UNIVERSITATEA „ALEXANDRU IOAN CUZA”
DIN IAȘI

JOURNAL OF EXPERIMENTAL AND MOLECULAR BIOLOGY

TOME XX, Number 1 & 2 **2019**

Editura Universității „ALEXANDRU IOAN CUZA” din Iași

EDITORIAL OFFICE

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CONTENT

THE EFFECT OF *RHIZOPHAGUS IRREGULARIS* AND *MESORHIZOBIUM LOTI* CO-INOCULATION ON *LOTUS JAPONICUS*

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Received: 24th of March 2019 / Revised: 10th of April 2019
Accepted: 22nd of June 2019 / Published: 20th of September 2018

Keywords: tripartite symbiosis, plant-microbe interactions, legumes, arbuscular mycorrhizal fungi, rhizobium

Abstract: Plants establish symbiotic relationships with soil bacteria or fungi, which colonize the plant root and provide the plant with inorganic nutrients, in exchange for photosynthesis products. Legume plants associate with both arbuscular mycorrhizal fungi (AMF) and the nitrogen-fixing soil bacteria called rhizobia. During the legume-rhizobium symbiosis, biological nitrogen fixation takes place in specific plants organs formed on the root, called nodules. Using the model legume *Lotus japonicus*, we studied the establishment of the legume-rhizobia-AMF tripartite symbiosis. We examined how the AM fungus *Rhizophagus irregularis* and the rhizobium *Mesorhizobium loti* affected one another during the colonization of the same legume roots, by performing co-inoculations. Moreover, we monitored the effect of the co-inoculation on the general plant performance. According to our results, the presence of *M. loti* had no effect on the root colonization by *R. irregularis*. However, root colonization by *R. irregularis* had a positive effect on the formation of root nodules. This study aims to enhance our understanding on how the plant selects, combines and controls its symbionts, towards to a more efficient use of legume plants in agroecosystems.

INTRODUCTION

Many plant species form associative relationships with a diverse range of microorganisms that result in interactions that are beneficial to both partners. During symbiotic associations the microorganisms typically capture nutrients that limit plant growth and provide the plant with these nutrients in exchange for carbon deriving from plant photosynthesis. Plants that engage in such symbiotic interactions require lower amounts of synthetic chemical fertilizers, therefore enhancing the sustainability of agricultural systems by reducing the economic and energy costs and protecting the environment.

Of the many associations formed between plants and microbes, arbuscular mycorrhizal (AM) symbiosis, in which plants and fungi of the Glomeramycota engage, seems to be the most ancient and widespread. The dominant role of AM symbiosis appears to be nutrient acquisition, as the AM fungi provide access to phosphorus (which is poorly mobilised in the soil) and, to a lesser extent, nitrogen and other mineral nutrients. In addition to that, AM symbiosis benefits the plant by providing disease resistance and tolerance to abiotic stress (Smith and Read, 2008). The plant facilitates fungal colonization into the inner root cortex, where arbuscules, the symbiotic organelles of the AM symbiosis, develop and mediate nutrient delivery. The arbuscule, is a highly branched exchange structure that forms within the root cortex surrounded by the invagination of the host cell plasmalemma (Bonfante and Genre, 2010). Arbuscules have a short lifetime, then they collapse and disappear, and the plant cell returns to its original state and can be re-colonized by a new arbuscule (reviewed in Gutjahr and Parniske, 2017).

Legumes are recognized as pioneer plants due to their capacity for initiating nutrient cycling in non-vegetated land and poor soils, *via* their symbioses with microbes (Graham and Vance, 2003). The association of legume plants with the soil bacteria called rhizobia represents one of the most celebrated mutualistic plant-microbe interactions. The legume-rhizobia symbiosis leads to the formation of novel organs on the plant root, termed nodules. Rhizobia occupy nodule cells and differentiate into bacteroids that fix atmospheric dinitrogen and provide it to the plant in a reduced form that the plant may assimilate (Oldroyd et al, 2011). By the application of rotation, intercropping and agroforestry techniques, this unique legume-rhizobia symbiosis may also improve nitrogen acquisition from non-leguminous crops in the agroecosystem.

Nodulation in legumes evolved from the much more ancient symbiotic association of plants with AMF (Parniske, 2008). Components of the rhizobial signaling pathway are also required for mycorrhizal signaling (Oldroyd and Downie, 2004), suggesting that the signaling pathway initially functioned in mycorrhizal signaling and was later recruited in legumes for recognition of rhizobia. The signaling molecules (lipochitinoligosaccharides, LCOs) produced by both AMF and rhizobia can activate a common symbiosis pathway. At least seven genes that are required for both the AM symbiosis and the root-nodule symbiosis with rhizobia have been identified in legumes (Kistner et al, 2005).

Such mutualistic plant-microbe interactions have been extensively studied over the past years, however, most of these studies are pairwise approaches, focused on a specific type of interaction. In nature, though, simultaneous microbial interactions usually take place on the same host. In this study we follow an integrated approach, in order to simulate the natural conditions, where different microbes co-exist in soils. We study the development of the tripartite symbiosis between the model legume *Lotus japonicus*, its microsymbiont, the *Mesorhizobium loti*, and the well-studied AM fungus *Rhizophagus irregularis*. The effect of concurrent symbiotic relationships on the general plant performance is examined, whereas, nodulation and mycorrhizal root colonization phenotypes are observed in order to understand how one symbiotic interaction affects another.

MATERIALS AND METHODS

Plant material and growth conditions: *Lotus japonicus* ecotype Gifu B-129 seeds were surface scarified, sterilized and imbibed for 3 hours at room temperature, then germinated on sterile wet filter paper for 7 days. Plants were then grown in pots filled with 2:1 v/v mixture of sterile sand and vermiculite for 5 weeks. The plants were fertilized with modified Long-Ashton nutrient solution (Hewitt, 1952). Growth chamber conditions were 16-h day and 8-h night cycles at 21°C.
AMF strain isolation and identification: *Rhizophagus irregularis* was isolated from a certified organic farm in Greece. The AMF strain was initially propagated in *Plantago lanceolata* plant grown in 2:1 v/v mixture of sterile sand and vermiculite. For the molecular identification of the strain, the extract of crashed spores was used directly as template in a PCR reaction. The PCR reaction was performed using the AM fungal specific primers AML1 (5'-ATC AAC TTT CGA TGG TAG GAT AGA-3') and AML2 (5'-GAA CCC AAA CAC TTT GGT TTC C-3') targeting the small subunit rRNA gene (Lee et al, 2008). The PCR program involved initial denaturation at 94ºC for 3 min, followed by 30 cycles at 94ºC for 1 min, 50ºC for 1 min and 72ºC for 1 min, and a final extension period at 72ºC for 10 min. The PCR product was cloned into pGEM-T Easy Vector (Promega) and transformed into *Escherichia coli* DH5a. Ten clones were selected randomly and sequenced.
Mycorrhizal inoculation: For root colonization by AMF, two spoonfuls (almost 60 spores plus hyphae) of *Rhizophagus irregularis* inoculum were added in each pot containing four plants.
Rhizobia inoculation: For bacterial inoculation, *Mesorhizobium loti* cv. R7A liquid cultures were grown for 2 days and diluted to an optical density of 0,02 at λ=600. One ml of bacterial suspension was applied directly to each root.
Quantification of mycorrhizal colonization: Mycorrhized roots were stained with 5% ink in 5% acetic acid solution (Vierheilig et al, 1998). Arbuscular mycorrhizas were quantified by microscopic examination of slides. Each slide was prepared from a sub-sample of 4 root systems and contained almost 20 cm of root. The (%) percentage of mycorrhizal colonization was estimated by the examination of at least 100 eye-pieces per slide. Four slides were examined per treatment, corresponding to 4 biological replicates.
Statistical analyses: For nodulation and mycorrhizal colonization, statistical analyses were performed by Student's t-test. A significance level of 5% was applied. For differences in the shoot biomass between treatments, statistical analysis was performed by one-way ANOVA followed by Tukey's honestly significant difference (HSD) post hoc test ($\alpha = 0,05$). Distinct letters indicate significant differences. All statistical analyses were performed in R statistics platform v. 3.5.2 (R Core Team, 2013) and graphs were generated with the R package ggplot2 (Wickham, 2009).

RESULTS AND DISCUSSION

To monitor the development of the legume-rhizobium-AMF tripartite symbiosis we performed co-inoculations of *Lotus japonicus* plants with the AM fungus *Rhizophagus irregularis* and the *L. japonicus* microsymbiont *Mesorhizobium loti*, under low nitrate and phosphate conditions. We used microbes that are known to interact with *L. japonicus* to expose plants to realistic microbial interactions they could encounter in nature. The co-inoculations were carried out simultaneously in order to simulate the natural conditions where the different microbes co-exist in soils.

To evaluate the effect of the concurrent symbiotic relationships on the general plant performance, we monitored the growth of the co-inoculated plants and compared it to the growth of plants inoculated with either *M. loti* or *R. irregularis* alone. Specifically, we examined the growth of the above-ground tissues of these plants by measuring the fresh and dry weight at 5 weeks post inoculation. Although the plants inoculated with *R. irregularis* appear to perform

better than the plants inoculated only with *M. loti*, the differences of the fresh shoot weights between treatments were not statistically significant (Fig. 1A). However, a significant weight increase was detected when the dry shoot biomass of co-inoculated plants was compared to the dry shoot biomass of plants inoculated with *M. loti* alone (Fig. 1B).

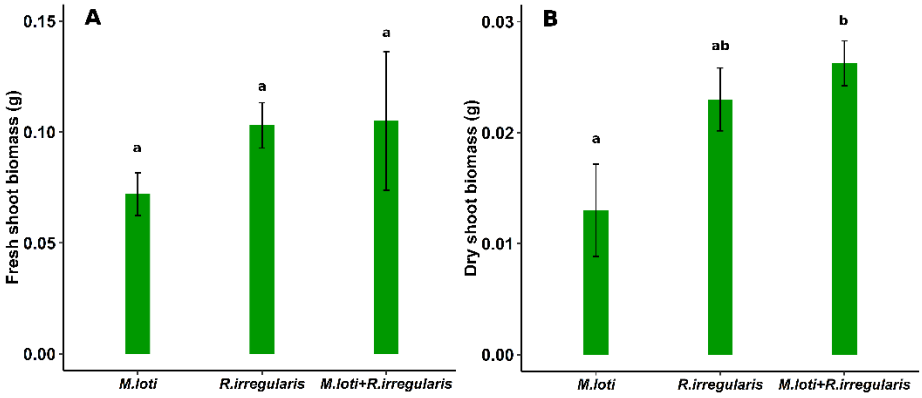

Figure 1. *M. loti* and *R. irregularis* co-inoculation improves the performance of *L. japonicus* plants. Shoot fresh (A) and dry (B) weight were measured in plants five weeks post single inoculations or co-inoculations with *M. loti* and *R. irregularis*. Bars show means (+SE) of four biological replicates (n=4).

The high dry shoot biomass presented by the co-inoculated plants indicates that *M. loti* and *R. irregularis* have synergistic effects on *L. japonicus* plant performance. To test whether the observed plant growth benefit is related to a positive response of the *L. japonicus* roots to nodulation in the presence of inoculation with *R. irregularis*, we counted the number of nodules five weeks post single inoculation with *M. loti* or co-inoculation with *M. loti* and *R. irregularis*. We observed that the number of nodules was significantly increased in co-inoculated plants compared to plants inoculated with *M. loti* alone (Fig. 2). Interestingly, co-inoculation with *R. irregularis* resulted in the production of more mature nodules (Fig. 2), suggesting an increased biological nitrogen fixation rate in these plants.

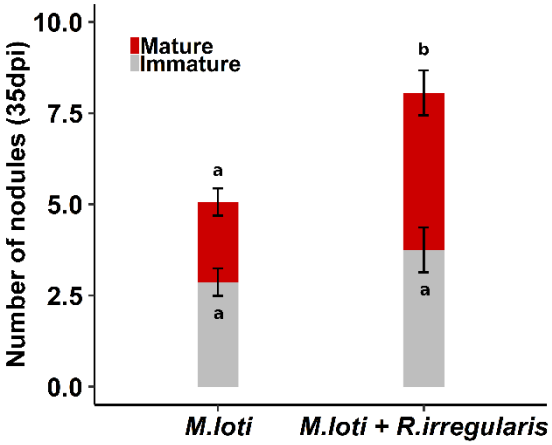

Figure 2. Nodulation increases when *L. japonicus* plants are co-inoculated with *R. irregularis*. Bars show means (+SE) of 16 plants.

Synergistic effects of multiple mutualists on plant performance reported before are in line with our results. In the model legume *Medicago truncatula*, co-inoculation of plants with *Ensifer meliloti* and *R. irregularis* resulted in higher above-ground biomass compared to single inoculations (Afkhami and Stinchcombe, 2016). Strong synergistic effects of AMF and rhizobia inoculation on plant biomass production have also been seen in the prairie legume *Amorpha canescens* (Larimer et al, 2014), and similarly, inoculation with rhizobia and AMF dramatically increased the shoot dry weight in soybean (Wang et al, 2011).

Regarding nodulation, the presence of *R. irregularis* enhanced nodulation in *M. truncatula* (Afkhami and Stinchcombe, 2016), and AMF inoculation increased nodulation in the prairie legume *A. canescens* (Larimer et al, 2014) and soybean (Wang et al, 2011). We therefore propose that the positive effect on aboveground plant biomass observed in the presence of *R. irregularis* in our experiments is directly related to enhanced nodule formation and activity in the *L. japonicus* roots induced by the presence of the fungus.

To test the effect of nodulation on the mycorrhizal root colonization, we quantified the root colonization by *R. irregularis* in the presence or absence of *M. loti*, five weeks post inoculation. The (%) percentage of mycorrhizal colonization was enhanced in the presence of rhizobia, however, this difference was not statistically significant (Fig. 3).

According to these results, *M. loti* does not affect *R. irregularis* root colonization in *L. japonicus*. In the prairie legume *A. canescens*, rhizobia inoculation decreased AMF hyphal colonization of roots (Larimer et al, 2014). On the contrary, co-inoculation with AM fungi and rhizobia resulted in higher AMF colonization than inoculation with AM fungi alone in soybean (Wang et al, 2011). It seems that the different AMF species used in these studies are affected by rhizobia in different ways. Alternatively, the different plant species respond differentially during co-inoculations and according to their special needs and growth conditions.

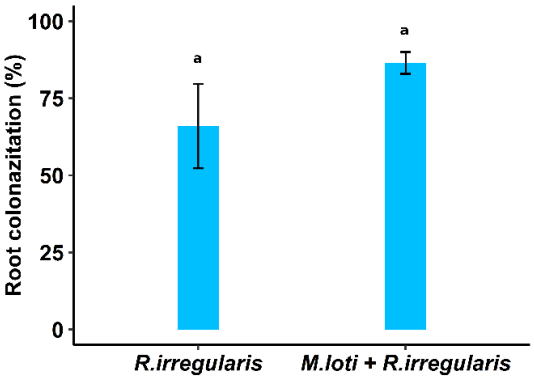

Figure 3. The presence of *M. loti* does not affect *R. irregularis* root colonization in *L. japonicus*. Bars show means (+SE) of four biological replicates (n=4).

CONCLUSIONS

In the present study the effect of *M. loti* and *R. irregularis* co-inoculation was examined in the model legume *L. japonicus*. According to our results, rhizobia and AM fungi co-inoculation improved the plant performance as indicated by the increased above-ground biomass recorded in the co-inoculated plants. Inoculation with *R. irregularis* had a positive effect on nodulation, as indicated by the formation of more mature nitrogen-fixing nodules in the co-inoculated *L. japonicus* roots. On the other hand, the presence of rhizobia had no effect on the *L. japonicus* root colonization by the AM fungus *R. irregularis*, under the specific experimental setup and the time lapse.

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Acknowledgements
The post-doctoral research was carried out with an IKY scholarship funded by the "Reinforcement of Post-doctoral Researchers" from "Human Resources Development, Education and Lifelong Learning" sources, with priority axis 6,8,9 and co-funded by the European social fund and the Greek government.

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GENETIC VARIABILITY OF PHARMACOKINETICS AND PHARMACODYNAMICS OF ANALGESICS (LAYERED MEDICINE) - PART II

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Received: 2nd of April 2019 / Revised: 20th of May 2019
Accepted: 23rd of June 2019 / Published: 20th of September 2018

Keywords: analgesics, single nucleotide polymorphism, pharmacogenomics

Abstract: Pain therapy, the most widely spread disorder, tends more as other diseases, to administration of drug molecules targeted to the affected tissue at the right dose, or to the patient or patient groups (personalized medicine). A decisive determinant of this strategy is the genetic one, which is to some extent the basis of the variability of pharmacokinetic and pharmacodynamic response to analgesics in the patient population. The differences in action and response to analgesics are due in these cases to hyperfunctional or nonfunctional uni-nucleotide gene polymorphisms encoding enzyme-modified transport proteins or receptors involved in the biotransformation and dynamics of analgesics. Genomic testing increases therapeutic efficacy and avoids adverse effects especially in patients with long-term therapies.

INTRODUCTION

Pain, especially chronic, affects a large number of patients and can become itself by its persistence a self-contained disease with significant costs to society and varying degrees of disability for patients with professional, social, and family consequences. Population studies conducted in the United States have shown that the number of prescriptions for painful sufferings in 20 years (1991-2011) has risen dramatically from 76 million to 219 million (Reuben, 2015). The approach over the past two decades to combat both chronic and acute pain on genetic basis, justifies the hopes of the effectiveness of this type of therapy and the reduction of costs allocated by the population and the state for analgesics. One of the open pathways for this is represented by the therapeutic transfer of viral or non-viral vectors, RNA or DNA segments encoding antinociceptive factors involved in the natural pain control mechanism (endogenous opioids, neurotransmitters, neurotrophins, growth factors, immunomodulators) which injected into target tissues or peripheral tissues, are conveyed by natural transport systems in the neurons and glia of the spinal nerve networks responsible for pain (Goins, 2012; Kibaly, 2016). The second therapeutical pathway on genetic basis is personalized therapy not only of chronic and acute pain but also of other diseases in which it has proved effective (diabetes, cancer, hypertension, tuberculosis, etc.). Successful treatment of pain or other suffering in certain patient groups, with certain drug molecules and in personalized doses, is part of the new concept of layered or personalized medicine (genomic medicine). Initially, pharmacogenetics deciphered individual allele-based pathological abnormalities of perceptions for painful sensitivity due to presence of some restricted gene polymorphisms (specific and typically rare in the population such as certain familial hereditary diseases). Then, as the genotyping acquisitions proceeded, the localization and other genes continued to decrypt the causes of variability in the patient's response to different classes of analgesics, from optimal response to those of unexplained failure of the same therapies with the same doses. Personalization of genotyping means the separation of patients on well-tailored therapeutic groups based on the results of genetic tests, regarding the identification and evaluation of interindividual allelic variability of single nucleotide polymorphisms (SNP) polymorphisms present in genes. This is reflected in patients' response to medication. Personalized medicine is important both for effective pain therapy in adults and the pediatric population (Schiavone, 2017). In addition to the known rarely genetic major defects family inherited, referred above, genetic influences in the population with painful disorders contribute synergistically or antagonistically to the phenotypic expression of the response to analgesic therapy. Research shows that although in the mass of the population a frequent situation is the mutual canceling of polymorphisms and genetic effects, the frequency of the *functional* mutant gene variants involved in the pharmacokinetics and pharmacodynamics of analgesics is still between 10-50% (Lotsch, 2009). This makes that the genetic differences with an impact on patients' response to analgesics, especially first-line drugs like opioids, to be increasingly taken into account when choosing a strategy for analgesic therapy, a motivated option otherwise by observations from medical practice. It is known, for example, that the success rate for morphine is only 65% at the same dose and affection (Mogil, 1999). The lack of favorable response to opioids for the other 35% of patients is based on both genetic and cumulative contribution of epigenetic factors, both of which being still insufficiently investigated and evaluated. It is also proven that the human population is divided into morphino-sensitive and morphino-insensitive individuals (Buchsbaum, 1977) and that there is a demonstrated

concordance between the rate of identical pain-related features in monozygotic as opposed to dizygotic brothers (Martin, 1997). On the other hand, other examples show that, depending on the target receptors, men's analgesia is *mu*-opioid (men respond better to *mu*-receptor opioids), whereas in women analgesia is *kappa*-opioid, and stress analgesia is mediated in men by glutamate and in women through non-glutamic mechanism. Taking into account the differences, the administration of the targeted drug to the affected individual and tissue ("magic bullet") and no adverse effects is no longer far away. Blakey (2011) affirmation remains valid: "Each prescription written constitutes an excursion into the unknown that is fundamental to the balance of risk or benefit in medicine. Will the patient respond fully, partially or not at all? Will there be unacceptable or even life-threatening adverse effects? ". It is true that for the time being, only relatively small groups of patients can benefit from genetically guided therapy, but the basis for expanding this concept is now being opened by decoding the human genome, reducing the cost of DNA sequencing associated with increasing the number of accessible genetic tests that can select the appropriate drug molecule in the appropriate patient at the appropriate doses and times. One of the most widely used predictive pharmacological tests (first approved in the US and the EU) is *AmpliChip CYP450* on DNA microarrays with rapid scanning of thousands of markers that identify the polymorphisms of the CYP2D6 and CYP2C19 genes, principally responsible for metabolic liver enzymes, with an important modulating role of drug response (Covic, 2012).

GENETIC MODULATION OF ANALGESICS PHARMACOKINETICS

The pharmacokinetics of analgesics includes a sequence of treatments to which all the drug substances in the body are subjected: absorption (bioavailability), biotransformation (metabolism), transport, distribution and excretion of the administered substances (Goodman Gilman, 1992).

INFLUENCE OF THE GENETIC FACTOR IN THE ANALGESICS METABOLISATION

A crucial process with significant impact in the treatment of pain is the *metabolisation of analgesics* that is performed in two phases (I and II). Phase I involves oxidation, reduction and hydrolysis, processes that can result in inactive, weakly active or more active metabolites than the primary drug. In the phase II, are taken place combinations/conjugations of the metabolites with a substrate (glucuronoconjugation, glutamino- and sulfoconjugation, acetylation or methylation) are carried out in the presence of catalytic enzymes: N-acetyltransferase, UDP-glucuronosyltransferase (GGT) and glutathion S-transferase (GSTs). Analgesic drugs commonly used in medical practice include *opioid* substances (morphine and morphinomimetics) and *non-opioid* substances. The most commonly used of these are antiepileptic/non-steroidal anti-inflammatory NSAIDs. Their action is inhibitory to cyclooxygenase-COX enzyme synthesizing prostaglandin (PG) enzymes with algic and/or inflammatory activity (especially PG2). NSAIDs commonly used are *non-selective* COX or with reduced selective inhibitors (diclofenac), COX-1 *selective* inhibitors (ibuprofen, naproxen, acetyl salicylic acid, indomethacin) and *selective* COX-2 inhibitors (celecoxib, parecoxib, etoricoxib). Non-opioid analgesics include also serotonin (triptans) and neuroleptics (co-analgesics) agonists such as anticonvulsants and antidepressants. Many substances used in pharmacological therapeutics and implicitly in pain therapy are metabolised in Phase I by oxidation by cytochrome P450 hemoprotein enzymes superfamily, which determined the focus of pharmacogenomics research on genes responsible for P450 synthesis. Some of the enzymes in this family process oxidative the molecules involved in pain mechanisms, both endogenous molecules synthesized by the body such as pain mediators (substance P, serotonin, histamine, cytokines, bradykinins, prostaglandin E2) or pain suppressors (own opioids) and also exogenes (analgesic and co-analgesic drugs). For the synthesis of these protein-enzymes are responsible 57 functional cytochrome P (CYP) genes and 58 non-functional CYP genes (Muralidharan, 2011). Mutations by single nucleotide polymorphism (SNP) substitutions or deletions occurring in the genes responsible for the synthesis of P450 enzymes induce changes in metabolism (biotransformations) of administered analgesics. Thus, the decrease in enzyme metabolism of analgesics prolongs their effects by prolonging their duration of action and delaying elimination from the body (eg NSAIDs). Other analgesic drugs (codeine, tramadol, metamizole, parecoxib, tilidine) increase their action over the pain even through catabolites resulting from metabolisation. Genomic polymorphic variants with a proven and currently proven role in drug metabolism are those responsible for the synthesis of CYP1A2, **CYP2D6**, CYP2C9, CYP3A4, CYP2E1, CYP2A6 enzymes. The genes responsible for the synthesis of mentioned enzymes may have each their own distinct sub-variants. For example, the synthetizing CYP2D6 gene of the metabolizing enzyme CYP2D6 has over 80 polymorphic sub-variants corresponding to extended varieties of enzymatically expressed phenotypes among the population. Numerous DNA sequencing meta-analyzes associated with clinical data have eventually led to the acceptance of the possible classification of SNP polymorphisms in carrier populations among various populations of the globe in 4 groups depending on the metabolic effects of the synthesized enzymes. It has been agreed to choose as the metabolic behavioral prototype the

CYP2D6 gene responsible for expression of the following variants of enzymatic phenotypes: a) *poor* metabolic action (PM) enzymes due to the presence of 2 deficiency alleles with deficit in analgesic hydroxylation; b) *intermediary* action (IM) with a non-functional allele; c) *extensive* action (EM) with a functional allele and d) *ultrafast* action (UM) with two functional alleles. The existence and level of action of citocrom P450 in the patients mass with pain has a particular clinical and therapeutic significance since the predominant coding of one or other of the enzymatic variants enumerated above depends on the variability of the degree of pain abolition at the same usual dosage of the analgesic substance. Substances carrying allelic variants that synthesize weakly-metabolizing (PM) enzymes may, for example, exhibit low oxidative processing, persistent high plasma concentrations of the administered analgesic and slow elimination with good analgesic effect but which can induce by the high levels also undesirable consequences (eg. adverse effects, sometimes risky). On the contrary, the individuals at the other extremity, with duplications or gene multiplications in the UM metabolisation position type, can induce a rapid hepatic metabolisation of the analgesic substance, resulting in sub-therapeutic, inefficient plasma levels. The CYP2D6 prototype gene influences the pharmacokinetics of a large number of drug substances, approximately 25% of commonly used drugs, including opioid, anti-emetic, antiarrhythmic, antipsychotic (Zhou, 2009). At the same time, the distribution of over 100 known functional gene variants of the above-mentioned CYP450 enzymes is important. From this point of view, there are significant differences in the frequency of variant carriers depending on their belonging to different *ethnic groups*, which should be taken into account in the medical practice in order to obtain the maximum therapeutic efficiency, while avoiding the risk of toxic accidents. Thus, a significant percentage of the Arabian and Ethiopian populations (29%) are carriers SNP polymorphic variants of the UM CYP2D6 gene type, whereas 7-10% of the Caucasian populations are affected by the presence of the autosomal recessive character of the CYP2D6 non-functional allele, while in Asians their presence is only 0.5%. Other examples are offered by the CYP2D4 variant, which has a frequency of 20% in Caucasians but is absent in the Chinese. Also, CYP2D6*-10 is specific to Asians, and CYP2D6*-45 and 46 variants are characteristic of African populations (Stamer, 2010). On the other hand, interindividual genetic variability also involves other types of related changes in patient behavior towards certain drug molecules. For example, in carriers with functional deficiency of the CYP2D6 gene with poor enzyme variant (PM), the beneficial effect of protection against opioid dependence is proven (Tyndale, 1997).

MODULATION OF METABOLIC PHASES I AND II OF OPIOID ANALGESICS (CODEINE, TRAMADOL, MORPHINE)

In metabolic phase I, codeine (methylmorphine) is strongly transformed by cytochrome P450 CYP2D6 isoenzyme into codeine-6-glucuronide intermediate products + nor-codeine (together with 83% from codeine) and morphine-6-glucuronide-M6G (6%). The analgesic effect of codeine is given by the M6G metabolite which has a 200-fold higher affinity for opioid miu receptors compared to morphine. The CYP2D6 enzyme is known to have many functional isoforms corresponding to the polymorphic gene variants which respond by their synthesis and functionally evidenced by the metabolic enzyme decrease, increase or metabolic-enzymatic inactivity on codeine. Thus, enriched gene-type polymorphic enriched versions of EM for CYP2D6 may present exaggerated effects at common doses of codeine, with predisposition to intoxication or even life-threatening by potentiating the side effects of the M6G metabolite (eg depression of breathing) being reported deaths in this way (Ciskowski, 2009). A high SNP gene variability is from this point of view the Caucasian populations in which treatment of pain with 60 mg of codeine that should reduce pain intensity by 50% finishes in 1 of 7 patients with failure or toxicity (Lotsch, 2009). Moreover, in such cases, adverse reactions may precipitate in genetically uninvestigated patients to who are filled opioids with non-opioid analgesics or in individuals with associated deficiencies in the excretion of metabolites (eg renal impairment). Other isoforms with metabolic action on codeine are CYP3A and UGT2B7. A codeine-like behavior is presented by tramadol (an opioid metabolised by the CYP2D6 enzyme), by its O-desmethyl-tramadol derivative (O-DSMT) derivative, which in turn has an affinity for the *miu* opioid receptors of 200 times higher than tramadol (Stamer 2003, 2007). Patients carrying PM variants with poor enzyme activity on the tramadol substrate (eg CYP2D6*10 homozygous Chinese) require high doses to achieve the analgesic effect (at risk for adverse effects in undetected genetically patients), unlike those who are carriers of at least one wild allele (Wang, 2006). Also, in carriers of gene-rich variants with extensive metabolism, the increase of O-DSMT favors the risk of side effects. Regarding the phase II metabolisation of analgesics represented by conjugation, an increasing interest has been shown since 1990 on the UGT enzyme (uridine diphospho- glucuronosyl-transferase) with the UGT2B7 isoform (from UGT-A1-10, UGT2B-4, 7,10,11,15,17,28 family). The isoform UGT2B7 is a special enzyme that has demonstrated strong glucuronidation actions of analgesic drugs such as morphine, codeine, buprenorphine, fluribiprofen, nonsteroidal anti-inflammatory drugs and anticonvulsants. It catabolizes morphine in two compounds with opposite effects: morphine-6-glucuronide (M6G) with analgesic effects (and with a 42-fold more expanding than morphine variability coeficient) and morphine-3-glucuronide (M3G-70%) with antianalgesic, excitatory effects. Various gene polymorphisms (SNPs) of the UGT2B7 gene in the nontranslational region induce morphine-derived metabolite alterations, e.g., M6G decrease, while UGT2B7*2/2 variant combined with the CYP2D6 enzyme has reversed effects.

CONTRIBUTION OF GENE VARIABILITY IN THE METABOLISM OF NON-OPIOID ANALGESICS

In pain-relieving analgesic pharmacology, not only opioids, but also non-opioid drugs have an important place to combat chronic pain, post-surgery pain, or frequently associated with opioids in the treatment of acute postoperative pain, or in addition to opioid medication in major surgery. They complete the analgesic action, reduce the side effects of opioids by reducing their doses and reduce the risk of opioid addiction. There is a certain genetic influence on the effectiveness of non-opioid analgesics, a class of drugs that include: synthetic NSAIDs and some neuroleptics, antiemetics, antiarrhythmics or serotonin reuptake inhibitors. Thus, NSAIDs contribute to pain relief by inhibiting CYP2C9, CYP2C8 and CYP2C19 genes responsible for the formation of catabolic enzymes. Contributions of genetics to modification of inhibitory actions on genes and enzymes were first suggested by highlighting in some experimental animals, but also in some patients, a high degree of sensitivity to NSAIDs or paracetamol, while others, on the contrary, found the presence of increased resistance to this therapy. In recent years, it has been shown that response differences are due to the variability of action (gene-dependent) of metabolizing enzymes on the drug substrate. For example, it is now known that the synthesis of enzymes which metabolise synthesis NSAIDs (diclofenac, ibuprofen, naproxen, piroxicam, celecoxib, parecoxib, etoricoxib, valdecoxib) significantly responds to the CYP2C9 gene (33 sub-variants), CYP2C8 and CYP2C19. The existence to polymorphic genic variants carriers of gene CYP2C9 PM type (metabolic / enzymatic weak effect on NSAIDs) allows to CYP2C9*3 homozygotes slowing down the elimination of the analgesic (COX-1 and 2 inhibitors) and consequently increase of their concentration in plasma over 2 fold compared to CYP2C9*1/1 wild-type carriers (Tang, 2001; Stamer, 2007). Delayed excretion of NSAIDs in CYP2C9*3 allele carriers is associated with an increase in pharmacodynamic activity with significant clinical effects but which not exclude careful monitoring of the risk of adverse effects on these substances. Catabolism of other substances used to combat pain in association with opioids (with the advantage of lowering their dosages especially in the treatment of chronic pain) such as co-analgesics represented by adjuvants neuroleptics such as antidepressants (dextromorphan, trimipramine or paroxetine), antipsychotics (haloperidol), antiemetics (metoclopramide) or antiarrhythmics (amiodarone) are made by enzymes synthesized under the control of the CYP2D6 gene, which also controls opioid metabolism. For example, carriers of the CYP2D6 polymorphic (SNP) gene variant with a reduced degree of synthesis (PM type) of the metabolizing isoenzyme exhibit high exposure to the drug by slowing metabolism associated with a risk of adverse reactions. In contrast, duplicates of the CYP2D6 gene with ultra-fast enzyme metabolism (UM type) of antidepressants or antipsychotics can result in ineffective sub-therapeutic concentrations (Kirchheiner-2003, 2004). Antidepressants used in the treatment of chronic pain are substrates for other genes by the enzymes they synthesize, such as the CYP2C19 and ABCB1 genes.

GENETIC INFLUENCE OF THE ANALGESICS TRANSPORT

An important role in the pharmacokinetics of analgesics is represented by the drug-carrier molecules family in brain regions with key roles in the central mechanisms of pain. Most transporters are glycoproteins that modulate the hemato-encephalic and neuronal transmembrane passage, and finally the intracellular concentration of the therapeutic molecules. One of the most well-known transporters group is the ABC family with 7 subfamilies and 49 members (Muralidharan, 2011; Mercer, 2011), the most studied transporter being B1 glycoprotein P (P-gp). The P-gp transporter is encoded by the ABC B1 gene and acts as a transmembrane pump for the active transport of some drug molecules, implicitly analgesic. It has over 50 mutational SNP variants with numerous polymorphisms (by insertions / deletions) identified in the ABC B1 gene that increase or decrease the concentration of analgesics in the brain by their transport with variable velocity through the blood-brain barrier. In this way, for example, modifications of the polymorphic encoded SNP positions of the ABCB1 gene shows in both animal and clinical experiments in cancer patients treated with morphine, significant associations with the variability of analgesia and the reduction of adverse effects (Fujita, 2010). The P-gp transporter mediates the effects of opioids and antidepressants through its structural functional variants genetically coded. It has thus been shown in heroin-dependent patients undergoing methadone replacement therapy that the doses may be reduced to carrier of ABCB1-2435 C>T variants that decrease the expression of the P-gp transporter resulting in slowing of the methadone flow out of the brain . The transport also modulates in this way methadone-induced analgesia, as evidence of increased anti-antalgic effects following the pharmacological blockade of the P-gp transporter (Coller, 2006; Lotsch, 2009; Mercer, 2011). SNP gene variants responsible for carrier synthesis modulate the variability of patient response to other molecules also involved in analgesic mechanisms, for example the SLC6A2 and SLC6A4 genes for noradrenaline and serotonin.

GENETIC MODULATION OF PHARMACODYNAMIC ACTION OF ANALGESICS

The biochemical and physiological effects, as well as the mechanisms of action of the analgesics, are part of their pharmacodynamics and are influenced by the variability of genotypes in the population mass as well as the pharmacokinetic processes mentioned above. The decisive factor of the pharmacodynamics of analgesics depends on the ability and their effectiveness of coupling to neurons receptors in the spinal and cerebral target areas, with a role in pain perception. Poor binding on membrane receptors or low number of active receptors induce poor and unsatisfactory therapeutic effects on pain. Receptors, as protein structures, are genetically encoded, in the first line of the analgesic efficacy being the opioid receptors: miu, delta, kappa, epsilon and the orphan receptor FQ (nociceptin). All of mentioned opioid receptors manifest their actions by modulating G protein (pGm) in their structure, in this case the inhibiting variant of protein G (Gi) with the reduction of AMPc formation. The OPRM1 gene responsible for the encoding of the opioid miu receptor is highly SNP polymorphic, mutant variants being due either to alteration of the extracellular receptor domains or to the intracellular neurons (Shabalina, 2009). In the first case, was identify the decrease of the extracellular glycosylation site of the protein-miu receptor (replacement aspartate with asparagine at position 40) with reduction of the analgesic effects of the opioids (see note I). In the second case, is better known the OPRM1 mutant gene variant which affects the 3rd intracellular loop of the receptor followed by cascade events: decrease in G protein binding capacity, decrease in receptor signaling capacity and decrease in therapeutic effects or even inefficiency of the analgesic. This mutation affects precisely those cerebral neuronal areas densely populated by miu opioid receptors involved in natural pain control and stimulation of reward behavior, associated in the same time with important participations in the mechanisms of perception and pain feeling: the somatosensory cortical area I and II, the anterior cingulated cortex, the posterior islet cortex and the *accumbens* nucleus. The patient carriers of OPRM1 gene mutation need to reduce or suppress pain high doses of opioid analgesics: morphine, M6G, alfentanil, methadone (Oertel, 2009). The alteration of the pharmacodynamic intimate mechanism is related to two key factors of miu neuronal receptor function: (1) G-protein - kinase receptors (GRK) on surface neuronal membrane regulating Gi protein by phosphorylation and 2) protein G - K^+ rectifying channels (GIRK), couples factors being primary post-synaptic effectors in central nervous system neurons. In the first case (GRK), mutant coding of modulators signal proteins arrestin *beta*-1 and 2 block the action of the Gi protein by inducing desensitization (lack of activation) of the phosphorylated miu opioid receptor (Ferguson, 1996). In the second case (GIRK), normally the GIRK ionic channels present in the heart (involved in the mechanism of cardiac pain), spinal cord, cerebral zones activated by coupling with the G protein opens, followed by an influx of K^+ ions from the extracellular space in the cytoplasm, activating in turn the miu opioid receptors that induce analgesia by blocking the transmission of pain. In contrast, in carriers of ion channels genetically modified GIRK, mutations in the intracellular domain of GIRK decrease their sensitivity for the Gi receptor protein. Under these conditions, the binding of the Gi protein to a neuronal receptor ligand (the ligands may be for miu receptors and nociceptin FQ receptors or for muscarinic receptors, adrenergic alpha2, serotonin 1A, cannabinoid, adenosine 1A), results in the reduction of K^+ inside the cell, to which is added the depression of N-type Ca^{2+} channels, which ultimately induces opioid receptor desensitization (Nishizawa, 2014). GIRK1 channels (encoded by the KCVJ3 gene) participate in opioid analgesia (Marker, 2004), and GIRK2 channels (CKNJ6 gene) contribute to modulation of opioid-induced tolerance (Saland, 2008). GIRK1,2 and GIRK3 (encoded by the KCNJ9 gene) strongly modulate painful sensitivity, gene *knockout* mice developing hyperalgesia (Marker, 2004; Smith, 2008). The GIRK2 and GIRK6 ion channels are involved in inflammation and allodynia by lowering the excitability threshold and increasing pain sensitivity (Eijkelkamp, 2009). Two of the SNP mutant variants (Cs2835859) of the CKNJ6 and KCNJ9 genes have recently been identified as being responsible for the analgesic and pain sensitivity associated with the predisposition for nicotine, alcohol and cocaine dependence and can serve as useful markers in medical practice (Nishizawa, 2014). Desensitization of neuronal opioid receptors promotes the occurrence and installation of pain, primarily being targeted bulbar neurons from *locus coeruleus* area and cerebrospinal gray substance from the brainstem, major inhibitory areas in ascending pain transmission through spinal tangles to the thalamus and cortical areas somato-sensitive and vegetative projection of painful sensitivity pathways.

CONCLUSIONS

The pharmacokinetic and pharmacodynamic advantages of analgesic pain therapy based on pharmacogenomic molecular mechanisms are primarily due to the increase in efficiency by individualization treatment under the conditions of dose reduction, the ideal of any drug therapy (it is known that "in the usual classical treatment, patients take biger doses for smaller effects "- Stamer, 2007). In addition, is followed the avoidance of adverse side effects and toxicity (100,000/year deaths in the USA by adverse effects), reducing the risk of addiction, avoiding the

loss of time to maximize efficacy doses and appropriate drug associations, avoiding polypragmasia and drug interactions which can amplify (or block) the metabolic effects of enzymes and especially the occurrence of adverse effects in particular in fragile individuals such as elderly with multiple sufferings and associated therapies or young children. Since the phenotype of pain and response to treatment are also influenced by epigenetic factors (environmental factors, lifestyle, diseases and associated medications), in the near future, it will be more accurately highlighted how and where these factors induce subtle changes summed up in the genome of individuals. Today the recent acquisitions, as well as the benefits of pharmacogenomics, widen the possibility of applying these data and results to pain management strategies in order to maximize the efficiency gains on this basis. It is true that there are complex difficulties due to the redundancy and pleiotropism characteristic of the biological systems, which require time to solve and that the therapy of acute and chronic pain can not yet be personalized satisfactorily (depending on the patient's genotype) for large populations. Many times, only the accentuated adverse effects or the failure of the therapy can direct the physician's prediction to a genetic motivation. But predictive analgesic therapy, guided and genetically individualized, remains for the very near future the most effective strategic option for well-defined patient groups.

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TEXTILE DYE BIOREMEDIATION POTENTIAL OF SOME RHIZOBIAL STRAINS AND THEIR HEAVY-METAL AND HIGH SALINITY TOLERANCE

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Received: 20th of March 2019 / Revised: 3rd of June 2019
Accepted: 26rd of June 2019 / Published: 20th of September 2018

Keywords: rhizobial strains, bioremediation, textile dyes, heavy metals, salinity, Danube –Delta Biosphere Reserve

Abstract The discharge of untreated textile dye effluents enriched with toxic pollutants including dyes, heavy metals and other hazardous materials may cause negative impacts on the entire ecosystem. The proposed work aimed to isolate, molecularly identify and characterize the native rhizobial strains with textile dye biodegradation potential in relation with their tolerance to high salinity and heavy metals (usually meet in high concentrations in the textile dye effluents). Native rhizobial strains were isolated from various terrestrial ecosystems originated in Danube – Delta Biosphere Reserve. Most of the strains tolerated ≥ 2.0% NaCl. Our data showed that 3 strains (*Agrobacterium* sp.CR-B19; *Rhizobium giardinii* CR-B22 and *Ensifer* sp.CR-B26) were able to tolerate 15 ppm concentration of cadmium (Cd^{2+}), whereas all strains identified as *Rhizobium* sp. (except *R. leguminosarum* CR-B10), and *Agrobacterium* sp. could tolerate 70 ppm of chromium (Cr^{6+}). Moreover, 3 indigenous strains (*Rhizobium giardinii* CR-B13; *Rhizobium* sp.CR-B15 and *Agrobacterium* sp. CR-B19) tolerated a concentration of 200 ppm of lead (Pb^{2+}). In regard to azo-dye degrading potential, only *Rhizobium leguminosarum* CR-B10 was able to degrade the Reactive Orange 16 dye (90.18% decolorization) in stationary conditions, at 30°C. Comparatively, *Agrobacterium sp*. CR - B19 strain removed Reactive Orange 16 (sulphonic azo-dye) (78.92 % decolorization) and Reactive Blue 4 (antraquinonic dye) (12 % decolorization) by adsorbtion. Based on their bioremediation potential, the newly isolated rhizobial strains could be further used (in pure culture or in consortia) to develop a new environmental friendly and cost–effective biotechnology in order to reduce the toxicity of textile dyes effluents.

INTRODUCTION

In the last decades, the severe environmental pollution has been associated with fast industrialization and discharge of large amounts of waste waters without pretreatment into water bodies. In some cases, the difficulty in treating industrial waste waters by conventional treatment methods has been reported (Van der Zee, 2001). Synthetic dyes and heavy metals are ones of the major xenobiotic pollutants of textile, pharmaceutical, cosmetics and food industries. Many reports confirmed the recalcitrant nature of synthetic dyes and their carcinogenic or mutagenic properties (Grover, 1999; Phugare et al, 2011). Also, soil and water contamination with heavy metals containing effluents received an increasing concern due to their direct toxicity to animals, plants and microorganisms (Hamilton and Wetterhahn, 1988; De Flora et al, 1990) and irreversible immobilization in soil components (McGrath and Lane, 1989). Textile dye effluents containing various synthetic dyes and heavy metals could induce toxic effects on agricultural plants and soil microorganisms by reducing soil fertility and agricultural output ((Puvaneswari et al, 2006). Based on environmentally friendly characteristics of bioremediation process (low cost, less sludge volume, environmentally safe) many bacteria, molds and yeasts (live or dead microbial cells, in pure culture or in consortia, free or immobilized cells) have been used for industrial waste water bioremediation (Ali, 2010; Ali et al, 2009; Allam, 2017).

Some studies showed that species of *Agrobacterium spp., Phyllobacterium spp., Rhizobium spp., Mesorhizobium spp., Ensifer spp.* and *Bradyrhizobium spp.* could detoxify the industrial waste waters or soil due to their resistance to heavy metals and their abilities to degrade organic pollutants (Ahmad, 1997; Carasco et al, 2005; Stan, 2011; Teng et al, 2015). Species of *Rhizobium* spp. și *Bradyrhizobium* spp are ones of the most frequent strains isolated from contaminated area (Teng, 2015). Also, strains of *Agrobacterium radiobacter* were isolated from activated sludge (in sewage treatment plants) (Drysdale et al, 1999; Singh et al, 2004).

In this study, the textile dye bioremediation of some soil rhizobial strains was investigated. Since the presence of elevated level of salts and heavy metals in textile dye effluents can significantly reduce the efficacy of dye biodegradation process due to their toxicity to microbial cells, a preliminary screening of bacterial resistance to salinity (NaCl) and heavy metals, as chromium (Cr^{6+}), cadmium (Cd^{2+}) and lead (Pb^{2+}) was also performed. A new biotechnology – based bioremediation technique could be next developed and applied to detoxify the toxic waste waters used for agronomic practices.

MATERIALS AND METHODS

Sampling sites and sample collection. Soil samples from various sites and soil type of Danube - Delta Biosphere Reserve (DDBR) were collected from the soil depth (10 -15 cm), packed in sterile bags, transported to the laboratory, homogenized and stored at 4°C for later use. The slurry was obtained by adding 1 g soil to 50 ml of sterile distilled water and mixed on an orbital shaker at 200 rpm for 1 h. Then, aliquots (0.1 ml) were plated on on yeast – mannitol-agar (YMA) supplemented with Congo red dye (25µg/ml). Rhizobial strains were then isolated and purified according to standard protocols (Vincent, 1970) and further characterized. The purified isolates were maintained at -80°C in yeast – mannitol broth (YMB) containing 20% glycerol.

Phylogenetic analysis of 16S rDNA sequences. Bacterial strains were grown in liquid YMB medium and incubated at 28ºC on a rotary shaker. Equal aliquots of bacterial cultures were collected by centrifugation and total genomic DNA was isolated using Bacteria DNA Preparation kit (Jena Bioscience, Germany) according to manufacturer instruction. The purity and concentration of genomic DNA was checked by Nano Drop measurements and electrophoresis on 0.8% agarose gel. The fD1and rD1universal primers (Weisburg et al, 1991) were used to amplify conserved region of 16S rDNA as previously described (Efrose et al, 2018). PCR amplification products were purified and directly sequenced on both strands using the same primers as for PCR (CEMIA, Greece) and deposited in GenBank/NCBI database for the selected bacterial strains. For phylogenetic analysis, sequences were corrected and assembled using DNA Baser v. 3.5.4 program. The sequences obtained from the newly isolated rhizobia together with the sequences retrieved from the NCBI/GenBank database were aligned using the CLUSTAL W software as implemented by implemented by MEGA7 *v*.7.0.*26* software package (Kumar, Stecher and Tamura, 2016), trimmed to the same length and used in the phylogenetic analysis. Phylogenetic tree was built with the Neighbor-Joining method based on Kimura's two-parameter model. Bootstrap confidence levels were calculated for 1000 replicates.

Nucleotide sequence accession numbers. The GenBank accession numbers for the 16S rDNA sequences obtained from the two bacterial strains which exhibited multiple biotechnological potential (*Rhizobium leguminosarum* CR-B10 and *Agrobacterium sp.* CR-B19) are MH456791 and MH456793, respectively. Accession numbers of the related reference strains are individually specified in the corresponding phylogenetic trees.

Stress tolerance. The bacterial cultures (10µl) were point - inoculated into YMA plates supplemented with NaCl (w/v) (0.1; 0.5; 2.0; 4.0, and 8.0 %). Heavy metal resistance was determined on YMA plates supplemented with the different heavy metals: Cr^{6+} ($K_2Cr_2O_7$) and Cd^{2+} ($CdCl_2$) at concentrations in a range from 0.1 to 70 ppm and, also Pb^{2+} ($Pb(NO_3)_2$) at concentrations in a range from 15 to 600 ppm. The readings were made after 3 days of incubation at 30°C. The highest concentration of NaCl and heavy metal salt supporting strains growth on YMA plates were defined as the maximum tolerance level.

Screening of dye degrading bacteria. Different liquid culture media have been used in order to assess the dye biodegradation potential of newly isolated rhizobial strains, as follow: V1 culture medium (mineral salt medium – 1.0 g L^{-1} NH4Cl, 0.28 g L^{-1} $(NH_4)_2SO_4$, 0.067 g L^{-1} KH_2PO_4, 0.04 g L^{-1}, $MgSO_4.7H_2O$, 0.022 g L^{-1} $CaCl_2.2H_2O$, 0.005 g L^{-1} FeCl3 and trace elements solution (10 ml L^{-1}); the trace element solution contained: 10.0 mg L^{-1} $ZnSO_4.7H_2O$, 3.0 mg L^{-1} $MnCl_2.2H_2O$, 1.0 mg L^{-1} $CoCl_2.6H_2O$, 2.0 mg L^{-1} $NiCl_2.6H_2O$, 3.0 mg L^{-1} $NaMoO_4.2H_2O$, 30.0 mg L^{-1} H_3BO_3 and 1.0 mg L^{-1} $CuCl_2.2H_2O$ - supplemented with glucose 10.0 g L^{-1} and yeast extract 10.0 g L^{-1}); V1.1.culture medium (mineral salt medium supplemented with 10.0 g L^{-1} glucose and yeast extract reduced to 3.0 g L^{-1}); V2 culture medium (TY medium – 10.0 g L^{-1} tryptone, 5.0 g L^{-1} NaCl and 3.0 g L^{-1} yeast extract); and V2.1. (TY medium supplemented with yeast extract reduced to 1.5 g L^{-1}). Synthetic dyes used in the experiments were azo-dye Reactive Orange16 (commercial name: Bezactiv Orange V-3R – λ_{max}= 495 nm) and antraquinonic dye Reactive Blue 4 (commercial name: Procion Blue MX-R - λ_{max}= 595 nm).The stock solution of dyes (1000 mg L^{-1}) were prepared, filter sterilized (Millipore filter, 0.22µm, Millipore Corp., Bedford, USA) and diluted properly before use.

The decolorization assay has been performed as follow: 250-ml flasks contained 100 ml of different culture media, supplemented with 20 ppm textile dyes (Reactive Orange 16 and, respectively Reactive Blue 4) were inoculated with 2% (v/v) suspensions from newly isolated bacterial strains and incubated at 30°C in stationary conditions. The control flasks containing the same mediums without inoculums were also kept as control. At the maximum visible dye decolorization time (72 -120 h), 5 ml of liquid media was centrifuged at 12,000 rpm for 10 min and the supernatant was analyzed for remaining dye content. The experiments were carried out in triplicates.

The decolorization efficiency of dyes was determined by measuring the absorbance of culture supernatant at their λ_{max} for each textile dye (λ_{max}= 495 nm (RO16) and λ_{max}= 595 nm (RB4), respectively). The decolorization percentage (%) was calculated as follow:

$$\text{Decolorization (\%)} = \frac{\text{initial absorbance-final absorbance}}{\text{initial absorbance}} \text{x} 100$$

UV–Vis analysis. The culture supernatants were analyzed by spectral scanning between 200 and 800 nm using a UV–Vis spectrophotometer (BekmanCoulter-DU730) and changes in the absorption spectra were recorded in order to analyze treated dyes degradation compared with non – treated dyes.

RESULTS AND DISCUSSIONS

Isolation and identification of bacterial strains

For bacteria isolation, soil samples were collected from various terrestrial ecosystems located in the Danube - Delta Biosphere Reserve (DDBR). Most of the strains (CR-B1; CR-B5; CR-B6; CR-B10-13; CR-B15; CR-B17-18) were isolated from agricultural soil originated in Chilia Veche - Pardina.

Table 1 - Molecular identification and geographical origin of bacterial strains with biotechnological potential, isolated from various ecosystems in DDBR -Romania

No.	Bacterial isolates	Geographical origin	Identity* (%)	Identified species**
1.	CR-B1	Chilia Veche - Pardina	99.8	*Rhizobium giardinii*
2.	CR-B5	Ostrovu Tataru	100	*Rhizobium giardinii*
3.	CR-B6	Ostrovu Tataru	99.7	*Ensifer* sp.
4.	CR-B9	Chilia Veche - Pardina	100	*Phyllobacterium bourgognense*
5.	CR-B10	Chilia Veche - Pardina	100	*Rhizobium leguminosarum*
6.	CR-B11	Chilia Veche - Pardina	100	*Ensifer adhaerens*
7.	CR-B12	Chilia Veche - Pardina	100	*Rhizobium* sp.
8.	CR-B13	Chilia Veche - Pardina	99.9	*Rhizobium giardinii*
9.	CR-B15	Chilia Veche - Pardina	99.8	*Rhizobium* sp.
10.	CR-B17	Chilia Veche - Pardina	99.6	*Ensifer* sp.
11.	CR-B18	Chilia Veche - Pardina	99.8	*Ensifer* sp.
12.	CR-B19	Dunavatu de Jos	100	*Agrobacterium* sp.
13.	CR-B22	Dunavatu de Jos	99.9	*Rhizobium giardinii*
14.	CR-B26	Murighiol - Sf. Gheorghe	100	*Ensifer* sp.

*Estimates of evolutionary identity between 16S rDNA sequences of the test strains and recognized rhizobia species, based on pair-wise analysis of the obtained sequences.

** The GenBank accession numbers for the sequences obtained from CR-B10and CR-B19 rhizobial strains are MH456791 and MH456793, respectively.

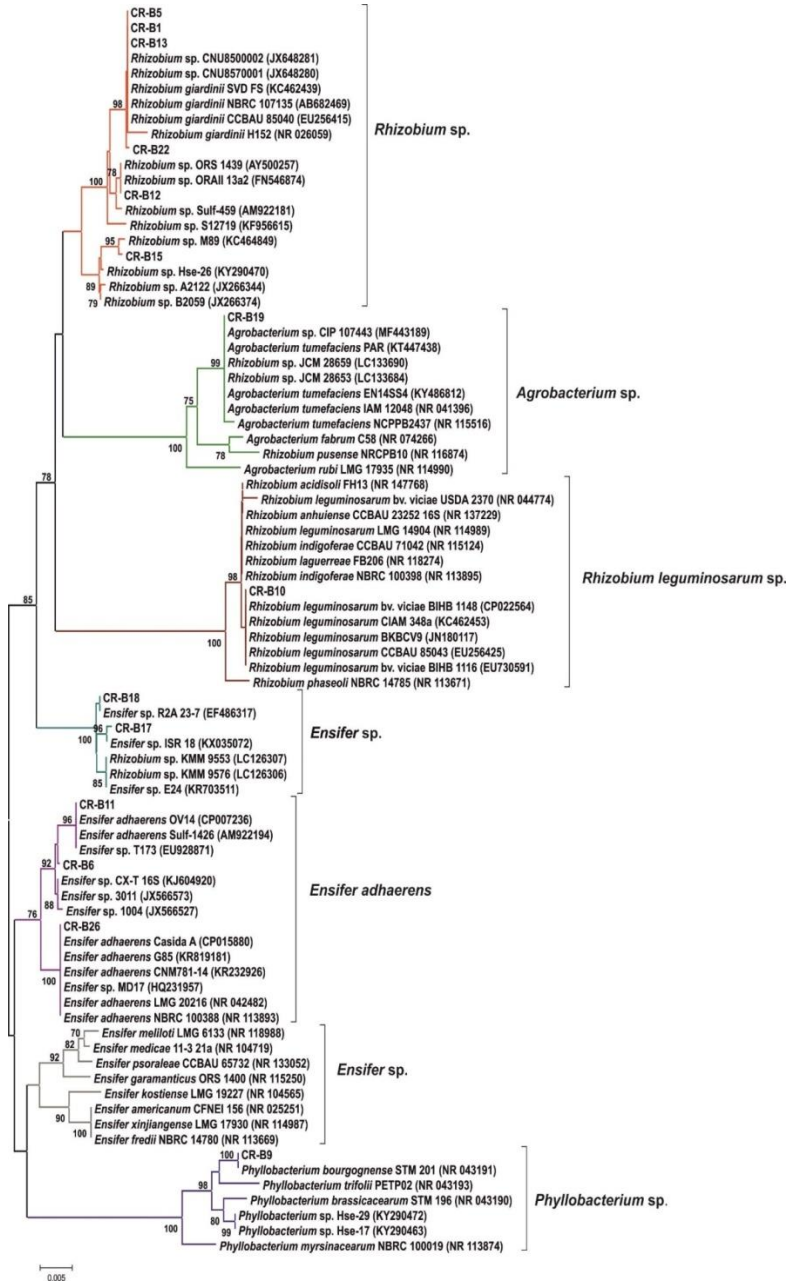

Figure 1. **Phylogenetic trees of the 16S rDNA sequences** showing the relationships of the representative bacterial strains isolated from various habitats from DDBR with selected reference strains for defined bacterial species. The Neighbor-Joining dendrogram was constructed using the Kimura 2-parameter model. Bootstrap values (based on 1000 replicates) below 70% are not shown. The scale bar represent 0,005 nucleotide substitutions.

The fields were cultivated with corn, wheat, barley and rape (Chilia –Veche – Pardina) or alfalfa (Ostrovu Tataru). Three strains (CR-B19; CR-B22 and CR-B26) were isolated from natural habitats (e.g. alluvial soils) located in Murighiol – Dunavatu de Jos area and Sf. Gheorghe branch (Saraturi Lake) (Table 1). The newly rhizobial strains were selected based on typical colonies appearance on Congo Red supplemented culture medium (Kuykendall, 2015). The taxonomic identification of the isolates and their relatedness to known bacterial strains was assessed by the sequence analysis of 16S rRNA gene (Figure 1; Table 1). Phylogenetic analysis revealed that the newly isolated strains clustered in seven well defined phyletic groups highly similar with previously described bacteria strains belonging to the Order of *Rhizobiales*.

Salinity and heavy metal tolerance of soil rhizobia

Most of the bacterial isolates could tolerate concentrations up to 2.0 % NaCl. However, the isolates identified as *Rhizobium giardini* (CR-B5) and the *Ensifer* sp. (CR-B6), originated in agro ecosystems (sodium saline alluvial soil type) as well as *Rhizobium leguminosarum* (CR-B10) showed increased tolerance to salinity (4% NaCl) (Table 2).

Table 2 - Salinity tolerance of selected rhizobial strains

Rhizobial strains	**NaCl (%)**			
	0.5	**2.0**	**4.0**	**8.0**
CR-B1 (*Rhizobium giardinii*)	+	-	-	-
CR-B5 (*Rhizobium giardinii*)	+	+	±	-
CR-B6 (*Ensifer* sp.)	+	+	±	-
CR-B9 (*Phyllobacterium bourgognense*)	+	-	-	-
CR-B10 (*Rhizobium leguminosarum*)	+	+	+	-
CR-B11 (*Ensifer adhaerens*)	+	+	-	-
CR-B12 (*Rhizobium* sp.)	+	+	-	-
CR-B13 (*Rhizobium giardinii*)	+	+	-	-
CR-B15 (*Rhizobium* sp.)	+	+	-	-
CR-B17 (*Ensifer* sp.)	+	+	-	-
CR-B18 (*Ensifer* sp.)	+	+	-	-
CR-B19 (*Agrobacterium* sp.)	+	+	-	-
CR-B22 (*Rhizobium giardinii*)	+	+	-	-
CR-B26 (*Ensifer* sp.)	+	+	-	-

+ good growth; ± weak growth ; - no growth

Since undiluted wastewaters from dyestuff industries usually contain salt concentrations up to 15 - 20 % (EPA, 1997) a high salinity tolerance of newly isolated bacteria should be considered as a prerequisite for those strains that are intended to be used as bioremediation agents for textile dye waste waters. For example, *Stentrophomonas maltophilia* RSV – 1 was able to decolorized (82.72 %) a mixture of dyes (Blue RR, Black B, Red RR and Yellow RR) within 6 days of incubation in the presence of 3% NaCl (Rajeswari et al, 2013).

The newly free – living bacterial isolates presented different responses to heavy metal stress (Table 3). For example, for those originated in soils collected from flooded areas (natural ecosystems), the highest tolerance to Cd^{2+} was 15 ppm (*Agrobacterium sp*. CR-B19*; Rhizobium giardinii* CR-B22 and *Ensifer sp.* CR-B26) (Table 3). In terms of tolerance to Cr^{6+}, bacterial strains belonging to the species *Rhizobium* sp. and *Agrobacterium* sp. were tolerant at maximum concentration of 70 ppm. Most strains tolerated 100 ppm Pb^{2+} concentration. Much adapted to heavy metal stress, the isolates *Rhizobium giardinii* CR-B13, *Rhizobium sp*. CR-B15 and *Agrobacterium* CR-B19 were able to tolerate 200 ppm Pb^{2+}.

Table 3 – Highest heavy metal tolerance of selected rhizobial strains

Rhizobial strains	Cd^{2+} (ppm)		Cr^{6+} (ppm)		Pb^{2+} (ppm)		
	5.0	**15**	**50**	**70**	**70**	**100**	**200**
CR-B1 (*Rhizobium giardinii*)	+	-	+	-	-	-	-
CR-B5 (*Rhizobium giardinii*)	+	-	+	+	±	-	-
CR-B6 (*Ensifer sp*.)	+	-	+	-	+	+	-
CR-B9 (*Phyllobacterium bourgognense*)	+	-	+	+	+	+	-
CR-B10 (*Rhizobium leguminosarum)*	+	-	±	±	+	+	-
CR-B11 (*Ensifer adherens*)	+	-	-	-	+	-	-
CR-B12 (*Rhizobium sp.)*	+	-	+	+	+	+	-
CR-B13 (*Rhizobium giardinii*)	+	-	+	+	+	+	+
CR-B15 (*Rhizobium sp*.)	+	-	+	+	+	+	+
CR-B17 (*Ensifer sp*.)	+	-	-	-	+	+	-
CR-B18 (*Ensifer sp*.)	+	-	+	-	+	+	-
CR-B19 (*Agrobacterium sp.)*	+	+	+	+	+	+	+
CR-B22 (*Rhizobium giardinii*)	+	+	+	+	+	+	-
CR-B26 (*Ensifer sp*.)	+	+	+	-	+	+	-

+ good growth; ± weak growth ; - no growth

This behavior is encouraging since some heavy metals as cadmium (Cd) and lead (Pb) are usually quantified in industrial effluents in high concentrations, being widely used for production of colour pigments of textile dyes. The above mentioned heavy metals have no known biological and/or physiological, functions (Gadd, 2010), but their presence could strongly inhibit the dye decolorization process by living microorganisms (Gadd, 1992). Abd - Alla et al. (2012) reported isolation and characterization of a heavy metal resistant isolate of *Rhizobium leguminosarum* bv. *viciae*, as a potentially efficient biosorbent for Cd^{2+} and Co^{2+}. The new isolate was resistant to 10 ppm Cd^{2+}. Comparatively, chromium (Cr) is an essential trace element, but higher levels are considered toxic and mutagenic in humans, animals and plants (Léonard and Lauwerys, 1980; De Flora et al, 1990) and can significantly reduce also, the efficacy of biological sewage treatment.

Smith and Giller (1992) first isolated strains of heavy metal - resistant *R. leguminosarum* from soil contaminated sewage sludge. Raaman (2012) reported that *R. leguminosarum* could detoxify a medium containing chromium by adsorption, but also by reduction of CrVI to CrIII. As result, bacteria can grow and could be a potential agent for the bioremediation purposes (heavy metals and dyes contaminated waste waters).

Screening of bacterial isolates with bioremediation abilities

The bioremediation capacity of the RO16 and RB4 textile dyes by the newly isolated rhizobial strains is diverse as efficiency and mechanism, mainly due to the differences in the chemical structure of the dyes. From a total of fourteen tested bacterial strains belonging to four bacterial genera (*Rhizobium* spp., *Ensifer* spp., *Agrobacterium* spp. and *Phylobacterium* spp.) only two strains, namely *Agrobacterium* spp. CR-B19 and *Rhizobium leguminosarum* CR-B10, showed a decolorization capacity over 78 % against Reactive Orange dye 16 within 120 hr of incubation (Table 4). The increased efficiency, in terms of shortest the decolorization time, was obtained by reducing the quantity of yeast extract (source of nitrogen and vitamins) from the composition of culture media (Table 5). The best results have been obtained on V2.1 (up to 90.18 % decolorization - *R. leguminosarum* CR-B10) culture media within 72 hr of incubation at 30°C, under stationary conditions (Table 5). As referring to the RB4 bioremediation process, only *Agrobacterium* sp. CR-B19 strain exhibited an adsorption capacity of the dye (up to 12 % decolorization on V1.1 culture medium). The decolorization percent (%) was not increased with prolonged incubation time. Also, no other new tested bacterial strains have demonstrated the potential to remove the RO16 and RB4 dyes from the aqueous media.

Table 4 - Maximum textile dyes decolorization potential (%) of some rhizobial strains isolated from soil (DDBR - Romania) after 120 hour of incubation at 30°C

Rhizobial strains	**Decolourization (%)**	
	Culture medium V1	Culture medium V2
	Azo – dyes (Reactive Orange 16 – 20 ppm)	
CR-B9 (*Phyllobacterium bourgognense*)	10.21	13.29
CR-B10 (*Rhizobium leguminosarum*)	21.12	78.56
CR-B19 (*Agrobacterium* sp.)	95.05	61.41
	Antraquinonic dyes (Reactive Blue 4 – 20 ppm)	
CR-B19 (*Agrobacterium* sp.)	10.23	5.89

Table 5 - Remediation of RO16 and RB4 dyes (20 ppm) by some rhizobial strains isolated from soil, after 72 h of incubation at 30°C

Rhizobial strains	**Decolorization (%)**	**Cell growth (OD_{640nm})**	**pH**	**Color of biomass**
	Reactive Orange 16 (20 ppm) - medium V1.1			
CR-B19 (*Agrobacterium* sp.)	78.92	1.583	3.14	orange
	Reactive Orange 16 (20 ppm) - medium V2.1			
CR-B10 (*Rhizobium leguminosarum*)	90.18	1.281	8.38	white
	Reactive Blue 4 (20 ppm) – medium V1.1			
CR-B19 (*Agrobacterium* sp.)	12.00	1.023	5.85	blue

UV- Vis spectrum analysis (200-800 nm) was performed in order to elucidate the mechanisms involved in the bioremediation of RO16 and RB4 dyes by the most efficient bacterial strains. As was noticed, *R. leguminosarum* CR-B10 was able to degrade the RO16 dye: the characteristic peak at 495 nm almost disappeared within 72 hr of incubation and white

biomass was obtained at the final of the biodegradation process (Figure 2 and Table 5). Comparatively, *Agrobacterium spp.* CR-B19 removed the tested dye through adsorption: the colored biomass which was noticed at the final of the incubation time was correlated with no modifications into UV- Vis spectra of both dyes (except that the main peaks in the visible spectrum decreased gradually within 72 hr of incubation)(Figure 3 – 4 and Table 5).

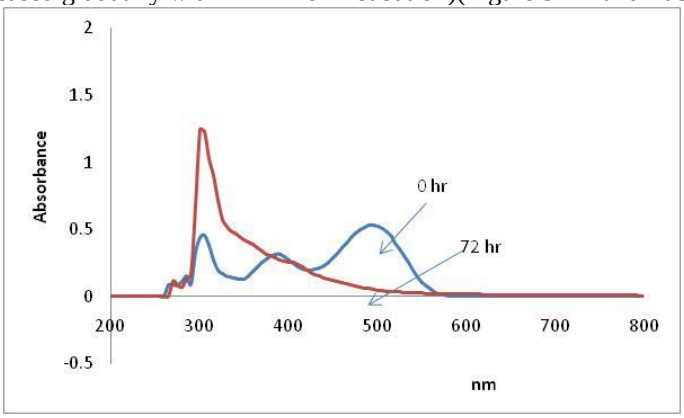

Figure 2 - UV-Vis spectra of Reactive Orange 16 (20 ppm) before and after bioremediation by *R. leguminosarum* CR-B10 in V2.1 culture medium

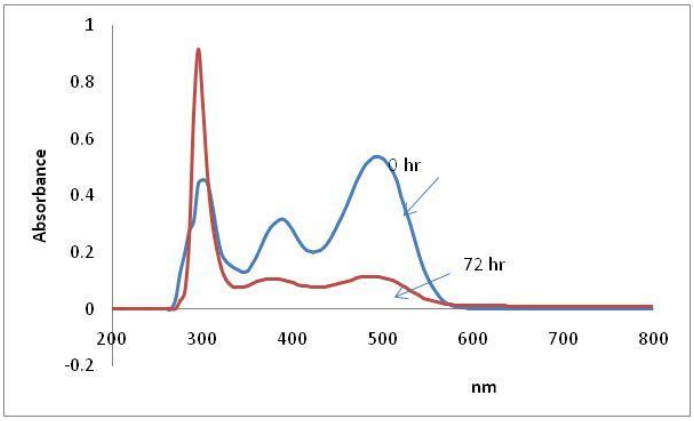

Figure 3 - UV-Vis spectra of Reactive Orange 16 (20 ppm) before and after bioremediation by *Agrobacterium spp.* CR-B19 in V1.1 culture medium

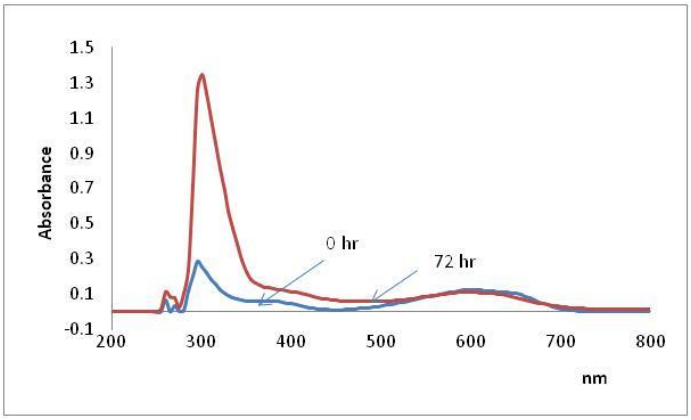

Figure 4 - UV-Vis spectra of Reactive Blue 4 (20 ppm) before and after bioremediation by *Agrobacterium spp.* CR-B19 in V1.1 culture medium

Other studies reported that *Agrobacterium radiobacter* cells induced maximum decolorization of Crystal Violet (10 mg/L) (triphenyl methane dye type) within 8 hr under static anoxic conditions, due to lacase and aminopirin N- demetilase activities (Parshetti 2011). The enhanced efficiency on heavy metal removal and decolorization of other textile azo-dyes (Methyl Orange and Congo Red) has been obtained by treating the industrial waste water with a bacterial consortium consisted of *Rhizobium radiobacter*, *Sphingomonas paucimobilis* and *Bacillus subtilis* (Allam, 2017). The experiment confirmed the importance of synergic activity of different metabolites of bacterial cultures in bioremediation of industrial effluents.

CONCLUSIONS

In the present study we have reported on isolation, molecular identification and characterization of the native rhizobial strains with textile dye biodegradation potential, in correlation with their tolerance to high salinity and heavy metals. Native rhizobial strains were isolated from various environments from Danube - Delta Biosphere Reserve and were grouped based on 16S rRNA gene phylogeny in seven well defined clusters.

In terms of their resistance to abiotic stress, most of the strains tolerated ≥ 2.0% NaCl and *R. leguminosarum* CR-B10 grew up until 4 % NaCl. The *Agrobacterium* sp. CR-B19, *Rhizobium giardinii* CR-B22 and *Ensifer* sp.CR-B26 strains were able to tolerate 15 ppm concentration of cadmium (Cd^{2+}), whereas the strains identified as *Rhizobium* sp. and *Agrobacterium* sp. could tolerate 70 ppm of chromium (Cr^{6+}). Three indigenous strains (*Rhizobium giardinii* CR-B13; *Rhizobium* sp.CR-B15 and *Agrobacterium* sp.CR-B19*)* tolerated a concentration of 200 ppm of lead (Pb^{2+}). Moreover, *Rhizobium leguminosarum* CR-B10 strain was able to degrade the RO16 (sulphonic azo-dye), while both dyes (RO16 and RB4) have been removed from aqueous medium by *Agrobacterium* sp.CR-B19 cells through an adsorbtion mechanism. Based on their bioremediation potential, the two newly isolated rhizobial strains could be further used to develop a new environmental friendly and cost–effective biotechnology in order to reduce the toxicity of textile dyes effluents.

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Acknowledgments: This work was supported by the Romanian Ministry of Research and Innovation through the NUCLEU program (BIODIVERS) and Program 1 - Development of the National R & D System, Subprogram 1.2 - Institutional Performance - Projects for Excellence Financing in RDI (Contract no. 22PFE / 2018).

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THE EPIGENETICS OF DIABETES, OBESITY, OVERWEIGHT AND CARDIOVASCULAR DISEASE

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Received: 20th of March 2019 / Revised: 3rd of June 2019
Accepted: 26rd of June 2019 / Published: 20th of September 2018

Keywords: chromatin, DNA methylation, microRNA. transcription factor, olfactory receptors, biomarkers

Abstract: The objectives of this review was once to understand the roles of the epigenetics in diabetes, obesity, overweight and cardiovascular disease. Epigenetics represents a phenomenon of altered heritable phenotypic expression of genetic records taking place except changes in DNA sequence. Epigenetic modifications can have an impact on a whole lot metabolic disease with the aid of special alteration of candidate genes based totally on the change of the target genes. In this review, I will summarize the current findings DNA methylation, histone amendment two in each types of diabetes, obesity, overweight and cardiovascular disease.
The involvement of histone adjustments and DNA methylation in the development of metabolic diseases is now broadly accepted .recently many novel genes are validated that has roles in diabetes pathway and it can be used for detection prediabetic however Over the modern-day years, mass spectrometry-based proteomics techniques positioned and mapped one-of akind range of histone modifications linking obesity and metabolic diseases. The checklist of these changes is evergrowing; however, their points and roles in obesity are no longer properly understood in obesity. Furthermore, epigenetic lookup in cardiovascular treatment revealed a massive quantity of modifications affecting the improvement and development of cardiovascular disease. In addition, epigenomics are moreover involved in cardiovascular risk factors such as smoking. the aberrant epigenetic mechanisms that make a contribution to cardiovascular sickness.

INTRODUCTION

Eukaryotic genomes are packaged in two established types of chromatin: gene-rich euchromatin and genetically inactive heterochromatin . Heterochromatin is a tightly packaged structure of DNA, and its foremost attribute is that DNA transcription is limited. Centromeres and telomeres are each heterochromatic .The euchromatin, in contrast, consists of 'active' chromatin: DNA sequences that are being transcribed into RNA (Dimitri 2005).Heterochromatin replicates in the S phase (synthesis phase) of the cell cycle later than euchromatin, most possibly keeping DNA structure during replication. Heterochromatin additionally keeps a compact and seen structure throughout mitosis consequently differing from euchromatin, which undergoes a ordinary cycle of condensation and unravelling at some stage in this process (Muhonen and Holthofer2008).
Epigenetics is the find out about of stable and heritable changes to the genome that can alter gene expression except altering the underlying DNA sequence. Epigenetic marks can be influenced through both the underlying genetic variation as nicely as exclusive environmental exposures (Non and Thayer, 2019). Epigenetic modifications are vital for X inactivation and genomic imprinting and direct normal developmental programming by using imposing distinct gene expression profiles of character cell kinds at particular developmental levels (Weksberg *et al.*, 2019).
Diabetes is a crew of metabolic illnesses characterised by way of hyperglycemia ensuing from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is related with long-term damage, dysfunction, and failure of special organs, mainly the eyes, kidneys, nerves, heart, and blood vessels(American Diabetes Association 2010). two The two most frequent varieties of diabetes are type 1 and type 2. Type 1 diabetes consequences from the autoimmune destruction of the pancreas's beta cells, which produce insulin. Persons with type 1 diabetes require insulin for survival; insulin may additionally be given as a daily shot or consistently with an insulin pump(Bullard *et al.*, 2018). In type 2 diabetes (adult onset diabetes), the pancreas makes insulin, however it either would not produce enough, or the insulin does no longer work properly. Nine out of 10 human beings with diabetes have type 2 (The National Institute of Diabetes and Digestive and Kidney Diseases 2018). It is usually prevalent that Type 1 is the end result of gene–environment interactions, but such speedy will increase in incidence are now not explained with the aid of Mendelian inheritance. There have been severa advances in our understanding of the pathogenesis of Type 1. Indeed, there has been a large wide variety of genes recognized that make a contribution to threat for this sickness and several environmental factors have been proposed (MacFarlane, Strom and Scott, 2009).
Obesity is a disorder characterised with the aid of extra adiposity that is a supply of considerable morbidity and mortality due to quite a number weight-related complications. Therefore, the diagnostic contrast ought to consist of an

anthropometric measure that displays elevated fats mass and an indication of the degree to which the excess adiposity is adversely affecting the health of individual patients (Garvey 2019). Overweight is normally due to more body fat. However, overweight can also additionally be due to greater muscle, bone, or water. People who have weight problems typically have too a whole lot body fats (Jensen *et al.,* 2013). The epigenome consists of DNA methylation, histone modifications, and RNA-mediated processes, and disruption of this stability may additionally cause numerous pathologies and make contributions to weight problems and type 2 diabetes (T2D) (Ling and Rönn, 2019).

Cardiovascular disease is main reasons of morbidity and loss of life in the Western society, accounting for a tremendous percentage of health care costs This disease share frequent danger factors, together with obesity, , lipid oxidation toxicity and low-grade inflammation, and they coexist in a super wide variety of patients (Roger *et al.,* 2011). The incidence of Cardiovascular disease, in particular coronary artery disease and diabetes is expanded in individuals with the metabolic syndrome , a cluster of metabolic abnormalities which include central obesity, hypertension and insulin resistance (Andreassi *et al.,* 2011). Emerging proof suggests a complicated new order regulated by using epigenetic mechanisms mark cardiac cell lineage. Indeed, molecular cardiologists are in the process of shedding light on the roles performed through ncRNAs, nucleic acid methylation and histone/chromatin changes in unique pathologies of the coronary heart (Al-Hasani, Mathiyalagan and El-Osta, 2019).

1 - Characteristics of metabolic syndrome:

Metabolic syndrome is a developing reason of morbidity and mortality worldwide. Metabolic syndrome is characterised by means of the presence of a range of metabolic disturbances along with obesity, hyperlipidemia, hypertension, and increased fasting blood sugar. Although the hazard for metabolic syndrome has mostly been attributed to adult way of life factors such as bad nutrition, lack of exercise, and smoking, there is now robust proof suggesting that predisposition to the improvement of metabolic syndrome starts off evolved in utero (Smith and Ryckman 2015). Epigenetic variants have been proven to expose vulnerability to diabetes and its complications. Although it has turn out to be clear that metabolic derangements, especially hyperglycemia, can impose a long-term metabolic memory that predisposes to diabetic complications, the underlying mechanisms continue to be to be understood (Xu, Natarajan and Chen, 2019).

2 - Epigenetic in type 1 of diabetes:

Epigenetic mechanisms have an effect on gene expression that ought to predispose individuals to the diabetic phenotype for the duration of intrauterine and early postnatal development, as properly as at some stage in adult life. Furthermore, epigenetic modifications may want to account for the accelerated prices of persistent and continual microvascular and macrovascular complications associated with diabetes(Keating and Osta 2013). An epigenetic phenomenon that is well-documented in human beings and might also be the first that springs to thinking is genomic imprinting, whereby all through germ mobilephone development, regulatory areas of certain genes are differentially methylated and expressed relying on whether or not the gene is inherited from the mother or father (Jaenisch, Bird 2003).Imprinting influences various genes, inclusive of some in which mutation of the expressed copy or disturbance of normal imprinting is involved in each most instances of the uncommon transient neonatal form of diabetes and, primarily based on latest evidence, curiously some cases of polygenic type 1 diabetes as properly (Pollin 2011). In T1D, epigenetic phenomena, such as DNA methylation, histone modifications, and microRNA dysregulation, have been related with altered gene expression. Increasing epidemiologic and experimental evidence helps the function of genetic and epigenetic alterations in the etiopathology of diabetes. (Stanko *et al.,* 2013).

3 - Epigenetic mechanisms in type 2 of diabetes:

Recently, epigenetic mechanisms had been proven to be concerned in endocrine cell differentiation and islet function. Genomic profiling of pancreatic islets in non-diabetic and diabetic states is wanted in order to dissect the contribution of epigenetic mechanisms to the

declining proliferation potential of β cells that we see with growing old or the β-cell failure determined in diabetes (Bramswig and Kaestner, 2012).type 2 diabetes, and has been proposed to end result from altered gene regulation patterns due to epigenetic changes of developmental genes. To decide whether or not epigenetic changes may additionally play a position in the improvement of adult diabetes following IUGR, we used a rodent model of IUGR that expresses decrease levels of Pdx1, a pancreatic and duodenal homeobox 1 transcription factor fundamental for β cell characteristic and development, which develops diabetes in adulthood (Park *et al.*, 2008).

Pdx1 is a pancreatic and duodenal homeobox 1 transcription factor that regulates pancreas development and β cell differentiation. Both genetic and obtained reductions in Pdx1 expression in human beings and in animal models have been proven to cause type 2 diabetes, β cell dysfunction (Kulkarni *et al.,* 2004). Epigenetic regulation of gene expression is one mechanism by means of which genetic susceptibility and environmental insults can lead to type 2 of diabetes. Therefore, therapeutic agents concentrated on epigenetic gene regulation can eventually be used to treat Type 2 of diabetes; however, there is a lot to be learned about genome-wide epigenetic programming of health and disease earlier than these treatments can be used in affected person care (Pinney and Simmons 2010).

4 - Novel candidate gene in diabetes:

There is proof that obese and diabetic human beings have a pattern of epigenetic marks unique from nonobese and nondiabetic individuals. The foremost long-term desires in this field are the identification and understanding of the position of epigenetic marks that may want to be used as early predictors of metabolic threat and the development of pills or diet-related treatments able to delay these epigenetic modifications and even reverse them(Martínez *et al.,* 2014). The 14 bp INS/DEL polymorphism in the 3′UTR of HLA-G can also have an effect on the susceptibility for diabetes and coronary artery diseases (CHD), therefore suggesting a novel candidate gene. DNA hypomethylation at HLA-G promoter may additionally be a putative beneficial medical biomarker for CHD onset. Up-regulation of soluble HLA-G isoform (sHLA-G) used to be detected in prediabetic and diabetic subjects, as a result suggesting a putative function in metabolic dysfunctions (Sommese 2019).on the different hand DNA methylation of eight genes chosen based totally on a literature review of candidates doubtlessly involved in Gestational diabetes mellitus and obesogenic pathways (IGF1, IGF2, H19, ARHGRF11, MEST, NR3C1, Adiponectin, and RETN) (Joyce *et al.,* 2019).

5 - Chromatin modification in diabetes:

Diabetic patients proceed to increase inflammation and vascular problems even after reaching glycemic control. This poorly understood “metabolic memory” phenomenon poses predominant challenges in treating diabetes. Recent research reveal a link between epigenetic modifications such as chromatin histone lysine methylation and gene expression. We hypothesized that H3 lysine-9 tri-methylation (H3K9me3), a key repressive and particularly steady epigenetic chromatin mark, can also be involved in metabolic memory (Villeneuve *et al.,* 2008). chromatin immunoprecipitation linked to promoter tiling arrays to profile H3 lysine-9 acetylation (H3K9Ac), H3 lysine-4 trimethylation (H3K4Me3), and H3K9Me2 in blood monocytes and lymphocytes bought from 30 DCCT traditional cure group topics (case subjects: suggest DCCT HbA1c level >9.1% [76 mmol/mol] and development of retinopathy or nephropathy via EDIC 12 months 10 of follow-up) versus 30 DCCT intensive therapy subjects (control subjects: imply DCCT HbA1c level <7.3% [56 mmol/mol] and except development of retinopathy or nephropathy) (Miao *et al.,*2014).

6 - Genes related to the overweight and obesity:

Obesity and metabolic issues are growing international and are related with intelligence atrophy and dysfunction, which are danger factors for late-onset dementia and Alzheimer's disease. Epidemiological research confirmed that modifications in lifestyle, along with the customary exercise of bodily workout are in a position to stop and treat no longer only obesity/metabolic disorders, however additionally to enhance cognitive feature and dementia. Several biochemical pathways and epigenetic mechanisms have been proposed to recognize the really helpful consequences of physical workout on cognition. (Barros *et al.*, 2019). These genes encompassed olfactory receptors (OR4D2, OR51A7, OR2T34, and OR2Y1) and quite a few downstream signaling molecules (SLC8A1, ANO2, PDE2A, CALML3, GNG7, CALML6, PRKG1, and CAMK2D), which notably regulated odor detection and sign transduction procedures inside the whole olfactory cascade, as published with the aid of pathway enrichment analyses ($p = 1.94 \times 10–10$). Moreover, OR4D2 and OR2Y1 gene methylation patterns strongly correlated with each day intakes of whole energy ($p < 0.0001$), carbohydrates ($p < 0.0001$), protein ($p < 0.0001$), and fat ($p < 0.0001$) (Ramos *et al.*, 2019).

7 - Roles microRNAs obesity and Histone alteration

The involvement of histone adjustments in the development of metabolic illnesses is now broadly appreciated. Over the current years, mass spectrometry-based proteomics methods located and mapped one-of-a-kind variety of histone changes linking obesity and metabolic diseases. The listing of these modifications is evergrowing; however, their features and roles in obesity are not nicely understood. Same as for the most properly studied histone modifications, specifically acetylation and methylation (Xu *et al.*, 2019). In the modern-day society, due to energy-rich diets, sedentary lifestyles, and environmental factors (such as pollution), many humans are overweight or obese (Romieu *et al.*, 2017). According to the World Health Organization (WHO), about 39% of the world's adult population used to be overweight and around one third of them (i.e., 13% of the world's adult population) had been overweight in 2016, and the global incidence of obesity has almost tripled considering 1975. In fact, obesity is mostly preventable. Weight loss can be completed with the aid of limiting consumption of fats and carbohydrates. Many dietary interventions had been proposed to combat the obesity epidemic (Austin *et al.*, 2011).

Recent research point out that obesity DNA and histone methylation levels, histone acetylation, and noncoding RNAs such as microRNAs (miRNAs) in oocytes and sperm. Several necessary genes, such as PPAR-α, Igf2, H19, Fyn, Stella, Sirt3, Sirt6, and Peg3 as nicely as miRNAs, such as let-7c, reportedly take part in the regulation of epigenetic changes in mammalian gametes (Ou, Zhu and Sun, 2019). In the remaining years, the preliminary discovery of epigenetic mechanisms represents the most applicable discovering to give an explanation for how the genome interacts with environmental factors and the ripple consequences on disease pathogeneses. Since then, all epigenetic process has been investigated via the scientific communities for almost two many years to decide which elements are concerned in this process. DNA/RNA methylation and miRNA are labeled as two of the most vital consultant lessons of such epigenetic mechanisms and dysregulated activity of such mechanism can definitely make contributions to disease pathogenesis and/or development specially in tumors((Ayers, Boughanem and Macías-González, 2019). Male obesity might also have intergenerational and even transgenerational consequences in mammals. Studies in rodents have published variations in energy metabolism and disease susceptibility in offspring of obese males, pointing to sperm epigenetic changes as likely causal factors (Duale *et al.*, 2019).

8 - Link between cardiovascular disease and epigenetics:

Epigenetics has been at the start studied in patients with cardiovascular disease for its outstanding role in irritation and vascular involvement (*Castro et al.,* 2003; Stenvinkel *et al.,* 2007). Furthermore, epigenetic research in cardiovascular remedy revealed a huge quantity of modifications affecting the development and progression of CVD. In addition, epigenomics are additionally worried in cardiovascular threat factors such as smoking (Buro-Auriemma *et al.,* 2013).

Epigenetic mechanisms encompass DNA methylation, histone modification, and microRNA alterations, which together allow the cell to reply rapidly to environmental changes. A quantity of cardiovascular diseaseEpigenetic threat factors, such as nutrition, smoking, pollution, stress, and the circadian rhythm, have been related with amendment of epigenet cardiovascular diseaseEpigenetic ic marks. Further examination of these mechanisms can also lead to beforehand prevention and novel remedy for cardiovascular diseaseEpigenetic (Ordovás and Smith, 2019). The genetic heritability of cardiovascular sickness can fluctuate significantly, relying on sex and on the situation in question. Data from some research recommend a extensive range of anywhere between about 40% to 80% genetic contribution to cardiovascular disease. Other factors regarded to have an effect on the occurrence of cardiovascular disease include environment-gene interplay (potentially mediated by using epigenetics), variations in gene imprinting, and traits of amniotic sac development, amongst others (Webster and Marsden 2013). Accumulating proof links cardiovascular getting old to epigenetic transformations encompassing a complicated interaction of DNA methylation, histone posttranslational modifications, and dynamic nucleosome occupancy ruled by means of severa epigenetic factors. Advances in genomics technological know-how have led to a profound understanding of chromatin reorganization in each cardiovascular growing older and diseases (Shirodkar and Marsden 2011).

Remarkably, each in utero programming and postnatal hypercholesterolemia can also have an effect on the epigenetic signature in the human cardiovascular system, thereby supplying novel early epigenetic-related pharmacological insights. Interestingly, some dietary compounds, consisting of polyphenols, cocoa, and folic acid, can modulate DNA methylation status, whereas statins might also promote epigenetic-based manage in cardiovascular disease prevention thru histone modifications (Sun *et al.,* 2013). Accelerated ageing', assessed by means of adult DNA methylation predicts cardiovascular disease . Adolescent accelerated getting older would possibly predict cardiovascular disease previously (Huang *et al.,* 2019). The rising quintessential nature of epigenetics for cardiovascular physiopathology and, importantly, the amenability to manipulation with pharmacological methods are an indication that epigenetics-based prognostic and therapeutics techniques would possibly be developed in the future (Elia and Condorelli, 2019).

9 - Histone alteration cardiovascular disease:

Chromatin is the complicated of chromosomal DNA related with proteins in the nucleus (Campos and Reinberg 2009). DNA in chromatin is packaged round histone proteins, in units referred to as nucleosomes. A nucleosome has 147 base pairs of DNA related with an octomeric core of histone proteins, which consists of 2 H3-H4 histone dimers surrounded by using 2 H2A-H2B dimers. N-terminal histone tails protrude from nucleosomes into the nuclear lumen. H1 histone buddies with the linker DNA positioned between the nucleosomes. Nucleosome spacing determines chromatin structure, which can be widely divided into heterochromatin and euchromatin two Chromatin shape and gene accessibility to transcriptional machinery are regulated through modifications to both DNA and histone tails (Fedorova and Zink 2008).

Histone modifications lead to adjustments in chromatin structure to render it energetic (euchromatin), in which DNA is reachable to transcriptional factors, or inactive (heterochromatin), in which DNA is inaccessible to transcriptional factors. Eight distinctive kinds of modifications catalyzed through wonderful enzymes have been described. The 2 most broadly studied histone changes are methylation and acetylation; much less properly studied modifications encompass phosphorylation, sumoylation, ubiquitination, ADP ribosylation, deimination, and proline isomerization (Kouzarides 2007). In addition, miRNA performs a numerous function in the pathological manner of cardiovascular disease. Numerous research have determined that some cardiac-specific miRNAs have achievable as certain diagnostic biomarkers and treatment goals for cardiovascular disease. the aberrant epigenetic mechanisms that make a contribution to cardiovascular disease will be discussed (Duan *et al.*, 2019).

CONCLUSIONS

There is no doubt that histone modification and DNA methylation as two main types of epigenetics are can affect to developed metabolic disorder and there different genes for each disease can be changes.

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