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# INCREASED SURVIVAL CHANCES OF THE SPECIES QUERCUS PETRAEA IN TERMS OF POLLUTION WITH CD AND CU BY USING MICROBIOTA-BENTONITE SYSTEMS

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**Abstract:** *Quercus petraea*, a native tree species belonging to the regional flora of Baia Mare area, was tested for its resistance to heavy metal pollution in combination with systems composed of microorganisms amd bentonite. Native strains of microorganims (*Aspergillus niger, Sarcina flava*), from the flotation tailings of Bozanta Mare tailing pond were isolated, cultured and concentrated, and then used in pure cultures or in combination with bentonite. The strains of microorganisms, and the mixture of microorganims with bentonite, respectively, were included in the substrate of *Quercus* seedlings roots, the subjected to artificial pollution of Cd and Cu ions. It measured the concentrations of metal ions in the seedlings of different plant organs of experimental variants. Related to witness without any microorganisms or bentonite, the system bentonite-*Aspergillus* induced a decrease in the Cd concentrations in roots with 84.53%, in stem with 99.65% and in the leaves with 99.77%. The mixed system consisting in bentonite and bacteria drastically reduces copper availability for the plant. Use of this ”material” significantly reduces the concentration of Cu in plants with: 89.2% in the root, 79.69% in the stem and 90.86% in the leaves.

**Keywords:** Quercus petraea, Sarcina sp., Aspergillus, $Cd^{+2}$, $Cu^{+2}$, heavy metal resistance, native flora.

## 1. INTRODUCTION

Throughout the world there is growing concern that the heavy metal content of soils are increasing as the result especially of mining and industrial activities (Vega et al., 2004); Sastre et al., 2006; Wan-Xia et al., 2009). For the areas situated near of metallurgical platform where soils have different uses, heavy metal concentrations exceed normal concentrations and the allowable maximum limit, especially for Pb, Zn and Cu (Damian et al., 2010a). Heavy metals are difficult to remove from the environment. These metals can not be chemically or biologically degraded, and are ultimately indestructible.

Remediation of these lands contaminated with heavy metals including, among other processes, bioremediation. Bioremediation is an umbrella concept that covers various layers of multiscale complexity involved in polluted land cover with a green coated and/or the disposal of toxic waste from the contaminated sites (Lorenzo, 2008). The potential use of trees as a suitable vegetation cover for heavy metal-contaminated land has received increasing attention over the last years. (Pulford, 2003). Trees have been suggested as a low-cost, sustainable and ecologically sound solution to the remediation of heavy metal-contaminated land (Punshan et al., 1999). Trees have massive root systems, which help to bind and stabilise the soil (Stomp et al., 1993), and the litter add to the surface of areas an organic cover over the contaminated soil. In addition, transpiration of water by the trees reduces the overall flow of water down through the soil, thus, helping to reduce the amounts of heavy metals that are transferred to ground and surface waters. In this vein, using native species of trees can be the cleanest solution, ensuring observance of polluted areas in the natural landscape. Otherwise, the use of alien species, even if they are resistant to heavy metal pollution, can produce a new form of biological pollution in the area. Benefits can arise mainly from stabilisation of the soil or mining waste, named *sterilum*. Before these benefits can be realised, the trees must become established on a site. On highly contaminated soils, or on mining wastes, tree establishment may be inhibited by high concentrations of heavy metals (Schippers et al., 2000; Jelea et al., 2007). Under such conditions root immobilisation, which would normally protect a plant, may not be able to prevent toxic amounts of metal being translocated to the aerial parts of the plant. Other factors such as: macronutrient deficiencies, physical conditions, water or aeration deficit may limit plant growth (Pulford & Watson 2003). Using of soil bacteria (often plant growth-promoting bacteria) can significantly facilitate the growth of plants in the presence of high (and otherwise inhibitory) levels of metals. In the reported studies, which encompass a range of different plants, metals, soils and bacteria, the effect of adding bacteria is typically to facilitate plant growth. These studies are done with the expectation that the selected metal resistant bacteria will be able to both proliferate and promote plant growth in the presence of high levels of toxic metals (Glick, 2010).

We consider that the consolidation of a protective vegetal layer can be adequately helped by soil microorganisms: bacteria, fungi and yeasts. For this purpose we tested the influence of types of microorganisms, alone and in the systems associated with the bentonite on the level of heavy metals absorption by plants.

## 2. MATERIALS AND METHODS

### 2.1. Soils used in experiment

The soil used was taken from the South of Maramures County and is the type eutricambisol. Eutricambisolul was prelevated from a oak forest, typical of tested species. The soil was prelevated from the same place where acorns were collected (Codrului's peak, a region of oak forested hills) and used experimentally. Eutricambosoil is structurally less differentiated, with small differences between horizons. Chemical reaction is moderately acidic (pH: 5.5-6). Soil is moderately to acid reaction, low in humus, mediocre in total nitrogen, total phosphorus as mediocre, in poorly supplied with phosphorus and low in mobile potassium. Size analysis revealed that the predominant soil mass fractions of coarse sand and fine crystalline shale remaining after disintegration, still unspoiled, so much texture profile is the middle. In the soil absorbing complex, predominates $H^+$ ions, which falls in the category of unsaturated in base soil. Analytical data show that this type of soil provides favorable ecological conditions for forestry species such as oak.

### 2.2. Bentonite used in experiment

Source of bentonite is Orasu Nou, a village in Satu Mare county. The samples were taken from the point Mujdeni. Literature characterized bentonite used. From geological point of view, the area belongs to the Neogene period, the basement consisting of sedimentary and volcanic deposits (Damian et al. 2010b). Sedimentary rocks are marine deposits (marls and fine sandstone). Volcanic rocks have a thickness of up to 300 m and consist of rhyodacites in ignimbritic facies, lapilic tuffs and pyroclastic rhyodacites, perlite. Piroclastic rocks and perlite are altered, and thus generates bentonite deposits.

Bentonite, hosted in the volcanic formation, is in the form of lenses, with thicknesses ranging from 3 to 8 m, with lengths of 100-400 m and widths of 50-250 m. The actual bentonite deposit is followed by unaltered perlite, clay and a soil blanket. Transformation of piroclastic rocks and perlite in bentonite is related with deuteric weathering with an alkaline pH, favored by porosity of rocks. Bentonite are generally white, fine and compact grain, preserving or not, the perlite structure.

In the terms of mineralogy, the mineral clays consist in montmorillonite, subordinate, kaolinite, illite, cristobalite, carbonates, zeolites (clinoptilolite), iron oxides and hydroxides as relicts of primary minerals, quartz and felspat.

The proportion between the montomorillonite and kaolinite group minerals varies with the degree of transformation of the original rock. The content of the montmorillonite is between 20-85%, depending on the stage of rock alteration and can reach up to 95%. The chemical composition of the bentonite from Orasu Nou is as follows: $SiO_2$- 60.62-71.46; $TiO_2$ – 0.15-0.36; $Al_2O_3$- 15.40-23.55; $Fe_2O_3$ – 0.84-2.34; CaO – 1.18-1.86; MgO – 0.54 – 2.05; $Na_2O$ – 0-0.3; $K_2O$ – 0.25 – 2.16; PC. 8.69 – 12.4. (Măicăneanu et al., 2009).

### 2.3. Vegetative experiment

*Quercus petraea* seedlings, with the same genetic origin, germinated in vitro in the laboratory of biotechnology at the University of Baia Mare, were used. At 30 days after germination, 135 of seedlings were transferred to sterile soil and acclimatized to the conditions of 22$^{o}$C, humidity of 75% and duration of 14 hours in room lighting vegetation Sanyo MLR 351H. Each pot contained 250 g soil and one plant individual.

After 60 days of acclimatization, during which the seedlings have reached sizes of 25-30 cm, in the soil were incorporated cultures of microorganisms, system of microorganisms and bentonite, respectively, in following experimental variants (each consist in 15 individuals):

a. soil with bacterial cultures
b. soil with bentonite-bacteria system
c. soil with fungi cultures
d. soil with bentonite-fungi system
e. witness without fungi or bacteria

After 7 days of the inclusion of micro-organisms in soil systems, has been done on artificially heavy metal pollution. Four experimental versions have been polluted with cadmium ions, and four have been polluted with Cu ions. Experimental pollution was done with Cd acetate solution in a concentration of 500 mg/l. For each plant were given 10 ml of solution every 48 hours for 18 days. Also, the experimental variants polluted with Cu (in $CuSO_4$ solution), dosage and administration were identically.

*Quercus* specimens were grown on type eutricambisol soil, characteristic of oak forests, coming from the Codru's region, Maramures County.

### 2.4. Preparation of microorganisms biomass and the systems bentonite-microorganisms

The microorganism strains used in the studies were isolated from native microbiota from tailing pond, located in Bozanta Mare (Maramures County). Tailing samples were taken from horizon surface and from the rhizosphere. The samples were brought into solutions, were inoculated on Thorthon medium, obtaining colonies of bacteria, fungi and yeasts. Colonies of bacteria and fungi were isolated, cultured, multiplied on agarose first, and then in the liquid medium. To obtain the bentonite – bacteria, bentonite-fungi respectively, the cultivation of the strains of microorganisms was done on a medium consist of 15 g bentonite in 25 ml nutritive medium, during 72 hours. The cultures were maintained on a rotary shaker (50 rpm), 23$^{o}$C. For bacteria strain the growth medium was Thorthon medium and for fungi, we used a medium with yeast extract.

The harvested biomass of pure culture of bacteria and fungi were incorporated in the soil with the liquid medium, for each plant 25 ml medium containing microorganisms. The systems bentonite-microorganisms were washed with distilled water and than, have been incorporated into the soil to the roots of trees (25-30g system bentonite-microorganisms for each individuals).

### 2.5. Analitical procedure

All samples solutions for vegetal samples were analyzed with AAS. The vegetal samples (roots, stems and leaves) were taken from every experimental variant, washed with deionized water, dried at 60$^{o}$C for 72 hours, brought into the stage of dust. 100 g of dust were taken from each sample subjected to mineralization and then brought into solution. The samples were diluted, filtrated (prepared according to ISO11466) and analyzed from the point of view of heavy metals (Cu, Cd) through atomic spectrophotometry according to AAS 800. A witness sample without vegetal material was also analyzed.

### 2.6. Statistical analysis of data

Data were analyzed using Past Paleontological Statistics Version 1.99 and tested for detrended correspondence analysis principal coordinates analysis (DCA) for a measure of variance and a distance between different groups of samples. DCA may be used for simple reduction of the data set to only two variables (the two most important components), for plotting purposes. For heavy metal content, three analyses with three repeats were carried out for each experimental variant of plant. Detrended correspondence analysis (DCA) is a procedure for finding hypothetical variables (components) which account for as much of the variance in your multidimensional data as possible, (Oxanen & Minchin, 1997). These new variables are linear combinations of the original variables. DCA is specialized for used in ecological data sets and may be used for simple reduction of the data set to only two variables (the two most important components), for plotting purposes. One might also try to hypothesize that the most important components are correlated with some other underlying variables (Oxanen & Minchin 1997).

## 3. RESULTS AND DISCUSSIONS

*Quercus petraea* belongs to the regional flora, without being considered a plant resistant to high concentrations of heavy metals. Studies on resistance to heavy metals are very few in the case of this species. Cadmium (Cd) and copper (Cu) are of highly bioactive and toxic elements, there presence at elevated levels in soil or in water, could be dangero us for plants, animal and human. The literature does not record experiments on *Quercus* resistance to the high concentration of Cd.

Table 1. Present the concentrations of $Cd^{+2}$ and $Cu^{+2}$ in the vegetative organs of experimental variants. Each value means an average of the three measurements made.

| Vegetal sample | Witness without pollution mg/l M1 | | Witness without microorganisms mg/l M2 | | Bacterian concentrated Mg/l B | | Bentonite-bacteria sysyem Mg/g B-B | | Fungi Mg/g F | | Bentonite-Fungi system Mg/g B-F | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cd | Cu | Cd | Cu | Cd | Cu | Cd | Cu | Cd | Cu | Cd | Cu |
| Root | 0.004 | 0.001 | 4.21 | 6.31 | 2.265 | 4.34 | 0.006 | 0.681 | 3.52 | 5.466 | 0.651 | 3.535 |
| Stem | 0.003 | 0.000 | 1.43 | 3.12 | 0.492 | 2.181 | 3.275 | 0.883 | 0.008 | 2.191 | 0.005 | 1.586 |
| Leave | 0.000 | 0.000 | 0.76 | 1.04 | 0.000 | 0.01 | 2.478 | 0.095 | 0.001 | 0.005 | 0.001 | 0.002 |

Usually, excess Cd causes toxic symptoms in plant, e.g growth retardation, inhibition of photosyntesys, induction an inhibition of enzymes, altered stomatal action etc. (Cosio et al., 2006). Studies on other wood species (e.g. *Salix viminalis*) indicate that the toxic simptoms increased progressively with incresing the concentration of $Cd^{+2}$ in solution between 5-200 µM. (Cosio et al., 2006).

By comparison, *Quercus petraea*, proves, at least in the early stage of growth, resistance of $Cd^{+2}$. The tolerant mechanisms of Cd tolerant plants have been reported previously (Sun et al., 2008). They included two strategies: exclusion and accumulation. In this case it is about exclusion, not about the accumulation. Cd concentrations alowed by the experimental plants used are reduced even more as supplements with the microorganisms and bentonite are used.

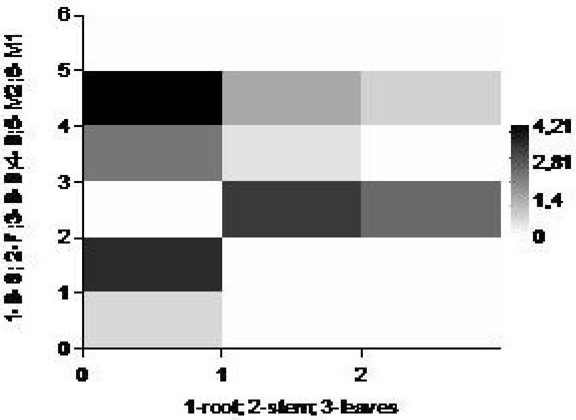



Figure 1. Matrix of Cd concentrations in the experimental variants

Stability is enhanced by the administration to the plants root of microorganisms or bentonite-microorganisms systems. As shown in the table 1, the absorbtion of Cd in roots and its accumulation in plants is lower in the experimental variants against witness M2.

As seen in figure 1 a lowest level of Cd are recorded in the experimental variants treated with the pure culture of *Aspergillus* and bentonite-*Aspergillus* system, respectivelly.

*Aspergillus niger* is an important microorganism in biotechnological applications. Waste biomass of *A. niger* is used to remove hazardous heavy metal ions, such as cadmium, lead, chromium and copper from aqueous solution. Studies cited in the literature demonstrates that live strain of *Aspergillus niger* are able to adsorb amounts of Cd ranging from 1.31-3.43mg/g DW. (Wang & Chen 2009). This study demonstrates that *Aspergillus* strains are not only an efficient biosorbent material, but also a partner that can support the growth of trees under stress of Cd.

Related to witness M2, the system bentonite-*Aspergillus* induced a decrease in the Cd concentrations in roots with 84.53%, in stem with 99.65% and in the leaves with 99.77%. Using a strain of *Aspergillus* supplement was also effective in reducing the absorbtion of Cd. Compared with untreated plants (M2), the individuals helped by the fungi have absorbed less: with 16.38% in the root, with 99.44% in the stem and with 99.86% in the leaves (Fig. 2).

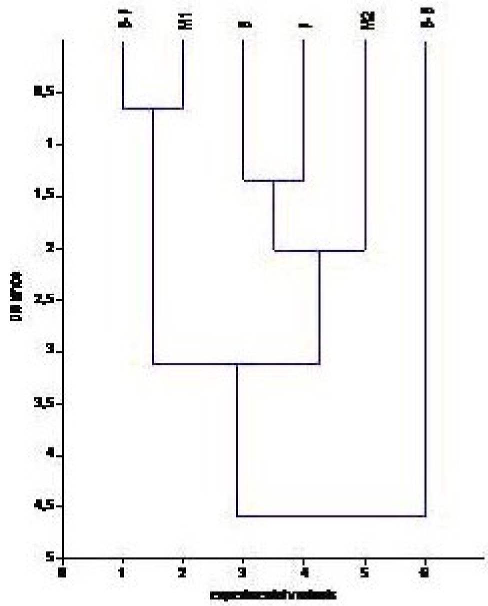


Figure 2 Cluster analysis according Euclidian distance index

Studies of literature (Prasad, 1995; Wang & Chen, 2009) cites a number of bacteria able to retain large amounts of cadmium ions, such as: *Pseudomonas* (cca 278 mg/b biomass), *Staphylococcus xylosus* (250mg/g), *Aeromonas caviae* (155.3 mg/g) etc.

We isolated from the contaminated area, and used a *Sarcina flava* strain. This specis has shown to retain cadmium, decreasing the level of ions available to plants.

Addition of pure bacteria culture diminished the acces of Cd ions into the plants. In particular, bacteria retained soluble cadmium and consequently, decreasing the cadmium level in the stems and leaves. In this case, the Cd concentrations in the plants are: 53.80% in the root, 43.40% in the stem and 0% in the leaves, compared with the control.

The system consists in of bentonite and bacteria has proved ineffective in retaining cadmium. Bentonite inactivated bacteria and cadmium remained available to enter into the plant. This explains the high levels of Cd in stem (with 229.02% more than witness) and leaves (with 326.05 more than M2) with in the experimental variant treated with bentonite-bacteria system.

Cluster analysis performed on the Euclidian distance index, emphasizes the betonita-fungi system and culture of fungi efficiency, in support of *Quercus* individuals in term of Cd pollution. Index distance between B-F and M1 (unpolluted) versions is less than unity (between 0.5 and 1), and from the polluted witness (M2), record an amount of over three (between 3 and 3.5) of 5 – maximum level.

Copper is hydrosoluble ion, than penetrates the plant and often, comes up in the leaves, affecting chlorophyll and, thus photosyntesis. Copper distribution in the root, stem and leaves follows a univariate model (in the all experimental variants), as shown in the figures 5-6.

In the all experimental variants analyzed, the concentration of copper in the plants organs, is lower than that of M2 (Figs. 3 and 4). This finding indicates the efficiency of both, the pure strains of microorganisms, and mixed systems, in support of species on polluted land.

In comparative terms, both the absolute concentrations (Fig. 3), and statistical analysis (Euclidian and detrended correspondence analysis) (Fig. 5) showed that mixed system consisting in bentonite and bactera drastically reduces copper availability for the plant. Use of this ”material” significantly reduces the concentration of Cu in plants with: 89.2% in the root, 79.69% in the stem and 90.86% in the leaves. Near as efficiency is mixed bentonite-fungus system, while the pure strains of microorganisms (both bacteria and fungi), sequester copper in a lesser degree.

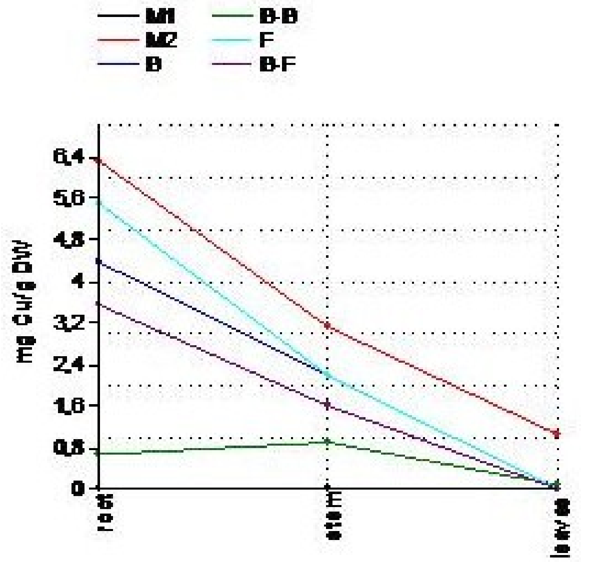



Figure 3 The concentration in the different exerimental variants.

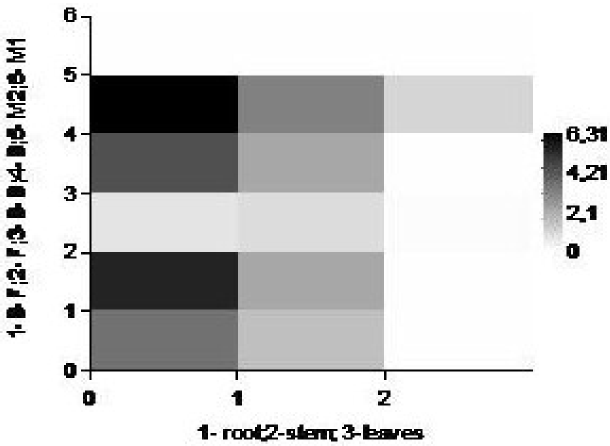
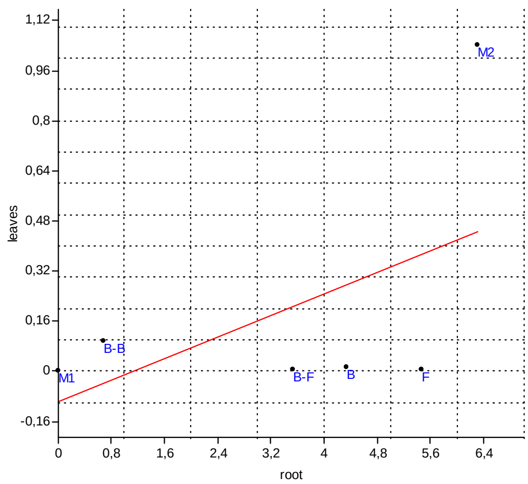



Figure 4 The matrix of Cu distribution in the vegetativ organs in the different experimental variants




Figures 5 The univariat model of $Cu^{+2}$ distribution between root and leaves

The results converge to those presented by the literature, according to which the copper is adsorbed by the microbial biomass in amounts of: 381 mg/g (*Bacillus firmus),* 96.9mg/g (*Pseudomonas putida)*, 66.7mg/g (*Streptomyces coelicolor*) (Wang & Chen 2009). The results of tested plants confirmed the tests done on copper adsorbtion by microorganisms isolated, respectively by the composite systems in solution with concentrations of 250 and 500 mg/l.

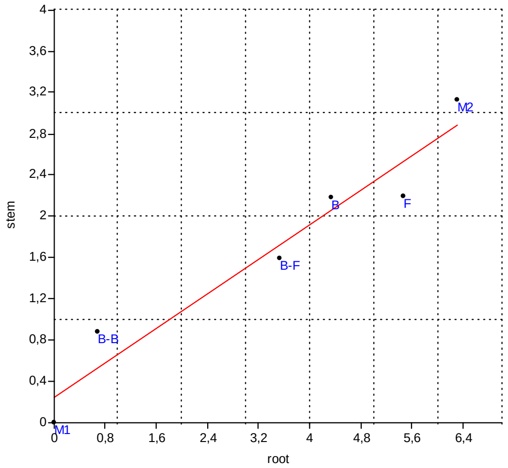



Figure 6 The univariat model of $Cu^{+2}$ distribution between root and stem

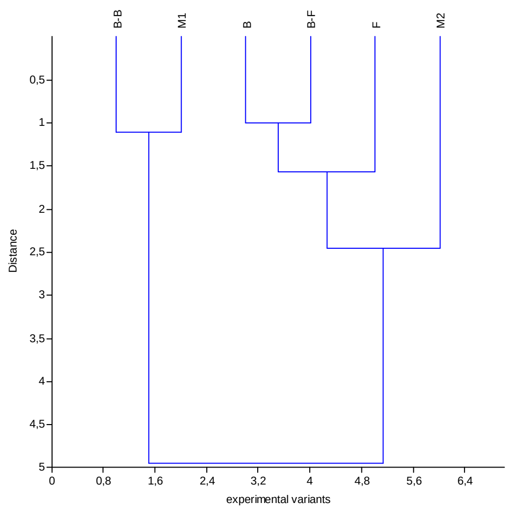



Figure 7 The cluster analysis based on Euclidian index of Cu concentration between experimental variants

Just as in the pure solutions (where retained more than 60% than initially level), and incorporated into the soil, the system composed of bentonite and bacteria, retains most of Cu ions, reducing the levels accesible to the trees roots.

This demonstrate the possibility of using composite materials of bentonite and native strains of microorganisms, increasing the chances of survival in some species of trees belonging the regional flora, in the contaminated areas.

Results of laboratory experiments support the observations ”in situ”, according to which, even considered susceptible tree species, aided by microorganism, may participate in the formation of ecosystems on land contaminated with heavy metals (Margui et al., 2007; Marian et al., 2010).

Statistical processing of experimental results, highlights that the Euclidian distance index, applied of copper concentrations in the experimental variants, has a minimum value when using bentonite-bacteria sysyem. The value between B-B and M1 samples being close to 1, and between B-B and M2 approaching to the maximum (Lorenzo, 2008) (Fig. 8).

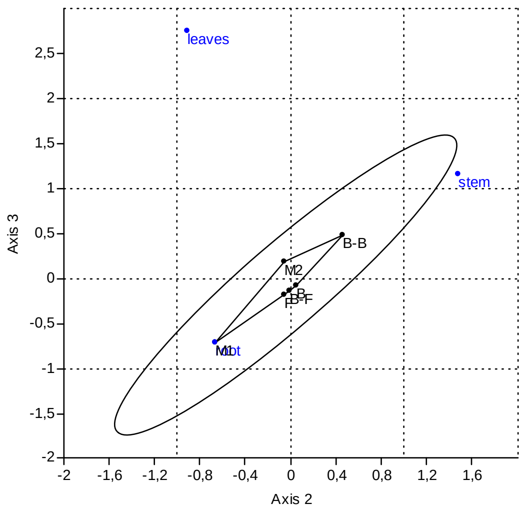



Figure 8 The detrended correspondence analysis for the Cu concentration between experimental variants

The detrending correspondence analysis expressed in the figure 7, suggests both the distance between the analysed samples, and grouping them accprding to the reporting of the same factor, the final concentration of copper in the plant organs.

## 4. CONCLUSIONS

As indicated in the results, the pot culture experiment, conducted with contaminated soils, indicated that *Quercus petraea* had capability to tolerate high levels of Cd and Cu, even in the initial stage of development, when the seedlings are fragile and sensitive. Resistence of trees to the pollution by Cd and Cu can be significantly increased by using materials that adsorb and retain large amount of heavy metals.The cultures of bacteria and fungi selected from the polluted sites, pure or in combination with bentonite, reduce the concentration of heavy metlas available to plants, contributing to the ecological restoration of polluted sites in a manner as close to natural ecoystems and regional native flora.

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