and 2-MS1 cell lines the altered chromosome 7 lost the terminal euchromatic segment (7D type). These results suggest that the cell bearing the original Df-Dp chromosome 7 (7B or 7C types) were highly adapted in culture and that the new types of chromosome 7 (7D and 7E types) detected were derived from the original altered chromosome (7C type) through new events of delayed separation at the terminal knob and

breakage at the knob. On the other hand, the chromosome 7F observed in the 42-month-old subculture of 1-MS1 is a new alteration of the normal chromosome. This chromosome could have been originated through a delay in sister chromatid separation on K7S at anaphase. If the duplicated knob in sister chromatids did not separate and a breakage occurred at an adjacent euchromatic region, an amplified knob would appear. The absence of K7L in the chromosome 7F could also be explained by a delay in separation of chromatids at the knob site and breakage eliminating the knob.

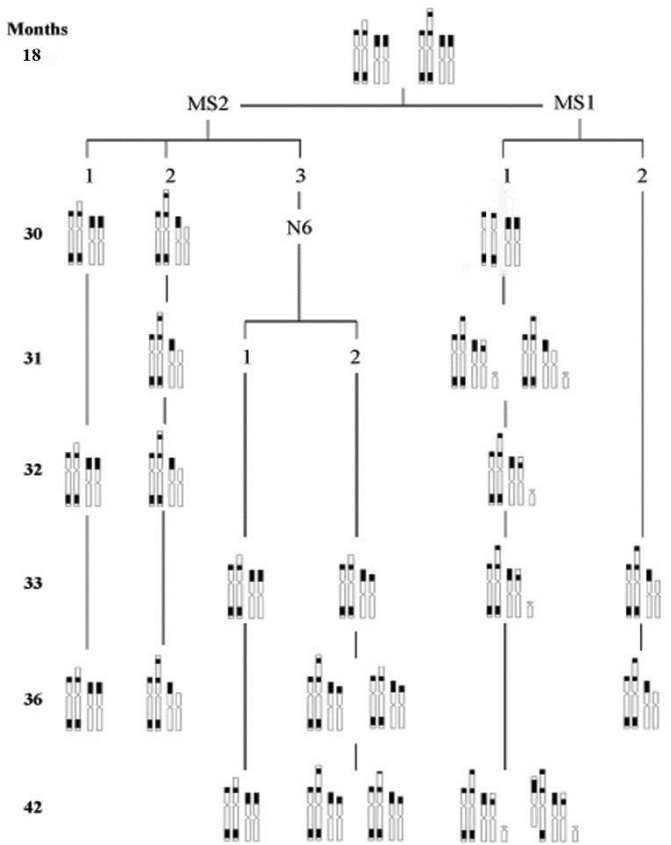

Figure 6. Cell line pedigree analysis showing the types of chromosomes 7 and 9 observed in C-banded metaphases in different subcultures of the six cell lines. (Santos-Serejo and Aguiar-Perecin, 2016. Genome 59, 367-378).

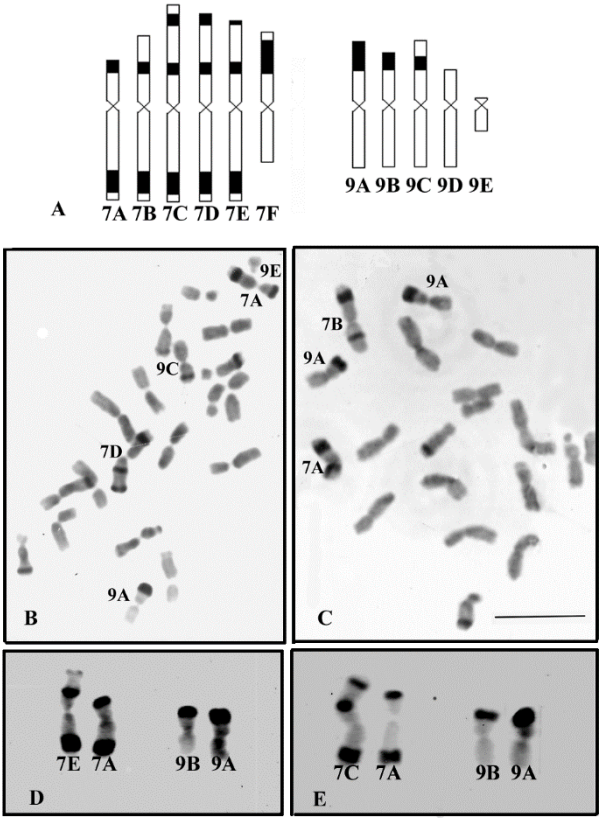

Figure 7. Types of chromosomes 7 and 9 observed in C-banded metaphases of the cell lines **(A),** a metaphase cell of the 1-MS1 cell line **(B)** and 1-MS2 **(C)**, and chromosomes 7 and 9 of the 2-N6 cell line **(D** and **E)**. Scale bar = 10 µm. (Santos-Serejo and Aguiar-Perecin, 2016. Genome 59, 367-378).

The chromosome 9 suffered alterations in most cell lines, except for 1-MS2 and 1-N6 in which the normal chromosome (9A) was observed. In the 2-MS2 and 2-MS1 cell lines, the K9S was not detected (9D type), and the 2-N6 cell line presented a partial deletion of this knob (9B type). A delayed separation of chromatids at the K9S in anaphase and a breakage that totally or partially eliminated the knob would explain the origin of these deletions. An interesting observation was made in the 1-MS1 cell line, in which a chromosome 9 displaying a subterminal small knob

(9C type), a chromosome without knob (D type) and a minichromosome (9E type) could have originated from the mechanism showed in Figure 8.

The analysis of the pedigree (Figure 6) showed that in most cell lines the altered chromosomes were stable in different samples, thus giving evidence that they were healed.

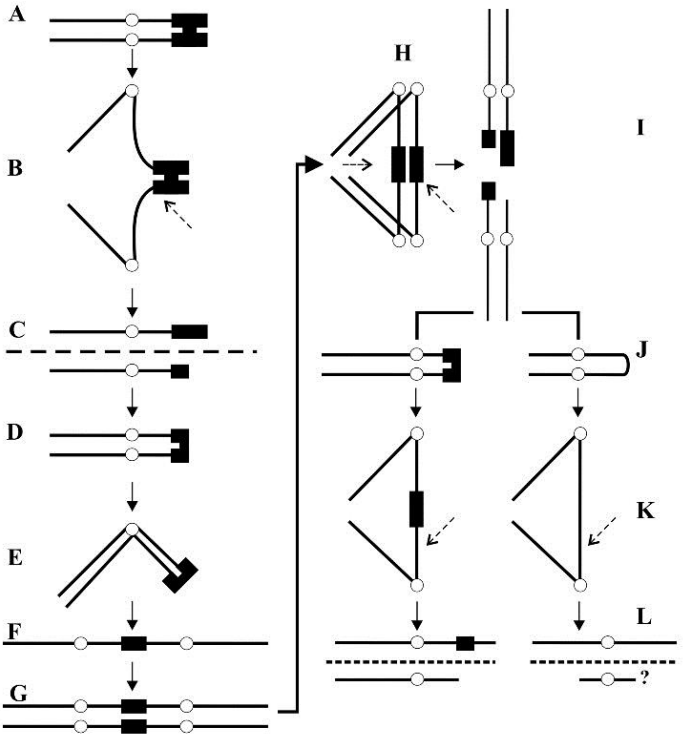

Figure 8. Mechanism that would originate different types of chromosome 9 in the 1-MS1 cell line: normal chromosome **(A),** anaphase with delayed separating chromatids and breakage at K9S **(B),** the resulting chromatids, one normal and the other with a deficient knob **(C)** fused after replication **(D),** nondisjunction of sister chromatids **(E)** resulting in a dicentric chromosome **(F, G)**, double bridge **(H)** giving rise to a chromatid with a deficiency at K9S and another chromatid without a knob **(I)**, which, after duplication and fusion **(J)**, suffered breakage in the next anaphases **(K)**, resultant chromosomes: one with a subterminal deficient K9S, another without the knob and a minichromosome originated after further deletions whose mechanism is unclear **(L).** Arrows at the anaphases indicate breakpoints. (Santos-Serejo and Aguiar-Perecin, 2016. Genome 59, 367-378).

Another evidence of chromosome healing was observed in the analysis of anaphase abnormalities in this study of cell lines (Santos-Serejo and Aguiar-Perecin, 2016). The total abnormalities, i.e., the presence of delayed separating chromatids, bridges, broken bridge and fragments, varied from 0.67% to 10%. They gave evidence of the occurrence of BFB cycles. It is interesting to note that the frequency of abnormalities did not increase with time in culture. They tended to decrease in each of the cell lines scored. Then, BFB cycles did not accumulated because broken chromosomes acquired a new telomere.

The frequency of anaphase abnormalities was evaluated in a study of four families of JD lines homozygous for the knobs K6L2, K6L3, K7L, K8L1, K8L2 and variable for the presence of K3L, K5L, K7S, K9S (Fluminhan and Aguiar-Perecin, 1998). The knob at the long arm of chromosome 5 (K5L) had been interpreted as localized in the chromosome 2 in this publication. But a further study using FISH (Mondin *et al*., 2014) showed that this knob was localized on the chromosome 5. In the study of Fluminhan and Aguiar-Perecin (1998), the frequency of delayed separating chromatids and of bridges at anaphase was not strictly correlated with the knob content of the genotypes evaluated. Perhaps only the larger K7S, K7L and K9S led to delayed separation of the sister chromatids. Rhoades and Dempsey (1973) reported the occurrence of a similar case of bridge formation to explain the elimination of chromatin from knob-bearing chromosomes in the presence of B chromosomes. The knob replication would be delayed or suppressed in the presence of B chromosomes at the second microspore division. The chromosomes possessing large knobs would be involved more frequently.

Besides the occurrence of chromosome breakage followed by BFB cycle, unequal crossing over was also detected in callus cultures, giving origin to knob amplification. This was evidenced by the presence of C-bands with different sizes in sister chromatids of chromosome 7, in short-term callus cultures (Santos-Serejo *et al*., 2018).

In addition to showing altered chromosome stabilization in callus cultures, the results reported here point to mechanisms of chromosomal evolution related to heterochromatin that might occur in plants.

CONCLUSION

Maize is a model plant, and the development of knowledge on the structure and function of the chromosomes using methods of molecular cytogenetics and biology is important for the understanding of mechanisms of chromosomal evolution with an impact on the investigation of maize global variability.

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Frontiers for the permission to use data of the article Karyotype variability in tropical maize sister inbred lines and hybrids compared with KYS standard line. *Front Plant Sci* 5: 544 http://frontiersin.org/ doi/10.3389/fpls.2014.00544 (Mondin, M., Santos-Serejo, J. A., Bertão, M. R., Laborda P., Pizzaia D., Aguiar-Perecin M. L. R., 2014) in the present review.

Canadian Science Publishing for the permission to use data of the article Breakage-fusion-bridge cycles and de novo telomere formation on broken chromosomes in maize callus cultures. *Genome* 59, 367-378 (Santos-Serejo, J. A., Aguiar-Perecin, M. L. R, 2016) in this review.

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BIOGRAPHICAL SKETCH

Margarida L. R. Aguiar-Perecin

Affiliation: Luiz de Queiroz College of Agriculture (ESALQ), University of São Paulo

Education: Bachelor in Natural History by the Faculty of Philosophy, Sciences and Letters, University of São Paulo. PhD by Luiz de Queiroz College of Agriculture (ESALQ), University of São Paulo

Business Address: Rua Padua Dias, 11, 13418-900, Piracicaba, SP, Brazil.

Research and Professional Experience: Full Professor

Professional Appointments: Senior Professor at ESALQ, University of São Paulo

Honors: Member of the Academy of Sciences of São Paulo State

Publications from the Last 3 Years:

Mondin, M., Santos-Serejo, J. A., Bertão, M. R., Laborda P., Pizzaia D., Aguiar-Perecin M. L. R., (2014) Karyotype variability in tropical maize sister inbred lines and hybrids compared with KYS standard line. *Front Plant Sci* 5: 544 http://frontiersin.org/doi/10.3389/fpls.2014.00544.

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Chapter 2

GENETIC ENGINEERING AS A STRATEGY TO IMPROVE RHIZOBIAL SYMBIOTIC PERFORMANCE

José Rodrigo da-Silva*, Ana Paço, Ana Alexandre, Clarisse Brígido and Esther Menéndez
Laboratório de Microbiologia do Solo,
Instituto de Ciências Agrárias e Ambientais Mediterrânicas (ICAAM),
Instituto de Investigação e Formação Avançada (IIFA),
Universidade de Évora, Évora, Portugal

ABSTRACT

Some rhizobia are able to establish effective symbiosis with legume species that have high agronomic value, such as soybean, chickpea or common bean, amongst others. This symbiotic relationship is a powerful alternative to the nitrogen fertilizers applied in cultivated soils, which besides being expensive, are often responsible for environmental

* Corresponding Author Email: jrsilva@uevora.pt.

problems, such as groundwater contamination. Therefore, rhizobial strains that are simultaneously highly efficient in nitrogen fixation, able to compete with native soil populations, persistent on the soil as well as adapted to endure different stress conditions in the field, have been selected over the years to be used as inoculants for legumes plants, aiming to improve their yields. Studies on the genetic manipulation of rhizobia, with the purpose to improve their symbiotic performance, are building up important knowledge that can be used to optimize these symbioses. Enhancing the rhizobia symbiotic performance by the overexpression of specific genes to improve their symbiotic effectiveness, nodulation efficiency, competitiveness and/or stress tolerance might represent a strategy to accelerate the development of more efficient and resilient inoculants. This chapter will present an overview of reports addressing the successful development of rhizobial strains symbiotically more efficient by overexpression of single or multiple genes, directly or indirectly associated to the symbiotic process, such as nodulation, nitrogen fixation, stress response and phytohormone production genes among others.

Keywords: symbiotic effectiveness, genetic manipulation, biofertilizers, plant growth promotion, inoculants, legume-rhizobia symbioses

1. INTRODUCTION

The ever-growing human population together with the modern models of intensive farming and the Global Climate Change form a vicious circle that compromises the Earth resources in a near future. Nowadays, food supply became the biggest concern for our society. The primary sector, animal husbandry and above all, agriculture, is the key to sustain the growing human population food demand. However, agricultural practices contributed to climate change, as well as climate change had effects on agriculture systems.

In the 20th Century, Fritz Haber and Carl Bosch discovered the process that converts atmospheric N_2 into ammonia, a nitrogen compound that can be used by the plants. In this chemical process, the atmospheric nitrogen (N_2) reacts with hydrogen (H_2) using an iron catalyst, under high temperatures (300°C to 600°C) and pressures (200 atm to 800 atm),

resulting in the production of ammonia (Hoffman et al., 2014). The application of this process became the key for increasing yields in agriculture, being the synthesis and field application of nitrogen-based chemical fertilizers a commonality in the past last decades.

The economic cost for the chemical synthesis of nitrogen fertilizers is high, requiring the use of fossil fuels, which are a non-renewable source of energy (Pimentel, 2009). This practice has harmful side effects, resulting on the water and air pollution, as well as soil damage (Savci, 2012). These represent only a part of the environmental problems associated with the widespread use of chemical N-fertilizers, which have been a rising concern in present-time agriculture. The farmers usually maintain high levels of crop production by the application of large amounts of chemical nitrogen fertilizers. However, this practice has high interference with the nitrogen cycle, raising concerns regarding the emissions of nitrogen oxides, soil acidification and water eutrophication (Jones et al., 2014). This environmental degradation compromises resources for future generations and corresponds to an unsustainable way to produce food.

Although this production of chemical N-fertilizers has been supporting food production for an increasing world population, alternative ways of supplying N to agricultural crops, are urgent in order to reduce both economical and environmetal costs (Biswas and Gresshoff, 2014). These facts lead to the crucial need for achieving higher productions through sustainable agricultural practices that grant the preservation of resources for next generations. In 2015, the United Nations launched the Agenda 2030 for a Sustainable Development, which summarized 17 global goals that should be achieved in order to erradicate any kind of poverty, keeping a sustainable equilibrium among environment, economy and the global society. The end of poverty and hunger are the first two goals of the Agenda 2030. Therefore, research aiming to increase crop production, in a more sustainable way, is at high priority nowadays. More recently, in the last report from the Food and Agriculture Organization (FAO) strategic framework (2017), "*The future of food and agriculture – Trends and challenges*", this specific concern is pointed out as a major global challenge, since the increase of the world human population was included

as a major concern and a globally recognized problem (FAO, 2017). In a plain language, the big question is: *How are we going to produce enough food to feed the World without compromising the planet's resources*?

One of the most accepted sustainable alternatives to mitigate damages and problems associated with the application of chemical nitrogen fertilizers and to increase crop production is the use and application of the natural-occurring Biological Nitrogen Fixation (BNF), which represent a powerful tool to provide N into the agricultural systems in a sustainable way. BNF is an enzymatic process that transforms atmospheric N_2 into ammonia, which is performed by archaeal and/or bacterial nitrogenase. The N_2 fixed by diazothropic microorganisms compensates the losses caused by the denitrification, helping to restore the nitrogen content of the environment (Olivares et al., 2013). As nitrogen generated by BNF is utilized *in situ*, it is less prone to leaching and volatilization, therefore this process constitutes an important and sustainable N-input into agriculture (Dixon and Kahn, 2004, Capone, 2001).

Diazotrophic bacteria exist as free-living soil bacteria in the rhizosphere or associated with plant species, colonizing their external and/or internal tissues, which performed BNF naturally (Serrato, 2014). One of the most studied group of diazotrophic bacteria is the Rhizobia, which establish mutualistic symbiotic relationship with members of the FaFaCuRo clade (Fabales, Fagales, Cucurbitales and Rosales) (Doyle, 2016). These microorganisms are able to metabolize atmospheric nitrogen and convert it into nitrogen compounds that can be absorbed by plants. In FaFaCuRo plants, this process takes place in specialized structures on the plant roots or shoots, commonly called nodules. In return, rhizobia benefit from carbon substrates derived from photosynthesis in the plant (Lu et al., 2017). Thus, the use of these symbiotic associations represent an efficient alternative and eco-friendly way to meet the N needs of the plants, reducing the use of nitrogen fertilizers.

Contrarily to the application of nitrogen fertilizers to the crops, the inoculation of plants with rhizobia has economic advantages, as well as environmental benefits. The use of native rhizobia, applied as inoculants, allows a natural N supply to leguminous plants and to subsequent crops in

the case of agricultural practices based on crop rotations, cover crops, amongst related land managements.

Rhizobial-based inoculants should be simultaneously effective in nitrogen fixation, persistent in soil, competitive with native populations as well as adapted to the field environmental conditions (Stephens and Rask, 2000), in order to be able to establish successful and effective symbioses. Thus, these microorganisms have become a target for genetic modifications, with the aim of making them even more efficient in its symbiotic relationship with plants. The overexpression of rhizobial specific genes, directly or indirectly involved in the symbiotic process, may be used to improve the performance of rhizobial inoculants, namely by improving their nodulation efficiency, competitiveness or stress tolerance.

2. The Use of Rhizobia as Inocula on the Fields to Increase Crop Production – Rhizobial-Based Biofertilizers

As reflected in the previous section, agriculture practices that take advantage of the symbiotic relationship plant-diazotrophs became essential in order to mitigate the negative effects already produced by the widespread use of nitrogen fertilizers. In fact, native soil microorganisms, which will have high efficiency in their symbiotic relationships with plants even in stressful conditions, might be targeted as biofertilizers for improving the crop yield.

Biofertilizers are formulations containing one or many microbial strains, which when applied to the soil, seeds or plant surfaces, are able to promote plant growth by increasing the availability of primary nutrients on soil, as nitrogen or phosphorus, by favoring the production of plant growth hormones, by environmental stress relief, or by the prevention of plant diseases (Barman et al., 2017, García-Fraile et al., 2015). Curiously, the use of biofertilizers is not a newly developed agricultural practice. Ancient Greeks, Romans or Egyptians used agricultural practices such as crop

rotation with legumes, to improve the yield of other crops; it is now known that these benefits are due in part to the symbiotic nitrogen fixation.

The first patented and commercialized inoculum for leguminous plants based on *Rhizobium* was Nitragin, which dated from from the late 19th Century (Nobbe and Hiltner 1896). After that, the studies regarding rhizobial inocula benefits (single inoculum or a consortium with more than one species) and their applications to different crops raised a paramount importance, setting precendents for the formulation of a wide range of biofertilizers, many of them currently in the market (García-Fraile et al., 2017, García-Fraile et al., 2015).

By 2022, it is expected that biofertilizers marketing will reach the amount of 2,305.5 Million USD (Markets and Markets, 2016). North America, Europe and Asia are the three regions of the World with an economically well-developed biofertilizer bussiness network (Timmusk et al., 2017). From the different regions of the world, Europe and Latin America are the higher users of bacterial biofertilizers, possibly due to the rigorous policy imposed on the use of chemical fertilizers. These regions are followed by countries from Asia-Pacific region. In fact, it is estimated that the use of bacterial biofertilizers reduces the need for chemical fertilizers in an extent between 20 and 100% (Masso et al., 2015). In this context, it is understandable that the demand for bacterial biofertilizers is increasing worldwide, leading governments from different countries to legislate with the goal of protecting the environment, and also subjecting the biofertilizer formulation to a tight quality control. It is noteworthy that there is a great disparity of legislation regulating the marketing and application of these biofertilizers (Garcia-Fraile et al. 2017).

In the last decade, many studies showed the beneficial effects of bacterial biofertilizers and enumerated a high number of bacterial genera that by themselves or forming a consortium could be applied on different crops (Berg, 2009, Bashan et al., 2014, Babalola, 2010, García-Fraile et al., 2015, Jiménez-Gómez et al., 2017, Menendez et al., 2016, Celador-Lera et al., 2018). Nevertheless, the use of bacterial biofertilizers has yet less acceptance than chemical fertilizers. Survival of the formulation,

compatibility and competitiveness with native strains in a wide range of different climatic conditions, geographical regions and/or soil edaphic factors, amongst others, are concerns that might compromise the worldwide use of biofertilizers by farmers (Malusà et al., 2016, Panda, 2013). In this way, despite the numerous advances in the development of bacterial biofertilizers, more studies are still required to identify additional suitable strains and to develop better production technologies and quality control measures for its wider use.

There are two ways to achieve a more efficient rhizobial inoculant or biofertilizer in highly environmental stressful conditions: i) selection of symbiotically efficient native bacterial strains in non-stressful/stressful conditions and, ii) the use of genetic engineering tools to "design" suitable inoculant strains.

Currently, researchers devote an enormous effort in the development of efficient bacterial biofertilizers that will be compatible with a wide range of soils and plants, by genetic engineering the bacteria. However, the release into the environment of genetically modified microorganisms included in biofertilizer formulations, which is regulated by the biosafety laws of the different countries, generated controversy (García-Fraile et al., 2015). In these modified bacteria, one or more genes have been introduced *de novo* using recombinant DNA technology, allowing them to be more efficient in promoting plant growth in comparison with their wild-type parental strains. Thus, before suggesting genetically modified microorganisms for large-scale use in agriculture, it is necessary to guarantee that these engineered microorganisms do not have any unfavorable effects on the environment. Specifically, it is of paramount importance to evaluate the potential for horizontal gene transfer between the introduced and the indigenous bacteria on the soil (Hirsch, 2005).

In 1997, a genetically modified strain of *Ensifer meliloti (*RMBPC-2) was approved for limited commercialization by the United States Environmental Protection Agency (Wozniak et al., 2012). These bacteria were modified with an additional copy of both *nifA* and *dctABD* genes, which enhance nitrogen fixation and the C4 - dicarboxylic acid transport, respectively. This modified strain was able to increase the yield of alfalfa

crops (Bosworth et al., 1994). After that, other genetically modified bacterial inoculants have been released and tested in field conditions in a number of European countries under the terms of the EU Directive 90/220/EEC, which is no longer in force (repealed in 2002 by the EU Directive 2001/18/EC). One of the strains tested under the EU Directive 90/220/EEC was *Pseudomonas fluorescens* CHA0-Rif (pME3424), which was genetically altered to overexpress antifungal metabolites, being capable of controling efficiently the damping-off of cucumber, caused by *Pythium ultimum*. The data from these field experiments indicated an absence of significant unfavorable effects on crop yield, soil biomass and soil fertility (European Commission, 1999). Amarger (2002) reviewed the survival and spread of modified bacteria introduced in the field, as well as their interactions with the native microorganisms. The available data indicated that the spread from the site of their release was limited and the ecological modifications in the resident microflora were apparently transient and less pronounced than modifications resulting from traditional agricultural practices.

Besides the controversy in the release of engineered microorganisms on the soil due to the fear to alter genetically native soil bacteria by horizontal gene transfer, generally these microorganisms also present weak ability to compete for soil nutrients with indigenous soil microorganisms, not increasing the plant productivity under uncontrolled soil conditions (Wozniak et al., 2012). Nevertheless, despite all these issues that difficult the field application of biofertilizers containing modified microorganism, it is expected that biotechnology will allow to increase agricultural productivity on the next future, leading to more environmentally friendly practices, which will contribute tremendously for a sustainable agriculture.

3. Engineering Rhizobia by Overexpression of Specific Genes

Overexpression of genes in bacteria is a strategy based on the use of genetic manipulation to introduce features of interest or enhance those

already present with the aim of conferring a wide range of potential advantages to the modified strain. In addition, this technique can also cause different phenotypes, providing geneticists with an alternative yet powerful tool to identify pathway components that might remain undetected using traditional loss-of-function analysis (Prelich, 2012). This chapter will demonstrate evidence of the use of overexpression as well as heterologous expression of specific rhizobial genes in the ability of these modified strains to promote leguminous plant growth and/or on their symbiotic performance.

3.1. Nodulation Genes

The symbiotic process occurring between rhizobial members and leguminous plants involves the formation of specialized structures that provide plants with an environment suitable for nitrogen fixation, the nodules (Oldroyd and Downie, 2008). Nodulation genes (e.g., *nodABC*) encode enzymes responsible for the biosynthesis and secretion of Nod Factors (NFs), which are lipochitooligosaccharides (LCOs) that interact with plant flavonoids; thus, these NFs are important for determining the *Rhizobium*-legume pairing (Oldroyd, 2013, Via et al., 2016). Different rhizobia species can have different *nod* genes, therefore producing LCOs with varied structures (Limpens et al., 2015). These different structures contribute to the specificity of rhizobia-host plant interaction (Perret et al., 2000b).

Regarding the overexpression of *nod* genes, several studies have showed different effects either in both the transformed strain and the inoculated legume. For example, *E. meliloti* containing different number of copies of a DNA region including *nodD1* or *nodABC* introduced by specific DNA amplification, enhanced the symbiotic abilities in *E. meliloti* strains which received moderate number of copies and resulted in an increase in NFs production, nodulation, nitrogen fixation (acetylene reduction activity) and growth of alfalfa plants under environmentally controlled conditions (Castillo et al., 1999). The repetition on the genome

of a 30kb region containing *nodD3* amplified from pSym plasmid by random DNA amplification (RDA) (Banfalvi et al., 1981, Rosenberg et al., 1981) generated a more competitive strain of *Rhizobium tropici* for nodule formation in *Macroptilium atropurpureum* plants (Mavingui et al., 1997). The expression of *nodD* in *R. leguminosarum* significantly increased the nitrogen fixation during symbiosis with *Vicia sativa* and *Trifolium repens* (Spaink et al., 1989).

Nod genes overexpression is supposed to increase the number of active nodules as well as the symbiotic performance; however, some studies have shown that the overexpression of *nod* genes does not necessarily improve symbiotic parameters, on the contrary, it seemed to cause a negative effect. Machado and Krishnan (2003) showed that the addition of *nodD1* or *nodD2* copies caused delayed nodulation and reduced the number of nodules. Extra copies of structural nodulation genes (*nodABC*) added in *R. leguminosarum* reduced its nodulation capacity on *Vicia* plants (Knight et al., 1986). A hybrid *nodD* containing 75% of the *nodD1* gene of *E. meliloti* at the 5' end and 27% of the *nodD* gene of *R. leguminosarum* (formerly *R. trifolii*) induced pseudonodules on *Macroptilium atropurpureum*, *Lablab purpureus*, and *Leucaena leucocephala* and affected differently the nitrogen fixation when introduced into different rhizobia (Spaink et al., 1989).

Beside the well-known genes involved in the NFs formation in rhizobia, many other genes with important functions in nodulation have been studied, for example, *nodPQ*, *nodX*, *nodEF* and *noe* genes, which are involved in the synthesis of NFs substituents (Geurts and Bisseling, 2002, D'Haeze and Holsters, 2002). In *E. meliloti*, the *nolR* gene is involved in the synthesis of NF core (Cren et al., 1994). This gene was also present in the *E. fredii* genome (Vinardell et al., 2004). Multiple copies of *nolR* gene were introduced in *E. fredii*, showing altered amounts of NFs. Furthermore, *nolR* overexpression increased the amount of EPS produced and form less nodules on *Glycine max*, but nodulation capacity was improved on *Vigna unguiculata* (Vinardell et al., 2004).

3.2. Nitrogen Fixation

Once the plant nodules are formed, rhizobial bacteria start to fix atmospheric nitrogen and converting it into ammonia. Genes involved in the nitrogen fixation process include those that encode the NifA regulator and nitrogenase enzyme (*nifHDK*), responsible for the capture and conversion of atmospheric nitrogen into ammonia (Kaminski et al., 1998). Furthermore, other genes involved in the nitrogen fixation also play an important role in this process as the case of *fixLJ*, and *fixK* that encode transcriptional regulators and *fixABCX* involved in the electron transport chain to nitrogenase (Dixon and Kahn, 2004).

Several studies reported the use of extra copies of *nifA* genes from some genera of the *Enterobacteriaceae* family in rhizobia; however, these studies are restricted mainly to the genus *Ensifer*, formerly known as *Sinorhizobium*. Sanjuan and Olivares (1991) showed that the overexpression of *nifA* gene from *Klebsiella pneumoniae* in *E. meliloti* increased strain competitiveness for nodulating alfalfa plants. Nevertheless, a latter study showed that the expression of *nifA* from *K. pneumoniae* does not affect the *E. meliloti* competitiveness (van Dillewijn et al., 1998). In contrast, a similar study with *E. fredii* reported that extra copies of the *nifA* gene of *K. pneumoniae* accelerated the nodulation and increased competitiveness on soybean (Jieping et al., 2002). Another study described that the *nifA* gene overexpression in *E. meliloti* 1021 improved the nitrogen-fixing efficiency in *Medicago sativa* root nodules to a greater extent than that observed upon transfer of the *Enterobacter cloacae nifA* gene (Chengtao et al., 2004).

Interestingly, field trials testing alfalfa inoculated with *E. meliloti* modified with extra *nifA* and *dctABD* copies resulted in an increased alfalfa biomass by 12.9% compared with the yield achieved with the wild-type strain in soils with low nitrogen and organic matter contents (Bosworth et al., 1994). NifA regulator is also responsible for the activation/repression of other genes; therefore, higher expression of NifA also induces higher expression of other genes involved in nodule development, bacteroids persistence and/or competitiveness, such as *nfe* (nodule formation

efficiency) and *mos* (rhizopine synthesis) in *E. meliloti* and *groESL3* in *B. japonicum* (Fischer, 1994).

The overexpression of the whole nitrogenase enzyme (*nifHDK*) regulated by the *nifHc* promoter in *Rhizobium etli* increased nitrogenase activity in 58% compared with the wild type. Greenhouse trials with commom bean (*Phaseolus vulgaris*) inoculated with this modified strain increased the plant weight in 32% and nitrogen content in 15%. The seed yield was 36% higher in plants inoculated with the transformed strain, in addition, higher nitrogen content (25%) and nitrogen yield (72% on average) were detected in seeds (Peralta et al., 2004).

3.3. Stress Response Genes

Genes more related to rhizobia lifestyle and not directly involved in the symbiosis process may also play an important role in the interaction with the host plant. Besides allowing these bacteria to survive in the challenging soil conditions, stress response genes have also been reported to be involved in rhizobia colonization and life inside the root nodules. Thus, the development of highly effective rhizobial strains to be used as field inoculants must consider the importance of stress tolerance. If the inoculant formulation is not able to survive to abiotic stresses, its performance in the field may be significantly compromised.

Stress conditions such as heat, salinity, drought and oxidative stress or the presence of biotic stresses are important causes of plant production losses, hence several studies have addressed the effects of overexpressing stress response in the symbiotic performance of rhizobial strains.

3.3.1. Heat Stress

The chaperone system GroEL-GroES, which is known as central component of the heat shock response, has been shown to be involved in other functions in many bacterial species (Henderson et al., 2013). In rhizobia, which typically harbor multiple copies of these genes in their genomes, GroEL has been reported to play other roles in addition to stress

response, namely participating in central aspects of the symbiosis as the formation of functional NodD and the functioning of the nitrogenase complex (Fischer et al., 1999, Ogawa and Long, 1995, Alexandre and Oliveira, 2013, Yeh et al., 2002). Accordingly, a recent study showed that higher levels of a particular *groEL* gene copy is able to improve symbiotic effectiveness of a *Mesorhizobium*-transformed strain in approximately 50% compared with the wild type strain in chickpea plants (da-Silva, 2017).

The involvement of chaperones in the rhizobia-legume symbiosis is not restricted to GroEL. The chaperone ClpB was found to be involved in chickpea root nodulation, specifically through the analysis of a *clpB* knockout mutant, which showed a delay in the onset of the nodulation process (Brígido et al., 2012). The ability of this chaperone gene to improve the symbiotic performance of rhizobia was reported by Paço et al., (2016). The symbiotic performance of the modified strain *M. mediterraneum* UPM-Ca36^{T} harboring extra copies of the *clpB* gene was improved under control and under stress conditions (acidity). The nodulation kinetics analysis showed a higher rate of nodule development as well as a higher number of nodules in plants inoculated with the *clpB*-transformed strain. This improvement in the symbiotic phenotype was proposed to be related to an increased expression of the nodulation genes *nodA* and *nodC*, which are 3-fold more expressed in the *clpB*-transformed strain (Paço et al., 2016).

3.3.2. Salinity and Drought Related Stresses

Modified rhizobia strains harboring extra copies of genes related to protection of bacteria from salt stress has contributed to the improvement of the symbiotic performance under stressful environments. One strategy for minimizing the adverse effects of osmotic stress is the intracellular accumulation of low-molecular-weight organic solutes, such as betaines or trehalose. For example, an *E. meliloti* strain overexpressing the *betS* gene, which is involved in the rapid acquisition of betaines by cells exposed to osmotic shock, showed a better maintenance of nitrogen fixation activity in salinized alfalfa plants than the wild-type strain (Boscari et al., 2006). The

enzyme trehalose-6-phosphate synthase, encoded by the *otsA* gene, is involved in the biosynthesis of trehalose (Elbein et al., 2003). The overexpression of *otsA* from *E. meliloti* in *Mesorhizobium ciceri* increased the growth of the *otsA*-overexpressing strain in saline media (Moussaid et al., 2015). Moreover, in chickpea plants grown in the presence of NaCl, the inoculation of this *M. ciceri* harboring extra *otsA* copies improved the nodules formation and shoot biomass accumulation. Similarly, common bean inoculated with *R. etli* overexpressing *otsA* had an improvement in the number of nodules, with increased nitrogenase activity, and higher biomass compared with plants inoculated with the wild-type strain. In addition, only plants inoculated with the *otsA*-overexpressing strain fully recovered from drought stress (Suarez et al., 2008).

Studies on nodule metabolism under stress conditions showed that proline accumulates in these structures under salinity and drought conditions (Kohl et al., 1991, Fougère et al., 1991). Proline may be converted into glutamate by a proline dehydrogenase enzyme encoded by the *putA* gene (van Dillewijn et al., 2001). PutA activity was demonstrated to be required for colonization, nodulation efficiency and competitiveness of *E. meliloti* on alfalfa roots (Jimenez-Zurdo et al., 1997). The overexpression of *putA* increased the competitiveness of *E. meliloti* in nonsterile soil with alfalfa plants subjected to drought stress. In addition, nodule occupancy was improved for the modified strain with extra *putA* copies in an earlier stage of the symbiosis (van Dillewijn et al., 2001).

3.3.3. Oxidative Stress

Root nodules have a limited functional life that varies among different legume species, and although the underlying mechanisms are not complete understood, it is consensual that the reactive oxygen species (ROS) have a role on it. With this in mind, strategies to delay nodule senescence which would lead to an increase in nitrogen fixation and ultimately, to enhance legume productivity were attempted by different researchers. One strategy was the overexpression of flavodoxins, which are electron-transfer proteins known to be involved on oxidative stress response (Gaudu and Weiss, 2000), and therefore, their presence in the rhizobial cells may constitutes a

way to relieve bacteroids from ROS toxication in root nodules. For instance, alfalfa plants inoculated with rhizobia overexpressing flavodoxin displayed a delay in nodule senescence (Redondo et al., 2009). Moreover, the overexpression of flavodoxin genes was able to protect free-living *E. meliloti* cells from cadmium toxicity and reduced the negative effect of cadmium stress on the nitrogenase activity (Shvaleva et al., 2010). Similarly, under salinity conditions, the decline in nitrogenase activity was significantly less in *E. meliloti* overexpressing flavodoxin genes then in the wild type nodules (Redondo et al., 2012).

As stated before, ROS production has a role on the symbiotic process, being necessary for infection initiation. However, an unbalance of ROS levels and the prolonged exposition to these molecules are unfavorable for the plant-rhizobia interaction (Tóth and Stacey, 2015). In this sense, a recombinant *E. meliloti* strain, overexpressing *katB* gene, which is related to ROS and encodes for a catalase with high affnity for H_2O_2 displayed aberrant infection thread formation and delayed nodulation of *Medicago sativa* plants (Jamet et al., 2007), probably leading to a growth-decreased plant phenotype.

In order to limit the amount of oxygen in bacteroids, which irreversibly inactivates the rhizobial nitrogenase enzyme, the legume host synthesizes leghaemoglobin protein, which has a high affinity for oxygen (Downie, 2005, Hill et al., 1981). On the other hand, to supply the high amount of oxygen required to generate the energy needed for the nitrogen reduction process, bacteroids produce a high-affinity cytochrome cbb3-type oxidase to cope with the low oxygen concentration in the nodule (Preisig et al., 1996). The inoculation of *P. vulgaris* plants with a *R. etli* strain having enhanced expression of *cbb3* oxidase in bacteroids reduced the sensitivity of *P. vulgaris-R. etli* symbiosis to drought (Talbi et al., 2012). In addition, other studies have also reported that genetically modified rhizobial strains overproducing cbb3 oxidase are more efficient in nitrogen fixation under optimal conditions compared to their parental strains (Yurgel et al., 1998, Soberon et al., 1999).

Overall these reports highlight the fact that the expression levels of stress response genes, whose main function is not the symbiosis process

directly, may limit the symbiotic performance of rhizobia strains, particularly under more adverse conditions.

3.4. Plant Growth Promoting Traits

Although rhizobia are mostly known for their ability to convert atmospheric nitrogen to ammonia and make it available to leguminous plants, these soil bacteria may possess other mechanisms that directly or indirectly promote the host plant growth. For instance, an assessment of a collection of native Portuguese chickpea *Mesorhizobium* isolates for plant growth-promoting traits revealed that, in addition to being diazotrophic, all isolates were also able to synthesize indoleacetic acid (Brigido et al., 2017). Although less frequently, other plant growth-promoting mechanisms, such as siderophores production, phosphate solubilization, acid phosphatase or cytokinin activity, were detected in some rhizobia isolates (Rodriguez et al., 2006, Brigido et al., 2017).

3.4.1. Phytohormone Biosynthesis

Phytohormones are small molecules involved in the signaling pathways that control plant growth and development, therefore modulation of the hormone levels, namely auxin, cytokinin, gibberellin, and ethylene, on plant tissues is a suitable strategy used by some bacteria and fungi to directly control the plant growth and development. Besides the role of auxins, like indoleacetic acid (IAA), on plant growth and development, IAA is also involved in the genesis and development of root nodules (Desbrosses and Stougaard, 2011), playing an important role in rhizobia-legume symbiosis (Spaepen et al., 2009). Studies on the effects on the rhizobia-legume symbioses through the overexpression of this auxin in different rhizobial strains have been performed under control and stress conditions. For example, the inoculation of alfalfa plants with an IAA-overproducing *Rhizobium* strain resulted in higher tolerance to drought conditions (Defez et al., 2017). Similar results were obtained with an *E. meliloti* strain, harboring an additional pathway for the synthesis of IAA,

which showed an increased tolerance to several stress conditions, such as UV, high salt and low pH and under phosphate-starvation (Bianco and Defez, 2010, Bianco and Defez, 2009). Additionally, *Medicago truncatula* plants were inoculated with this transformed-strain showed reduced symptoms of senescence, lower expression of ethylene signaling genes, lower reduction of shoot dry weight due to stress and better nitrogen-fixing capacity (Bianco and Defez, 2009). Moreover, further studies using this transformed-strain showed that IAA overproduction was responsible for significant increases in both shoot and root fresh weights of *M. truncatula* plants grown under phosphate-limitation (Perret et al., 2000a). IAA-overproducing *E. meliloti* strain also doubled the number of nodules per plant in *M. truncatula*, which indicates that nodule formation may involve auxin transport regulation (Pii et al., 2007). Moreover, this strain improved the nitrogenase activity in nodules and stem dry weight (Imperlini et al., 2009). In another study, an IAA-overproducing *R. leguminosarum* strain, by addition of *iaaM* (from *Pseudomonas savastanoi*) and *tms2* (from *Agrobacterium tumefaciens*) genes, was able to produce 60-fold more IAA in vetch root nodules than the wild type strain. Furthermore, the lower number of nodules formed per plant were heavier, showing an intensification in nitrogenase activity and an elongated and more active meristematic zone (Camerini et al., 2008). Possible explanations for the results mentioned above may be related with the main findings of a transcriptional study comparing a *E. meliloti* 1021 and its IAA oveproducer derivative, where the overproduction of IAA in *E. meliloti* under free-living conditions induced the transcriptional changes that normally occur in nitrogen-fixing root nodule (Defez et al., 2016) or, may also be associated with the upregulation of genes related with the response to particular stress conditions. For example, an *E. meliloti* strain overexpressing genes related with IAA production, under P-deficient conditions, was able to release larger amounts of P-solubilizing organic acid than wild type strain and significantly increased the shoot and root fresh weights of those plants (Bianco and Defez, 2010).

Similarly to IAA, cytokinin acts as a signal in the induction of root nodule formation (Heckmann et al., 2011). For instance, the cytokinin

production by the *Bradyrhizobium* sp. strain ORS285 was responsible for faster nodule formation and alteration of size and number of nodules developed in *Aeschynomene* plants (Podlešáková et al., 2013). Moreover, although no differences on nodulation or nitrogen fixation were detected, the higher amounts of cytokinin produced by transformed *Ensifer (Sinorhizobium)* strains contributed for an higher tolerance of alfalfa plants to severe drought stress (Xu et al., 2012).

In contrast, ethylene, gibberellin and abscisic acid inhibit the cortical cell divisions induced by cytokinin. Ethylene is produced by plants in response to several environmental stresses and can negatively affect nodulation (Bari and Jones, 2009, Middleton et al., 2007). Curiously, some rhizobia may possess mechanisms to counteract the ethylene effects on nodulation through the modulation of plant ethylene levels locally. These mechanisms, namely the production of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Ma et al., 2003) and the synthesis of the rhizobitoxine (Yuhashi et al., 2000), have beneficial effects not only on the *Rhizobium*-legume symbiosis but also on the host-plant growth. The ACC deaminase enzyme (encoded by the *acdS* gene) regulates the levels of ACC, which is the immediate ethylene precursor. This gene is found in some rhizobial species from different genera that expressed it under free-living conditions, as is the case of *R. leguminosarum* bv. *viciae*. An *E. meliloti* strain modified with the *acdS* gene and its regulatory gene *lrpL* from *R. leguminosarum*, showed to improve both nodulation and shoot dry weight of alfalfa plants as well as strain competitiveness, in comparison with the wild type strain (Ma et al., 2004). A more recent study showed that under copper stress conditions, *M. lupulina* plants inoculated with a *E. meliloti* strain overproducing ACC deaminase showed higher biomass than plants inoculated with the wild-type strain (Kong et al., 2015).

Although some strains belonging to *Mesorhizobium* genera also possess *acdS* gene, its expression seems to be confined to symbiotic conditions (Nascimento et al., 2012). Engineering *M. ciceri* strains (salt-sensitive and salt-tolerant) to express an exogenous *acdS* gene (from a strain of *Pseudomonas putida*) under free-living conditions induced a significantly higher chickpea growth, compared with the wild-type strain,

in saline conditions (Brigido et al., 2013). Moreover, the *acdS*-transformed salt-sensitive strain was able to induce nodules in the same extent as the salt-tolerant strain under salinity (Brigido et al., 2013). Another *M. ciceri* strain, also expressing the *acdS* gene, was inoculated in chickpea plants growing in non-sterilized soil displaying biotic and abiotic constraints to plant growth (Nascimento et al., 2012a). The modified *M. ciceri* strain significantly increased the nodulation performance as well as the total biomass of chickpea plants, when compared to the wild type (Nascimento et al., 2012a). In addition, chickpea plants inoculated with this *M. ciceri* strain were less affected by fungal produced diseases. Similar results were found for chickpea plants inoculated with the same strain under waterlogging conditions (Nascimento et al., 2012b). Besides the demonstrated beneficial effects of ACC deaminase on the α-proteobacteria rhizobia-legume symbioses, a higher activity of this enzyme was also beneficial for ß-proteobacteria rhizobia-legume interaction, namely on the nodulation process by *Cupriavidus taiwanensis* STM894 of *Mimosa pudica* plants (Nascimento et al., 2018).

3.4.2. Phosphate Solubilization

Due to the low Phosphorus (P) mobility in the soil and its high capacity to react with calcium, aluminum and iron forming precipitates, this nutrient usually limits the plants growth (von Wandruszka, 2006). However, it has been reported that some bacteria produce enzymes able to solubilize phosphorus and make it readily available for plant uptake (Richardson et al., 2001, Peix et al., 2001). The phytase enzyme, encoded by *appA*, is responsible for solubilizing phytates, the major form of organic P (Rao et al., 2009). The overexpression of *appA* from *E. coli* in *E. meliloti* increased the phosphatase and phytase activity compared with the strain harboring the empty vector. In addition, maize plants inoculated *in vitro* with the modified strain showed a growth improvement in a medium containing Na-phytate as sole P source (Sharma et al., 2016). The overexpression of *appA* from *Citrobacter braakii* were evaluated in several rhizobacteria and the results showed a high constitutive phytase activity (from 10- to 538-fold higher than the respective control), increased

P content and shoot dry weight of mung bean (*Vigna radiata*) plants (Patel et al., 2010).

3.4.3. Siderophore Production and Antagonism-Related Genes

Several mechanisms are responsible for the antagonistic activities in bacteria, among them the inhibition of pathogen growth via antibiotics, toxins, surface-active compounds (antibiosis), and extracellular digestive enzymes such as proteases, cellulases and chitinases (De Souza et al., 2003). Trifolitoxin (TFX) is a potent antibiotic produced by *R. leguminosarum* that inhibits many α-proteobacteria growth (Triplett and Barta, 1987). A *R. etli* strain containing the TFX-encoding genes inserted in the chromosome showed an increase in its ability to compete for rhizosphere colonization and root nodulation in sterile, non-sterile soil and field condition (Robleto et al., 1997, Robleto et al., 1998). The overexpression of *tfx* genes was previously evaluated in *R. leguminosarum* and the modified strain occupied significantly more nodules from inoculated clover plants than a near isogenic nonproducing strain when co-inoculated with a TFX-sensitive strain (Triplett, 1990).

Iron is the fourth most abundant element on the surface of the Earth, however, the use of this element by plants and microorganisms is not always facilitated, since it is practically insoluble due the presence of ferric iron-hydroxide complexes under aerobic environments and neutral pH (Ratledge and Dover, 2000). Siderophores are molecules synthesized by microorganisms mostly under iron-defficiency conditions, which work quelating insoluble forms of iron. The siderophore production is a plant growth promoting trait, which helps to make iron available to plants as well as unavailable for other microorganisms, including pathogens (Saha et al., 2014).

In order to permit some rhizobia to use alternative sources of iron, the addition of specific genes was tested. *Rhizobium* spp. strains were transformed by receiving the *fhuA* gene from *E. coli*, which is able to uptake Fe^{3+}-ferrichrome. The modified strains increased pigeon pea plants growth as well as nodule density. In addition, the plants showed an higher shoot fresh weight, nodule number per plant, chlorophyll content of leaves

and effective nodule symbiosis when compared with plants inoculated with the control strains (Geetha et al., 2009). The *fegA* gene (encoding ferrichrome receptor) from *Bradyrhizobium japonicum* 61A152 was introduced in *Rhizobium* sp ST1 that was then used to inoculate pigeon pea seedlings. These plants showed an increase in growth parameters, furthermore nodule occupancy from plants inoculated with the transformed strains was larger then on those inoculated with the wild type (Joshi et al., 2009). *Mesorhizobium* sp. GN25, which is unable to utilize ferrichrome as iron source, was transformed by receiving the same *fegA* gene from *Bradyrhizobium japonicum* 61A152. Peanut plants inoculated with this mesorhizobial strain increased several plant growth parameters and these plants showed a larger nodule occupancy (Joshi et al., 2008).

3.5. Other Genes

Besides the genes that are known to be involved in the symbiosis process and stress response, or those which can promote directly the plant growth, it has been reported that the overexpression of several genes involved in other mechanisms may also improve the symbiotic performance of rhizobial strains and their relationship with plants (Janczarek et al., 2015, Menéndez et al., 2017) Menendez et al., 2016). In this section, we will give some examples of such genes.

Genes involved in rhizobial colonization and adhesion to plant surfaces are essential for the proper establishment of the symbiotic interaction and therefore, for the symbiotic performance of these strains under a wide range of conditions. Amongst those genes, the *pssA* and *rosR* genes are genes involved in the biosynthesis and regulation of exopolysaccharides (EPS) in *Rhizobium leguminosarum* bv. trifolii (Janczarek and Skorupska, 2007, Janczarek et al., 2001). It is known that exopolysaccharides are essential for the symbiotic relationship between rhizobia and leguminous plants (Marczak et al., 2017). The overexpression of EPS-related genes (*pssA* and *rosR*) in several *R. leguminosarum* bv. *trifolii* strains isolated from nodules, increased the EPS production, as well as competitiveness

and induced more nodules on clover plants than the wild type strain. In addition, plants inoculated with these transformed strains showed a higher shoot dry weight and occupied a larger nodule zone in the nodule (Janczarek et al., 2009). Another study also with genes related with EPS production showed that *E. meliloti* overexpressing *exoY* improved the levels of EPS succinoglycan. Moreover, *Medicago truncatula* A17 inoculated with this enhanced strain showed a higher shoot fresh weight and shoot length (Jones, 2012).

Rhizobial adhesines promote rhizobial attachment and aggregation (Frederix et al., 2014, Ausmees et al., 2001). In this sense a study using a *R. leguminosarum* bv *trifolii* overexpressing *rapA1* gene, which is involved in the bacterial adhesion to biotic surfaces, showed an increase in competitiveness and also, displayed an increase in nodules occupation in red clover plants (Mongiardini et al., 2009).

Another gene related with polysaccharide production and cleaving in *R. leguminosarum* bv trifolii is the *celC* gene, which encodes for a family 8 Glycosyl hidrolase or cellulase CelC2. On the contrary of genes improving symbiotic performance, the overexpression of this particular cellulase leads to a reduction of biofilm formation, aberrant infection phenotypes, a delay on nodulation and a decrease in *Trifolium repens* plant development, due to the extensive degradation of non-crystalline cellulose, the specific substrate of this enzyme (Robledo et al., 2011, Robledo et al., 2012). The heterologous expression of this *celC* gene on *E. meliloti* also displays similar phenotypes in *M. truncatula* nodules (Robledo et al., 2018).

Aminoacid and other molecules transport-related genes are also important for a successful symbiotic relationship. The *dctABC* operon encodes the proteins responsible for the transport system (Dct) in *E. meliloti*, which promotes the movement of succinate, fumarate, malate, and aspartate across the cell membrane (Yarosh et al., 1989, Jiang et al., 1989). The nitrogenase activity of *E. meliloti* transformed with a *dctA* gene from *Salmonella typhimurium* measured by acetylene reduction assays showed a higher rate of nitrogen fixation in *Medicago sativa* plants inoculated with the transformed strain (Rastogi et al., 1992). A previous study also showed

that *Bradyrhizobium japonicum* expressing the *dct* gene sequences from *E. meliloti* also improved its nitrogenase activity (Birkenhead et al., 1988).

Other genes that at first are not related with plant-microbe interactions might have some co-lateral effects. DNA methylation is somehow involved in the symbiotic process, but the underlying mechanisms for that are not yet well-known. In this sense, Ichida et al., (2009) evaluated a *ccrM*-overexpressing strain of *Mesorhizobium loti*, observing a delay in nodule development. CcrM is a bacterial DNA methyltransferase, which at least in the latter study contributes in an uncertain way to the regulation of the early phase of establishment of the symbiotic relationship between *Mesorhizobium* and *Lotus* plants.

CONCLUSION

To ensure the food supply for a growing human population without favouring the damage of the environment, the development of a more efficient and sustainable agriculture is needed, representing one of the greatest societal challenges. The combination of plant growth promoting bacteria-based biofertilizers with chemical fertilizers could be a first step for significantly reducing the use of chemical-based fertilizers. Nevertheless, the ultimate goal should be to completely eliminate the use of the chemical fertilizers by applying biofertilizers and implementing sustainable agricultural pratices.

Although most of developed countries in Europe and in North America have policies that do not allow the use of biofertilizers that include genetically modified microorganisms, they in fact have a powerful potential to improve the yields of agronomically important crops, mainly leguminous crops. The benefits imprinted to crops by the application of bacterial inoculants are recognized over the years; however, the replacement of chemical fertilizers is still limited mainly by these biosafety regulation policies and the lack of interest of farmers. An extra effort on "educational programs" about the use of biofertilizers for stakeholders is paramount (Pagaria, 2014, García-Fraile et al., 2017). Nevertheless, the

application of native soil bacteria, without any genetic modifications, as a way to reduce the use of chemical fertilizers, is being implemented by short steps. The use of genetically modified microorganisms by overexpression of symbiotically related genes is still controversial but will possibly become a reality in the near future. In addition, different levels of genetic modification should be considered. For example, introducing extra copies of an existing gene in a given genome is a distinct genetic manipulation from introducing a gene from an unrelated species.

Several of the bacterial genes presented in this chapter encode proteins involved in multiples metabolic pathways directly or indirectly associated to the symbiotic process, contributing to expand the symbiotic effectiveness, shoot and root dry weight of inoculated plants, nodule formation and occupancy, resistance to disease, abiotic stress tolerance, nutrients uptake, amongst others. In particular, the overexpression of *nif* genes is an interesting approach, because it is supposed to improve symbiotic efficiencies exerted by the rhizobial strains in the tested plants. Despite the potentialities of this approach, currently, the trend in research regarding nitrogen fixation genes is to transfer this capacity to plants, especially to cereals, engineering plants that are able to fix their own nitrogen, *ergo* the transference of whole nitrogen fixation complex into the genome of plants, keeping workable all the gene functionalities (Good, 2018, Curatti and Rubio, 2014, Vicente and Dean, 2017, Geddes et al., 2015).

The understanding of the molecular mechanisms behind the increase of the symbiotic performance of rhizobial strains is still a very important research line. This generates knowledge that will be useful to be applied when the limitations in the use of genetically modified (GM) bacteria on the fields are less restricted. Certainly, this molecular knowledge will be used to engineer efficient bacterial strains in the near future. The restrictions on introducing GM plants are changing and in the same way, the restriction on delivering GM beneficial bacterial will probably change soon. Genetic edition tools as CRISPR-Cas9 in plant growth-promoting organisms is already a reality (Basu et al., 2018) and will be essential for the upcoming research on the field. Therefore, some of the genes presented

in this chapter are strong candidates to be overexpressed or edited in rhizobia by biotechnological approaches to improve the symbiotic performance of these bacteria, which after these genetic modifications may be applied on the fields, when compliance withbiosafety regulations is guaranteed.

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Chapter 3

WHICH LEAF VARIABLE IS THE BEST INDICATOR OF THE PERFORMANCE OF OLIVE VARIETIES?

Masmoudi Chiraz[1,*], Marrakchi Olfa[2,†], Oueslati Amira[2,‡], Ben Abdallah Sirine[1,§] and Boujnah Dalenda[3,#]

[1]Sfax University, Olive Tree Institute, Unit of Tunis, Tunisia
[2]Carthage University, Department of Physic and instrumentation, High Institute of Applied Sciences and Technology, Tunis, Tunisia
[3]Sfax University, Olive Tree Institute, Unit of Sousse, Tunisia

[*]E-mail of the corresponding author: masmoudi.chiraz@yahoo.fr. Address: 17 Avenue Ahmed Tlili. 2091 Carnoy, Tunisia.
[†]olfa.charfi@gmail.com.
[‡]oueslatiamoura@gmail.com.
[§]benabdallah.syrine@yahoo.fr.
[#]dalenda.boujnah@yahoo.fr.

ABSTRACT

This work was made on 42 olive (*olea europaea*) varieties cultivated in northern Tunisia. The main objective is to evaluate their performance in low water conditions (LWC) and to infer the relationships between the leaf traits and the leaf and soil water variables. In line with these objectives, measurements and estimates of 15 leaf variables were made over two successive years (2015-2016), including the relative water content (RWC, %), the foliar tissue density (D, g/kg), the succulence (S, mg H_2O/cm^2), the sclerophylly (S_c, g/m^2), the water content at saturation (WCS, g H_2O/g DW), the specific leaf area (SLA, cm^2/g) and the water saturated deficit (WSD,%). Data (15 initial variables) were subject to the *Principal Component Analysis* (with the MATLAB program) to select the best indices. The variables S_c, WSD, WCS, RWC, S and D are found to be suitable for most varieties to describe accurately their response to LWC. Comparative results showed that olive varieties use different means to cope with LWC. The cultivars Gerboui, Coratina and Souri are the most performant; they have high S_c, RWC and S values and low WSD and WCS records, which makes them highly performant. Manzanilla has high D, medium S_c but low RWC. The cultivars Chamchali, Chemlali and Ascolana showed low performance in this area, suggesting that the means used to withstand the lack of water are less effective. Arbequina provided low S_c and D records, which makes it less protected against water loss, but it has low leaf area, which contributes to balancing out the other negative features. On the other hand, multiple relationships were observed between the leaf indices and the water variables, which could be used in the future to identify the spatial variability of responses of olive to LWC. Relationships established between the leaf indices and the other water variables (leaf or soil) showed that most varieties follow the general trends observed between RWC and D, LA and leaf weights and D and S, with some exceptions (Verdal, Coratina...). These varieties behave differently under LWC. Results also showed that the volumetric soil moisture (H_v, %) is poorly correlated to RWC and S_c but it interacts well with SLA, which can accurately traduce the soil water status. To conclude, the findings of this study provide supplementary information about the performance of olive varieties cultivated intensively under LWC and what leaf indices are the best to evaluate their response to the lack of water. We consider that further research is needed to see if these performant varieties are also competitive at a productive level.

Keywords: *olea europaea*, varietal collection, leaf water indices, classification, relationships, low water availability, *Principal Component Analysis*, soil water content.

1. INTRODUCTION

Olive tree has a great ability to withstand the limited water conditions (LWC). It develops several morpho-anatomic, physiological, eco-physiological and structural adaptive changes to avoid the excessive loss of water (Hsiao, 1973; Larcher et al., 1981; Chaves et al., 2002; Bacelar et al., 2004 and 2006; Guerfel et al., 2007; Boussadia et al., 2008 and 2013; Boughalleb and Hajlaoui, 2011; Ben Rouina and Ben Ahmed, 2013; Sikaoui et al., 2014; Nardini et al., 2014b; Ben Hassine et al., 2017). The major changes is observed on leaves, which are the central organ by which the tree regulates its water status (Giorio et al., 1999; Jorba et al., 2000; Guerfel et al., 2007 and 2009; Martin et al., 2014; Essaghi et al., 2016). Comparatively to stems and roots, leaves are found to be more flexible in their responses to water, reflecting more clearly the variability of the soil water content (Niinemets, 2001; Bacelar et al., 2004; Chehab et al., 2014; Ayachi-Mezghani et al., 2012; Saleem et al., 2013).

To quantify the leaf changes, several variables can be determined. The leaf area (LA) is commonly used for this purpose. Under limited water availability, leaf area is reduced to protect leaves against the excessive loss of water, although it leads to significant reduction in the biomass production (Villalobos et al., 1995; Aïachi Mezghani et al., 2016). Accordingly, the genotypes of varieties coming from dry areas are found to have smaller leaves than those of humid regions. The relative water content (RWC) of leaves is considered as a useful indicator of the plant water status because it is independent of the leaf anatomy and changes in RWC are proportional to changes in leaf turgor (Hsiao, 1973; González and González-Vilar, 2001; Saleem et al., 2013; Sikaoui et al., 2014). It expresses the absolute amount of water that plants require to reach cellular hydratation. Normal values range between 98% (for transpiring leaves) and 40% (for stressed leaves). High RWC sustains the shoot growth and the photosynthesis rates and allows adequate thermal regulation (Jorba et al., 2000). Water Content at Saturation (WCS) and Water Saturated Deficit (WSD) are thought to give more accurate results about the leaf water status, because they integrate in their formula the leaf weights. Abd-El-

Rahman et al., (1966) reported extremely low WCS for olives (1.59g H_2O/g DW) in comparison with fig (5.77 g H_2O/g DW) and grape (5.85 g H_2O/g DW). An optimum value of WSD of 17% is suggested to reach adequate transpiration rate. Also, varieties may react to adverse field conditions by increasing their sclerophylly (S_c), which does not change substantially through time, in mature leaves (Bussoti et al., 2002). Bacelar et al., (2004) reported values of 183.9 and 234.2 gm^{-2} in Cobrançosa and Blanqueta olive cultivars, respectively. These values are higher than those found for grape. High S_c value is a consequence of an increase of the density of the foliar tissue (D). It is observed in varieties that have thick cuticle layer and small intercellular volume (Jorba et al., 2000; Chehab et al., 2009b). Leaves with high foliar tissue density are more able to cope with drought because they present high resistance to the physical damages which occur to leaves when they desiccate. In such leaves, greater amount of water per unit leaf area can be stored; this characterizes the succulent species (Mantovani, 1999). Varieties that have high succulence index (S) cope with severe water shrinkage by increasing the leaf cuticle and epidermis thickness (Witkowski and Lamont, 1991).

In *olea europaea,* several adaptative mechanisms are used by trees to maintain the cell turgor (Jorba et al., 2000; Guggi et al., 2002; Boussadia et al., 2013). However, one variety may present a specific response (Proietti, 2000; Hagidimitriou and Pontikis, 2005). For example, Gucci et al., (2002) observed under severe water deficit more pronounced physiological effects on cv., Frontoio than in Leccino. In Portugal, Bacelar et al., (2004 and 2006) observed for Negrinha, Manzanilla and Cobrançosa cvs., more morphological and structural leaf adaptation forms than in other cultivars. In Cobrancosa cv., leaves have thick epidermis, high stomatal density, low specific leaf area (SLA, 5.13 m^2/kg) and high D values (554.8 g/kg). These features make it well protected against water loss. Cobrancosa is found to be more capable than Verdeal to cope with LWC. The cultivars Verdeal, Madurel and Cobrancosa afford LWC by providing low S values (15.5, 15.7 and 16.1 mg H_2O/cm^2 respectively). Leaves of the cultivar Arbequina present thinner trichome layer, this makes them less protected anatomically against the loss of water comparatively to the cultivars Negrinha,

Manzanilla and Cobrançosa. However, the small size of leaves in Arbequina contributes to balancing these negative anatomical features at the whole-plant level. In its native area, Arbequina was found to have low RWC (77%) and high WSD (12.5%) which likely inhibits the process of photosynthesis during the summer period. In Morocco, Sikaoui et al., (2014) obtained for the cv., Menara growing under deficit irrigation regimes high leaf RWC. Among the Spanish cultivars, only Manzanilla seemed to be well protected at the anatomical level, although it has medium leaf size. It has low WCS, WSD of around 8% and high S index. Under hard environment, it enhances its sclerophylly thanks to the protective leaf parenchymatous tissue, which is thicker than that observed for Blanqueta (234.2 gm^{-2}) and Cobrançosa (183.9 $g.m^{-2}$) (Bacelar et al., 2004).

However, although the impact of water shortage on olive is now well documented, and all results converge, revealing the different levels of susceptibility of olive varieties to LWC, few of them aimed to correlate the leaf parameters to other variables. Guggi et al., (2002) and Guerfel et al., (2009) reported significant relationships between the leaf water content and the specific leaf area (SLA, cm^2/g), which is itself highly correlated to the soil water content; this latest was found to be related to the olive production and growth (Masmoudi-Charfi et al., 2010).

In line with these findings, we intend, through this study to evaluate the performance of several olive cultivars originating from different provenances, to low water availability. We aim to provide a preliminary classification of varieties following their responses to LWC based on a limited number of variables. Besides, as we thought that water relation parameters were not studied extensively for *Olea europaea*, we intend through this study to investigate the possible relationships between the leaf parameters and the most significant water variables in order to identify the changes that enable olive varieties to cope with the lack of water.

2. MATERIAL AND METHODS

2.1. Site of Experiment

The experiment was carried out in 2015 and 2016 on a varietal olive collection planted in 2003 (Project RESGEN/IO/COI2002) at the *Research Station of the National Institute of Researchers in Rural Engeneering, Water and Forests* (INRGREF), NE of Tunisia (36.5ºN, 10.2ºE). In this area, the climate is semi-arid (Thornwaite AI is 0.29), with an average annual rainfall of 468 mm (Masmoudi-Charfi and Habaieb, 2014) and a Penman-Monteith reference evapotranspiration ($ET_{0\text{-}PM}$) of 1200 mm/year. Climate is dry and hot from May to end of August and rainy from September to April. During the years 2015 and 2016, annual rainfall amounts reached 360 and 420 mm, respectively.

2.2. Vegetative Material and Crop Management Practices

The experimental set-up is based on 42 olive varieties, local and foreign (3 trees/variety), grown on sandy-loam soil (70-80%S) at 6mx6m spacing. The orchard is plowed 3-4 times a year. Trees were trained following a cup-shaped form and irrigated during the fruit growth period (May to September) to complement the rainfall amounts.

During the two years of the experiment, the effective rainfall amounts recorded between May and September were insufficient to cover the monthly crop water needs (ET_c, mm), which were determined following the FAO climatic method as ET_c (mm) = ET_0 x K_c x K_r (Allen et al., 1998). The crop coefficient K_c was taken equal to 0.5 for 12-13 years old trees and the coefficient K_r relative to the soil coverage (26-31%) was equal to 0.7. The reference evapotranspiration (ET_0) was computed following the Penman-Monteith (PM) formula (Allen et al., 1998). Monthly values of ET_0 were used to establish the irrigation program. Amounts of water applied each month are given in Table 1; the lowest was applied in May

and September, representing 44 and 38%ETc. During the first campaign, as the amount of available water was limited, irrigation was applied each week during three days only. In addition, as the irrigation system didn't allow application of different doses of water to meet the variety's crop water needs with regard to its specific soil coverage, water was equally distributed to trees. This management procedure is considered as the "minimal technical package" that can be applied in the intensive olive groves.

The volumetric soil moisture (H_v, %) was determined at irregular intervals, when workers are available. Measurements were made at two different sites: (1) at the intersection point of 4 trees (non irrigated area, 4 replicates) and (2) under the canopies, along the line of emitters (irrigated area of 6 olive trees belonging to different varieties with and without olive production, Eastern direction). Soil samples were taken at 40 cm outside the irrigation ramp. For this first step of the study, we looked for the global trend of H_v evolutionary considering its average value. In the following step, individual H_v values will be analyzed separately with regard to the variety's behavior (fruit load, vigor...).

Table 1. Monthly values of reference evapotranspiration (ET_0, mm), crop water needs (%ET_c, mm) and irrigation amounts for the period May-September. Varietal olive collection of Nabeul, NE of Tunisia

	ET_0 (mm/month)	ET_c	Amount of irrigation water	
			mm/month	m^3/tree
May	123	43	18	0.68
June	157	55	26	1.01
July	163	57	24	0.94
August	161	56	22	0.83
September	110	39	15	0.54

NB: All weather variables were obtained from the National Institute of Meteorology and its website.

2.3. Leaf Measurements and Computation of the 'Leaf Indices'

Leaf indices were determined at different growing stages from early spring 2015 to autumn 2016. Monitoring was made on a pool of 42-monovarietal, mature and fully expanded leaf samples, each representing a variety, with 5 replicates per variety. A preliminary visual inspection showed that each leaf was in good physical shape. Measurements and computation of the leaf indices were made as shown in Table 2.

Table 2. Leaf indices followed during the campaigns 2015 and 2016 and methods used for their computation for 42 olive varieties grown under LWC

Variable	Method of computation
Length of the leaf (L, cm) and its width (l, cm)	Measured at different periods of the growing cycle during the years 2015 and 2016
Area of the leaf (LA, cm^2)	LA = 0.735 (L x l) + 0.125 (Tattini et al., 1995) (R^2 = 0.987)
Specific leaf area (SLA, cm^2/g)	SLA is derived from LA / SLA = LA/DW (Hummel et al., 2010)
Fresh (FW, g) and dry weight (DW, g)	Determined by using a digital weight balance (1/100 g accuracy) and an oven-dried (fixed at 70°C for 48 hours)
Weight at saturation(W_{sat}, g),	Measured on leaves rehydrated in demineralized water and placed in the dark for 24 hours at 4°C.
Succulence (S, mg H_2O/cm^2)	When water content is expressed relative to unit leaf area, it defines the succulence: S = (FW-DW)/LA.
Relative water content (RWC, %)	Amount of water contained in the leaf relative to DW. Determined on fully expanded leaves which were harvested before midday. RWC = (FW-DW) / (W_{sat}-DW) x 100 (González and González-Vilar, 2001).
Density of the foliar tissue (D, g/kg)	D (g/kg)= (DW/FW) x 1000 (Groom and Lamont, 1999)
Schlerophylly (S_c, g/m^2)	S_c (g/m^2) = DW / LA (g/m^2) (Denaxa et al., 2012)
Water content at saturation (WCS, gH_2O/gDW) and Water saturated deficit (WSD, %)	WSD = (W_{sat} – FW) / (W_{sat} – DW) x 100 WCS = (W_{sat} – FW) / DW (g H_2O/g DW). Bacelar et al., (2004 and 2006)

2.3. Statistical Analysis

Data collected on each sampling day were averaged for a single cultivar across the five replications and the means were combined for all cultivars across the entire data to run analysis for a particular year. At the same, the relationships between the leaf traits and the water variables were investigated. During this first step of the study, we looked for the global trends and the fine analysis will be performed later, considering each variety apart (production level and vigor). Only relationships that have physiological or practical implications are presented in this paper. Note that at this step of the study, we used the term 'relationship' instead of 'correlation' because results presented herein are averages of all varieties. Data relative to the 15 variables are subject to the *Principal Component Analysis* (PCA) (Asno, 2005). We used the *MATLAB* program to decorrelate the initial leaf variables. This analysis allowed selection of a limited number of parameters, which were used to classify varieties, based on the main PCA components. For each variable, varieties are organized in three groups: low, medium and high following the importance of their values.

The statistical PCA was made following these steps:

- Leaf data are organized in matrix of n = 42 varieties and p = 15 descriptive variables, for which variances and covariances are determined. When the difference between the observations and the average values are low, the variables are poorly related, but they are not necessary independent. The following steps of the PCA aimed to decorrelate the leaf variables.
- Determination of the matrix D representing the diagonal form of the eigenvectors (V) with values ordered decreasingly. The diagonal coefficients represent the variances of the 15 new *Principal Components* (CP) (data not shown). Characterization of this distribution is made with an orthogonal basis presenting the matrix of the 15 decorrelated components**.** The highest values are the most significative.

- Criteria of evaluation: each component has its own contribution (ratio of its own value to the sum of the eigenvalues of the other components, %). Those having high contribution are selected to classify olive varieties. This allows reduction of the number of the descriptors used to characterize olive varieties.

3. RESULTS

3.1. Average Data and *Principal Component Analysis* (PCA)

Table 3 presents the main components issued from the PCA relative to the leaf variables affected each by the corresponding contribution. It shows that the cumulated energy input of the first six components, *i.e.*, S_c, WSD, WCS, RWC, S and D is greater than that of the remaining ones, giving 100% of the information. Thus, classification of varieties can be made on the basis of these six components, only.

Table 3. Main components (leaf variables) and the corresponding contributions (%)

Main variables	S_c (g/cm^2)	WSD (%)	WCS (gH_2O/gDW)	RWC (%)	S (mgH_2O/cm^2)	D (g/kg)
Contribution (%)	90.4	5.76	3.64	0.14	0.05	0.002

3.2. Classification of Varieties Following the Main Leaf Water Variables

Table 4 reports the classification of the olive varieties relative to S_c, showing that a great number of varieties have medium records, ranging between 4.5 and 5 $g.dm^{-2}$. The varieties Souihli-Rkhami (local varieties) and Koroneiki, Arbequina, Picholine and Ascolana (most common foreign varieties) have low S_c values. The highest records for the local varieties are observed for Gtar, Gerboui, Barouni and Oueslati.

Table 4. Classification of olive varieties following the S_c variable ($g.cm^{-2}$)

$S_c < 4.5$ g/dm^2	$4.5 < S_c < 5.0$ g/m^2	$S_c > 5$ g/dm^2
Galega-Koroneiki-Dahbia-Leccino-Rkhami- Arbequina-Souihli-Zarrazi-Ascolana- Malarato-Picholine	Sigoise - Doukhar- Chamchali-Gemri- Franjivento- Madurel-Chemlali-Verdal-Madurel- Changlot Real-Sayali-Vera-Chétoui- Meski-Lucques-Besbassi-Zarzane Tounsi-Marsaline-Manzanilla- P.Marocaine	Beldi-Azeitera-Gtar- Gerboui-Coratina-Branquita-Barouni-Oueslati-Souri-Ayvalik- Conserva

Table 5. Classification of olive varieties following the WSD (%), WCS (gH_2O/g DW) and RWC (%) leaf indices

WSD > 23.5%	WSD: 20-23%	WSD < 20%
WCS > 0.33 gH_2O/gDW	WCS: 0.26-0.31 gH_2O/g DW	WCS < 0.25 gH_2O/g DW
RWC < 76.5%	RWC: 78-80%	RWC > 80%
Besbassi-Oueslati- Gtar- Ayvalik-Marsaline-Picholine-Madurel-R'khami-Manzanilla-Chamchali-Malarato- Doukhar-Beldi-Souihli-Gemri-Ascolana- Marocaine-Koroneiki-Tounsi-	Arbequina-Lucques-Dahbia-Franjivento-Zarrazi- Sigoise-Verdal-Meski-Sayali-Barouni-Conserva-Branquita-Leccino-Chemlali	Gerboui-Chétoui-Souri-Zarzane-Galega-Coratina-Vera-Azeitera-Changlot Real

NB: With the exception for the varieties Sigoise, Beldi, Meski and Branquita which provided RWC < 76.3% and WCS < 0.33gH_2O/g DW.

Table 6. Classification of olive varieties following the succulence index (S, mg H_2O cm^{-2})

S > 100 (mg H_2O/cm^2)	S: 86-100 (mg H_2O/cm^2)	S< 86 (mg H_2O/cm^2)
Gerboui-Chétoui-Souri- Coratina-Vera-Gtar- Verdal-Meski-Arbequina- Ayvalik–Zarzane-Madurel-Tounsi-Branquita-Conserva	Lucques- Dahbia- Franjivento-Zarrazi- Sigoise-Leccino-Galega-Changlot Real- P.Marocaine-Chemlali-Doukhar- Ascolana-Gemri-Barouni-Rkhami-Marsaline-Souihli-Picholine-Chamchali- Koroneiki- Oueslati	Besbassi-Manzanilla-Azeitera-Beldi-Sayali Malarato- Sigoise

Table 7. Classification of olive varieties following the density of the foliar tissue (D, $g.kg^{-1}$)

D > 530 (g/Kg)	530 < D < 470 (g/Kg)	D< 470 (g/Kg)
Beldi-Azeitera-Besbessi-Barouni-Oueslati- Gtar Manzanilla- Sayali-Marsaline	Sigoise – Lucques- Coratina- P.Marocaine – Gemri –Ayvalik – Chamchali- Malarato-Verdal- Zarzane- Ascolana- Changloreal-Zarrazi –Conserva- Tounsi- Meski- Gerboui-Souri-Liban- Chétoui- R'khami- Picholine-Vera-Leccino- Branquita- Chemlali- Madurel-Souihli- Galega- Franjivento- Koroneiki	Dahbia-Arbequina-Dhoukar

Table 5 provides a combined classification for the WSD, WCS and RWC variables. The varieties Picholine, Manzanille, Ascolana and Koroneiki, among others, have high WSD (>23.5%) and WCS (>0.33 gH_2O/gDW) and low RWC (<76.5%). The varieties Arbequina and Chemlali, as well as Meski and Zarrari have medium values of WSD and WCS and RWC ranging between 78 and 80%. The cultivars Chétoui, Gerboui, Coratina and Azeitera have low values of WSD and WCS and high RWC (>80%). Among these varieties, Gerboui, Coratina and Azeitera have also high S_c values.

Table 6 shows that most olive varieties have high to medium S indices. The highest are observed for Gerboui, Souri, Coratina and Gtar, which have high S_c records. The varieties Meski, Chétoui and Arbequina have high S but low to medium S_c. The lowest S values are recorded for Besbassi, Sayali and Manzanilla, among others. We extend the list of the selected variables to the density of the foliar tissue, although it has low energy intake (0.002%), because it is frequently cited in the bibliography as useful index to evaluate the performance of olive varieties to LWC.

In Table 7, varieties are classified as follows: Group 1 includes, among other varieties Beldi, Oueslati, Manzanille and Gtar (high to medium S_c but low RWC), with D value exceeding 530 g/kg. In group 2, were classified the varieties Picholine, Chamchali and Chétoui with D value ranging between 470 and 530 g/kg. The cultivar Arbequina provided low D values of less than 470 g/kg.

Results relative to LA estimates showed high records for Ascolana (8.5 cm^2) and Coratina (7.9 cm^2). The varieties Manzanilla (4.9 cm^2) and Arbequina (4.8 cm^2) provided medium values. Lower LA estimates are obtained for Oueslati (3.9 cm^2), Chétoui, Dahbia (4.2 cm^2) and Rkhami (4.7 cm^2).

With this classification, it is possible to assign to each variety a characterization taking into account the significant indices that allow an assessement of its general level of 'performance' in the face of water availability. Indeed, Chamchali, Chemlali and Ascolana showed low performance in this area (low to medium D, S_c, S, RWC, high WSD and WCS), while Gerboui, Coratina and Souri showed high performance (high S_c, RWC and S and low WSD and WCS observations). Chétoui has high S and RWC values, while Manzanilla provided high D, as well as Besbassi, Marsaline and Oueslati and medium S_c. Manzanilla has low RWC and S values. The cultivars Arbequina, Meski and Rkhami (which is a secondary variety of NE of Tunisia) provided high to medium S, while Beldi and Barouni have high S_c and D records. This classification shows that olive varieties use different means to cope with LWC.

3.3. Leaf Water Parameter's Relationships

An average downward trend of RWC is observed when D increases (when D increases from 450 to up to 550 g/kg, RWC declined from 89 to 57%, Figure 1a).This general trend is observed for most varieties (28/42) when they are, each, considered separately. The varieties Changlot Real, Verdal, Zarrazi, Conserva, Lucques, Zarzane, Barouni, Coratina and Sayali showed different behavior. They provided high values of both parameters (D and RWC) at the same dates. For Chemlali, Meski, Picholine and Madurel, values of D and RWC evolved also in the same direction but with low records for both variables. These varieties are thought to use other means to cope with LWC. The relationship is significantly improved (Figure 1b) when these varieties are discarded (14/42).

Figure 2a shows that LA and W_{sat} evolve in the same direction. This means that varieties that reach high weights at saturation are those with large leaves. Such leaves are able to store greater amount of water per unit leaf area. Results showed also good relationships between LA and DW, LA and FW (Figure 2b) and the leaf length and W_{sat} and the leaf width and FW (Figure 3). We observed also high values of S_c and S for leaves with high FW; better relationships are obtained when DW is used instead of FW (data not shown).

An average ownward trend of D is observed when S increases (when D increases from 450 to 550 g/kg, S decreases from 125 mg/cm^2 down to 70). This general trend is observed for most varieties when they are each considered separately, except the cultivars Ayvalik, Verdal, Coratina and Gtar which provided at the same date high values for both variables. Exception concerns also the cultivars Leccino, Changlot Real, Koroneiki, Dahbia, Galega, Souihli, Rkhami and Ascolana for which, D and S reached their lowest values at the same date. The relationship is improved when these varieties (12) are discarded (data not shown).

SLA reached high values (>12 cm^2/g) when D is down to 500 g/kg DW (data not shown). This general downward trend is observed for most varieties when they are each considered separately, except the varieties Tounsi, Gerboui, Zarzane, Sigoise, Branquita, Vera and Changlot Real, for which D and SLA evolved in the same direction, what means that these varieties may use different means to cope with LWC.

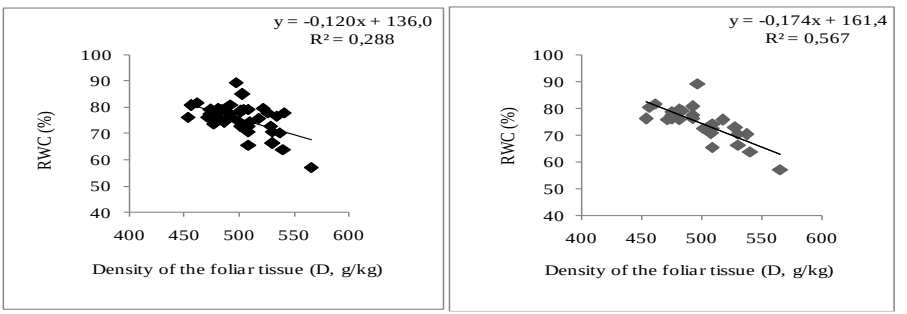
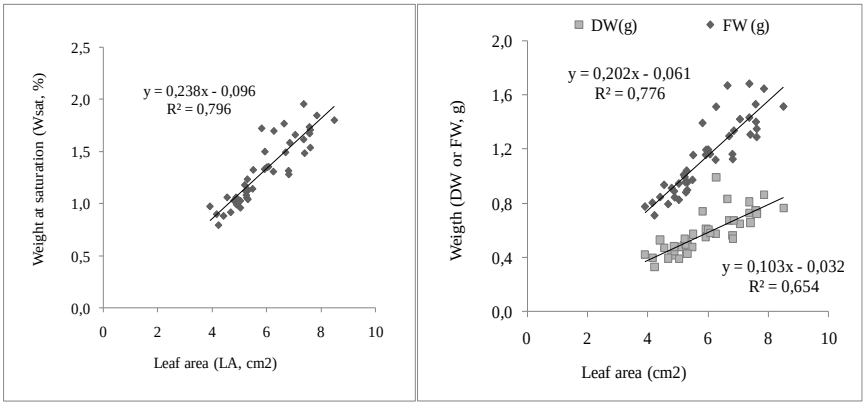
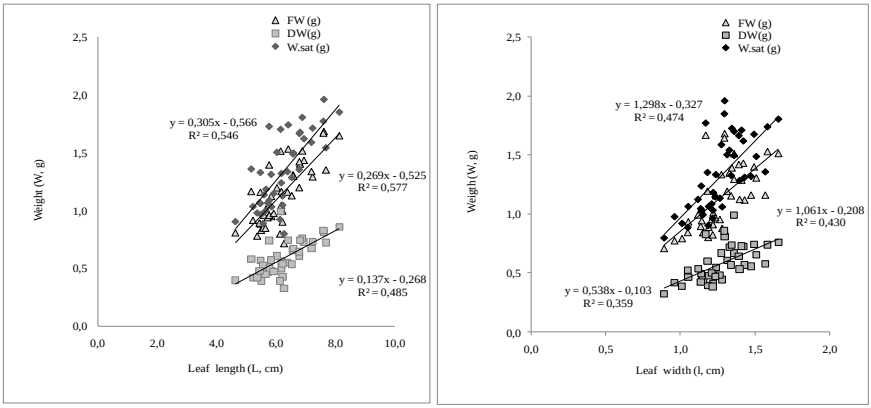


Figure 1. Relationships between RWC (%) and D (g/kg). (a): left: Considering all varieties of the olive varietal collection, and (b): right: The varieties for which D and RWC evolved in the same direction are discarded.



(a) (b)

Figure 2. Relationships between (a): LA (cm^2) and W_{sat} (g), (b): LA and weights (DW and FW, g) considering all varieties.



(a) (b)

Figure 3. Relationships between the leaf treats (L and l, cm) and the leaf weights (W_{sat}, DW, FW, g) considering all varieties of the olive varietal collection.

3.4. Soil Moisture

During the year 2015, soil moisture decreased consistently during the month of August to an unacceptable level due to the breakdown of the irrigation system for two successive weeks. During the year 2016, distinct

differences are observed between the sites of measurements. The highest soil moisture was recorded by the end of May 2016 (Figure 4). Results, also showed that the samples taken under the trees that have high fruit load or vigorous vegetation do not necessarily have the lowest H_v values (data not shown). Apparently, other factors, endogenous and/or exogenous interfere as reported by Walter and Schurr (2005). Thus, more investigation is needed to clarify this relationship. On the other hand, measurements of H_v made at the intersection point of 4 olive trees (non irrigated area) showed for one given depth, little variation between the sites of sampling during the autumn-winter period but large differences during the spring period (data not shown).

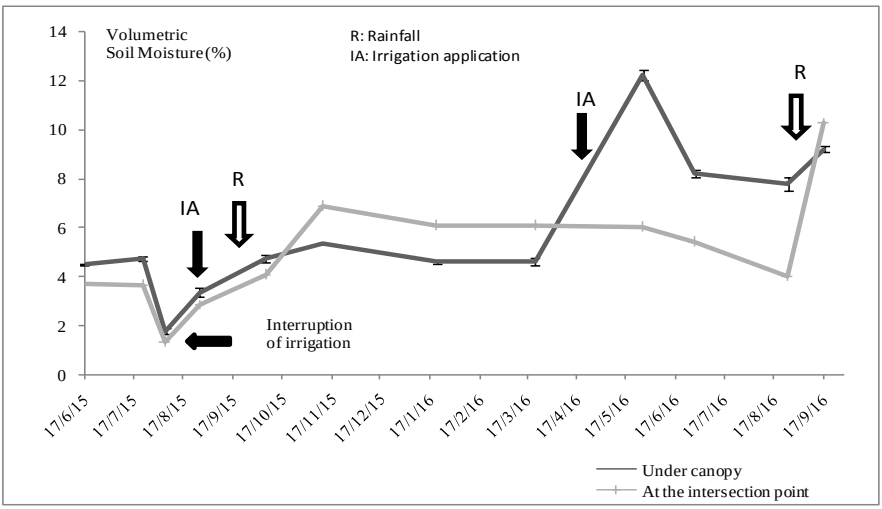


Figure 4. Evolution of the volumetric soil moisture (H_v, %) with time and site of measurement: Under the canopy (irrigated area) and at the intersection point of 4 trees (non irrigated area). Values shown are means ± se.

3.5. Relationship between the Volumetric Soil Moisture and the Leaf Parameters

Soil moisture (H_v) is well related to SLA, L, WCS, DW, LA and S_c, but it is poorly related to RWC. The best interaction is observed between

H_v and SLA (Figure 5), what means that SLA is good indicator of the soil water content. For high H_v measurements, we observed low records of SLA and high values of DW and S_c.

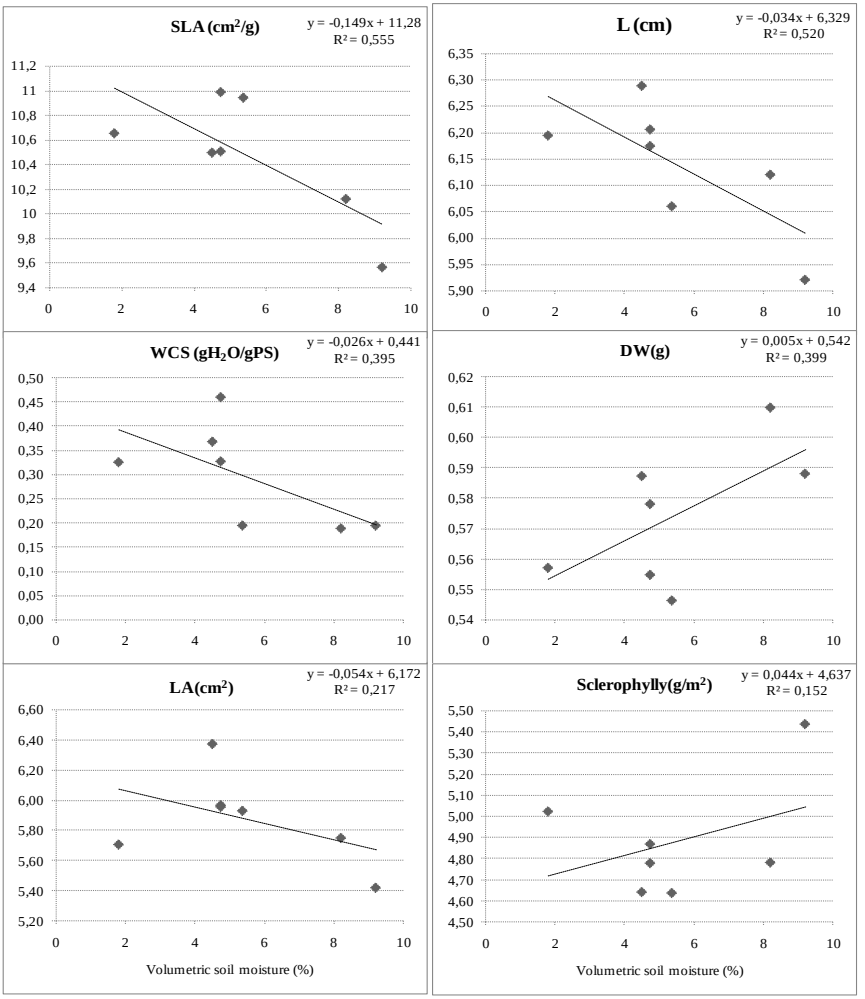


Figure 5. Linear relationships between the volumetric soil moisture (H_v,%) and the leaf indices, with R^2 of up to 0.5 for the SLA/H_v and L/H_v relationships. Data are relative to the period going from July 2015 toward May 2016.

4. DISCUSSION

In order to infer the superiority of some olive varieties for their ability to cope with LWC, a new experiment is made in northern Tunisia on a varietal olive collection including 42 local and foreign varieties. Through this comparative study, we aimed to classify varieties following their response to water availability and to investigate the potential relationships between the leaf traits and the leaf and soil water variables. Only relationships that have a physiological or/and a practical importance and signification are presented herein. As expected, results showed that varieties provide different responses. Distinct behavior was clearly observed for some of them, which have great performance in their native area (Chemlali) to cope with LWC. These results raise many questions:

(1) Which leaf water indices should be considered to classify the cultivars?
(2) How do varieties behave when they are cultivated far from their native area?
(3) Are the established relationships stable? How can we explain them? And how can these relationships be used at field level?

Relative water content: In most research works dealing with the aptitude of olive trees to cope with low water availability, RWC is determined to compare the performance of olive varieties. Results provided herein showed that RWC is, effectively, one of the selected indices (PCA), although it provides low energy intake (0.14%) comparatively to S_c and WSD. Classification of varieties based on this variable, showed that most of them have low to medium RWC (<80%). Manzanilla has RWC of less than 76.5%, while Gerboui, Chétoui, Galega, Coratina and Azeitera have high records that allow them to cope adequately with LWC. Indeed, according to Jorba et al., (2000), high RWC sustains tree growth and photosynthesis and allows adequate thermal regulation; it is considered as a useful indicator of the plant water status because its changes are proportional to changes in leaf turgor (González and González-Vilar, 2001;

Saleem et al., 2013; Sikaoui et al., 2014). Values of RWC recorded in this experiment are concordant with those cited in the litterature (Angelopoulous et al., 1996; Chartzoulakis et al., 1999; Giorio et al., 1999). All of them remained in the range of the standards, i.e., ranging between 98% (for transpiring leaves) and 40% (for stressed leaves) (Jorba et al., 2000). For example, Niinemets (2001) found for Arbequina average RWC of 77% in its native area, which is associated with some loss of water. He explains that the rapid changes observed in water potential for a given change in RWC, suggest that this cultivar is less protected against the loss of water than other varieties. However, this negative feature is thought to be balanced by the relative small size of its leaves (4.8 cm^2). In our experiment, Arbequina has value of RWC of the same order. The variety Chemlali provided an average value of RWC which is concordant with the those observed in its native area (69-86%), where it is found to be able to maintain high level of hydratation (Guerfel, 2007). However, in spite of the large use of RWC, it is criticized for the lack of continuous spatial coverage and time consuming when it is measured by using the traditional methods. Sepulcre-Cantó et al., (2006) recommanded the use of multiple sensors to detect even the minor changes of the physiological variables in order to assess more precisely the variations of the plant water status. According to these authors, remote sensing is found to be an effective alternative to field sampling, being non-destructive and providing continuous spatial coverage over large area. In this experiment, we observed that although RWC has low contribution comparatively to other indices, results converge to state that RWC, under low water availability, can be a useful tool to evaluate the plant water status. Values obtained herein are comparable to those measured in other areas for the same varieties.

Sclerophylly: High S_c records are observed for Beldi, Azeitera, Gtar, Gerboui, Coratina, Branquita, Barouni, Oueslati and Conserva (>5g/cm^2). The varieties Manzanilla, Meski, Chétoui and Chemlali have medium S_c records, while Arbequina has one of the lowest. This means that Arbequina would not provide much protection against the loss of water and so, it is anatomically less protected against water depletion. Based on the S_c values,

Manzanilla seem to be more able to cope with LWC than Arbequina, corroborating previous findings, following which, Manzanilla, has the capacity to withstand LWC by enhancing its sclerophylly thanks to its protective leaf parenchymatous tissues (Bacelar et al., 2004 and 2006). In this experiment, we have recorded also low S_c (<4 g/dm^2) for Koroneiki, Picholine, Ascolana (foreign varieties) and Rkhami, Souihli, Zarrazi (local varieties) which apparently use other means to withstand LWC.

Leaf succulence S (mg H_2O/cm^2) is used to assess the ability of olive leaves to cope with LWC, although olive is not a succulent species. The highest values were obtained for Gerboui, Chétoui, Souri, Coratina, Gtar, Meski, Arbequina and Conserva. Note here that the varieties Gerboui, Souri, Coratina, Gtar and Conserva have also high sclerophylly (> 5 g/dm^2). Besbassi, Azeitera, Sayali, Manzanille and Koroneiki have low records, down to 86 mg H_2O/cm^2. For Manzanilla, Hopkins, (1995) and Bussoti et al., (2002) found that it withstands LWC by enhancing its succulence, which would reduce the transpiration rate.

Water Saturated Deficit WSD (%) ranged between 42.9 and 10.6% with low records obtained for Gerboui and Chétoui. Larcher (1981) proposed an optimal value of 8%, which allows an easy CO_2 absorption and high transpiration rate. This value is too low compared to ours. Chartzoulakis et al., (2000) obtained low values for Cobrancosa leaves, which have compact arrangement of cells and high mesophyll surface area/unit leaf area. This arrangement facilitates the CO_2 uptake and maintains the photosynthesis rate under LWC. Main varieties have high to medium values.

Leaf area (LA) is the most used criteria to evaluate the response of olive to the lack of water. In several papers, it is sad that the most typical anatomical modification found in response to water deficit, besides acting on stomatal closure, is reduction in LA, resulting in reduction in the transpiration rates. Balaygue (1996) and Albouchi (1997) consided LA as a basic ‘morphological mechanism’ used by the olive trees to protect leaves against water loss. Results provided herein relative to LA estimates, showed high records for Ascolana (8.5 cm^2) and Coratina (7.9 cm^2) which are traditionally cultivated in the northern area of the Mediterranean basin.

Manzanilla (4.9 cm^2) and Arbequina (4.8 cm^2) provided values different from those observed in their native area (Fernandez and Moreno, 1999; Proietti, 2000...). Lower LA estimates are obtained for Oueslati (3.9 cm^2), Chétoui, Dahbia (4.2 cm^2) and Rkhami (4.7 cm^2), all of them originate from North Africa. These results are concordant with others, showing that genotypes coming from dry areas have smaller leaves than those of humid regions. Besides, Chemlali, which is the reference cultivar of Tunisia, characterized by its great ability to withstand the arid climate, provided average LA of 5.2 cm^2, which is higher than that recorded by Guerfel (2007) under the arid climate of Centre Tunisia. This author observed an increase of LA for the irrigated trees of +2.9% and +3.5% for Chemlali and Chétoui, respectively, and comparatively with the control. However, in spite of the convergence of these findings, LA is not considered in this experiment as a main component (by PCA).

The specific leaf area (SLA) is determined in other research papers because it is thougth to be more accurate than LA, because it takes into account the leaf dry weight (DW) (Dijkstra, 1989; Witkowski and Lamont, 1991). The findings of Nardini et al., (2014a) suggest that variation in SLA lies to modification in leaf thickness, which could limit the passive loss of water. This means that varieties, which are able to cope with LWC, have greater density of the foliar tissues (Essaghi et al., 2016). Veneklaas et al., (2002) observed low SLA in 'tolerant' varieties, which are able to increase the thickness and/or the foliar tissue density of their leaves. High D value observed for the cvs., Besbassi, Oueslati, Manzanilla reveals normally more protection against the lack of water (Witkowski and Lamont, 1991), giving the same dry mass within a smaller LA and higher turgor maintenance ability that may help these cvs., to maintain the expansion of leaves when water is less available. The varieties Chétoui and Chemlali have values of 493 g/kg and 481 g/kg, which are lower than those recorded by Guerfel (2007) under the arid climate of southern Tunisia, where D values exceeded 500 g/kg, with the highest recorded for Chemlali. According to Witkowski and Lamont (1991), leaves with high D are able to survive to more severe water shortage conditions than those having low D values because they have greater resistance to deshydratation, and thus,

they are well protected against the physical damages that may occur to the foliar tissues under LWC. Guerfel (2007) and Niinemets (2001) noted that leaves with high D values have long shelf lives.

However, as varieties showed variable responses, we thought that a complementary anatomical and ecophysiological study is necessary to provide accurate recommendations. In fact, our large review of bibliography, showed that the leaf anatomical features are related to other changes occuring at other levels. For example, the thin trichome layer in Arbequina makes it less protected against water loss, but the small size of leaves that it develops contribute to balance the negative cultivar's anatomical feature at the whole-plant level. The cv., Cobrancosa, which was cited to have the lowest SLA estimates and the highest D values, cope with LWC thanks to the small volume of the intercellular spaces. Chemlali was found to avoid water deficit deleterious by regulating its stomata operture during the first hours of the day (Guerfel et al., 2007; Ben Rouina et al., 2007 and 2013), while Chétoui procedes to stomata regulation later and so, it is exposed to higher water loss.

On the other hand, investigations made in this study showed multiple relationships which can be used to identify the spatial variability of olive responses to LWC, and then to predict the appropriate intervention that can be made to limit the negative effects of the lack of water at fine scale time (phenological stages) and specific level (each variety apart). Results relative to the relationships between the leaf indices and the water variables, showed a first level of interaction between LA and W_{sat}, which evolve in the same direction. This means that varieties with high weight at saturation have large leaves and important LA; they are able to store greater amount of water per unit leaf area. Mantovani (1999) explains that if two leaves have the same water storage capacity, the more succulent would be the one allocating water in a smaller area, which would provide reduced transpiration. Accordingly, Guerfel et al., (2009), Ben Rouina and Ben Ahmed (2013) found that leaf expansion in Chemlali leaves is predominantly driven by the cell turgor, which is correlated to the soil water potential. In another work, leaf turgor was found to be correlated to the leaf temperature, which is normally lower in the turgid leaves (Yiwei et

al., 2009). At high saturation deficit, Pantin et al., (2011) found that leaf expansion and water potential are reduced. In their recent review, Hummel et al., (2010) showed that low water availability leads to carbon accumulation and explains that the reduced LA is always associated to the limited local starch availability or transfer (Chehab et al., 2009 a and b). On the other hand, results showed low levels of D for high levels of RWC (in Dahbia and Arbequina for example), corroborating Jiang and Carrow (2005), according to which D is well correlated with RWC ($r = –0.76$ to $–0.78$). Additionally, D is found to evolve for most varieties following an ownward trend when the S index increases, with exception for some of them (Verdal, Coratina...). In previous works, good correlations were observed between the S index and the mesophyll thickness ($R^2 = 0.875$), suggesting that this could favor the hydraulic status as the leaf expands (Jorba et al., 2000; Bussoti et al., 2002, Chehab et al., 2009b; Martin et al., 2014). One of the most interesting relationships is that found between DW and the volumetric soil moisture, showing that the increases in the soil water content can traduce effectively the increases of the leaf water content, corroborating Giorio et al., (1999) who find significant relationship between the soil water content and the leaf water status. Results also showed good relationships between H_v and LA. However, the best relationship is that observed between H_v and SLA, which seems to traduce accurately the soil water status. SLA decreases when H_v increases. However, Ben Rouina et al., (2007) found that H_v was best predicted with soil and leaf water potentials rather than by other leaf water parameters because they allow prediction of the water stress before the development of the symptoms, leading to more efficient management of water at field level.

Results obtained herein are consistant with others. Indeed, these results and the large review of bibliography that we do showed that olive varieties use different means to cope with LWC. Together, our results fit with the general view that olive trees use an arsenal of responses when they are cultivated under low water availability. Based on the studied variables and the results of the PCA, our study shows that among varieties, Gerboui, Coratina and Souri are the most performant. They have high RWC, S and

S_c values and low WSD and WCS observations. Manzanilla withstands the LWC in this experiment with medium S_c and high D as well as Besbassi, Marsaline and Oueslati while Chétoui afford LWC with high S and RWC values. Chamchali and Chemlali showed low performance in this area. The cv., Arbequina provided low D and S_c records that makes it less performant to afford the loss of water but it has high RWC and relatively low LA that can balance the other negative features (low D).

CONCLUSION

Our results complement knowledge on the ability of olive varieties to counter/confront water shortage conditions, which is a matter of debate in many countries, and studies revealed the complexity of the responses of olive to LWC. Determination of leaf indices is important because data based only on leaf water content are informative in assessing the available tree reserves. Although they are an indirect measure of the plant water status, these indices are easy to make and beneficial for site-specific water management, particularly for locations that show large spatial rainfall variability and low water resources. The principal component analysis allowed reduction of the number of descriptive variables to six (S_c, WSD, WCS, RWC, S and D), which were used to classify the studied olive varieties. With this classification, it is possible to assign to each variety a characterization taking into account the significant indices that allow an assessement of its general level of 'performance' face to water availability. Accordingly, Chamchali, Chemlali and Ascolana showed low performance in this area (medium D, S_c and S, low RWC, high WSD and WCS), while Gerboui, Coratina and Souri showed high performance (high S_c, RWC and S and low WSD and WCS estimates). Chétoui has high S and RWC values, while Manzanilla provided high D, as well as Besbassi, Marsaline and Oueslati- and medium S_c. Manzanilla has low RWC and S values. The cultivars Arbequina, Meski and Rkhami (which is a secondary variety of NE of Tunisia) provided high to medium S, while Beldi and Barouni have high S_c and D records.

Investigations made in this study showed multiple relationships which can be used to identify the spatial variability of olive responses to LWC. The best is observed between H_v and SLA, which seems to traduce more accurately the soil water status (SLA decreases as H_v increases).

The findings of this study provide supplementary information on the performance of olive varieties and what indices can be used to evaluate their response to low water conditions. Also data obtained herein support the general view that olive trees use an arsenal of means to cope with LWC. It appears that S_c, WSD, WCS, RWC, S and D are good features and suitable for most varieties to characterize their performance.

The ability of some foreign varieties and those known as secondary (Tunisian) to withstand LWC represents a strong advantage for Tunisia, which is confronted to the increase of the water scarcity. Varieties with high S_c, D, RWC and low LA are normally able to compete with others, which are largely cultivated in the northern or southern areas. However, we should make sure that these cvs., (foreign and secondary) are also performant at productive levels and further investigation is required at anatomical level to establish the relevance of these results.

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Chapter 4

SYMBIOTIC CORN CAN IMPROVE YIELD, REDUCE MYCOTOXINS, AND PRESERVE NUTRITIVE VALUE

G. Masoero[1,2,*], L. Rotolo[2], L. Gasco[2], I. Zoccarato[2,1], A. Schiavone[3], M. De Marco[3], G. Meineri[3], G. Borreani[2,1], E. Tabacco[2], G. Della Casa[4], V. Faeti[4], P. M. Chiarabaglio[5], C. Lanzanova[6], S. Locatelli[6] and R. Aleandri[7]

[1]Academy of Agriculture, Torino, Italy
[2]Department of Agriculture, Forestry and Food Sciences, University of Torino, Grugliasco, Italy
[3]Department of Veterinary Sciences, University of Torino, Grugliasco, Italy
[4]Council for Agro-Economical Research, San Cesario Sul Panaro, Italy
[5]Council for Agro-Economical Research, Casale Monferrato, Italy
[6]Council for Agro-Economical Research, Bergamo, Italy
[7]Council for Agro-Economical Research, Roma, Italy

[*] Corresponding Author Email: giorgioxmasoero@gmail.com.

ABSTRACT

A chained set of symbiotic trials has been conducted with the aim of studying whether and how corn fertilised by Arbuscular Mycorrhiza (AM) and microbial consortia could influence crop yields, affect mycotoxins, and modify poultry and pig meat production, in the short term or after a long period of storage. Two experiments conducted in corn fields treated with a commercial bio-fertiliser have shown that the yield can be improved by +4 to +30% and that the resistance to fungal attacks had significantly increased. The secondary metabolites, fatty acid composition, NIRS properties, and electronic nose profiles were also modified, with a substantial reduction in the oxidant power of -47% in the grain flour and -19% in the feed. The productive performances, as well as the slaughter and meat colour, were not modified after a fresh corn utilization by broiler poultry and heavy pigs, but the blood biochemical parameters in the poultry revealed a clear amelioration of the physiological functions and of the serum antioxidant capacity. After a twenty-one-month delayed utilization, substantial nutritive differences, related to shelf life and palatability, emerged, since the broilers fed the control diet showed a reduced intake of -26.7%, with a final body weight reduction of -27.7%. Therefore, a symbiotic farming provided by AM and microbial fertilisation may be considered a strategic tool for agronomic sustainability, resilience and for the nutritive usefulness of maize crops.

Keywords: corn, symbiotic, mycorrhiza, yield, mycotoxins, antioxidant, poultry, shelf life, NIRS, vibrational spectroscopy, electronic nose

INTRODUCTION

"Producing more food with fewer resources may seem too good to be true, but the world's farmers have trillions of potential partners that can help achieve that ambitious goal. Those partners are microbes" (Reid and Green 2012). Arbuscular Mycorrhizae (AM) and microbial fertilisers, a symbiotic farming, need to be closely involved in a road map of sustainable agriculture. Masoero and Giovannetti (2015) found, in corn fertilised by a Micosat F® (Sym) complex consortium, that the in vivo raw pH of the plant is acidified according to a degradient from the roots (pH -7% in symbiotic corn) to the stem at an ear height (-4%). AM have

important effects on colonized plants, including an increase in nutrient absorption, particularly for phosphorus (Berruti et al. 2015; Wang 2016), with a consequent stimulating effect on plant growth and an enhancement of their resistance to biotic and abiotic stresses (Chu et al. 2013; Nuti and Giovannetti 2015). Any qualitative modifications of the seeds will have a great effect on the primary (Berta et al. 2013) and secondary compounds, that is, antioxidants (Raiola et al. 2015; Migliorini et al. 2018). Moreover, the presence of mycotoxins in kernels is a severe problem for the corn-based feed industry, due to the potential health hazard to livestock farming (Milani 2013) and considering that corn is the main ingredient of pig and poultry diets. All this information constitutes an important reason to study different approaches to guarantee an adequate corn production and contextually mitigate the mycotoxin contents. An important point that needs further information is the achievement of the best nutritional qualities, but it is also important to obtain knowledge to increase the shelf life for medium-long storage. Considering these considerations, a sequential set of researches was undertaken to study whether and how corn fertilised by Sym could influence the crop yield, affect mycotoxins, and modify animal production over a short term, or after a long period of storage.

MATERIAL AND METHODS

Two contemporary agronomic corn production trials (Corn1 and Corn2) were carried out in 2011, and these were followed by three animal utilisation trials, two on poultry broilers (Poultry1-fresh from Corn1, and Poultry2 from Corn2 after its long storage) and one on heavy pigs with fresh grain from Corn2. The Corn1 trial was carried out at the experimental farm of the University of Turin on corn stands of *NK Famoso* (FAO class 600 and Pioneer Hi-Bred Italia in Gadesco-Pieve Delmona, Cremona, Italy) and harvested as grain at physiological maturity (black layer). The soil was a sandy-loam textured alluvium soil with a pH, measured in water, of 7.6 and a Martonne aridity index value of 33. The sand, silt, and clay

contents of the soil were 470, 440, and 90 g kg^{-1}, respectively, at a depth of 0–30 cm. Two factors were evaluated: i) inoculation (INO) with a Sym consortium, as described in Zoppellari et al. (2014), vs. control (C); ii) irrigation (IRR) with rainfed (RF) vs. irrigated (IR), for a total of four treatments in a completely randomized block design with four replications (four blocks with 13.5 m x 45 m plots): control rainfed (C_RF) vs. irrigated (C_IR), inoculated without irrigation (Sym_RF), and inoculated with irrigation (Sym_IR). Sym was distributed at seeding at a rate of 12 kg ha^{-1}. Corn stands were planted at a theoretical planting density of 67,000 seeds ha^{-1}. Fertiliser was applied at a rate of 40 kg ha^{-1} of P_2O_5 and 55 kg ha^{-1} of K_2O immediately before planting. An additional 160 kg ha^{-1} of N was top-dressed as urea at the six-leaf stage. Irrigation was provided by a sprinkler irrigation system on two events at a rate of about 600 m^3 water ha^{-1}. The harvesting was carried out in early October, using a Wintersteiger plot combine harvester. The dry matter (DM) yield was determined by harvesting the two central rows of each plot to a length of about 25 meters, for a total surface collection of 37.5 m^2. A total of 500 kg of dried grain was utilised for each treatment in the feeding trial. The grain was dried immediately at a mild temperature (50°C) to a 10% moisture content and was then kept at 18°C indoors, where it was protected from damage by rats and insects.

The Corn2 trial was carried out at the CREA-SUI experimental farm near Modena (44°34’N, 11°2’E, altitude 41 m a.s.l.) on an *NK Famoso* cultivar, and a *DK6666* cultivar, FAO class 600, maturity d 132 (Dekalb, Monsanto Agricoltura Italia S.p.A. in Milano, Italy). Mineral fertilisation was provided by applying potassium sulphate 50% (100 kg ha^{-1} of K_2O), triple superphosphate 46% (92 kg $ha^{-1}P_2O_5$), and Entec 46 (120 kg ha^{-1} N in two fractions). Glyfosate was used at a dose of 3 l ha^{-1}. The two main fields were split into two subfields and seeded with 6.6 plants m^{-2}. Sym was utilised at a rate of 13 kg ha^{-1}. Checks on the yields were carried out in the four subfields in three homogeneous subplots, ranging from 5080 to 15240 m^2. The whole amount of corn harvested from each of the four large plots was weighed directly in the field to assess the yields and, after a thorough drying with a movable drier, to a humidity of less than 7%, it was

stored in 900 kg bags. *DK6666* was not utilised in the animal trial, while the *Famoso* corn was used twice: fresh in the Pig trial and in the Poultry2 trial, after transportation from Modena to Torino, and after a long period of storage for twenty-one months, without the addition of any chemical substances.

Corn Analyses

Ten corn samples were taken at harvesting and then divided into subsamples: a) 250 g of corn was packed in a Nallophan bag, sealed and stored at -20°C until being sent to the CREA-PLF unit in Casale Monferrato, AL, Italy, for subsequent near-infrared reflectance spectroscopy (NIRS) and electronic nose (EN) analyses; b) an aliquot of the grain from the Corn1 experiment was dried and analysed for crude protein (CP), ether extract (EE), ash, starch, neutral detergent fiber (NDF) and acid detergent fiber (ADF), according to Borreani and Tabacco (2014), as well as for the following mycotoxin compounds: aflatoxin B1 (AFB1), deoxynivalenol (DON), fumonisin B1 (FB1), ochratoxin A (OTA), T-2 toxin (T-2) and zearalenone (ZEA), which were analysed by means of an ELISA test.; c) 250 g of grain from the Corn2 experiment, after having been oven-dried at 60°C to a constant weight, was packed in bags under vacuum and kept until it was ground in a Cyclotec mill (Tecator, Herndon, VA, USA) to pass a 1 mm screen in order to determine, in duplicate, the dry matter (DM), CP, EE, ash, phosphorus, NDF, ADF, and lignine, according to Van Soest et al. (1991); a fatty acid profile of the *Famoso* cultivar was determined, as reported in Della Casa et al. (2010); d) an aliquot of 500 g of grain from the Corn2 experiment was dried in an oven at 60°C to a constant weight, and then vacuum packed and transferred to the CREA-MAC laboratory in Bergamo, Italy, for the prediction of the following constituents, via NIRS models, as described by Brenna and Berardo (2004): starch, lutein, zeaxantin, and carotenoids. The antioxidant activity of the Corn1 grain from the Poultry1 experimental diets was determined according the DPPH method, as reported by Prola et al. (2011),

and expressed as Trolox Equivalent 100 g^{-1}. Instrumental analyses of the raw dried grain were performed using an EN PEN2 device (Win Muster AirSense Analytics GmbH, Schwerim, Germany), as described in Torri et al. (2013) and a portable UV-Vis-NIR spectrometer (Model LSP 350-2500P LabSpec Pro, ASD; Analytical Spectral Devices, Inc.; Boulder, CO, USA), equipped to collect spectra from 350 to 2500 nm). Flour corn samples were scanned by means of a FOSS NIRSystem 6500 monochromator (NIRSystems Inc., Silver Spring, MD, USA).

Poultry Trials

The two feeding trials were performed at the Torino University experimental farm. In the Poultry1 trial with the fresh Corn1, 240 one-day-old female broiler chicks (*Ross 308*) were randomly allotted to twenty-four pens. The feeding programme was biphased (Table 1). A 2×2 factorial experiment was designed to evaluate the effect of the Sym corn and the irrigation with the four previously identified groups: C_RF, C_IR, Sym_RF, and Sym_IR. Six pens were replicated. The Poultry2 trial was conducted in 2013 with long-term stored Corn2, using 120 *Ross 308* broilers, allotted to twelve pens. The feeding programme consisted of a single period, that is, until thirty-three days of age (Table 1).

Pig Trial

Forty *Duroc* x *Large White Italian* cross-bred pigs, with a starting live weight of 52 kg, divided into two experimental groups, that is, the control (C) and Sym groups, each consisting of ten castrated males and ten entire females, were utilised. The diets were formulated (Table 1) according to the live weight, with the highest possible corn content (over 80%). The feed was wet fed with a water, feed ratio of 2.5: one twice daily for a total of thirteen meals per week. The head ration was scheduled as follows: initial ration 1.68 kg d^{-1}, an increase of 140 g d^{-1} for eight weeks, an

increase of 50 g d^{-1} for five weeks, up to the maximum administered dose of 3.05 kg d^{-1}. The animals were all slaughtered at 165 kg of live weight.

Table 1. Ingredients in the Diets for the Two Poultry Trials and in the Pig Trials as well as the Chemical Composition of the Feeds in the Control (C) and Corn-Inoculated (Sym) Groups

Ingredients (%)		Poultry1		Poultry2	Pig		
		#Starter	£Finisher	#Unique	Live Weight kg		
		1-21 d	22-33 d	1-33d	40-80	80-120	120-165
Corn meal	%	60.0	60.0	60.0	79.5	84.0	88.5
Soybean meal 50%	%	33.3	31.2	32.3	17.0	12.5	8.0
Vegetable fat	%	2.5	5.0	3.8	0	0	0
Dicalcium phosphate	%	1.3	1.24	1.33	1.12	1.14	1.15
Calcium carbonate	%	1.15	1.12	1.13	1.14	1.15	1.17
Sodium chloride	%	0.23	0.22	0.23	0.40	0.40	0.40
Sodium bicarbonate	%	0.13	0.15	0.14	0	0	0
DL-methionine	%	0.39	0.25	0.32	0.02	0.01	0
L-lysine	%	0.20	-	0.20	0.24	0.22	0.20
Threonine	%	0.08	0.11	0.08	0	0	0
L-thriptophan	%	0	0	0	0.03	0.03	0.03
Vitamin and mineral premix	%	0.50	-	0.50	0.55	0	0
Vitamin and mineral premix	%	-	0.50	-		0.55	0.55
Choline chloride	%	0.10	0.10	0.10	0	0	0
3-phytase; (E-300; natuphos)	%	0.10	0.10	0.10	0	0	0
Analyses (g kg^{-1} DM)	Group						
Crude Protein	C	223	228	226	162	143	125
	Sym	225	227	226	165	145	124
Ether Extract	C	49	49	49	32.9	33.5	33.5
	Sym	49	49	49	33.5	34.4	33.3
Ash	C	64	68	66	44.3	42.2	41.6
	Sym	65	68	67	43.9	42.4	41.1

#Starter premix kg^{-1}: 3500000 IU vit A; 6000 IU vit E; 1000 mg vit K3; 600 mg vit B1; 1200 mg vit B2; 500 mg vit B6; 6 mg vit B12; 40 mg biotin; 4000 mg Ca panthothenate acid; 150 mg folic acid; 15000 mg vit C; 8000 mg vit PP; 15000 mg Zn, 15800 mg Fe; 14230 mg Mn; 5500 mg Cu; 185 mg I; 70 mg Co; 54 mg Se; 40 mg Mo; 25000 mg DL-methionine; 25000 mg BHT.

£Finisher premix kg^{-1}: 2500000 IU vit A; 1000000 IU vit D3; 10000 IU vit E; 700 mg vit K3; 400 mg vit B1; 800 mg vit B2; 400 mg vit B6; 4 mg vit B12; 30 mg biotin; 2800 mg Ca panthothenate acid; 100 mg folic acid; 15000 mg vit C; 5600 mg vit PP; 10500 mg Zn, 10920 mg Fe; 9950 mg Mn; 3550 mg Cu; 137 mg I; 50 mg Co; 70 mg Se; 30 mg Mo; 25000 mg DL-methionine; 25000 mg BHT.

Blood Biochemical Analyses

In the Poultry1 trial, samples of blood were collected before slaughtering from twelve broilers per group, via wing vein puncturing, using a Veno Jet tube (Terumo, Leuven, Belgium), with a 19-gauge pin, according to Good Veterinary Practices. The blood was allowed to clot and was then centrifuged at 3000 rpm for ten minutes at room temperature. The serum was separated and stored at -70°C until analysis. An ILab Aries analyzer (Instrumentation Laboratory, Milan, Italy) was used to analyse the serum levels of the cholesterol, triglycerides, liver enzyme alanine aminotransferase (ALT), albumin, and glucose. The antioxidant capacity of the serum was evaluated by means of an anti-ROMs test (Diacron s.r.l., Grosseto, Italy). In this test, the colour intensity increases proportionally according to the quantity of iron reduced by the antioxidants present in the sample. This test enables the concentration of the so-called "fast antioxidants," determined at the start by the instrument, that is, those which are fast-acting, such as Vitamin C or Vitamin E, to be discriminated from the concentration of "slow antioxidants," subsequently determined by the instrument, such as the thiol-SH groups, uric acid, polyphenols, and anthocyanins. The results were expressed in μEq of reduced iron l^{-1}, using ascorbic acid as the standard.

Slaughter Procedures and Analyses

At the end of the trials, two chicks per pen were randomly selected and weighed individually. The birds were slaughtered by stunning and were then exsanguinated. The carcasses were plucked, eviscerated, and weighed without the head, neck, feet, and abdominal fat. At twenty-four hours after slaughtering, the pH of the breast and thigh muscles was measured with a Crison MicropH 2001 (Crison Instruments, Barcelona, Spain), using a combined electrode. At the same time, the colour of the meat on the breast skin, breast muscle, and thigh muscle was measured using a portable Minolta CR-331C Colorimeter (Minolta Camera, Osaka, Japan). The

colorimetric results were expressed in terms of lightness (*L**), redness (*a**) and yellowness (*b**) in the CIELAB colour space model (CIE, 1976). Forty-five minutes after slaughtering, the hot carcass weight, the lean meat content of the carcasses, as established by means of a Fat O Meter, the weight of the thighs, and the pH of the *semimembranosus* and *bicep femoris* muscles of the pigs were recorded. The weight of the cooled thighs and the pH of the muscles were measured after twenty-four hours, and the colour of the s*emimembranosus* muscle was recorded with a colorimeter Minolta CR-300, according to the CIELab system with illuminant C. Small pieces of three tissue: muscle *sternomandibularis*, rind and fat were sampled at slaughtering. The specimens, which overall amounted to less than 20 ml, were introduced in a 50 ml Falcon tube, and the tube was then filled up by ethanol (EtOH) 95% and kept in a fridge. After two hours of aeration, the EtOH specimen tissues were then examined by means of the ASD spectrometer, as described by Masoero et al. (2007).

Data Analyses

Several mono and bifactorial ANOVA models were used, with PROC GLM by SAS V. 9 software (SAS Institute, Cary, NC, USA), to evaluate the effect of the factors on the data, for each corn trial, as well as for the two subsequent poultry trials. The statistic elaboration of the spectra and EN traces was performed by conducting a discriminative analysis of the main factors, that is, Sym, Irrigation, and/or Cultivar, and was performed using the WinISI II, version 1.04, spectral analytical software (InfraSoft International LLC, State College, PA, USA). The adopted chemometrics involved a cross-validation process of equations, which were used to calibrate the fixed effects; the prediction capacity was then evaluated considering the 1−VR parameter and the relative prediction deviation (RPD). A similar partial least square (PLS) bivariate model was applied to discriminate the Sym vs. Control condition, based on the DPPH test values of the corn and of the derived diets.

RESULTS

Corn Field Trials

In the Corn1 trial (Table 2), the yield was on average 12.63 t ha^{-1}, that is, almost 50% greater than in the Corn2 trial (8.47 t ha^{-1}, Table 3). Microbial fertilisation was effective in improving the yield in both trials, albeit to different degrees, according to the farm, the water supply, and the cultivar; in fact, the increase in the high productive field (Corn1) was +4% in the rainfed and +6% in the irrigate conditions, respectively. In the less productive conditions (Corn2), a significant improvement was observed for Sym, for the *Famoso* cultivar (+8%), while a very high value (+30%) was recorded for the *DK* cultivar.

Preliminary assessments of the biodiversity effects were obtained from the NIRS and the EN instruments (Table 4). In the Corn1 experiment, no irrigation effect was apparent in the NIRS radiation values or in the EN profile of the integer corn grains, while a slight appearance (1-VR = 0.38 and 0.42) emerged for the Sym factor. However, when the relationships were studied separately, the irrigation condition appeared more favourable for an enhancement of the Sym factor in the NIRS radiation (0.67). In the Corn2 trial, the examination of the integer grain as well as of the flour ensured a perfect discrimination of the two cultivars (0.97 and 0.99, respectively), which also appeared high according the EN analyses (0.83). The Sym factor was instead lowered overall (0.20 and 0.42 for NIRS and EN, respectively), but when considered separately, according to the cultivar, the discriminative values rose more in the *DK6666* cultivar (0.57 and 0.82) than in the *Famoso* (0.39 and 0.43) one. After milling, the NIRS discriminative ability for the Sym factor became considerably higher, reaching levels of 0.73 and 0.85 for the two cultivars. In short, the average discrimination coefficient of the inoculated Sym from all the trials was 0.51±0.24 for NIRS and 0.49±0.16 for EN.

Table 2. Results of the Corn1 Field Trial for the Inoculation (INO: Control-C vs. Inoculated-Sym) and Irrigation (IRR: Rainfed vs. Irrig.) Factors

Parameters		LSMeans				RMSEE RMSE	P value		
		C Rainfed	C Irrig.	Sym Rainfed	Sym Irrig.		INO	IRR	INO* IRR
Grain yield	t DM ha^{-1}	12.1	12.5	12.6	13.3	1.42	**	*	
Moisture at harvest	g kg^{-1}	167	169	163	166	0.131			
Crude Protein (CP)	g kg^{-1} DM	83.8b	85.8ab	86.7a	86.5a	3.4	*		
Ether Extract (EE)	g kg^{-1} DM	32.9b	33.5b	34.2°	35.0a	17.0	**		
Ash	g kg^{-1} DM	11.2	11.6	11.4	11.6	0.3			
Starch	g kg^{-1} DM	689	710	708	703	38.6			
Neutral Detergent Fiber (NDF)	g kg^{-1} DM	118	110	113	118	8.0			
Acid Detergent Fiber (ADF)	g kg^{-1} DM	28	27	28	29	1.7			
DPPH corn (Trolox Equivalent)	100 g^{-1} DM	0.71c	1.05a	0.62d	0.90b	0.014	***	***	**
DPPH diets (Trolox Equivalent)	100 g^{-1} DM	1.24b	1.41a	1.11c	1.11c	0.025	***	***	***
Aflatoxin (AFB_1)	ppb DM	<1.00	<1.00	<1.00	<1.00	-			
Zearalenone (ZEA)	ppb DM	35.5b	26.94c	36.12a	35.34b	3.35	***	***	**
Fumonisin (FB_1)	ppm DM	1.57a	1.32a	0.76b	0.92b	0.17	***		
Deoxynivalenol (DON)	ppm DM	0.17a	0.06c	0.10b	0.06c	0.02	*	***	
Ochratoxin (OTA)	ppb DM	1.53b	2.65a	2.41a	2.86a	0.43		*	
T-2 toxin (T-2)	ppm DM	< 0.025	< 0.025	< 0.025	< 0.025	-			

Means followed by the same letter are not significantly different, after Tukey's HSD test at $P < 0.05$; P>F: * <0.05; ** >0.01; *** < 0.001.

As far as the Corn1 trial is concerned, a chemical investigation of the primary and secondary compounds corroborated the preliminary instrumental analyses (Table 2). The CP and EE were increased in the Micosat-treated corn by 2% and 4%, respectively. However, the decrements in the mycotoxin complex, for Fumonsin (-72%) and DON (-44%), were even more important, while the ZEA toxin underwent an interaction among the factors. The rainfed regimen was clearly unfavourable for a healthy production, because of the high DON (+125%) and ZEA (+15%) occurrences, despite the somewhat reduced OTA (-28%).

The DPPH score measures the oxidant power of the matrix as a reciprocal of the antioxidant values: Sym reduced DPPH by -47% in the corn, which was furtherly less reduced to -19% when corn meal was incorporated in the poultry diets. It is important to observe that the oxidant power of the feed preparates was increased by +36% after milling, mixing, and storing, compared to the control, and by +67%, compared to the Sym groups.

Table 3. Results of the Corn2 Field Trial for the Inoculation (INO: Control-C vs. Inoculated-Sym) and Cultivar (CV: *Famoso*-FM vs. *Dekalb 6666*-DK) Factors

Parameters		LSMeans				RMSE	P value		
		C_FM	C_DK	Sym_ FM	Sym_ DK		INO	CV	INO* CV
Moisture at harvest	g kg^{-1}	141b	224a	144b	205a	13		***	
Grain yield	t DM ha^{-1}	8.12b	6.77c	8.81a	8.77°	0.65	**		
Crude Protein-CP	g kg^{-1}	84.8c	91.9a	85.6c	88.7b	1.8	**	***	***
Ether Extract-EE	g kg^{-1}	38.0a	38.3a	38.1a	38.1a	0.82			
Starch	g kg^{-1}	700a	696c	701a	698b	1.2	*	***	
Crude fiber-CF	g kg^{-1}	23.3a	20.7b	22.9a	21.0b	6		**	
NDF	g kg^{-1}	126.2a	92.5b	126.0a	126.1a	1.7	***	***	***
ADF	g kg^{-1}	29.0b	33.7a	34.2a	27.2b	4.6			***
ADL	g kg^{-1}	12.1b	18.7a	10.5b	12.9b	4.6	**	**	
Phosphorus	g kg^{-1}	1.43 b	1.53 a	1.43 b	1.50 a	0.03		***	
Carotenoids	g kg^{-1}	24.0a	11.0c	22.9a	13.9b	0.95		***	*
Lutein	mg kg^{-1}	24.5a	22.8c	24.0b	22.3d	3.78	**	***	
Zeaxantin	g kg^{-1}	6.26d	9.18a	7.49c	8.12b	7.19		***	***
Fatty Acids									
Palmitic 16:0	%	15.4a	14.9b	15.8a	15.2a	0.37		**	
Palmitoleic 16:1	%	0.14 ab	0.10b	0.17a	0.15a	0.04	*		
Stearic 18:0	%	2.49b	2.56a	2.45b	2.47b	0.04	**		
Oleic 18:1	%	30.0a	26.5b	30.2a	25.9b	0.63		***	
Linoleic 18:2	%	49.9b	54.0a	49.3c	54.4a	0.38		***	*
α–Linolenic 18:3	%	1.52a	1.43b	1.55a	1.46b	0.05		**	
Eicosenoic 20:1	%	0.27	0.26	0.24	0.26	0.03			
∑ saturated FA	%	18.0a	17.5b	18.3a	17.7b	0.38		**	
∑ unsaturated	%	81.9ab	82.4a	81.6b	82.2a	0.38		**	

Means followed by the same letter are not significantly different, after Tukey's HSD test at P < 0.05; P>F: * <0.05; ** >0.01; *** < 0.001.

Table 4. NIRS and Electronic Nose Results for the Two Corn Trials

Samples	Factor	Contrast	NIRS			Electronic Nose		
			No.	1-VR	RPD	No.	1-VR	RPD
	Corn1 trial - Whole grain - ASD Instrument							
All	Inoculation	C_1\Sym_2	45	0.38a	1.3	38	0.42a	1.3
All	Irrigation	IR_1\RF_2	40	0.03b	1.0	45	0.02b	1.0
Irrigated	Inoculation	C_1\Sym_2	37	0.67a	1.7	21	0.38	1.2
Rainfed	Inoculation	C_1\Sym_2	43	0.13b	1.1	22	0.44	1.3
	Corn2 trial - Whole grain - ASD Instrument							
All	Cultivar	FM_1\DK_2	262	0.97a	6.1	33	0.83a	2.4
All	Inoculation	C_1\Sym_2	291	0.20b	1.1	35	0.42b	1.3
FM	Inoculation	C_1\Sym_2	139	0.39b	1.3	22	0.43b	1.3
DK	Inoculation	C_1\Sym_2	142	0.57a	1.5	20	0.82a	2.4
	Corn2 trial - Flour- FOSS 6500 Instrument							
All	Cultivar	FM_1\DK_2	72	0.99a	12.8			
All	Inoculation	C_1\Sym_2	75	0.69b	1.8			
FM	Inoculation	C_1\Sym_2	38	0.73	2.0			
DK	Inoculation	C_1\Sym_2	37	0.85	2.0			

a > b in the columns; P<0.05. 1-VR: 1-variance ratio in cross-validation mode; RPD = standard deviation/standard error in cross validation.

To characterise the Sym corn, a partial least square (PLS) equation was developed to discriminate the Micosat-treated condition and to provide the DPPH values of the corn-final-feed couples:

Conventional (score 1) vs. Sym (score 2) =
6.799 - 1.197 DPPH_corn (ppm) -3.546 DPPH_feed (ppm)

(R^2 = 0.89). The cross validated reclassification was 100% success.

Figure 1 illustrates the biplot of the DPPH antioxidant values of the corn (X axis) and of the derived feeds (Y axis) and suggests how to testify the two origins of the corn, namely as Sym and Conventional. Unfortunately, no DPPH oxidant measurements of grain were conducted in the Corn2 trial; moreover, the Cultivar factor was very prominent, as can be seen from the vibrational and aromatic profiles, but it very often interacted with the Sym effects. In fact, after the biofertilisation treatment, the protein and starch appeared decreased and increased, respectively, but

the EE level did not change; marked increases affected the neutral cell wall constituents of the grain (NDF +13%), while the presence of lignin was reduced by 32%. Among the secondary compounds, a reduction in lutein (-2%), an increase in monounsaturated palmitoleic acid (+34%) and a decrease in saturated stearic acid (-3%) were noted.

Poultry Trials

The feed intake, conversion rate, and growth performances were very similar in the Poultry1 trial for the four groups (table 5). No statistically significant effects were observed for the carcass traits or for the quality, except for two interactions in the colour examination. On the contrary, the biochemical traits of the poultry showed a very different scenario: all the biochemical parameters were improved significantly after the intake of Sym corn. The following parameters were favourably modified: fast antioxidants (+20%); slow antioxidants (+14%); uric acid, an antioxidant in poultry (+22%); ALT, alanine amino transferase (-8%); cholesterol (-5%); triglycerides (-4%); glucose (-1%). Neither the chicken carcass nor the meat quality was influenced by the imposed treatments (data not reported).

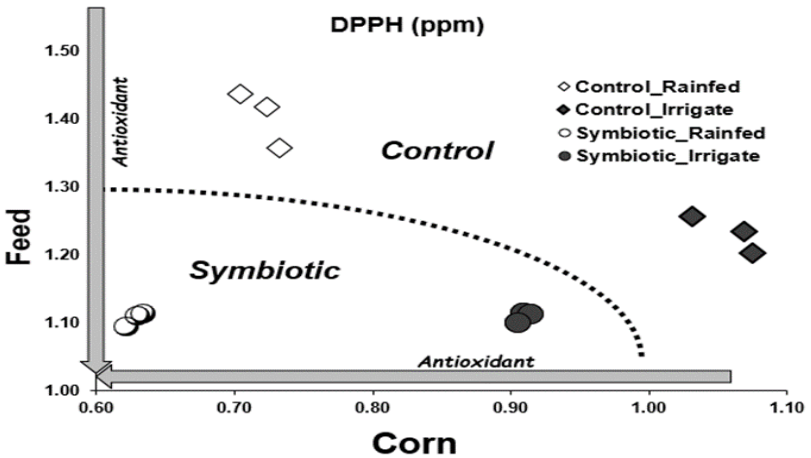

Figure 1. Biplot of the DPPH oxidant-antioxidant values of the corn and of the derived feeds.

Table 5. Results of the Poultry1 Trial for the Inoculation (INO: Control-C vs. Inoculated-Sym) and Irrigation (IRR: Rainfed vs. Irrig.) Factors

Live Parameters No. = 239		LSMeans C_RF	C_IR	Sym_ RF	Sym_ IR	RMSE	P value INO	IR R	INO* IRR
Body Weight	g	1805	1630	1858	1793	39.4			
Feed Intake	g	2553	2310	2620	2533	53.6			
Feed Conversion Ratio	g g^{-1}	1.45	1.46	1.44	1.45	0.01			
Biochemical No. = 40									
Fast antioxidants[£]	µEq $Fe^{+++}l^{-1}$	191	199	232	234	0.92	***	***	***
Slow antioxidants[§]	µEq $Fe^{+++}l^{-1}$	966	1009	1127	1128	1.21	***	***	***
Uric Acid	mg dl^{-1}	3.8	4.11	4.74	4.93	0.13	***	***	
ALT	U l^{-1}	33.82	32.1	30.72	29.61	0.93	***	***	
Cholesterol	mg dl^{-1}	176.4	172.2	165.3	164.5	0.73	***	***	***
Triglycerides	mg dl^{-1}	52.6	51.9	50.5	49.6	0.77	***	**	
Albumin	g dl^{-1}	2.03	1.99	2.05	2.03	0.08			
Glucose	mg dl^{-1}	221.7	221.5	220	220.1	0.93	***		

[£] First concentration of antioxidants determined by means of an anti-ROM test;

[§] Second concentration of antioxidants determined by means of an anti-ROM test.

Table 6. Results of the Live Traits of the Poultry2 Trial, after a Long Period of Corn Storage, for the Two Groups: Control *Famoso* (C_FM) vs. Micosat *Famoso* (Sym_FM)

Parameters No. =120		LSMeans C_FM	Sym_FM	RSME	P value
Final Body Weight	g	1354.1	1715.8	52.6	***
Feed Intake	g	2372.5	2981.5	105.2	***
Feed Conversion Ratio	g g^{-1}	1.81	1.78	0.04	

The growth model observed in the Poultry2 trial (Table 6) was very different. The feed containing the Sym corn was consumed as normal, while the C corn was consumed at a -26% rate. Therefore, a statistically high body weight, namely +26.7%, characterised the Sym group compared to the C group. The feed conversion index was very similar in both groups. As in the case of the Poultry1 trial, neither the relative composition of the chicken carcass nor the meat quality traits were influenced by the origin of the feeds.

Pig Trial

In the Pig trial, no effect was apparent for the live performances (Table 7), slaughter, or colour performances (data not reported). The presence of ontogenetic effects resulting from the NIR spectroscopy of four EtOH tissues (Table 8), is interesting. A high degree of difference was observed for the *sternomandibularis* muscle between the Sym and the Control (1-VR = 0.77). This difference was also significant in the dermis (0.42) and skin (0.33), while nothing emerged on the fat spectra. A sex effect was only found in the dermis (0.35).

Table 7. Results of the Live Stage Pig Trial for the Two Groups: Control *Famoso* (C_FM) vs. Micosat *Famoso* (Sym_FM)

Parameters No. = 40		LSMeans		RSME
		C_FM	Sym_FM	
Final Body Weight	kg	169	166.2	1.98
Average Daily Gain	g d^{-1}	840	820	572
Feed Conversion Ratio	g g^{-1}	3.18	3.26	0.091

Table 8. Results of the NIR Spectroscopy on the EtOH Tissues from the Pig Trial

EtOH-tissue	**C_1 vs. Sym_2**	**Female_1 vs. Castrated_2**
	1-VR	**1-VR**
Muscle	0.77	0.05
Skin (external rind)	0.33	0.00
Dermis (internal rind)	0.42	0.35
Fat	0.05	0.00

DISCUSSION

Concordant references for positive AM effects have been synthesised in a meta-analysis of field studies on the responses of wheat (Pellegrino et al. 2015), and microbial inoculation has been proposed as an effective

agronomic practice; aboveground biomass increases of around 20%, as assessed under Indian conditions by Mader et al. (2011), have been observed. The effect of Micosat on corn yield has already been reported in literature. In a low input experiment (Sabia et al. 2015), a Sym treatment at the half-milky line stage enhanced the total DM yield by around +18% and increased the P concentration (+29%). Under partial-field conditions, Berta et al. (2014) observed massive increases, ranging from +34 to +53% for AM and/or Pseudomonad. In field surveys, Masoero and Giovannetti (2015) observed a significant ($P< 0.02$) growth effect for Micosat on the stalk weight, with an improvement of +14%; a +12% increase in the ear was not considered significant ($P< 0.20$), while the total green mass yield (+12%) was nearly significant ($P< 0.07$). Celebi et al. (2010) confirmed that AM inoculation increased the corn silage yield, over a whole irrigation regimen, but also in the underrestricted water availability they observed a large increase in the leaf and stem parts. A similar result was reported by Zoppellari et al. (2014) for the *Famoso* cultivar. Results from a third research centre, published for the same concerted SOS-ZOOT project (Tripaldi et al. 2017), pointed out that the yield was not increased while the lipids, ash, and ADF were increased in Sym corn; however, NIR spectroscopy and the electronic nose profile indicated some physico-chemical differences between the inoculated and non-inoculated samples, with R-squares of 0.86 and 0.63, respectively, and this was even higher than the values of 0.51 and 0.49 found in the present work in the other two centres of the project. As far as secondary compounds in Sym durum wheat are concerned, Migliorini et al. (2018) observed a significant rise in antioxidant compounds (FRAPS +64%; ABTS + 24%) with higher contents of carotenoids (+43%) and phenols (+231%). Migliorini et al. (2018) observed significant increases in bound polyphenols (+13%) and bound flavonoids (+30%) in Sym wheat (cv. *Blasco*) but also a reduction in anthocians (-21%).

In this study, the data on growing chickens are consistent with those of previous broiler feeding trials reported in literature (Schiavone et al. 2008). The lack of significant effects, in both the starter and grower periods, indicates that the performances of the birds fed the different diets are

comparable. These outcomes were expected, in view of the results reported about corn grain quality and its mycotoxin content. It is possible to assume that although Sym had a significant effect on the percentage of both CP and EE, the magnitude of the range obtained for these results is restricted, and thus has no practical relevance in the formulation of chicken diets. Similarly, the mycotoxin results of the harvested grains were also affected by the experiment, but they were largely under the limits fixed for products intended for animal feeding and did not show any perceivable impact on the growth performances of the chickens.

As for the significant changes in the biochemical blood properties of poultry, it is possible to infer that the antioxidants of Sym groups may reduce cholesterol and triglyceride absorption, due to the interaction of these compounds with the cholesterol and triglyceride carriers and transporters that are active in the intestinal brush border membrane. In fact, a similar effect was described for the use of polyphenolic antioxidants in obese rats (Agouni et al., 2009). In the current study, a similar improvement to that of the liver enzyme ALT appeared; therefore, it is possible to state that Sym fertilisation can induce an indirect protective effect on the liver of poultry. The observed serum albumin level was in the standard range for poultry, which shows that the broilers had a normal nutrition state (Kaneko et al. 2008). Plasma glucose concentrations in bird species are 150%–300% higher than in mammals of a similar body mass and are resistant to the glucose-lowering effects of insulin (Braun and Sweazea 2008); the small but significant reduction observed for the Sym groups could be of interest. As far as shelf life improvement is concerned, the lower feed intake of chicks fed the C diet could be due to a decrease in feed palatability, which could be ascribed to the formation of unappetizing substances during the long period of storage (twenty-one months). The Sym corn seemed to have been protected from the formation of undesirable substances, and therefore resulted in having a very different shelf life. The thorough drying of corn applied in the Corn2 trial (water < 7%) may help explain why such a long storage period did not affect the health of the seeds without chemicals.

A similar result to that achieved in the Poultry2 trial was obtained in a Friesian cow trial using conventional and Sym corn derived from the SOS-ZOOT 3rd centre (Chiariotti et al. 2015), but after a long storage period: the results confirmed the relevance of Sym from the greater intake (+6%) and from the accentuated gain in weight during lactation (+118%) as well as from the milk quality improvement (+6% in protein content).

The results of the NIR Spectroscopy of EtOH in pigs are interesting; unfortunately, we did not perform any rheological tests, but the high differences pointed out for the Sym-corn were also found for different types of pigs by Cenci Goga et al. (2011) and for different types of cattle (Brugiapaglia et al. 2011).

In a trial on Piedmontese cattle meat, Peiretti et al. (2018) have observed new feed functionalities in Sym corn that have led to unexpected results. They found that the pentadecanoic and heptadecanoic odd FAs in the lipids of the longissimus thoracis muscle may appear as a sign of the adopted "*symbiotic*" feeding system, perhaps because of an enhancement of the ruminal factor. The improvement in the water-holding capacity quality traits, promoted by Sym corn, could be explained by considering other compounds that protect the proteins and lipids from oxidation processes and the muscular fibres from water relaxation.

CONCLUSION

The agronomic trials have shown the capabilities of symbiotic farming by using microbial fertilisation at a real scale, which should be considered as a key for sustainability in relation to a future decreasing mineral support for the intensive maize crop, especially as far as phosphate supply is concerned. The application of Micosat F® could hinder the advancement of mycotoxins and could enhance the levels of several secondary metabolites. Environmental conditions and the modality of AM inoculation could modulate the ontogeny of both primary and secondary metabolites.

The general trend verified in these chained trials is that of a substantial equivalence of the nutritive value of symbiotic corn and feeds, as obtained

from a contextual verification of animal performances and yields. However, a detailed investigation has revealed a reduction in the oxidant power of the corn and of the derived feeds, and that this antioxidant power can be transferred to animals to improve their health and welfare. When the utilisation of corn is delayed as a result of a long storage time, microbial fertilisation can improve its shelf life, and as a result the substantial equivalence will turn into a substantial improvement of animal performances.

In short, the hypothetically harmful and adverse effect of symbiotic corn on poultry or pig feeding has been excluded in the present trials. In fact, the functional conditions and poultry livability have instead shown signs of immediate improvement, which have improved even more after a long storage period.

Therefore, AM and microbial fertilisation may be considered a strategic tool for agronomic sustainability and for the nutritive usefulness of maize crops.

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Chapter 5

COSMETIC PERFORMANCE OF EMULGELS CONTAINING FATTY RAW MATERIAL FROM BRAZILIAN ORGANIC AGRICULTURE

Douglas Dourado[1,2,*], Myla Lôbo[1] and Neila de Paula Pereira[1]
[1]Department of Medicine, Federal University of Bahia, Salvador, Brazil
[2]Department of Health Sciences, Federal University of Rio Grande do Norte, Natal, Brazil.

ABSTRACT

In recent years, the cosmetics industry has been focusing on the development of natural and organic cosmetics, aiming to manufacture products with greater quality and effectiveness, and to fulfil the diverse requirements of cosmetics regulations (Regulation of the European Commission, 2009). Certifiers like the IBD and ECOCERT, produce

[*] Corresponding author: Rua General Gustavo Cordeiro de Faria, Petrópolis., CEP: 59012570 - Natal, Rio Grande do Norte – Brazil, E-mail: ddourado.farma@gmail.com.

guidelines for these products. Here in Brazil however, we have no institution equal to the Ministry of Industry and Commerce in France, which is respected in more than 80 countries (Higuchi, 2013). From 2008 to 2012, Brazil grew 7.4% without the organic and natural cosmetics segment (NEVES, 2010). Brazilian biodiversity products are undergoing a-n expansion process, following a worldwide trend of substituting synthetic products for agricultural raw materials. By organic cosmetics, we understand products composed of only natural ingredients, with a minimum of 95% raw materials, produced in accordance with the precepts of organic agriculture. There are also no preservatives, synthetic fragrances or petroleum derivatives used, as well as other components of non-natural origin (Kapsnet, 2007; Pereira and Dourado, 2017). In this context, the objective was to develop and evaluate the cosmetic performance of emulsions containing oils and vegetable butter from Brazilian organic agriculture, verifying the influence of these emollients on the physical-chemical and sensory stability of cosmetic formulations. Emulgels were formulated containing *Syagrus coronata* (Licuri), *Dipteryx alata* (Baru) and *Pachira aquatica* (Munguba) oils, from the Brazilian biomes Caatinga, Cerrado and Amazonia respectively, combined with *Virola surinamensis* (Ucuuba) vegetable butter, the thickener used for natural polymer xanthan gum,. These emulsion systems were submitted to accelerated stability tests (centrifugation, vibration and thermal stress), according to the cosmetic stability guide of the Brazilian National Health Surveillance Agency (ANVISA). The sensory potential (instrumental texture and *in vitro* scattering) of the emulgels in the post-preparation and post-aging accelerated periods was verified. pH and organoleptic characteristics were analyzed at the end of 90 days. All formulations developed were stable, with no presence of cream, flocculation or phase separation. The pH presented over the 90-day period was compatible with the body pH of the skin. Regarding sensory characteristics, the emulgels containing Licuri oil combined with Ucuuba butter showed greater spreadability as well as lower hardness and less adhesiveness. These results reflect a mild and pleasant sensory quality that is conferred by the greater low-level fatty acids in this emollient. Given the results observed, we can see that raw materials from Brazilian agriculture generate stable cosmetic products, which can be sensorially optimized, with characteristics that are inherent to the uniqueness of the grease composition. Additionally, this is the first study investigating these raw materials from Brazilian organic agriculture in emulgel-type cosmetic formulations.

Keywords: organic cosmetics, agriculture, raw materials, Brazil

INTRODUCTION: THE COSMETICS INDUSTRY AND SUSTAINABILITY

Sustainability is an increasingly present term in our daily lives in both social and economic contexts. The concept of sustainability first came to prominence during the 1980s, when sustainable societies were defined as those capable of satisfying their needs without compromising the chances of survival of future generations (Neves, 2008). Sustainability seeks to promote health, environmental balance, preservation of biodiversity cycles and biological soil activity. In addition, it emphasizes the use of management practices, excludes the use of agrotoxic and other materials that affect soil actions that are foreign to the ecosystem and uses local resources, thereby maximizing nutrient recycling (Vásquez, 2008). The cosmetics industry has adopted sustainable development practices for product manufacturing (Kates, 2010). Pereira 2009, states that the cosmetics industry showed the largest research investment into natural raw materials, especially in Brazil, where agroforestry production processes have been adopted to exploit Brazilian biodiversity by integrating plant production into the ecosystem, with positive environmental, social and economic outcomes (Schimaichel and Resende 2006). Brazil is currently ranked 4th in the global personal hygiene, perfume and cosmetics markets (ABIHPEC, 2017) and is looking for even greater expansion in this segment, with the cosmetics industry constantly developing innovative products that satisfy the desires of consumers without damaging the environment. In this context of constant innovation, the cosmetics industry remains in step with new market trends, with the current trend being ‘green’ cosmetics, manufactured using natural, organic and bio-cosmetic ingredients derived from raw materials that do not threaten biodiversity.

ORGANIC COSMETICS: CONCEPTS AND LEGISLATION

In recent decades, there has been an increase in interest in natural products, such that a consumer market called the “green consumer” has

emerged, especially in European countries. Studies show that the international market for personal care products developed from natural products has an estimated average annual growth of 8-25%. This situation, allied with the impact of the global environmental crisis, has led to a rethinking of the use of non-renewable resources, as well as an awareness of environmental problems and the risks they cause to the maintenance of human life. In this context, organic cosmetics are a new cosmetology concept that has recently been expanding, where raw materials from organic agriculture are obtained for the manufacture of cosmetics. The widespread acceptance of organic cosmetics is generally due to concerns about environmental degradation, consumer awareness and increased demand for natural products (Schimaichel; Resende, 2006). This reality gives consumers a responsibility both for environmental health as well as for their own. Consumers of these cosmetics are a large, dedicated group who prioritize not consuming cosmetic products that use synthetic substances, preferring those based on natural and/or organic products, as they are safer and more sustainable for the environment. Cosmetics classified as organic are those with at least 95% of the formulation components, other than water, being certified organic raw materials or raw materials that follow strict production, extraction, purification and processing standards. The remaining 5% of the formulation may consist of water, non-certified natural raw materials or extractives allowed in organic formulations (ECOCERT, 2003, IBD, 2013). Cosmetics based on organic raw materials should have a minimum of 70% and a maximum of 95% of components in the formulation certified as organic, discounting water and salt. The remainder of the components of the formulation can come from conventional agriculture and extraction. In the case of natural cosmetics, they have 5% certified organic raw material, while the other 95% of the formulation can be composed of uncertified raw materials but only those allowed for natural formulations (Sebrae, 2008). Certification is of utmost importance, since it gains the confidence of the consumer, providing certainty that the product is genuinely organic (Ambrosano, 1999). It includes procedures that follow the standards established by the certification agencies, guaranteeing a more reliable product for the

consumer (Ribeiro, 2009). However, there is still no official regulation for organic cosmetics, so the certification groups develop their own regulation standards (BISPO, 2008).

The International Federation of Organic Agriculture Movements (IFOAM) is a European international organization dedicated to the principles of organic agriculture, whose main function is to evaluate, standardize and disseminate standards for the marketing of organic products (Schimaichel and Resende, 2006). Several certification agencies develop their organic production standards based on IFOAM standards (Ribeiro, 2009). Among these, the Biodynamic Institute (IBD) is a certifying entity founded in Brazil in 1981, which acts in experimentation, research and publication. It is the largest certifier of organic and biodynamic products in Latin America and is recognized by the IFOAM (Ambrosano, 1999). In Brazil, the IBD agency recommends processes that do not cause changes in natural raw materials. The extraction processes permitted for raw materials are those that use cold distillation, pressure, water or steam, percolation and concentration by physical or mechanical methods. Processes that use extraction solvents, such as alcohol and glycerin, are permitted on the condition that they are organically obtained. Processes that use water, nitrogen and CO_2 are also allowed. Vegetable butters and oils, lanolin, natural dyes, essential oils, plant extracts (glycolics, dyes and dry extracts), minerals and natural polymers (xanthan gum, alginates and starches) are, for example, categories of materials - raw materials obtained through these extraction methods (IBD, 2010). According to the certification agencies, certification aims to verify the ingredients, processes, production, storage of raw materials, packaging, labelling, use of energy resources and waste management and certification of producers to ensure the quality of the final product. This means that certification agencies impose standards that must be met by the productive sector to ensure quality throughout the production cycle (ECOCERT, 2003).

BRAZILIAN AGRICULTURE AS A SOURCE OF RAW MATERIAL FOR ORGANIC COSMETICS

Brazil occupies a privileged position in terms of biodiversity in all concepts. Considering the more restricted aspect of species already catalogued around the world, Brazil has the largest total number (13%) and the second largest quantity of endemic species in absolute terms, nearly equal with Indonesia. It stands out in the group of the 17 mega-diverse countries in the world, known for its seven biomes: Amazonia, Cerrado, Caatinga, Atlantic Forest, Pampa, Pantanal and Maritime and Marine Zone (Pimentel et al., 2015). From this perspective, Brazilian biodiversity products are undergoing an expansion process, since they are a source of unique natural raw materials, whose characteristics give these products the particularities desired by the consumer market. Formulations containing components of organic origin have the natural ability to stimulate skin recoveryand accessories. They can also be softer for the skin and hair and more effective (Lyrio et al., 2011). Phytopreps such as oils, resins, butters, extracts and dyes are widely used in the manufacture of Brazilian organic cosmetics with scientifically proven topical and capillary benefits (ABIHPEC, 2012). Corte (2006) states that vegetable oils have good compatibility and penetration of the skin and are a source of vitamins and essential fatty acids. These emollients can positively influence the sensory performance of the product, reflecting greater scattering, stability and high replacement of fatty acids in the skin. Such characteristics are not conferred by synthetic raw materials.

In Brazilian biodiversity, Amazonian fruits are known for their chemical properties, specifically clinical and dermatological (SILVA, 2012). Pereira and Dourado (2017) briefly describe raw materials from Amazonian vegetation as sources to produce organic cosmetics. They include, *Euterpe oleracea* (Açai), *Orbignya martiana* (Babassu), *Theobroma grandi-florum* (Cupuaçu), *Carapas guianensis* (Andiroba), *Pentaclethra macroloba* (Pracaxi), *Copaifera landesdorffi* (Copaiba), *Platonia insignis* (Bacuri), *Theobroma cacao* (Cocoa), *Virola surinamensis*

(Ucuuba) and *Bertholletia excelsa* (Brazilian nuts). In addition, Brazil offers numerous natural source for organic cosmetics production in biomes little explored in terms of their potential. The present study reveals three plant species from Brazilian agriculture that can be used as raw materials for the manufacture of these cosmetic products; these include: the species *Syagrus coronata* (Licuri), from the Caatinga biome, whose chestnuts contain a high fixed oil content presenting a predominance of saturated fatty acids such as lauric, capric and caprylic acid (Gomes Neto, et al., 2008); the species *Dipteryx alata* (Baru) from the Cerrado biome, mainly containing oleic and unsaturated linoleic acids (Lima, 2012); and the species *Pachira aquatica* (Munguba) originating in the Amazonian biome, but also present in the Cerrado and Caatinga, presenting a predominance of palmitic and oleic acids (Jorge; Luzia, 2012). From this perspective, the objective of the present study was to develop and evaluate the cosmetic performance of emulgel-type formulations containing organic vegetable oils from Brazilian agriculture.

METHOD OF ANALYSIS

Obtaining of Organic Raw Materials

Syagrus coronata (Licuri) fixed oil was supplied by the Licuri Brasil industry. *Pachira aquatica* (Maranhão nut) oil, from *Dipteryx alata* (Baru) and *Virola surinamensis* (Ucuuba) vegetable butter were supplied by the Beraca industry.

Development of the Emulgels

Three emulgels were developed in oil (O/W) whose lipophilic phase contained the vegetable oils: (1) *Pachira aquática* (Munguba), (2) *Dipteryx alata* (Baru) (3) *Syagrus coronata* (Licuri) combined with *Virola surinamensis* (Ucuuba) vegetable butter. In the hydrophilic phase, the

natural xanthan gum polymer was slowly dispersed. Both the lipophilic and hydrophilic phases were heated to 75°C ± 5, dispersing the oil phase in the aqueous phase with continuous mechanical stirring at 500 rpm, obtaining different oil-in-water (O/W) emulsifying systems. In the table below, the composition of the formulated emulgels is described.

Stability Tests

To obtain information indicating the degree of relative stability of the products under the various conditions to which it may be subject from manufacture until the end of its validity, stability studies of the emulgels were performed for the parameters: pH, centrifugation, vibration, freeze/ thaw cycle and organoleptic characteristics (Brasil, 2004).

Organoleptic Characteristics

Color, odor, and coalescence aspects of the emulgels were observed at 30, 60 and 90 days after preparation.

Centrifugation and Vibration

The emulgels were centrifuged at 3000 rpm for 30 minutes (Centrifuge, Excelsa 208 N model, Brazil). Soon after, they were subjected to vibration in a Vortex (Lab Dander Bay model, IKA Brazil) based on ANVISA specifications (Brasil, 2004).

Table 1. Formulation of developed emulgels

Oil phase	Aqueous phase
Lanette N ® - 4%	Water -qsp 100mL
Vegetable butter-5%	Sorbic acid- 0.05%
Vegetable oil - 7%	Xanthan gum -1%

Freeze/Thaw Cycle

The freeze/thaw cycle consists of 6 alternating temperature cycles, a complete cycle consisting of 24 hours at 4°C and 24 hours at room

temperature (25°C), completing 12 days of testing, according to ANVISA specifications (Brazil, 2004). The test was performed in triplicate.

pH Analysis

The determination of pH was based on the method proposed by Borghetti and Knorst (2006), using samples diluted in distilled water (1:10 w/v), homogenized and read at a temperature of 25 ± 1°C. The pH meter (Model PH 300, ALPAX, Brazil) was calibrated beforehand, using standard solutions of pH 7.0 and 4.0. Testing of the emulgels was performed post-preparation after 30, 60, and 90 days of storage. Analyses were performed in triplicate.

Sensory Potential Performance

Spreadability in Vitro

Spreadability is defined as the expansion of a semi-solid formulation on a surface after a given period of time (Feltkamp et al., 1983; Borghetti and Knorst 2006). It is an important parameter for any cosmetic formulation, since it is linked to the sensory characteristics of the product. Therefore, the emulgels were gradually subjected to spreading *in vitro* under extrusion using stacked plates until the maximum weight spreadability was found (Ei máx.) (Knorst 1991; Milão et al., 2007). The spreading of the emulsions was analyzed over the storage time (30, 60 and 90 days after preparation) and after accelerated aging action (stability test). Spreadability is expressed by the formula below:

$$Ei = d^2 \, x \frac{\pi}{4}$$

where:

Ei = spreadability of the sample to the weight i (mm^2),

d = Average diameter (mm).

Study of Emulgel Texture

The texture test corresponds to the physical characteristics perceived through touch, which are related to the deformation caused by force. It is measured in terms of force, distance and time (Krambeck, 2009). In the development of preparations for cutaneous application, it is necessary to take into account certain attributes that contribute to the acceptance of the product and improvement of its effectiveness. These attributes mechanical properties such as adhesiveness and hardness are included. In the case of pharmaceuticals and cosmetics the penetration test is usually performed for texture analysis, in which the probe penetrates the sample at a given speed from a predefined distance, and subsequently returns to a position at a predetermined distance, above the sample.

The sensory performance of the formulations (post-preparation) was verified by means of the Texturometer (TA-XT Plus), in triplicate and under the following conditions:

- Circular Probe - 6mm
- Pre-test speed-5 mm/s
- Post-test speed-1mm/s
- Test speed-5mm/s
- Distance - 5mm
- Time - 5 s
- force- 5.1g

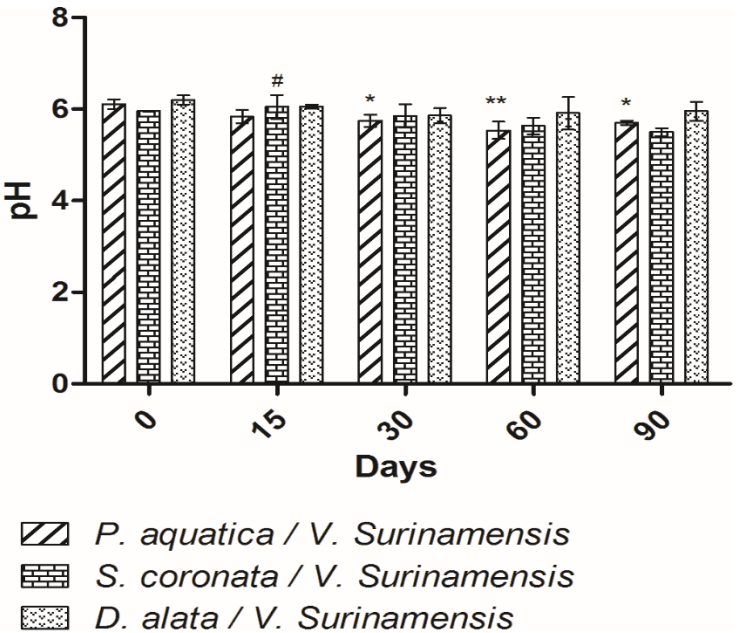
Statistical Analysis

The results were expressed in means and standard deviations of the triplicate assays. The data was processed using the one-way ANOVA test followed by the Tukey test for a suitable comparison of the means obtained, considering a 5% significance level ($p < 0.05$).

RESULTS

All formulations were stable during organoleptic analysis and stability tests, with no evidence of cream, colour change, odor and/or phase separation. PH measurements over 90 days revealed a slightly acidic profile of the emulgels between 5 and 6; skin pH in its turn should be maintained within the range of 4.5 to 6 (Clares et al., 2014). This fact shows that the formulations containing organic materials show near optimal pH for skin, revealing a similarity with the skin and biocompatibility characteristics for such an application. According to the pH measures evaluated over 90 days (Figure 1), statistically significant variations ($p < 0.05$) were observed for the emulgel containing Munguba oil (*P. aquatica*) at 30, 60 and 90 days when compared to post-preparation, and for the emulgel containing Licuri oil (*S. coronata*) only on day 15 when compared to day 90. Even so, the emulgels were stable, presenting values compatible with the skin, corroborating with the low instability of the systems developed, since high pH changes may be related to chemical reactions of degradation of oils such as the hydrolysis of fatty acid esters, which may lead to a sudden reduction of the pH of the formulations due to an increase of free fatty acids (Martini, 2005).

Referring to the spreadability profile of each sample for 90 days (Figure 2) revealed that the emulgel containing the Licuri oil (S. coronata) showed better spreadability performance post-preparation, with maximum spreading 6698.04mm^2, followed by the emulgel containing Munguba oil (*P. aquatica*) with 5570.18 mm^2 and Baru (*D. alata*) with 3908 mm^2. In simple cream emulsion systems combined with Licuri oil (*S. coronata*) and Ucuuba vegetable butter (*V. surinamensis*), Dourado and collaborators (2015) showed a maximum spreadability of 9072mm^2, higher than the emulgel studied here, which contained the same natural emollients. This performance was expected since the addition of a gel phase increases the viscosity of the formulation due to the thickening properties of the polymer, which directly leads to the stability, rheology and sensory values determined by the settling velocity according to Stokes law.

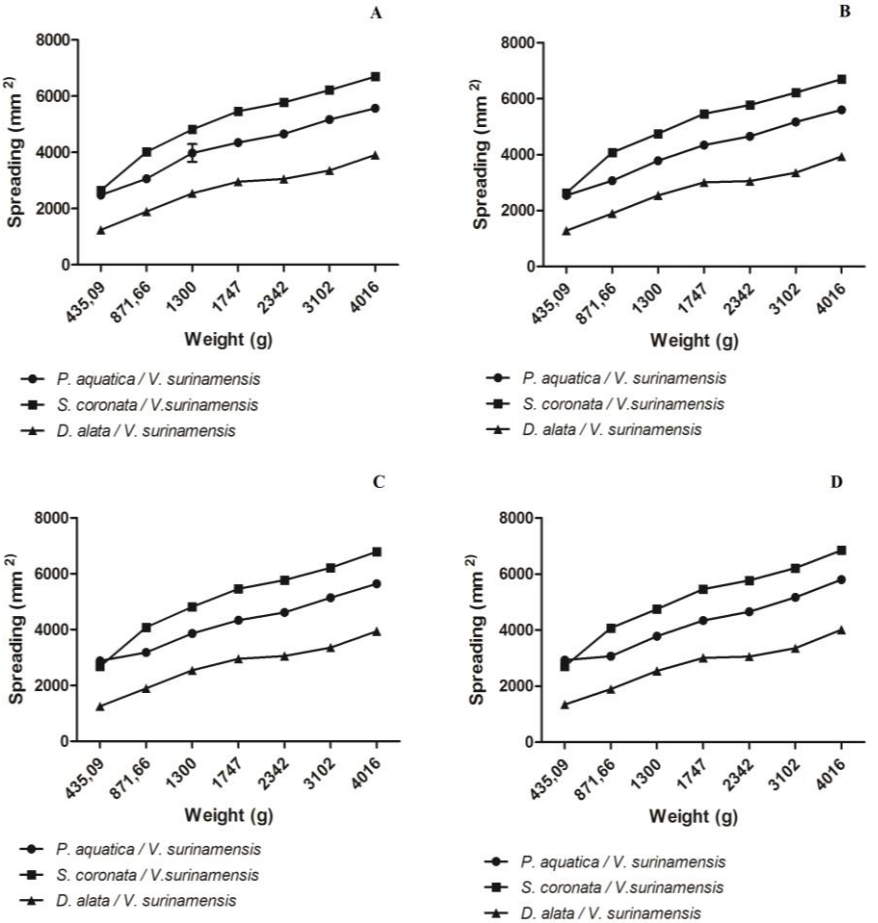


Legend: * ($p < 0.005$) when comparing day 0 with day 30 and 90; ($p = 0.0245$) when comparing day 15 with day 90. Results are means ± standard deviation ($n = 3$).

Figure 1. Evaluation of the pH of the emulgels containing organic raw materials from Brazilian agriculture during the 90 days of study.

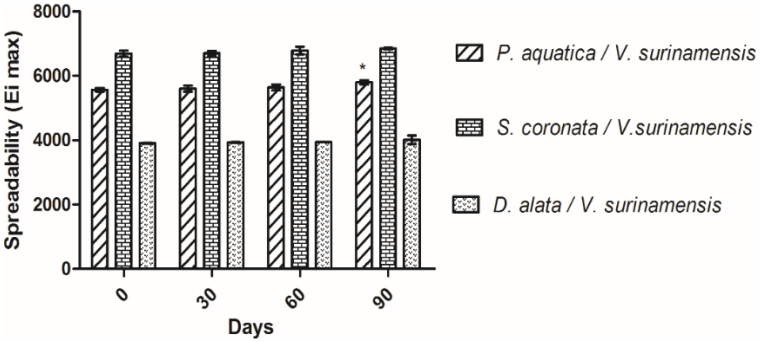
When analyzing the spreadability profile at the other times, after 30, 60 and 90 days, the post-spreading profile was determined, with the observation of an increase in spreadability being possible in all formulations in the study. This behaviour can be better observed in Figure 3, where the maximum spreading of each emulgel was compared over the 90-day study period. A better spreading of emulgel containing Licuri oil (*S. coronata*), followed by Munguba oil (*P. aquatica*) and Baru oil (*D. alata*) was observed. The differences between the spreading of each emulgel were significant ($p < 0.0001$), including an increase found over the 90 days. When comparing the maximum spreadability on each test day, variation was only statistically significant for the emulgels based on Munguba oil (*P. aquatica*) in the post-preparation spreadability and at 30 days when compared to 90 days. For the other species, variation was not significant during the study. These results may be related to the viscosity

of each emulgel, so that the higher the lowest spreading the more viscous the formulation tested. Therefore, the spreading profile of the emulgel based on the Licuri oil (*S. coronata*) showed lower viscosity when compared to the others, which can be confirmed using other sensory tests.



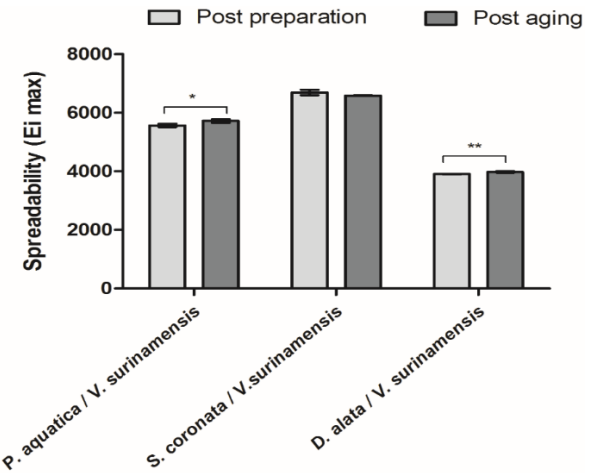
Legend: A = post-spreading; B = spreading on day 30; C = spreading on day 60; D = spreading on day 90. Results are means ± standard deviation, n = 3.

Figure 2. Spreading profile of the emulgels containing organic raw materials from Brazilian agriculture over 90 days.



Legend: * (p = 0.0220) when comparing the spreadability at 0 days and 90 days and between 30 and 90 days. On the other days and in the other species, variation was not significant over the 90 days. The changes in spreadability between species were significant on all test days (p < 0.0001). Results are means ± standard deviation, n = 3.

Figure 3. Graph of maximum spreadability of the emulgels containing organic raw materials from Brazilian agriculture, over 90 days.



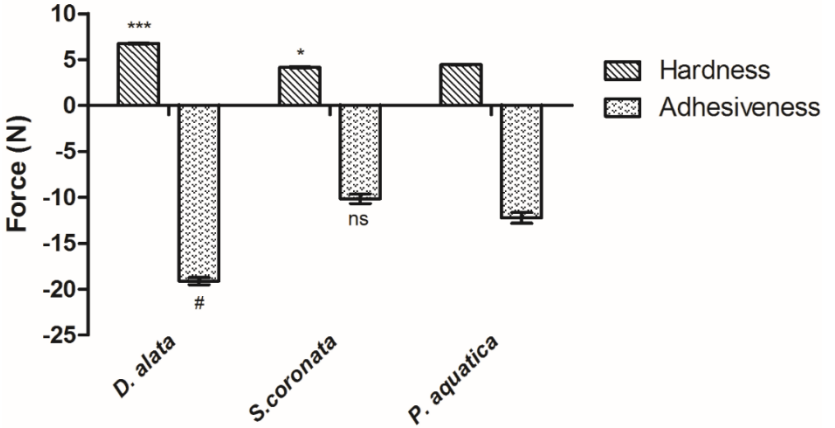
Legend: *(p = 0.0310) when compared to post-preparation spreadability and after accelerated aging. **(p = 0.0097) when compared to post-preparation spreadability and after accelerated aging. For the *S. coronata*/*V surinamensis* sample, the change was not significant (p = 0.1682). Results are means ± standard deviation, n = 3.

Figure 4. Graph of comparison between maximum spreadability post- preparation and after accelerated aging test.

The spreadability of the emulgels was also verified after aging, in which the samples were submitted to accelerated stability tests. When comparing the maximum spreadability of each sample in the post-preparation and after accelerated aging (Figure 4), it was possible to observe a significant increase ($p < 0.05$) for Munguba oil (*P. aquatica*) containing 5,570.18 mm^2 to 5,722.65mm2 and with Baru oil (*D. alata*) from 3.908mm^2 to 3.979mm^2, whereas for the emulgel containing Licuri oil (*S. coronata*) the variation was not significant ($p > 0.05$).

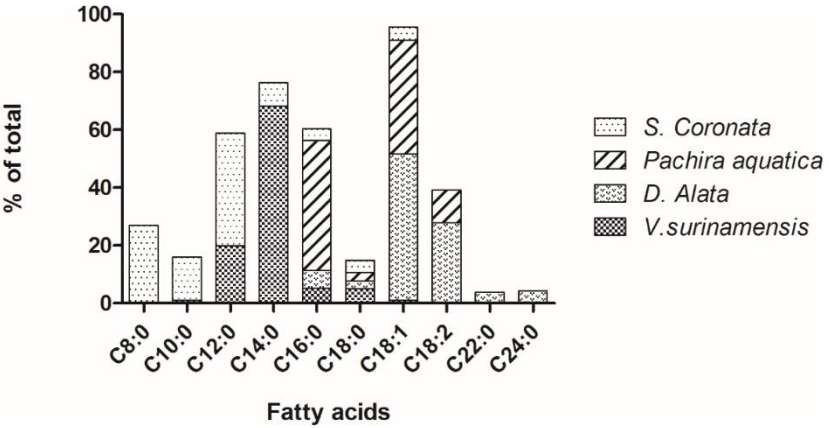
These results reveal the stability of the formulations regarding the maintenance of their sensory profile, even with the stresses to which the samples were subjected in the accelerated stability test. Therefore, the emulgels based on organic raw materials from Brazilian agriculture were able to maintain their scattering profile even after aging.

Complementing the study of the sensory potential, the texture tests are consistent with the performances found in the spreading profile evaluations, since the formulation containing Licuri oil (*S. coronata*) was the one with the lowest hardness, followed by the emulsions containing Munguba (*P. aquatica*) and Baru (*D. alata*) oils. In Figure 5, it is possible to visualize the result of the *in vitro* texture test, which revealed significantly different hardness values when all the emulsifiers were compared to each other ($p < 0.05$). When correlating the two *in vitro* sensory tests - spreadability and texture, these can be corroborated, since for the emulgel to spread further, it needs a lower strength of resistance, that is, less hardness. Additionally, adhesiveness is another important parameter in the sensory texture test. This parameter was high in the emulsion containing Baru oil (*D. alata*) and low in the emulgels containing Licuri oil (*S. coronata*) and Munguba oil (*P. aquatica*). Comparing the adhesiveness of the formulations, only the emulsion with Baru oil presented significantly higher values than the others ($p < 0.0001$). Pharmacotechnical presentations with high adhesiveness tend to have higher viscosity and impart a sticky sensory impression, a characteristic that is not desirable for a product. Therefore, the emulsion based on Licuri oil (*S. coronata*) presented the best sensory *in vitro* performance, considering the two tests performed.



Legend: ***($p < 0.0001$) when comparing *D. alata* and *S. coronata* and *D. alata* with P. *aquatica;* *($p < 0.05$) when comparing to *S. coronata* with *P. aquatica*; #($p < 0.0001$) when comparing *D. alata* with *S. coronata* and *D. alata* with *P. aquatica*; ns = not significant ($p > 0.05$) when *S. coronata* and *P. aquatica* were compared. Results are means ± standard deviation (n = 3).

Figure 5. Texture of the emulgels containing organic raw materials from Brazilian agriculture.



Legend: C8: 0 = Caprylic acid, C10: 0 = Capric acid, C12: 0 = Myristic acid, C14: 0 = Lauric acid, C16: 0 = Palmitic acid, C16: 1 = Palmitoleic acid, C18: 0 = Stearic acid, C18: 1 = Oleic acid, C18: 2 = Linoleic acid, C18: 3 = Linolenic acid, C22: 0 = Behenic acid, C24: 0 = Lignoceric acid.

Figure 6. Profile of fatty raw materials from Brazilian agriculture.

Among the physical-chemical characteristics of the raw materials that may influence the spreadability and textural behaviour of the formulations is the grease composition of the vegetable components used. In analyzing the fatty acid composition of Licuri (*S. coronata*), Baru (*D. alata*) and Munguba (*P. aquatica*) oils and Ucuuba (*Virola Surinamensis*) vegetable butter reported in the literature (Gomes Neto, et al., 2008; Lima, 2012; Jorge; Luzia, 2012) and represented in Figure 6, we can suggest that the medium and short chain saturated fatty acids, such as lauric (C12: 0), capric (C10: 0) and caprylic (C8: 0) major in Licuri oil, produce high spreadability and low hardness formulations. Kim and collaborators, (2008) report high spreadability and permeability for formulations containing long chain fatty acids such as oleic (C18: 1) and palmitic (C16: 0) as the major emollients. In a prior study, Dourado and collaborators, (2015), showed greater spreadability for formulations containing short chain C12: 0, C10: 0 and C8: 0 short chain fatty acids in comparison to those containing emollients in which long chain fatty acids such as C18:1 and C16:0 are more common. However, in the emulsions studied, there is significant influence from the myristic acid (C14:0) present in Ucuuba butter, an emollient combined with all the oils in this study that can configure synergy or not with the composition of the evaluated oils. It is noted that the emulgel containing the baru oil combined with the Ucuuba butter revealed low spreadability and high hardness and adhesiveness when compared to the others. In this case, it is suggested that the high content of linoleic acid present in its composition confers an unsatisfactory sensory profile, not observed in the other oils, which do not contain the fatty acid in their composition or only in low quantities.

CONCLUSION

There is a growing change in social behaviour in relation to the consumption of cosmetic products, with a move towards natural and organic products. This has led the industry to adapt to the requirements of this new niche market. It is possible to observe that raw materials coming

from Brazilian organic agriculture produce stable cosmetic products, with characteristics and profiles that are not found in synthetic products but that are peculiar to these natural resources. Natural emollients have greater skin compatibility, in addition to the greater frequency of short chain fatty acids which obtains a lighter sensory performance consequently generating greater consumer satisfaction. This profile was observed in the emulgel containing the Licuri oil originating from the Caatinga biome, a vegetation exclusive to Brazil, whose potential has yet to be tapped by the cosmetics industry. It is also worth mentioning that this was the first study to investigate these raw materials from Brazilian organic agriculture for cosmetic formulations of the emulgel type. Their contribution in the present study proved to be valuable in the design of new products from the organic scenario. In addition, the study of new raw materials from Brazilian agriculture establishes sustainable systems and valorization of natural resources, where an environmentally-conscious production chain is developed, from manufacturing to consumption, following the global trend towards sustainability. Therefore, the use of natural and organic products in cosmetics represents an innovation while exploiting Brazilian agricultural biodiversity, which still presents several biomes and raw materials with untapped potential for such applications.

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BIOGRAPHICAL SKETCHES

Douglas Dourado Oliveira

Affiliation:

1. Department of Medicine, Federal University of Bahia, Salvador, Brazil.
2. Department of Health Sciences, Federal University of Rio Grande do Norte, Natal, Brazil.

Education: Graduated in Pharmacy from the Federal University of Bahia (UFBA), with a sandwich degree - CAPES at the Ecole des Mines d'Albi Carmaux, France, Master 2 Bio Santé ingénierie (Master in Pharmaceutical Engineering), parcours l'industrie pharmaceutique. PhD student in in pharmaceutical nanotechnology.

Research and Professional Experience: He carried out research, development and innovation activities of galenic formulations linked to CNRS (National Center for Scientific Research of France) on the GALA Platform, an advanced galenic industrial platform located in France in a project associated with the Laboratory of Molecular Interactions of Chemical Reactivity and Photochemistry) at the Université Paul Sabatier-Toulouse and RAPSODEE Center. Develop research on the Laboratory of

Research in Medicines and Cosmetics (LAPEMEC), proposing innovative cosmetic and therapeutic lines from raw materials from the Bahian semiarid. He has experience in semi-solid formulations, solid and complex, evaluating rheological, kinetic, sensorial, pharmacological and physico-chemical parameters

Professional Appointments:

Cosmetology and Pharmaceutical Technology

Publications from the Last 3 Years:

Lobo, M.; Dourado, D.; Ribeiro, P. L. L.; Pereira, N. P.; Druzian, J. I. Nanoemulsions For Cosmetic Applications: What Innovation Status? *Recent Patents on Nanotechnology*, 2018.

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Myla Lôbo de Souza

Affiliation: Masters at the Research laboratory in medications and cosmetics, Deparment of Pharmacy of the Federal University of Bahia, Salvador, Bahia, Brazil.

Education: Graduated in Pharmacy from the Federal University of Bahia. Post-graduation lato-sensu, specialist in Pharmacotherapy and drug interactions in the clinical pharmacy by Facunicamps. Master's student in pharmaceutical sciences at the Federal University of Bahia.

Research and Professional Experience:

Experience in the field of community pharmacy, acting as manager and pharmacist responsible technician. He works in the area of Bioprospecting and drug planning, focusing on the development of complex emulsifying systems based on vegetable oils, for dermocosmetic purposes. She does research at the Laboratory of Research in Medicines and Cosmetics (LAPEMEC), with the following topics: fixed oils, chromatographic

methods, macro and nanostructured emulsion systems, physicochemical analyzes, stability of cosmetic formulations, biological tests. Integrates the EMBRAPA Passitec Research Network.

Professional Appointments:

Cosmetology and Pharmaceutical Technology

Publications from the Last 3 Years:

Lobo, M.; Dourado, D.; Ribeiro, P. L. L.; Pereira, N. P.; Druzian, J. I. Nanoemulsions For Cosmetic Applications: What Innovation Status? *Recent Patents on Nanotechnology*, 2018.

Dourado, D.; Meira, N. A. N.; Lobo, M.; Pereira, N. P. Development Of Multiple Photoprotective Emulsion Containing Pachira retusa OIL. *Brazilian Journal of Pharmaceutical Sciences*, 2017.

Neila de Paula Pereira

Affiliation: Coordinator of the research laboratory on medicines and cosmetics. Adjunct Professor of the Department of Pharmacy of the Federal University of Bahia, Salvador, Bahia, Brazil.

Education: Graduated in Pharmacy from UFRJ. She is a PhD in Pharmaceutical Sciences in the area of inputs, medicines and correlates by UFPR with an interdisciplinary thesis in phytochemistry applied to pharmacotechnics. He also holds postgraduate and master's degrees in Organic Chemistry from the Institute of Chemistry of UFRJ with research developed in the Laboratory of Evaluation and Synthesis of Bioactive Substances (LASSBio).

Research and Professional Experience: Has professional experience in the area of pharmacotechnics, cosmetology, in addition to extraction and

synthesis of bioactive. She works as a professor at the Federal University of Bahia (UFBA) coordinating two research projects in phytocosmetology and another teaching/extension in homeopathic manipulation at the Laboratory of Research in Medicines and Cosmetics (LAPEMEC), which is responsible. It develops academic and applied research with the following themes: fixed and essential oils, chromatographic methods, macro and nanostructured emulsion systems, gel systems, rheological parameters and biological tests. It operates within the scope of innovation, composing the inventors' framework of patent PI 1102277-9 registered at INPI. He guides and coordinates students in the Pharmacy Postgraduate Program at UFBA, integrates the Passitec Research Network of EMBRAPA and participates in the teaching staff of the interinstitutional programs of pharmaceutical assistance

Professional Appointments:

Cosmetology and Pharmaceutical Technology

Publications from the Last 3 Years:

Lobo, M.; Dourado, D.; Ribeiro, P. L. L.; Pereira, N. P.; Druzian, J. I. Nanoemulsions For Cosmetic Applications: What Innovation Status? *Recent Patents on Nanotechnology*, 2017.

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Chapter 6

NON-CONTACT MONITORING BIRD MASS IN POULTRY FARMS

Sergey S. Sarkisov* *and Avedik S. Sarkisov*
[1]SSS Optical Technologies, LLC, Huntsville, AL, US
[2]Department of Financial Management,
Gubkin State University of Oil and Gas, Moscow, Russia

ABSTRACT

Old-fashion automatic weighing platforms in the poultry industry require frequent maintenance and re-calibration and are intrinsically inaccurate because bigger birds occasionally avoid them. This chapter describes a new no-contact method of determining bird mass that is free of these deficiencies. The method is based on computer processing of digital top-view bird images automatically taken with image sensors mounted on the ceiling of a poultry house. Experimental data presented in this chapter prove that the average area of such images can be used to predict with good accuracy the average mass of birds. The chapter discusses: (1) hardware setting; (2) algorithm of computer processing of

* Corresponding Author Email: mazillo@hotmail.com.

bird images and determining the average image area; (3) lab testing with test objects imitating birds; (4) field testing in two production houses at a commercial poultry farm; and (5) future directions of research and development. The information provided in the chapter can be useful for agriculture professionals who are interested in: (a) inexpensive and maintenance free tools for continuous monitoring bird mass for timely adjustment of feeding process to keep it on target and thus maximize output and minimize feed conversion ratio and (b) paradigm change in bird's mass monitoring technology that reduces grower's maintenance burden and makes mass monitoring a daily routine in every poultry farm.

1. INTRODUCTION

Continuous monitoring mass of birds during feeding period would be very helpful in attaining high output in the poultry industry (Arbor Acres, 2014, p. 52; Butcher, 2009). Such parameters as average mass, daily gain, flock uniformity (coefficient of variation *CV%*), etc. provided to growers on daily basis can help to identify and timely correct the problems causing deviation from target mass and reduce house-to-house variations of feed conversion ratio (FCR) in poultry farms (Arbor Acres, 2011). Market of weighing equipment is filled up with numerous systems, manual, semi- and fully automatic, for weighing birds in poultry houses (JOFATAN, 2018; SKOV, 2018; Weltech, 2018; ACE Integrated Solutions, 2018). All these systems use the variations of scales that generate mass data when one or several birds step on. They suffer from two major problems: (a) inaccuracy of measurements due to continuous movement of birds on scales and litter deposits and (b) maintenance burden associated with necessary frequent cleaning and re-calibration. It has been also reported (Chedad, 2003) that heavy birds visit automatic weighing systems less frequently than lighter birds further compromising the data. This explains why growers rarely use bird weighing systems in their houses. As a result, they remain unaware of true day-to-day performance of birds in terms of mass gain and feed conversion until the final moment. There is therefore a great need for an inexpensive, maintenance free and possibly non-contact system for

monitoring mass of birds in poultry houses with a data flow rate of several times per day.

The proposed solution is based on the hypothesis that the area of top-view image of a bird can be used as a measure of its mass. Curiously enough, Chedad et al. (Chedad, 2003) computed the areas of top-view digital images of birds in poultry houses to prove that larger (heavier) birds have a tendency to avoid automatic scales. But Chedad and his team did not proceed further to hypothesize that visible area could be a measure of mass.

There have been attempts to obtain mass and other characteristics of animals from their images in the past. U.S. Patent No. 4,745,472 (Hayes, 1981) describes an apparatus and method for determining characteristics of animals, such as cattle or the like, confining the animal, producing at least one profile image and locating specific reference points on the profile image related to the characteristics to be determined, linear measurement of predetermined parts of the animal and utilization of the linear measurements to provide correlation between the linear measurements of a particular animal and other animals. In U.S. Patent No. 5,576,949 (Scofield, 1994) an animal is evaluated in a special arrangement as it moves through first and second scenes that correspond to different first and second fields of view in a chute guiding forward movement of animals. A plurality of selected parameters is ascertained that form selected animal indicia that are used to evaluate the animal. U.S. Patent No. 6,974,373 (Kriesel, 2002) describes a method and apparatus for measuring the physical characteristics of livestock animals such as cattle and hogs. The apparatus includes a plurality of strategically positioned cameras that are used to obtain data concerning volumetric, curvilinear (surface) and linear measurements of the animal and the full carcass thereof. U.S. Patent No. 7,128,024 (Doyle II, 2003) describes a system and method for measuring an animal that includes a light source and a photo camera. The light source, an array of monochromatic light emitting diodes, backlights one or more of the animal's legs. The camera opposes the light source and obtains an image that includes silhouettes of one or more legs of the animal. A computer determines measurements, such as the approximate skeletal trunk

length of the animal, from the silhouetted legs in the image. Additionally, ultrasound transducers are used to determine an approximate height and width of the pelvic region. U.S. Patent Application No. 2007/0110281 (Jurk, 2006) describes the computer software for determining physical characteristics of an unrestrained animal by enabling a user to interact with one or more images of the unrestrained animal on a graphical user interface connected to a computer. Using the graphical user interface, the user is able to designate piecewise linear and circumferential measurements of selected animal features on one or more images. The previous approaches have the following disadvantages: (a) one act of measurement is performed on one animal only; (b) an animal is kept restrained during the measurement or a complex software required the intense involvement of human operator in case of an unrestrained animal; and (c) statistical averaging requires multiple acts of measurement with logistical problems of maintaining the flow of animals to and from the measurement compartment. The method described in this chapter is free of such deficiencies.

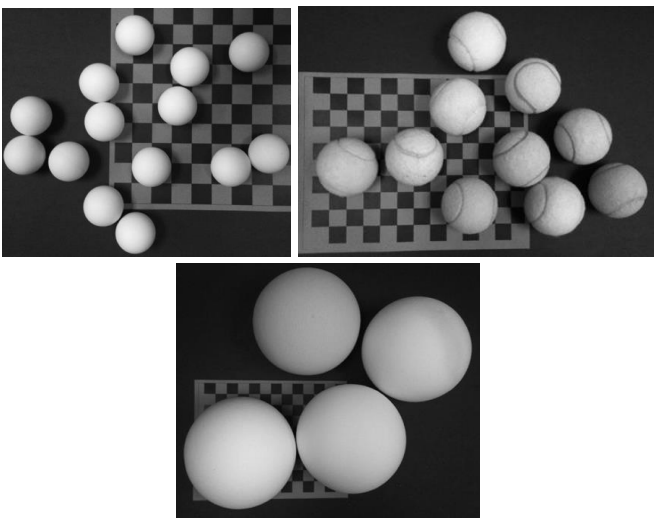

Figure 1. Examples of the images of groups of spherical objects used in the preliminary experiments. Top left: 38-mm-diameter ping-pong balls; top right: tennis balls; bottom center: 17-cm-dimater foam balls.

Early assumptions about feasibility of the method were derived from the following experiments. Top-view images of various spherical objects (simulating birds of different age) were taken with rudimentary iPhone digital camera at a distance of 0.5 to 1.5 m using room light illumination (Figure 1). A 2 x 2-cm-cell checkerboard was used as a length standard. The images were processed using the techniques that will be described in detail in the next section.

Since the balls had different mass density, instead of mass, the average volume $V_{average}$ was plotted against the average image area $A_{average}$ as shown in Figure 2. The nonlinear regression analysis showed that the data well fit in equation $V_{average} = C\, A_{average}^{3/2}$ similar to $V_{sphere} = [4/(3\pi^{1/2})]A_{cross\text{-}section}^{3/2}$, connecting the volume V_{sphere} of an ideal sphere to its cross-sectional area $A_{cross\text{-}section}$. The experiments thus indicated that the visible image area of oblong objects can be possibly used for determining their volume or mass (if the mass density is known).

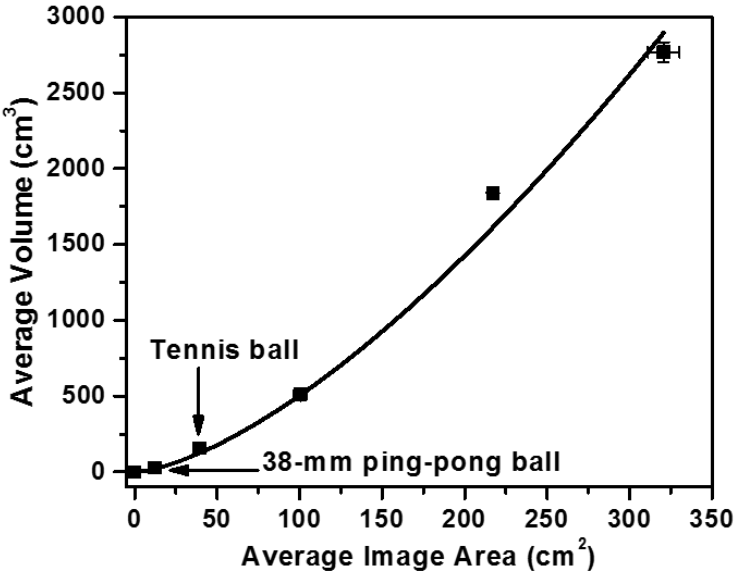

Figure 2. Volume of spherical test objects plotted versus the average image area. Solid red curve is the nonlinear fit $y(x) = Cx^{3/2}$, $C = 0.504 \pm 0.017$.

2. METHOD

The block-diagram of the proposed non-contact bird weighing system is presented in Figure 3. The system consists of image sensor 1, such as digital photo camera, computer 2, computer-controlled illuminator 3, and 2-dimensional length standard 4. Image sensor 1 is connected to computer 2. Illuminator 3 is also connected to the computer. The image sensor and the illuminator are mounted at some elevation in poultry house 5 above birds 6. Length standard 4 is placed on floor 7. The computer is located in control room 8.

The proposed method consists of the following steps:

1) *Capturing digital images of the birds.* A special program running in computer 2 triggers illuminator 3 and digital image sensor 1. Sensor 1 captures the top-view digital image of scene 9 (Figure 4) with birds 6 and length standard 4. The image is sent to computer 2 and stored in its memory. As alternative, the image of length standard 4 can be initially taken separate from the birds and, provided that the image sensor does not move (more likely the case), the images of the birds can be taken without the length standard.

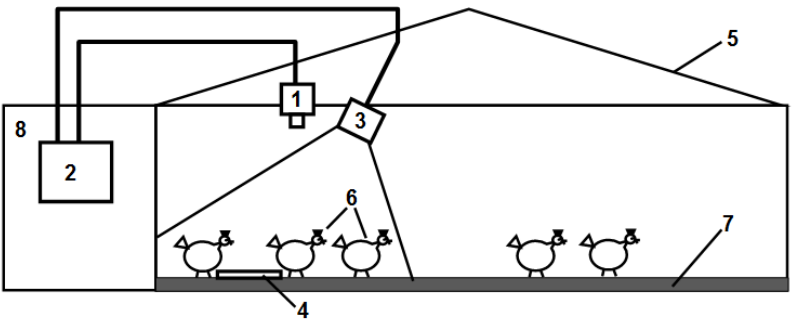

Figure 3. Schematic of the proposed system for monitoring mass of birds in a poultry house: 1 is the image sensor, such as a digital photographic or video-camera; 2 is the computer; 3 is the computer-controlled illuminator; 4 is the 2-dimensional length standard; 5 is the poultry house; 6 are the birds; 7 is the house floor covered with litter; 8 is the control room.

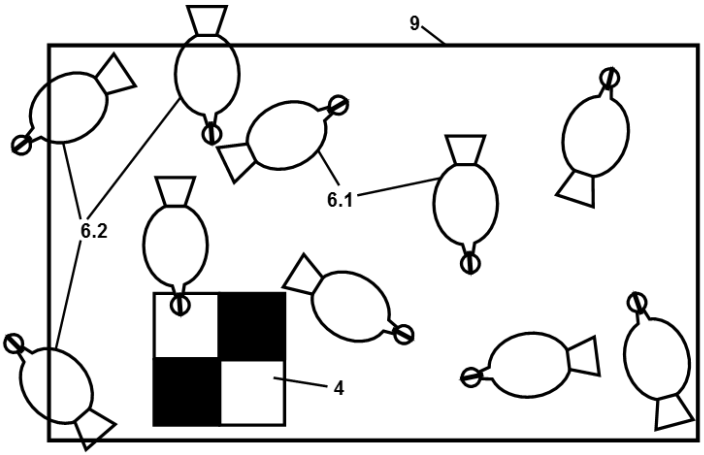

Figure 4. Digital top-view image of the scene with the birds and the length standard: 6.1 are the images of birds wholly in the frame; 6.2 are the birds, which are partially out of the frame; 9 is the framed digital image of the scene.

2) *Filtering the image from noise.*
3) *Calibration the size of the image in units of length.* The true size of scene 9 in units of length (cm) is obtained by counting number of pixels per unit of length of the image of length standard 4.
4) *Digital processing of the framed scene image*. Image 9 (Figure 4) is processed to obtain binary images of individual birds in a single scene (Figures 5 and 6).
5) *Computation of the average bird image area.* The total area of all the bird images A'_{total} in the scene is determined and the number of the images N is counted. The average bird image area $A'_{average}$ for the scene is computed using formula

$$A'_{average} = A'_{total}/N. \quad (1)$$

The images of K consecutive scenes are captured and processed. For each i-th scene the average image area $A'_{average\ i}$ is computed using Equation (1). Then the average image area $A_{average}$ over the whole number of scenes K is computed as

$$A_{average} = \frac{1}{K}\sum_{i=1}^{K} A'_{average\ i} \tag{2}$$

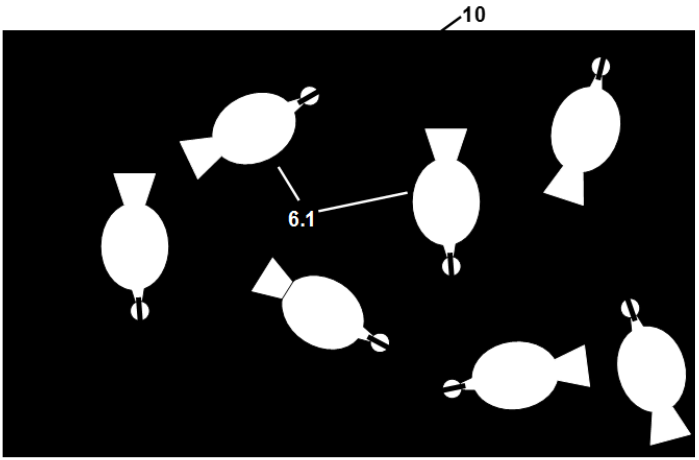

Figure 5. Binarized image 10 of the scene with the birds, which are wholly inside the frame.

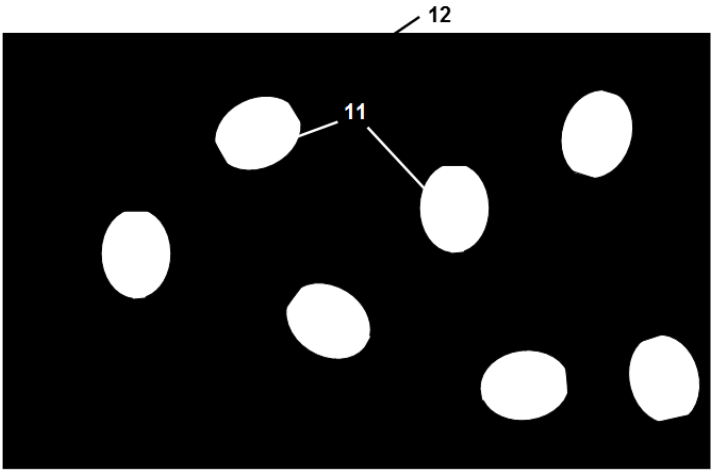

Figure 6. Oblong "blob" images 11 of the birds with optionally removed heads, necks, and tails in scene image 12.

6) *Calibration of the system.* During the period of feeding of one or several prior flocks the average bird image area $A_{average}$ is measured, preferably several times per day, using Steps 1 through 5. Concurrently the actual average bird mass $M'_{average}$ is computed for the same flock as

$$M'_{average} = \frac{1}{L}\sum_{i=1}^{L} M'_j, \tag{3}$$

where M'_j is the actual mass of the j-th bird measured with a conventional scale; L is the number of the weighed birds. The actual average bird mass $M'_{average}$ is plotted versus $A_{average}$ for the same flock obtained during the same measurement period. In case the experimental data points are least-square fit with a straight line, the corresponding linear regression equation has the form

$$M'_{average} \approx FA_{average} + B, \tag{4}$$

where F is the slope of the said fitting line; B is the intercept.

7) *Non-contact measurement of the average mass*. After parameters F and B are established, the average ("non-contact" or "computed") mass of birds $M_{average}$ for the ensuing flocks is computed from their $A_{average}$ using Equation (4).

In more detail, digital processing of image 9 (Step 4) is executed through the following sub-steps:

4.1) *Digital contrast enhancement.* Contrast between the bird images and the background (the floor and the length standard) is enhanced.
4.2) *Binarization.* Scene image 9 in Figure 4 is binarized (turned black and white). The binarization threshold is selected to turn everything in the scene, except the birds, black (Figure 5).
4.3) *Filtering off partial and clustered bird images.* Only bird images 6.1 (Figure 5) that are wholly inside the frame are left in scene image 10.

Additionally, images of bird clusters falling out of the expected range of the areas of single birds are also removed.

4.4) *Optional editing of the binary bird images.* In order to obtain more regular oblong "blobs" representing birds, such details as heads, tails, and legs can be removed from the bird images as shown in Figure 6.

3. EXPERIMENTAL RESULTS AND DISCUSSION

The method was experimentally verified at a commercial poultry farm near Arab, Alabama, USA. The experiment was conducted at two adjacent broiler houses with common feeder (Figure 7). The left house (House A) was populated with 26600 young chicks of both sexes of a single breed and the right house (House B) – with 26845 chicks (both sexes, 1/4 – the same breed as in House A, 3/4 – another breed) on June 10, 2017. By July 26, 893 birds died in House A and 1064 – in House B. The grown birds were removed from House A on July 28 (after 49 days of feeding), and from House B – on July 29 (50 days of feeding). Birds were weighed manually in Houses A and B once in three or four days (measurements in House B started 4 weeks later) at ~ 8 am in the morning. The necessary statistically representative minimal sample size n_{sample} for the average mass measurement was determined using equation (Wikipedia, 2018)

$$n_{sample} = 2(^{SD\%}/_{EM\%})^2, \tag{5}$$

where *SD%* is the relative standard deviation (*SD%* is equal to the flock coefficient of mass variation *CV%*) and *EM%* is the relative error margin. Equation (5) corresponds to 95% confidence. The coefficient of mass variation *CV%* for commercial poultry farms commonly does not exceed 12% (Arbor Acres, 2009). The sample size for relative error margin

EM = ± 5% would thus be n_{sample} ~ 23. In this experiment between 30 and 40 birds were taken for weighing from different locations in the houses thus keeping the sample size greater than the necessary minimum n_{sample}. Best effort was made to take birds of random sizes and different sexes.

During the same period of time the digital top-view images of the house floor mostly occupied by single birds and occasionally by groups of birds were taken manually with a consumer-grade hand-held digital camera using built-in flash light. The elevation of the camera over the house floor was kept between 0.5 and 1.5 m. A blue-color checker board (2 x 2-cm cell size) was used as a length standard. Blue color was chosen to simplify digital color-based filtering of the checker board out from the scene image in Step 1 of the method using conventional image processing software, such as Adobe Photoshop. Digital color filtering was tuned up to let the bird images with primarily yellow-orange color pass through. The captured and filtered images were stored in PC computer and digitally processed according to Steps 4 and 5 using the commercial Digimizer software package (Digimizer, 2018). The algorithm of image processing and computing image area is schematically presented by the flow chart in Figure 8. The algorithm starts from opening scene image 9 (Figure 4) stored in the computer memory. After opening the image and turning it gray stretching its brightness histogram is performed (Figure 9a). Histogram stretching helps to enhance the contrast between the bird images and background. Binarization threshold is automatically computed at the median brightness level (vertical line in the middle of the stretched brightness histogram in Figure 9b). For the sake of simplicity, the heads, necks, tails, and legs were not cut off from the images. The "good" images appropriate for further computing were selected to be those, which did not extend beyond image frame 9 (Figure 4) and corresponded to isolated single birds only.

Figure 7. View of the commercial poultry farm near Arab, Alabama, US used in the experiment. Left poultry house is House A, right – House B.

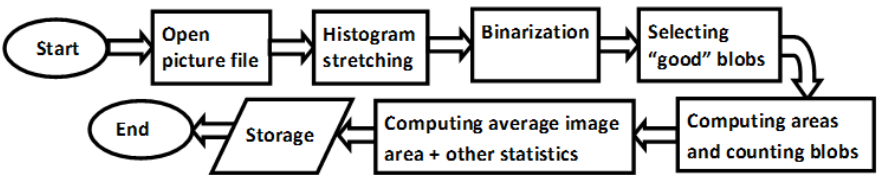

Figure 8. Flow chart of the algorithm of automatic computing the average image area of birds.

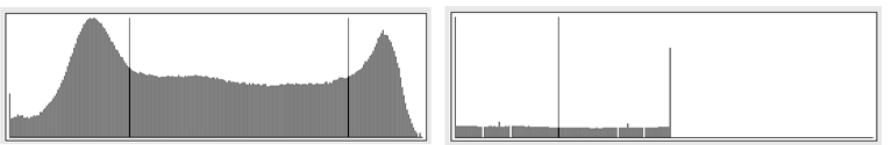

Figure 9. Typical brightness histogram of the scene image (a) before and (b) after stretching. Horizontal axis is the brightness (scaled from 0 to 255); vertical axis is the frequency of occurrence in arbitrary units. Vertical lines in (a) mark the margins of histogram stretching; all the image elements with brightness beyond the margins are turned either black (to the left from the left margin) or white (to the right from the right margin). Vertical median of histogram (b) marks the threshold of binarization: all the elements of the image with brightness to the left from median are turned black, to the right – white.

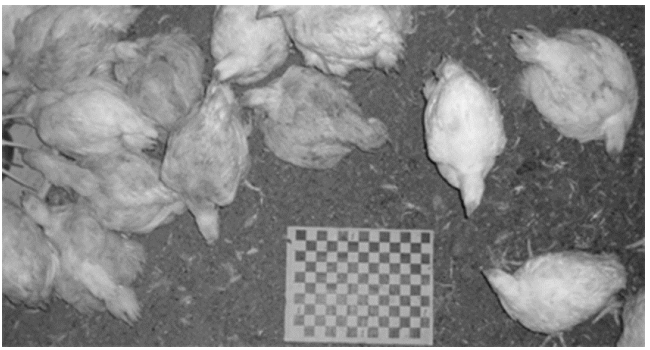

Figure 10. Typical picture with the birds flocking (to the left) and standing alone (to the right).

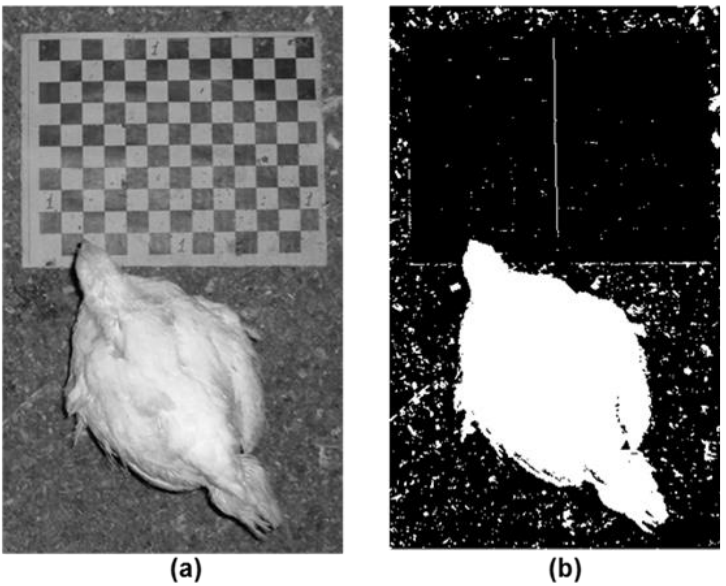

Figure 11. (a) Typical initial image of a single bird (33-day broiler in House A) and (b) its digitally edited and binarized version. The binarized image has an area of 322.25 cm^2.

Thresholds to the maximum and minimum image area were set (based on the preliminary measurements) to reject any "unusual" images created by small objects, such as shed feathers, or the birds flocking in clusters (Figure 10). Typical binary image of an isolated single bird is presented in Figure 11.

Furthermore, the Digimizer program was used to compute the image areas of individual birds, count the birds, and determine the average bird image areas (according to Equations (1) and (2)) and other statistical parameters (standard deviation, etc.). The standard deviation *SD%* for the bird average image area was experimentally determined not to exceed 14%. Using Equation (5), the sample size was determined as n_{sample} = 32 (for 95% confidence and ± 5% error margin). From 30 to 80 digital images of the randomly picked birds were used to compute the average bird image area of each flock each time (Step 5 of the method). This number of the images was thus of the order or exceeded the minimal statistically representative sample size. The data on the manually measured actual mass of birds were used to obtain the bird average mass according to Equation (3). The actual average bird mass was plotted versus the average bird image area for the entire period of feeding (Figure 12).

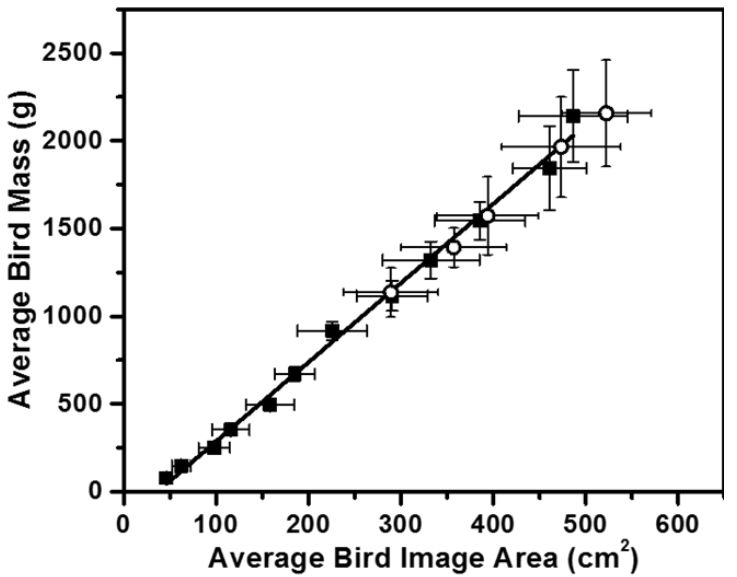

Figure 12. Calibration plot. The average actual mass of birds in House A (solid squares) and House B (open circles) plotted versus the average bird image area for the period of feeding from June 10 to July 28-29, 2017. Solid line is the linear fit of the mass vs area plot for House A. The linear fit slope F = 4.51 ± 0.11, intercept B = -162.69 ± 29.70, and the correlation factor r^2 = 0.9939.

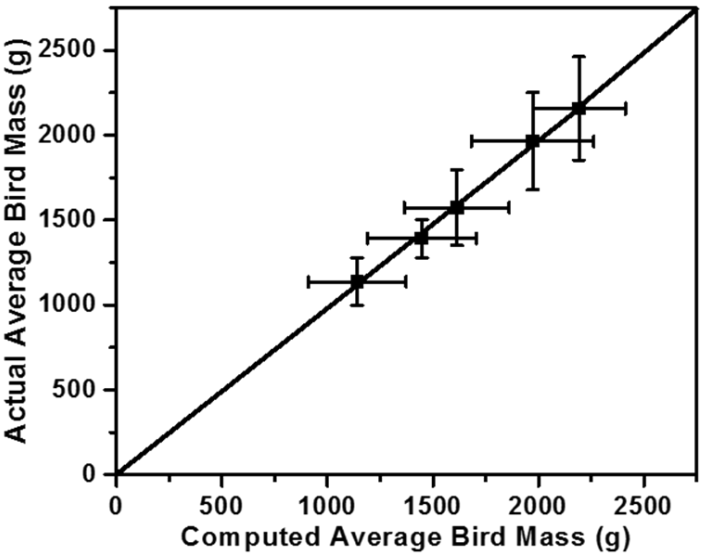

Figure 13. The actual average bird mass in House B plotted versus the average mass computed by plugging the average bird image area in calibration Equation (4) with the slope F and intercept B derived from the data collected in House A (Figure 12). Solid line is the linear fit with the slope 0.986 ± 0.006 and the correlation coefficient r^2 = 0.99982.

The average mass of the birds turned out to be a linear function of the average image area (solid line in Figure 12) described with good accuracy by linear regression Equation (4). This is a remarkable departure from the spherical objects (Figure 1) with mass M (proportional to volume V) being nonlinear function of image area A ($M = CA^{3/2}$, see Figure 2). It could be attributed to the distinctive features of the bird's body. The feather coat, contributes greatly to the bird image area, but little to the mass. As a result, the bird mass growth at a slower rate than the image area as compared to spherical objects. The linear regression analysis was applied first to the data obtained in House A (solid straight line in Figure 12). The parameters F and B of calibration Equation (4) for the flock in House A were obtained with the standard deviations for F and B not exceeding 2% and 18% respectively. The correlation coefficient r^2 was close to one proving the validity of linear fitting and the calibration equation. In order to verify if this calibration equation with parameters F and B defined for the flock in

House A could predict the mass of another flock, it was used to compute the average mass of the birds in House B based on their average image area. The actual average mass of flock B was plotted versus the average mass computed form the average image area using the calibration equation for flock A (Figure 13). The actual mass correlated well with the computed mass: the slope of the linear fit was almost 1.0, and r^2 was close to 1.0. And this was despite the fact that the calibration equation was derived for the flock grown in different house and with one breed instead of a mixture of two. This result proves the validity of the proposed approach.

Fully automatic implementation of the method would require placing several image sensors in different locations throughout a poultry house for taking the images of at least 32 (the sample size) birds in one cycle. If the young chicks will be initially placed in one-half or one-third brooder section of the house, the reduced number of the cameras covering the area will be compensated by the increased number of small-size young chicks falling in the camera's field of view. Taking into account a standard house stocking density of 0.065 m^2/bird (Arbor Acres, 2009), a camera with an area of the field of view of 1 m x m will thus be able to capture the images of ~ 15 isolated birds in a single shot (assuming their even distribution). Three or four cameras will thus would be sufficient to meet the sample size requirement.

CONCLUSION

It has been shown that:

- The proposed method of non-contact monitoring of the mass of birds using the top-view images is feasible in commercial poultry house environment.
- The average mass of birds is a linear function of the average area of the visible top-view images. The linear fitting error of the slope is of the order of 2%.

- The fact that the average bird mass is linearly proportional instead of being proportional to the 3/2-th power of the average image area (expected for oblong objects) can be explained by significant contribution of the relatively light feather coat to the image area specific for bird bodies.
- From 30 to 80 digital images of the randomly picked birds are sufficient to compute the average bird image area for each flock each time and establish the linear calibration plot of the average mass versus the average image area.
- Bird's body parts such as heads, necks, tails, and legs can be left in the final binarized bird image used to compute the average image area. They do not bring a substantial increase of the measurement error.
- Once the calibration of the non-contact measurements of bird mass is conducted for one flock, it can be used to determine the average masses for other flocks in the same or other houses. Correlation between the actual average bird mass and the mass obtained using the proposed non-contact method was proven to be strong: the linear regression fit had a slope of 0.986 ± 0.006, and a correlation coefficient of 0.99982.

Further advancement of the method can be based on usage of the deep learning algorithm (LeCun, 2015) to improve the selection of the most suitable bird images through the following sub-steps:

4.1') Training a deep learning computer software/hardware (such as the Convolutional Neural Network – CNN or the Caffe Deep Learning Framework) using the collection of the stored most suitable images of the birds.

4.2') Using the deep learning algorithm to recognize and select the suitable bird images from the scene.

The unique feature of the proposed method is that it uses the images of relatively large number of birds: from tens to hundreds. The advantage of

the method with regard to previous attempts is that it makes possible to measure promptly the statistically meaningful average bird mass for the entire flock. The average mass can be monitored continuously and used to control the feed conversion ratio (FCR) and other meat production parameters for the entire flock on daily basis. The method uses a new concept of the average top-view image area as a measure of the average bird mass for a flock. The proposed approach has a potential to provide the poultry industry with long awaited bird weighing system that is inexpensive (~ $1K or less per house), non-contact, and maintenance free. It uses no weighing equipment on the floor that restricts free motion of birds and distorts measurement statistics.

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Chapter 7

SALACCA EDULIS: NUTRITIONAL COMPOSITION, BIOACTIVE COMPOUNDS AND HEALTH BENEFITS

Ola Lasekan*, Rafiat Shittu, Li Shing Teoh, Mee Gie Lin and Megala Muniandy
Department of Food Technology, University Putra Malaysia, UPM, Malaysia

ABSTRACT

Salacca edulis is known to possess flavonoids, flavanol, tannin, anthocyanin, ascorbic acid, alkaloid and antioxidants. This may make it important in disease prevention. The fruit extracts have high levels of phenolic compounds with anti-proliferative and antioxidant properties, including chlorogenic acid, (-)-epicatechin, singly-linked pro-anthocyanidins which exist as dimers through hexamers of catechin or epicatechin. General inspection of the literature suggests that *S. edulis* has the ability to diminish the generation of fibrinogen which could

* Corresponding Author Email: lasekan@upm.edu.my; olaniny56@gmail.com.

subsequently reduce the risk of coronary atherosclerosis. The fruit has also been accredited with the ability to inhibit proliferation and induce selective cytotoxicity and apoptosis in cancer cells. In another study *S. edulis* has been shown to possess strong anti-hyperuricemic capacity. However, the long-term impact of *S. edulis* intake on specific populations and their functionality claims has not been fully evaluated. Although several anti-proliferative effects which are based on epidemiological studies have been explained, the mechanism of their actions is not fully understood.

Keywords: *Salacca edulis*, palm tree, antioxidant, phytochemicals

INTRODUCTION

"Salacca edulis" also known as 'salak' or 'snake fruit' due to its reddish-brown scaly skin is an exotic tropical fruit which originates from Southeast Asia [1, 2]. The snake fruit is referred to as 'rakum' and or 'sala' in Thailand and in Malaysia and Indonesia as 'salak'. The fruit is highly rich in polyphenols and bioactive antioxidants such as vitamin C, pro-vitamin-A carotenoids and phenolic compounds with nutraceutical and functional food additive applications [3-8]. These dietary antioxidants are powerful phytochemicals associated with different biological activities. They prevent chronic degenerative diseases such as neurodegenerative illnesses, cancers, coronary atherosclerosis and cardiovascular diseases [9]. The antioxidant potential of snake fruit have been shown to positively affect plasma lipid levels and plasma antioxidant activity through in-vivo and in-vitro experiments in rats fed cholesterol-containing diets [3, 4]. Previous studies showed that the high level of bioactive antioxidants and anti-proliferative activities of snake fruit extract significantly correlated with phenolic compounds such as pro-anthocyanidins, chlorogenic acid and (-)-epicatechin [6]. The antioxidant extracts of the fruit could be used as a functional food ingredients or dietary supplements in the food industry, nutraceutical, pharmaceutical and cosmetic industries [10, 11]. The mature ripe snake fruit has a sweet to sub-acidic taste but the immature tastes astringent and sour. Nonetheless, the fruit has a short shelf

life due to its rapid ripening. It is commonly consumed fresh and can also be processed into various food products such as juice, wine, candies, jam, dried fruit, pickles canned in syrup and chips [12-17]. The young 'salak' fruit is used to make a type of salad called 'rujak' in Indonesia.

In this report, a brief review of the botanical, traditional application and current state of scientific knowledge in the areas of nutritional composition, bioactive constituents and potential health benefits of snake fruit are outlined.

BOTANICAL DESCRIPTION OF *SALACCA EDULIS*

Salacca edulis is a palm tree that belongs to the Arecaceae family that is about 1.5 - 5 m tall (Figure 1). The trees are short-stemmed, highly spiny and do not form a trunk. *S edulis* grows on a moist well-drained soil with high organic matter content [18]. The palm tree can be productive for 50 years or more [19]. They are widely grown as under-story palm in the low lands of tropical rain forests in Indonesia, Malaysia, and other Southeast Asian countries [16]. The leaves are pinnate which can reach up to 10 m long and 1.5 m wide with each leaf having a 2 m long petiole and numerous leaflets measuring about 20-89 cm long and 2-11 cm wide (Figure 1). The upper surface of the leaflets is dark green and shiny while the lower part is light green. The snake fruit has an oval shape with elongated end, scaly dark brown rind having an appearance similar to that of snake's skin from which the name snake fruit is derived. The white to creamy flesh consists of several segments similar to that of a large peeled garlic clove and a large dark brown inedible seed at the center. The fruit grows in clusters of about 15-40 fruits at the base of the palm (Figure 2). The palm is dioecious. The male inflorescences are packed closely in finger-like spadices of 50-100 cm in length, occurring in bunches of about 4-12 spadices. The female inflorescences are 20-30 cm long and are composed of 1-3 spadices [16, 19, 20]. In Indonesia, there are about 30 cultivars of snake fruit; however, three cultivars ('*Salak pondoh,*' '*Salak bali'* and '*Salak gular pasir'*) are most frequently obtained. Nonetheless,

the first two cultivars are the most common commercial fruit. '*S. pondoh,'* a native of Yogyakarta province on the island of Java, Indonesia is known to reveal superior nutritional and sensory quality due to its sweet flavor and intense aroma which is devoid of bitter or sour components as compared to other cultivars [21]. On the other hand, *S. bali* which is commonly found on the island of Bali, Indonesia is moist and crunchy in texture with a starchy mouth feel and a flavor reminiscent of lemon and pineapple [22].

Figure 1. Appearance of the overall plant of *Salacca edulis* showing the spiny leaf petioles.

(A) (B)

Figure 2. Matured *Salacca edulis* fruits (A), and clusters of immature fruits growing at the base of the palm (B).

PRODUCTION AND ECONOMIC VALUE

Snake fruit has received much attention as an exotic and prominent fruit with excellent potential for the export and domestic market. Its demand per annum is around 420 000 tons, including fresh consumption, processed fruit and export. In recent times, the demand for the fruit in Japan, China, United States, and Europe has increased. The popularity of the salak fruit has significantly increased since *S. pondoh* was discovered in 1980s and became a commercial fruit. This particular fruit is produced all year round in Indonesia. However, it's produced from June to August in Malaysia and Thailand. The *Salacca edulis* palm has been cultivated throughout the islands in Indonesia and the fruit is commonly used as fresh fruit. About 60-70% of the world's salak fruit production is from Indonesia which is equal to 334 000 tons and the country exports about 32 755 tons per annum [23]. The major commercial production areas cover the provinces of central Java, Yogyakarta, and Bali. According to the Indonesia Agricultural Quarantine Agency (IAQA) [24], the national production area for *Salacca edulis* in 2011 was 24,728 hectares with an annual production value of over one million tons. According to the Indonesia ministry of agriculture [25], snake fruit production in Indonesia increased from 423.5 tons in year 2000 to 862.5 tons in 2009. Fresh Indonesia snake fruits have been exported to United Kingdom, Singapore, Thailand, Saudi Arabia, and Hong Kong at the rate of 4-10 tons per week [26]. Bakar and Idris [27] and the Malaysian department of agriculture [28], reported steady increases in the hectares of salak fruit plantation from 561 hectares in 1995 to 1409 hectares in 2010.

S. pondoh from the Yogyakarta region of Indonesia is known to reveal superior sensory quality due to its intense sweet flavor. It is regarded as a promising and potential export fruit commodity. In Indonesia, there are about 30 cultivars of salak fruit, which are often distinguished by their place of origin, fruit texture, taste, or color [20, 29, 30]. In Thailand, salak fruit is grown in the southern and eastern parts of the country and the major commercial species cultivated are '*S. rumphii'* and '*S. wallichiana,'* which

are commonly referred to as ‘sala’ and ‘rakam’ respectively by the people [13, 31]. Whereas in Malaysia, snake fruit is cultivated mainly in three states which are Terengganu, Sabah, and Kelantan. The demand for the fruit has been increasing both locally and internationally. There are three major species of snake fruit grown commercially in Malaysia [27].

NUTRITIONAL COMPOUNDS

Snake fruit has a high amount of basic nutritional compounds [32]. It is an excellent source of the basic nutritional compounds such as dietary fibers, crude proteins, crude fats, and carbohydrates [32]. It is a good source of minerals such as sodium, potassium, calcium, zinc, magnesium, manganese, iron, sulfur, boron, and copper [12, 32, 34]. It also contains small amount of water soluble pectin. Glucose, sucrose, and fructose are the major sugar found in the snake fruit [2]. Salak has moderate ascorbic acid content (about 0.73–2.4 mg 100 g −1) [5, 12].

Table 1 showed the main minerals and some nutritional compounds of Snake Sumalee and Snake Noen Wong. The potassium/magnesium ratio for snake fruit was 18.68 to 21.10 while the magnesium/calcium ratio was from 2.76 to 1.98. Leontowicz et al. [3] have shown that the consumption of snake fruit could help in diminishing the generation of fibrinogen and compositions of electrophoretic protein bands in the range of 110 and 14 kDa; thus reducing the potential risk of coronary atherosclerosis and blood coagulation. The high content of polyphenols in snake fruit such as garlic acid and flavonols has been shown to inhibit proliferation and induce selective cytotoxicity and apoptosis in cancer cells [33]. The cytotoxic assay was performed using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethylphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) method in MCF and T47D cell lines to determine the IC_{50} of the β-sitosterol from snake fruit extract.

Table 1. Nutritional compounds and mineral content of *Salacca edulis*

Indices	Snake Sumalee	Snake Noen Wong
Total dietary fiber	71.3 ± 0.4a	69.8 ± 0.3b
Crude lipids	41.25 ± 0.4a	39.18 ± 0.3b
Crude protein	33.17 ± 0.2a	30.91 ± 0.2a
Carbohydrate	711.32 ± 0.6a	684.15 ± 0.5b
Water soluble pectin	13.91 ± 0.6a	10.12 ± 0.5b
Potassium	1161 ± 51a	896 ± 45b
Calcium	220 ± 9a	287 ± 12a
Magnesium	607 ± 31a	567 ± 25a
Sodium	231 ± 11a	220 ± 11a
Iron	12.0 ± 0.5a	12.9 ± 0.6a
Zinc	10.4 ± 0.3a	11.4 ± 0.4a
Copper	3.36 ± 0.2a	1.48 ± 0.1b

Values are mean ± SD of five measurements. Means in rows with different superscript letters are significantly different ($p < 0.05$).

Table 2. Effect of β-sitosterol in various concentrations on the number of cells in breast cancer cell lines

Samples (Concentrations) of β-sitosterol	Type of breast cancer	
	MCF7	T47F
FBS (β-sitosterol 0)	5968 ± 184e	5433 ± 338e
DMSO (β-sitosterol 0)	6161 ± 325e	5680 ± 379e
Starving (β-sitosterol 0)	5841 ± 279e	5311 ± 401e
β-sitosterol 100 µg/ml	1070 ± 40a	0.00 ± 0.00a
β-sitosterol 50 µg/ml	3894 ± 179b	0.00 ± 0.00a
β-sitosterol 25 µg/ml	4385 ± 103c	559 ± 133b
β-sitosterol 12.5 µg/ml	4562 ± 258c	1126 ± 40c
β-sitosterol 6.25 µg/ml	4925 ± 163d	1375 ± 86c
β-sitosterol 3.125 µg/ml	5100 ± 220d	2406 ± 43d

Table 2 showed the effect of β-sitosterol in various concentrations on the number of cells in breast cancer cell lines. From Table 2, concentration of β-sitosterol had a negative correlation on the number of both MCF7 and

T47D cancer cell viability. This means that higher β-sitosterol concentration led to stronger anticancer activity of the β-sitosterol. According to Afrianti [33], snake fruit is rich in terpenoids which act as cytotoxic agents that could trigger apoptosis through two signaling mechanisms, the activation and release of mitochondrial pro-apoptotic proteins known as caspases. Through his study, cytotoxic assay proved that proliferation and viability of MCF7-breast cancer line- (IC_{50} = 45.414 µg/ml) and T47D- breast cancer stem cell line- (IC_{50} = 1.1942 µg/ml) was inhibited in snake fruit extract [33].

BIOACTIVE COMPOUNDS

Salak fruit is an excellent source of natural antioxidants. The high level of antioxidant and anti-proliferative activities of 'salak' fruit extract were significantly correlated with phenolic compounds. The antioxidants in snake fruit were found to be (-)-epicatechin, chlorogenic acid and singly linked pro-anthocyanidins which existed mainly as dimers through hexamers of catechin or epicatechin [6]. The total polyphenols measured as mg of GAE/100 g of fresh weight and flavonoids as mg of CE/100 g of fresh fruit in snake fruit was found to be 217.1 and 61.2 respectively [35]. Similarly, Leong and Shui [5] found the L-ascorbic acid equivalent antioxidant capacity (AEAC) calculated on dry weight (mg/100g DW) for snake fruit to be 260. Antioxidant activity in µM TE/100 g of fresh snake fruit as measured by 2, 2-diphenyl-1-picrylhydrazyl hydrate (DPPH) and 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assays was 110.4 and 1507.5 respectively. In another study by Aralas et al. [12], it was revealed that the total phenolic and flavonoid contents of the snake fruit were in the range of 12.6-15.0 mg Gallic acid equivalent/g and 4.9-7.1 mg catechin equivalent/g of dry sample, respectively. The antioxidant activities of the extracts using DPPH assay were highly correlated with total phenolic and moderately correlated with flavonoid content. The reducing capabilities of the extracts using FRAP assay were moderately correlated with all phytochemicals tested. The results suggested that the

phytochemicals and antioxidant activity of salak was mildly affected by variety. The high content of phytochemicals and antioxidant properties of snake fruit indicated that it possessed potential health benefits.

In comparison with mangosteen which is also rich in bioactive compound and less investigated, the snake fruit has a significantly higher polyphenol contents (14.9 ± 1.5 and 9.2 ± 0.8 mg GAEg g^{-1}) and antioxidant capacity (46.7 ± 4.7 and 72.9 ± 7.4 μmol Trolox equivalent g^{-1}) than mangosteen [3, 4, 9]. The positive influence was higher in rats fed diet with supplemented snake fruit. Nonetheless, the positive results of this experiment on laboratory animals cannot be automatically applied to humans.

Clinical and epidemiological studies have consistently linked abundant consumption of fruits and vegetables to a decrease in the risk of developing several kinds of cancer. A study by Gorinstein et al. [32] compared a less known snake fruit with better-known kiwi fruit by means of fluorometry (FL), Fourier transform infrared (FTIR) spectroscopy, various radical scavenging and proliferative assay. It was revealed that there was similarity between snake fruit of Sumalee cultivar and kiwifruit of Hayward cultivar in terms of their contents of total polyphenols (8.15–7.91, mg GAE g^{-1} DW), antioxidant capacity by DPPH (11.28–10.24, μM Trolox equivalent g^{-1} DW), and anti-proliferative activities on both human cancer cell lines (SMU-601 for human gastric carcinoma and Calu-6 for human pulmonary carcinoma, 89.3–87.1%, and 90.5–87.6 cell survival, respectively). FTIR and 3D-FL can be used as additional tools for identification and comparison of bioactive compounds.

Priyatno et al. [1] studied the anti-hyperuricemic activity of snake fruit (cv. Bangkok) ethanol extract on Wister male rats. As shown in Figure 3, the ethanol extract at doses of 200 mg/kg body weight decreased serum uric acid level significantly ($p < 0.05$) compared to control group (CMC-Na 0.5%) at 6 and 7 h respectively after inducing with potassium oxonate intra-peritoneally simultaneously with uric acid orally. Meanwhile, ethanol extract administration at doses of 100 mg/kg body weight did not decrease serum uric acid level significantly ($p > 0.05$) compared to control group at 6 and 7 h. Besides, probenecid at a dose of 45 mg/kg body weight had

increased excretion of urine uric acid level. Hence, the mechanism of action of the snake fruit ethanol extract as an anti-hyperuricemia is suggested to bring about the inhibition of xanthine oxidase (XO) and subsequently decreased the synthesis of uric acid and increased the excretion of urine uric acid level. XO inhibitors could be used as potential therapeutic agents for treating hyperuricemia as they can be used to block the biosynthesis of uric acid [35]. Similarly, in another study by Priyatno, et al. [38], the XO inhibitor activity of two bioactive compounds; 3ß-hydroxy-sitosterol and 2-methylester-1-H-pyrrole-4-carboxilyc acid from ethyl acetate extract of snake fruit pulp that could help in the management of gout was elucidated. Inhibition of XO by the two compounds was investigated by the authors and 3ß-hydroxy-sitosterol was found inactive whereas the other compound i.e., 2-methylester-1-H-pyrrole-4-carboxilyc acid was found to be active with an IC_{50} value of 48.86 µg/mL.

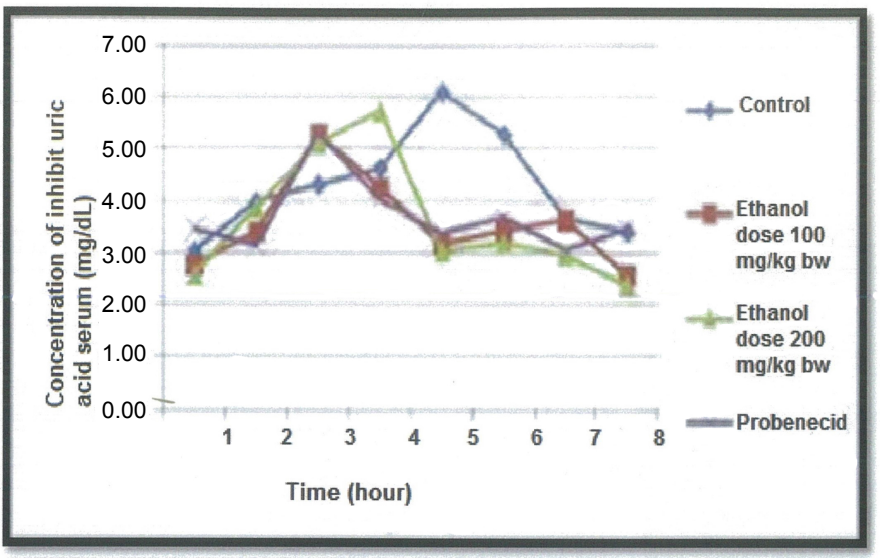
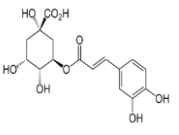


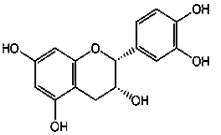
Figure 3. Anti-hyperuricemic Effect of Ethanol Extract of Snake Fruit (*Salacca edulis* Reinw.) var. Bongkok on Wistar Male Rat. Priyatno et al. [1].

Phytochemical Composition of *Salacca edulis*

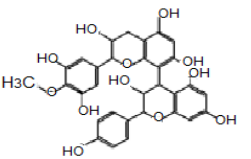
Earlier investigation of the phytochemical composition of snake fruit pulp showed the presence of a wide variety of bioactive compounds and high antioxidant potentials. A number of chemical compounds such as flavonoid, flavanol, tannin, anthocyanin, ascorbic acid, alkaloid, terpenoid, pyrrole, tannin, phenolic acid, carotenoid and quinone compounds have been isolated from the pulp of this fruit [9, 36, 37]. The contents of total polyphenols in GAE g^{-1} DW were in the range of 2.58 to 8.46 in water extracts of snake fruit and the DPPH antioxidant capacity values were in the range of 6.35 to 9.43 µMTE/g [9]. The chemical structures of some of the compounds found in the snake fruit are shown in Figure 4.



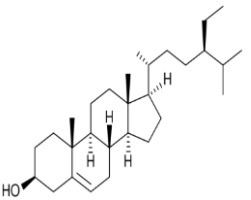
1. Chlorogenic acid



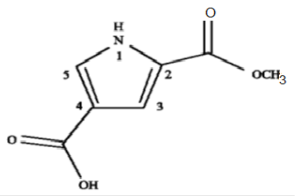
2. Epicatechin



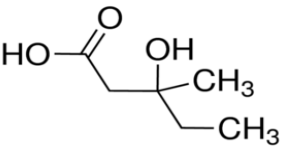
3. Pro-anthocyanidin



4. 3β-Hydroxy-sitosterol



5. Pyrolle-2,4-dicarboxylic acid methyl ester



6. Methyl 3-hydroxy-3-methylpentanoate

Figure 4. Chemical structure of some compounds isolated from the fruit pulp of *Salacca edulis.*

In a study conducted by Shui and Leong [6], the antioxidants isolated from salak fruit pulp were; (-)-epicatechin, chlorogenic acid, and singly linked pro-anthocyanidins which existed as dimers through hexamers of catechin or epicatechin. The chlorogenic acid was a slow reaction type antioxidant due to its slow reaction with free radicals pro-anthocyanidins or (-)-epicatechin. The snake fruit has an excellent free radical scavenger that showed a high correlation with flavonoids and total phenolic compounds [32]. The compounds of 3-hydroxystigmastan-5(6)-en (β-sitosterol) and pyrolle-2.4-dicarboxylic acid-methyl ester were isolated from snake fruit (cv. Bangkok) ethyl acetate extract [36].

CONCLUSION

Salacca edulis is an exotic tropical fruit and an excellent source of natural antioxidants. The rich phytochemicals and high antioxidant properties of the fruit indicates that it possess potential health benefits. The seed is considered as inedible and it is usually discarded after the consumption of its pulp. Supplementation of diets with exotic fruits such as snake fruit has been shown to positively affect plasma lipid profile and antioxidant activity in rats fed cholesterol-containing diets. Nonetheless, medical studies on human that could provide proof of the efficiency and effectiveness of the snake fruit are still very limited. Understanding the chemo-preventive activities of snake fruit can stimulate an interest in maximizing their utilization as a functional food in human diet. Although several anti-proliferative effects which are based on epidemiological studies have been explained, the mechanism of their actions is not fully understood. A better knowledge of some variables such as safety, efficacy, and interactions with other dietary components, must be stated.

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Chapter 8

ACID TOLERANCE OF NATIVE AND COMMERCIAL BRADYRHIZOBIA: THE EFFECT OF LIMING ACID SOILS ON GROWTH AND THE NODULATION OF SOYBEANS

Macharia Mary Wangui, Ezekiel Mugendi Njeru* and John Maingi
Department of Microbiology, Kenyatta University, Nairobi, Kenya

ABSTRACT

Within the rhizospheric soil, rhizobia frequently encounter various abiotic stresses that affect their growth, initial steps of symbiosis with legumes, and their capacity of nitrogen fixation. In sub-Saharan Africa (SSA), acidic soils that characterize many agroecosystems are a major impediment to biological nitrogen fixation. Moreover, this is exacerbated

* Corresponding author Email: njeruezek@gmail.com.

by poor soil health management practices and lack of soil testing services by resource limited smallholder farmers. Consequently, this has led to unabated poor crop production including soybean, which is an important cheap source of proteins to both man and livestock. Therefore, identification of most symbiotically effective and acid tolerant native bradyrhizobia isolates would lead to development of more efficient and low-cost inocula that are widely adopted by smallholder farmers in SSA. To realize this purpose, four native *Bradyrhizobium* isolates (NRI1, NRI2, NRI3, and NRI4) obtained from field trap cultures set in acidic soils of Embu and Tharaka Nithi County, Kenya and a commercial inoculant (USDA 110), were examined for acid tolerance on yeast extract mannitol agar at pH levels of 3, 5, 7 and 9. Moreover, the isolates were further examined for nitrogen fixation potential in greenhouse bioassays using limed and non-limed field soil. The experiment was set in a complete randomized design with liming as the main factor, inoculated soybean as the sub factor and replicated three times. After 28 days, the crop was harvested and assessed for nodulation, shoot and root dry weight. Interestingly, most native isolates tolerated pH of 5 while the commercial inoculant tolerated pH level of 9. All the isolates were sensitive to pH of 3. Soybean inoculated with the commercial bradyrhizobia showed significantly higher ($p<0.001$) shoot dry weight, nodule number and dry weight, compared to those inoculated with the native bradyrhizobia. Besides, liming of soil enhanced soybean nodulation and growth across all the bradyrhizobia inoculants. This study forms an important step towards the use of most effective rhizobial inoculants which are well adapted to different local agro-climatic conditions.

Keywords; pH**,** soybean, bradyrhizobia, nodulation, symbiotic nitrogen fixation, Kenya

1. INTRODUCTION

Globally, over half of the population currently lives in regions characterized by acidic soils, P fixation and aluminum toxicity (Yang et al., 2004) whose productivity is on the decline to meet the food requirements of the ever increasing population. This is especially evident in the tropics (Fageria and Baligar 2008). Soil acidity is a major yield-limiting factor for crop production worldwide. The land area affected by acidity is estimated

at 4 billion hectare, representing approximately over 50% of the world's total arable land (Zheng, 2010). In Kenya, acid soils cover about 35% of the total land area and are widely distributed in Central and Western Kenya regions. They cover over one million hectares under, legume, tea and coffee crops, grown by over 5 million smallholder farmers (Gudu et al., 2007). Liming is one of the most important and effective practice to ameliorate soil acidity constraints for optimal crop production (Ngabonziza, 2014).

Soybean (*Glycine max* L. Merrill) is a major source of food rich in protein, oil and carbohydrates and it forms part of balanced diet of farmers from Sub-Saharan Africa. It also helps in maintaining soil fertility via biological nitrogen fixation (BNF) with bradyrhizobia (Njeru et al., 2013). Soybean depends on their symbionts for a large part of their nitrogen requirement, for growth and increased dry matter production (Mugendi et al., 2010). Many biotic and abiotic factors affect the growth and persistence of symbiotically effective rhizobia strains in soil. Soil acidity is one of the factors restricting soybean production through its deleterious effects on ecologically important bradyrhizobia.

Soil acidity is a major limitation to symbiotic nitrogen fixation since it negatively affects rhizobia survival, persistence in soil and nodulation (Ibekwe et al., 1997). Nodulation failure under acid soil conditions is common especially in soils with pH level of less than 5. To augment BNF, rhizobia inoculation is a common practice in agricultural legume production (Catroux et al., 2001, Koskey et al, 2017) which requires survival and establishment of inoculated rhizobia in soil environments (Ozawa et al., 1999; Gicharu et al., 2013). However, a wide variation in the tolerance to acidic soil conditions has been reported among rhizobia strains isolated from diverse agriculturally important legumes (Appunu et al., 2005; Raza et al., 2001). Contemporary agriculture requires the implementation of efficient, sustainable and environmentally sound management practices (Fageria and Baligar, 2008). Liming is important in achieving optimum yield of all crops grown on acid soils since it neutralizes soil acidity and is a long term method of soil acidity amelioration (Kaitibie et al., 2002).

Here, we tested the hypotheses that varying the pH level influences growth of native and commercial bradyrhizobia inocula (USDA 110) *in vitro,* and addition of agricultural lime enhances nodulation and dry weight of soybean. The specific objectives of the study were:- 1) to determine acid tolerance of both native and commercial bradyrhizobia isolates *in vitro*, 2) to determine effects of agricultural lime in enhancing soybean nodulation by both native and commercial bradyrhizobia, 3) to evaluate effects of liming on shoot and root dry weight of soybeans.

2. MATERIALS AND METHODS

2.1. Bradyrhizobial Isolates Acid Tolerance

Four native bradyrhizobial isolates (NRI1, NRI2, NRI3, NRI4), and commercial bradyrhizobia (USDA110) were tested for pH tolerance *in vitro*. The native isolates were obtained from nodules of two promiscuous soybean varieties (SB8 and SB126) planted in four smallholder farmers' fields with no inoculation history. The trap culture experiments were carried out during long rain season (March to July 2015), in Embu (0.53° S, 37.45° E) and Tharaka Nithi (0.30° S, 38.06° E) counties of Kenya. Yeast extract mannitol agar was sterilized at 121ºC for 15 minutes. The pH level of the media was adjusted to obtain required values of 3, 5, 7 and 9 by using HCl or NaCl. The isolates were then streaked on the solidified agar, incubated at 25^0C and growth monitored for 5 days. Acid tolerance of bradyrhizobia was determined by checking growth of the isolates on the growth media.

2.2. Greenhouse Bioassay

Certified soybean seeds obtained from Kenya Seed Company Limited, Nairobi Kenya, were surface sterilized in 95% alcohol for 10 seconds followed by 3% sodium hypochlorite for 3 minutes, then rinsed with 6

changes of distilled water. They were later planted in pots containing sterilized limed soil (pH 6.5) and non limed soil under greenhouse conditions. The experimental soil obtained from Embu and Tharaka Nithi farms was sterilized by autoclaving at 72^{o}C for 12 hours before being put in clean sterile pots. The soil had the following physico-chemical characteristics; loamy, pH 5, N 0.37%, available phosphorus 23.00 ppm, exchangeable potassium (K^{+}) 0.95 cmol.kg^{-1} and organic carbon 2.96%. In each pot, one healthy soybean plant was maintained. Inoculation was done 5 days after germination with the native rhizobia isolates or the commercial isolate USDA110. Each plant was supplied with an exact quantity of 1 ml of broth having 10^{9} bradyrhizobial cells using a micro-pippete. The control pots were left untreated. The experiment was replicated thrice and laid out in a completely randomized design with liming as the main factor and bradyrhizobia inoculation as the sub factor. Plants were supplied with water twice a week and rotated regularly.

2.3. Plant Harvesting and Analyses

Plants were harvested after 4 weeks and washed gently to remove soil from the rooting system. The nodules were detached gently, counted and their fresh and dry weights recorded. The roots were separated from the shoot and their dry weight was recorded in mg^{-1} after oven drying at 65^{o}C for 24 hours.

2.4. Statistical Analyses

Data was tested for homogeneity of variance by Bartlett test. Data on *in vitro* acid tolerance, root dry weight, shoot dry weight, and nodulation was subjected to ANOVA using SPSS version 20 computer program. Wherever feasible, means were separated by Tukey's Honest Significance Difference (HSD) at $p < 0.05$.

3. RESULTS

3.1. Acid Tolerance of Native and Commercial Bradyrhizobium

The bradyrhizobia isolates were characterized with respect to their survival and tolerance response to different pH levels. Most native isolates were tolerant to pH of 5 and 7, sensitive to pH 3 and moderately tolerant to pH of 9. Commercial inoculant USDA 110 was tolerant to pH of 7 and 9, sensitive to pH of 3 and moderate to pH of 5. The results indicated that the isolates were sensitive to extreme low pH levels 3. At 24, 48, 72, 96 and 120 hrs the isolates were significantly ($p<0.05$) different, where native isolate NR14 recorded the slowest growth. Statistical analyses showed significant effect ($p<0.001$) of acid tolerance and ability to grow for both native and commercial bradyrhizobia (Table 1).

Moreover, there was significant bradyhizobia × pH interaction whereby growth of native rhizobia was more affected by pH level of 9 (Figure 1), while the growth of the commercial bradyrhizobia was more affected by pH level of 5 (Figure 1).

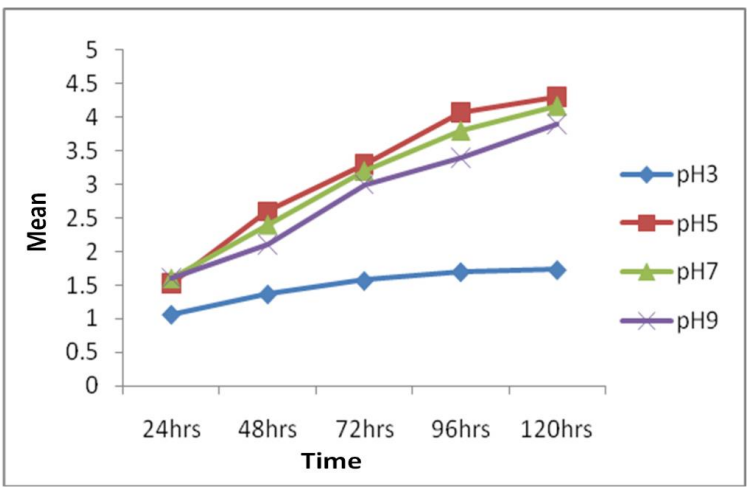

Figure 1. Effects of different pH values on the growth and tolerance of native bradyrhizobia isolates *in vitro*. Values are the means obtained. P-value < 0.05 for the interaction between bradyrhizobia isolate and pH level.

Table 1. Acid tolerance of native and commercial bradyrhizobia inoculant USDA 110, at different hours after inoculation

Bradyrhizobia isolate	24 hrs mean± S.E	48 hrs	72 hrs	96 hrs	120 hrs
NRI1	1.5±0.11b	2.2±0.15b	2.8±0.21a	3.4±0.32a	3.6±0.35a
NRI2	1.4±0.08b	2.2±0.22b	2.8±0.26b	3.4±0.32a	3.5±0.33a
NRI3	1.5±0.11b	2.4±0.19a	3.0±0.29b	3.4±0.34a	3.6±0.35a
NRI4	1.0±0.00c	1.2±0.07c	1.8±0.17b	2.5±0.24b	3.2±0.33b
BIO-FIX	1.8±0.17a	2.6±0.18a	3.4±0.29a	3.5±0.28a	3.7±0.28a
pH					
pH3	1.1±0.07b	1.4±0.08c	1.6±0.08c	1.7±0.07d	1.7±0.08c
pH5	1.5±0.10a	2.6±0.15a	3.3±0.14a	4.1±0.13a	4.3±0.10a
pH7	1.6±0.11a	2.4±0.20a	3.2±0.20a	3.8±0.14b	4.2±0.12b
pH9	1.6±0.12a	2.1±0.18b	3.0±0.25b	3.4±0.19c	3.9±0.12b
p- values					
Bradyrhizobia	0.0001	0.0001	0.0001	0.0001	0.0002
pH	0.0001	0.0001	0.0001	0.0001	0.0001
Bradyhizobia×pH	0.0072	0.0001	0.0001	0.0001	0.0001

NRI1-native rhizobia isolate one, NRI2-native rhizobia isolate two, NRI3-native rhizobia isolate three, and NRI4- native rhizobia isolate four. S.E - Standard error. Means in a column followed by same letter are not significantly different ($p<0.05$) by Tukey's Honest Significant Difference (HSD) test.

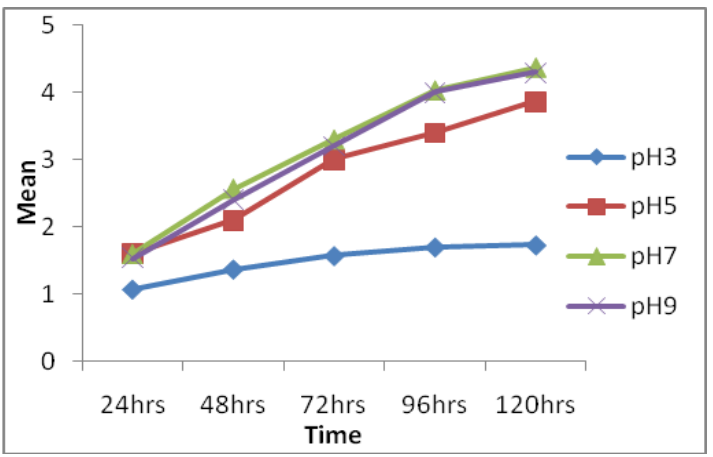

Figure 2.Effects of different pH values on the growth and tolerance of commercial *Bradyrhizobium* isolate *in vitro*. Values are the means obtained. P-value < 0.05for the interaction between bradyrhizobia isolate and pH level.

Table 2. Average means dry weight of shoot, roots, nodule and nodule number from the greenhouse

Isolate. Limed	SDW plant $^{-1}$ grams Mean ±S.E	RDW plant^{-1} grams	NN plant^{-1}	NDW plant^{-1} Milligrams
NRI1	0.6±0.02b	0.2±0.02a	18.4±2.06b	10.5±1.69b
NRI2	0.5±0.02c	0.2±0.02a	13.1±1.94b	5.7±0.88b
NRI3	0.5±0.02c	0.1±0.03a	12.9±1.25b	8.1±1.10b
Biofix	0.8±0.03a	0.2±0.02a	32.3±2.9a	36.5±14.91a
Control	0.2±0.05d	0.2±0.03a	0.0	0.0
Not. Limed				
NRI1	0.2±0.02a	0.1±0.01a	6.9±1.37b	2.3±0.55b
NRI2	0.2±0.04b	0.1±0.02a	6.4±1.60b	2.1±0.54b
NRI3	0.2±0.02a	0.1±0.02a	6.2±1.17b	3.1±0.43b
BIOFIX	0.2±0.02a	0.1±0.01a	12.3±1.63a	6.1±0.81a
Control	0.2±0.04b	0.1±0.03a	0.0	0.0
p-values				
Bradyrhizobia	0.0001	0.0001	0.0001	0.0006
Lime	0.0001	0.0001	0.0001	0.0032
Bradyrhizobia×lime	0.0001	0.0014	0.0001	0.0023

SDW- shoot dry weight, RDW-root dry weight, NN-nodule number, NDW- nodule dry weight. S.E- Standard error. Means in a column followed by same letter are not significantly different ($p<0.05$) by Tukey's Honest Significant Difference (HSD) test.

The soybean plants had distinctive variation in shoot color for all the treatments and the control. As expected, soybean plants inoculated with the bradyrhizobia had significantly ($P = 0.0001$) shoot dry weight compared to the uninoculated plants. Commercial inoculant USDA 110, showed a significantly higher nodule number and nodule dry weight compared to all the native isolates. Besides, there was a significant interaction ($p<0.05$) between the limed soil and the bradyrhizobia inoculation in improvement of plant nodule numbers (Table 2). The greatest effect of liming was observed following inoculation with USDA 110, where nodule dry weight plant^{-1} increased by 83.3% after lime application. There was no nodulation observed in the uninoculated plants.

4. DISCUSSION

Neutral pH supported maximum growth of all the isolates. Interestingly pH levels of 5 and 9 showed similar growth for native and commercial bradyrhizobia respectively. Native bradyrhizobia isolates were more tolerant to acidic pH which is typical of tropical soils, where the trap cultures were set. On the contrary, commercial bradyrhizobia performed well in pH level of 9, which is a characteristic of many agricultural soils worldwide. Therefore, native bradyrhizobia isolates form important candidates as potential low-cost inoculants for acid soils of most soybean fields to improve yield. The ability to grow in acidic soils provides the native rhizobia strains with competitive advantage over the commercial inoculants due to adaptation to the prevailing edaphic conditions (Manassila et al., 2012). Our observations regarding native isolates are in line with the previous findings of Aurag and Sasson (1992) who reported acid tolerance of native isolate *Rhizobium leguminosarum* bv. *phaseoli*.

Following bradyrhizobia inoculation, all the isolates nodulated with the tested soybean cultivar. However, the highest nodule number plant^{-1} was observed in the limed soil inoculated with commercial bradyrhizobia. Native isolates NRI1, NRI2 and NRI3 recorded moderate nodule numbers which were significantly higher in limed soils. These results confirm the greater effect of soil acidity on biological nitrogen fixation, irrespective of important agronomic practices such as legume inoculation. Soil acidity is also associated with Al^{3+} toxicity has a direct negative impact on rhizobia growth, persistence or nodule initiation and nitrogen fixation effectiveness (Indrasumunar et al., 2011). Moreover, acidity limits rhizobia survival and persistence in the soil and its subsequent root colonization, infection and nodulation (Graham et al., 1992; Bekere et al., 2013).

Infectivity of root nodule bacteria in nitrogen fixation was affected by compatibility among the bradyrhizobia, the soybean plant and the environmental conditions. Commercial bradyrhizobia in the limed soil was more compatible than the native bradyrhizobia due to the favorable pH and its resistant characteristics hence more productivity. This was evidenced by the increased nodule numbers, nodule dry weight, and shoot weight in the

limed soil pots treated with commercial inoculants. The native isolates did not significantly differ in nodulation. This is possibly because the isolates were from the same environmental conditions and a similar crop was used for trap cultures. In contrast to this study, soybean unlike other legumes may show poor growth in acidic soil due to aluminum toxicity rather than nodulation failure (Mubarik and Sunatmo, 2014). Therefore, it is suggested that effort to improve acid tolerance should be directed towards both the plant host and the bradyrhizobia bacteria, (Bhardwaj, 2014).

CONCLUSION

Modern agricultural production requires the implementation of efficient, sustainable, and environmentally sound agronomic management practices. In this study, commercial bradyrhizobia USDA 110 was more competitive and effective in enhancing nodulation and shoot dry weight of soybean under optimal pH requirements compared to the native isolates. However, the native isolates were more tolerant to acidic pH which is typical of most smallholder agroecosystems in SSA. Nonetheless, liming positively influenced the performance of both commercial and native bradyrhizobia, thus promoting nodulation and soybean growth. Therefore, to promote biological nitrogen fixation and performance of soybeans in smallholder farms in SSA, soil testing and liming of acidic soils to provide optimal microbial environment are imperative. Moreover, the native bradyrhizobia isolates should be further screened since they have great potential to provide affordable and efficient inocula that are well adaptable to the local agro-climatic conditions.

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Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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