# SPRUCE BARK EXTRACT AS MODULATOR IN RAPE PLANT COPPER BIOACCUMULATION

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The aim of this research work was to investigate the influence of spruce bark natural bioactive compounds on copper bioaccumulation in rape plants. To eliminate the influence of soil characteristics and microorganism interaction, germination tests and hydroponic culture experiments were carried out in the presence of different polyphenolic aqueous extracts and various copper ion concentrations (10, 25, 50 mg/L $CuSO_4$). To evaluate the effects of spruce bark polyphenolic extracts (0.580, 1.004, 1.912 g dry matter content/L), the following parameters were determined: content of chlorophyll **a** and **b**, concentration of copper ions at root level and their translocation to hypocotyls and cotyledons, as well as the bioaccumulation factor. The presence of spruce bark extracts in a copper contaminated environment stimulates the development of rape plantlets and blocks the heavy metal access to the plant.

***Keywords***: spruce bark, assimilatory pigments, bioaccumulation

## INTRODUCTION

Heavy metal contamination of agricultural soils is a major environmental problem that can reduce both the productivity of plants and the safety of plant products as foods and feeds. Remediation of metal contaminated soils is imperative since metals will persist for an almost indefinite period of time in the environment, as due to their stability.[1,2]

There are two possibilities to deal with heavy metal contaminated soil: phyto-extraction and phyto-stabilisation. Phyto-extraction (phyto-accumulation) is a nondestructive technique, aesthetically pleasing by its nature, developed to remove the trace elements from the soil through their uptake and accumulation by plants. This technique is only effective if plants accumulate large concentrations of metals/metalloids in shoots and have a reasonable biomass production.[3] Phyto-stabilisation (phyto-immobilisation) aims at establishing a vegetation cover and at promoting *in situ* inactivation of trace elements by combining the use of metal-tolerant plants and soil amendments that help reduce the mobility and toxicity of pollutants and, at the same time, may increase soil fertility and improve plant establishment. The plants involved in phyto-stabilisation should retain the metals at root level, with restricted transport to the aerial parts, to avoid further transfer into the food chain.[4]

Natural organic acids have been proposed to enhance phyto-extraction,[5] as due to their higher biodegradability, but they can also be a drawback for efficient phyto-extraction.[6] It was noticed that citric, oxalic, vanillic and gallic acids, applied at 10 or 20 mM/kg, solubilized significant amounts of Zn, Ni, and Cd from the soil.[7]

The present work was carried out to compare the effectiveness of spruce bark extract (in three different concentrations of natural bioactive compounds) applications on the availability and accumulation of copper by rape plants (*Brassica napus*).

## EXPERIMENTAL

### Polyphenolic aqueous extraction

Spruce bark was ground to a fine powder of 0.5 mm with a grinder 5, 10 and 20 g of dried

ground material were successively extracted (3 times) with 125 mL water in a water bath at 80-90 °C for 45 min. The aqueous filtrates were collected and completed to a volume of 500 mL with distilled water. The extracts were suggestively named SB5, SB10, SB20, corresponding to the extracted quantity of vegetal biomass (5, 10 and 20, respectively).[8]

The dry matter content in the extracts was determined by evaporation of 25 mL extract on a water bath and drying at 105 °C up to constant mass, according to the Polish Standard PN–90/A75101/03. After cooling at room temperature in a dessicator, the 105 °C dried sample was placed into a muffle furnace at 600 °C for 6 h, to quantify the organic matter content.

The total polyphenolic compounds (TPC) concentration was determined by the Folin-Ciocalteu colorimetric method.[9] Measurements were carried out in triplicate and calculations were based on a calibration curve obtained with gallic acid. TPC were expressed as mg of gallic acid equivalents (GAE) per L of aqueous extract.

**Rape plantlet experiments**

The rape seeds were germinated on wet filter paper in Petri dishes, under different treatment conditions. The main tested solutions were represented by 10, 25, 50 µg/mL copper ions and spruce bark extract in which $CuSO_4 \cdot 5H_2O$ was dissolved to obtain the same copper concentration. The Petri dishes were incubated for 7 days at dark and kept under controlled temperature, at 29 °C, then exposed to daylight for 3 days, for chlorophyll assimilation.

After 5 days, the healthy and uniform rape plantlets that germinated in distilled water (Control) were selected and transferred to a Hoagland nutrient solution (1 mM $KH_2PO_4$, 5 mM $KNO_3$, 5 mM Ca $(NO_3)_2 \cdot 4H_2O$, 2 mM $MgSO_4 \cdot 7H_2O$, 11.8 µM $MnSO_4 \cdot H_2O$, 0.7 µM $ZnSO_4 \cdot 7H_2O$, 0.32 µM $CuSO_4 \cdot 5H_2O$, 0.16 µM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 46.3 µM $H_3BO_3$, 5 µM Fe) in a hydroponic culture experiment under natural light. The nutrient solution was replaced once a week. The tested solutions were denominated as: Cu-10, Cu-25, Cu-50 (10, 25, 50 mg/L copper ions in a water solution) and SB5Cu-10, SB10Cu-10, SB20Cu-10, SB5Cu-25, SB10Cu-25, SB20Cu-25, SB5Cu-50, SB10Cu-50, SB20Cu-50 (10, 25, 50 mg/L copper ions in spruce bark aqueous extract obtained from 5, 10, 20 g vegetal raw material).[10]

**Plantlet analysis**

***Biometric and quantitative determination***

After harvesting, the plantlets were rinsed once in tap water and twice in distilled water, to eliminate any trace of adherent particles. Biometric measurements of rootlets, hypocotyls and cotyledons were done to estimate the growth index of *Brassica napus* (stem length/(root + stem length)) under copper stress conditions and spruce bark polyphenolic amendments. The plants were separated into roots, hypocotyls, cotyledons and weighed for Fresh weight (FW) determination.

***Photosynthesizing pigments***

Chlorophyll was extracted in 80% acetone and determined spectrophotometrically by reading the absorbance values at 646 and 663 fixed wavelengths. Specific extinction coefficients were used to quantify chlorophyll **a** and **b**.[11] Chlorophyll assimilation was expressed in µg/g green biomass.

***Determination of copper in plant tissue***

Once separated into roots, hypocotyls, the cotyledon rape samples were dried in an oven at 65 °C for 48 h until constant mass.[12] For Cu analysis, the plant tissues were digested with nitric acid and hydrogen peroxide (0.1 g in 8 mL $HNO_3$ and 2 mL $H_2O_2$) on a hot plate, for at least 5 h.[13,14]

The concentrations of heavy metals were determined on a GBC Avanta flame atomic absorption spectrophotometer (www.ch.tuiasi.ro/cercetare/PNCDI/MEDRES-LAB/index.php). The wavelength for Cu determination was of 324.8 nm. The following formula was used for calculating the parameters:

Translocation factor (TF) = ratio of metal concentration in shoots/ratio of metal concentration in roots;[15]

Bioaccumulation coefficient = copper content/g dry plant tissue/copper content mL/nutrient solution.[16]

## RESULTS AND DISCUSSION

Spruce bark aqueous extract characterization shows that the dry and organic matter content increase with increasing the quantity of extracted vegetal raw material (Table 1). The total polyphenolic content presents relatively constant concentration values (191, 190 mg/L extract) for SB10 and SB20 aqueous extracts.

The biometric measurements of roots, hypocotyls and cotyledons obtained by germination tests indicate a decreasing trend of rape plantlet length. Rootlet and hypocotyl length development was inhibited with increasing copper concentrations (Fig. 1) The presence of spruce bark extracts in the growth medium temperates the metal inhibitory effect, according to polyphenol and copper ion concentrations.

This effect could be better observed in hydroponic culture experiments (Fig. 2), where it is obvious that the polyphenolic

aqueous extracts stimulate root and hypocotyl elongation. The stimulatory effect on root elongation decreased with increasing metal concentrations. SB20 stimulate rape hypocotyl elongation even at higher contamination levels.

The growth index presents almost the same tendency for both germination tests and hydroponic culture. The most pronounced inhibition effect on rape plant growth and development was observed under the influence of SB10Cu-10 and Cu50 treatments, while a positive effect was registered in the presence of SB20 in the growth medium (Fig. 3).

Table 1
Dry matter, organic matter and total polyphenolic content of spruce bark aqueous extracts

| Extract type | Dry matter content (g/L extract) | Organic matter content (g/L extract) | Total polyphenolic content (mg/L extract) |
|---|---|---|---|
| SB5 | 0.58 | 0.57 | 130 |
| SB10 | 1.00 | 0.96 | 191 |
| SB20 | 1.91 | 1.79 | 190 |

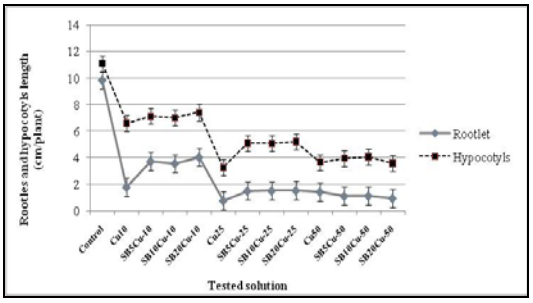

Figure 1: Root and hypocotyl elongation in germination tests

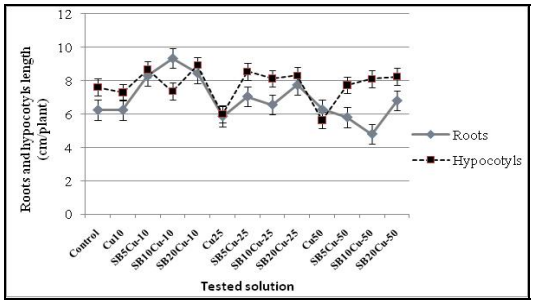

Figure 2: Root and hypocotyl elongation in hydroponic cultures

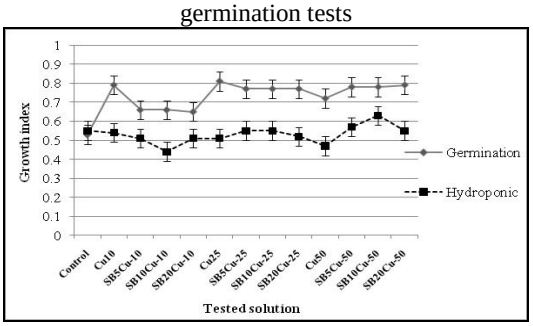

Figure 3: Growth index variations in germination tests and hydroponic cultures

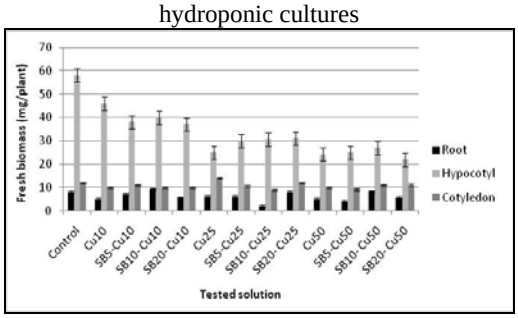

Figure 4: Root, hypocotyl and cotyledon fresh biomass in germination tests

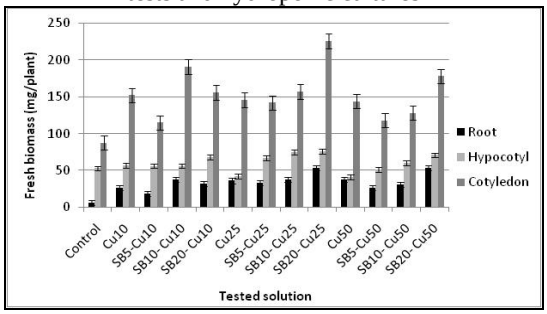

Figure 5: Root, hypocotyl and cotyledon fresh biomass in hydroponic cultures

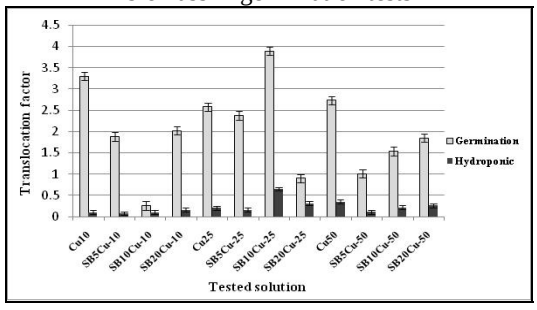

Figure 6: Translocation factor of copper ions obtained in germination tests and hydroponic cultures with different solutions

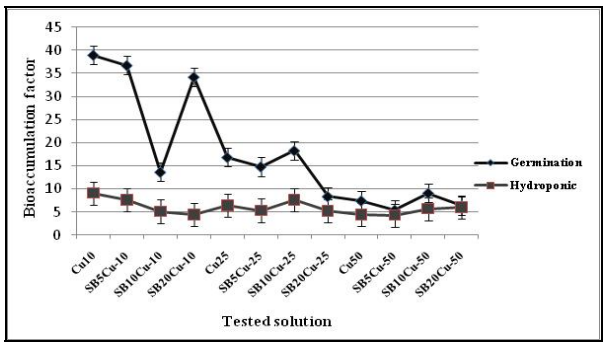


Figure 7: Copper bioaccumulation by rape plant in germination tests and hydroponic cultures

Rape plant fresh biomass yield was predominantly inhibited in the presence of higher copper concentration in the germination test, even with a spruce bark amendment solution. The lower quantity (mg/plant) of fresh biomass yield was recorded in the case of Cu-25 and Cu-50 contaminated environments (Fig. 4).

In hydroponic culture experiments, the green biomass yield was higher in all tested solutions, compared to the reference (Fig. 5). Significant stimulating effects on fresh biomass accumulation were observed under spruce bark amendments obtained from 10 and 20 g raw material.

In germination tests, pigments content decreases with increasing copper concentration. No significant stimulatory effects on chlorophyll **a** and **b** assimilation were provided by spruce bark extract amendments. When applying Cu-50 (germination tests) and Cu-10 (hydroponic culture) spruce bark extract solutions, stimulation effects were registered (Table 2).

Copper concentration in rape plant increases by increasing the copper contamination level in the growth medium. It was observed that, in the presence of spruce bark extract, the copper ions were predominantly retained at root level (Table 3). The polyphenolic extract blocks the access of copper ions to the upper level (hypocotyls and cotyledons) of rape, which explains why the translocation factor took minor values comparatively with Cu-10, Cu-25, CU-50 (Fig. 6). This could be explained by the possible complex formation of copper ions with polyphenolic compounds.

The copper bioaccumulation factor decreases with increasing copper concentration in the medium, as observed in Figure 7. The spruce bark extract obtained from 5 and 20 g raw material reduced copper accumulation, while SB10 contributed to a higher bioaccumulation factor. Such effects could be correlated with polyphenolic extract composition, and with the growth and development of the rape plant. The stimulating effect on copper bioaccumulation has a negative impact on rape growth index and on chlorophyll accumulation, as well.

## CONCLUSIONS

The results obtained showed that the presence of spruce bark extracts in a copper-contaminated environment stimulates the development of rape plantlets and blocks the access of the heavy metal to the plant. This process depends upon both copper concentration level and tested concentration of the total polyphenolic content of spruce bark aqueous extract.

It seems that the characteristic natural bioactive compounds of spruce bark aqueous extract complex with the copper ions, decreasing the availability of the latter in the soil, allow a proper development of rape plants, even in a heavy metal contaminated environment.

Depending on extract concentration and copper level contamination, the spruce bark extract could be used as both assisted phytoremediation amendment and plant protector. In the case of a 191 mg/L concentration in TPC, the spruce bark aqueous extract is able to modify the bioavailability of heavy metals in soils, and stimulate copper bioaccumulation. Therefore, it could be properly used as a natural amendment to improve phytoremediation.

The extracts of spruce bark with 130 and 191 mg/L TPC concentrations promote *in situ* inactivation, stimulate rape plant growth

and development, reduce copper bioaccumulation to the upper part of the plant, acting as a natural plant protection agent.

Table 2
Rape plant pigments assimilation in germination tests and hydroponic cultures

| Experiment type | Tested solution | Chlorophyll concentration (µg/g green biomass) | | | |
|---|---|---|---|---|---|
| | | Chl a | Chl b | Chl a+b | Chl a/b |
| Germination test | Control | 209.17 | 52.70 | 261.88 | 3.96 |
| | Cu 10 | 291.06 | 121.27 | 412.337 | 2.40 |
| | SB5Cu-10 | 102.73 | 127.67 | 230.40 | 0.80 |
| | SB10Cu-10 | 53.47 | 19.86 | 73.33 | 2.69 |
| | SB20Cu-10 | 122.43 | 51.26 | 173.70 | 2.38 |
| | Cu25 | 332.63 | 85.62 | 418.26 | 3.88 |
| | SB5Cu-25 | 159.49 | 96.62 | 256.12 | 1.65 |
| | SB10Cu-25 | 239.42 | 60.58 | 300.00 | 3.95 |
| | SB20Cu-25 | 66.84 | 100.86 | 167.71 | 0.66 |
| | Cu50 | 89.20 | 53.51 | 142.72 | 1.66 |
| | SB5Cu-50 | 176.39 | 62.34 | 238.73 | 2.82 |
| | SB10Cu-50 | 120.18 | 32.67 | 152.86 | 3.67 |
| | SB20Cu-50 | 159.58 | 92.03 | 251.61 | 1.73 |
| Hydroponic culture | Control | 439.20 | 142.97 | 582.18 | 3.07 |
| | Cu10 | 466.95 | 151.92 | 618.87 | 3.17 |
| | SB5Cu-10 | 511.08 | 170.80 | 681.88 | 2.99 |
| | SB10Cu-10 | 816.76 | 223.69 | 1040.45 | 3.65 |
| | SB20Cu-10 | 401.84 | 133.49 | 535.33 | 3.01 |
| | Cu25 | 585.88 | 192.47 | 778.36 | 3.04 |
| | SB5Cu-25 | 396.93 | 139.40 | 536.33 | 2.84 |
| | SB10Cu-25 | 293.91 | 98.12 | 392.04 | 2.99 |
| | SB20Cu-25 | 405.15 | 124.86 | 530.01 | 3.16 |
| | Cu50 | 555.28 | 177.77 | 733.05 | 3.12 |
| | SB5Cu-50 | 466.79 | 95.76 | 614.10 | 3.16 |
| | SB10Cu-50 | 318.05 | 95.76 | 413.82 | 3.32 |
| | SB20Cu-50 | 362.47 | 126.18 | 488.65 | 2.87 |

Table 3
Rape plant copper concentrations in germination tests and hydroponic cultures

| Experiment type | Tested solution | Concentration (µg/g dry weight) | | | |
|---|---|---|---|---|---|
| | | Root | Hypocotyls | Cotyledons | Plant |
| Germination test | Cu10 | 90.45 | 178.81 | 119.97 | 389.92 |
| | SB5Cu-10 | 127.36 | 117.66 | 122.02 | 367.06 |
| | SB10Cu-10 | 107.90 | 6.59 | 21.81 | 136.3 |
| | SB20Cu-10 | 113.10 | 121.92 | 106.85 | 341.88 |
| | Cu25 | 117.36 | 168.33 | 135.23 | 420.93 |
| | SB5Cu-25 | 109.46 | 161.20 | 99.57 | 370.24 |
| | SB10Cu-25 | 93.44 | 219.83 | 143.95 | 457.22 |
| | SB20Cu-25 | 110.11 | 44.22 | 54.88 | 209.23 |
| | Cu50 | 99.79 | 141.30 | 131.59 | 372.68 |
| | SB5Cu-50 | 135.27 | 73.30 | 64.59 | 273.16 |
| | SB10Cu-50 | 179.17 | 142.12 | 134.09 | 455.39 |
| | SB20Cu-50 | 112.40 | 108.84 | 108.17 | 321.41 |
| Hydroponic culture | Cu10 | 2475.50 | 157.50 | 96.37 | 2729.37 |
| | SB5Cu-10 | 1578.30 | 103.25 | 72.90 | 1754.45 |
| | SB10Cu-10 | 1391.50 | 101.00 | 47.44 | 1539.94 |
| | SB20Cu-10 | 1153.50 | 153.50 | 33.64 | 1340.64 |
| | Cu25 | 3975.00 | 795.00 | 70.43 | 4840.43 |
| | SB5Cu-25 | 3440.00 | 501.00 | 60.00 | 4001.00 |
| | SB10Cu-25 | 3475.00 | 2170.00 | 105.66 | 5750.66 |
| | SB20Cu-25 | 3015.00 | 905.00 | 54.35 | 3974.35 |
| | Cu50 | 4862.00 | 1590.00 | 118.33 | 6570.83 |
| | SB5Cu-50 | 5975.00 | 597.25 | 85.16 | 6657.41 |
| | SB10Cu-50 | 7010.00 | 1516.75 | 58.75 | 8585.50 |
| | SB20Cu-50 | 7112.50 | 1807.00 | 87.93 | 9007.43 |

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