Growth Test with *Lepidium sativum* L. for Soil Monitoring of Sites, Part of the Bulgarian National Soil Monitoring System.

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Abstract

The publication deals with the application of *Lepidium sativum* L. biotest for the study of soils from three sites included in the National Soil Monitoring Network. There were no exceedances of the limit values of the soil indicators in the monitored sites. The obtained results presented the existence of strong correlations between the bio test seeds and sprouts development and the variation in nitrogen content and the concentration of cobalt, nickel, zinc and copper in the soil. The results of this study have shown some possibilities for *L. Sativum* L. usage in soil monitoring, especially for the influence of soil nutrient elements (nitrogen) and microelements on the plants and thus for determining soil functional capacity and ability to provide material and support services.

Keywords: soil monitoring, biotest, *Lepidium sativum* L., functional soil diagnistics, germination energy

Introduction

The National Soil Monitoring System of Bulgaria is a part of the National Environmental Monitoring System (NEMS) and includes the collection, assessment and summarization of soil information as well as the maintenance of an information system on soil conditions and its modification (Ordinance № 4, 2009). As a methodological manager, the Executive Environment Agency (EEA) develops and implements the program, maintains the monitoring network and databases, evaluates and analyzes the state of the lands and soils. In 2004 a new soil monitoring program, organized on three levels, was developed and approved by the Minister of Environment and Water. Level I - Observations (large-scale monitoring) are carried out on a regular network of 16x16 km at 397 points and provide data for the assessment of soil status by the following indicators: 9 heavy metals and metalloids, total nitrogen, phosphorus, organic carbon, active soil reaction (pH) electrical conductivity, nitrate nitrogen, total carbon and persistent organic pollutants -16 PAH, 6 PCBs, 15-chlorine organic pollutants, bulk density (Figure 1). The periodicity of observation is 5 years. Observations at Level II are concentrated to regional manifestations of degradation processes - acidification (54 polygons) and salinization (12 polygons). The erosion processes caused by water and wind are observed through specially developed mathematical models for estimation and prognosis. Soil sealing is assessed on the basis of statistical data and mapping of land cover (Corine Land Cover Project, https://www.eea.europa.eu/data-and-maps/). The Observations at Level III are identified with the so-called local soil contaminations within which inventory of contaminated soil areas is carried out. The inventory is still partial and irregular, based on available data. In 2007, a special Ordinance was adopted to the EEA for the inventory and surveys of areas with polluted soil, the necessary restoration measures, as well as the maintenance of the restoration activities, and in 2016 a methodology for the preliminary and detailed surveys and the establishment of a public inventory register on areas with contaminated soil was adopted (http://eea.government.bg/bg/nsmos/soil). Periodicity of observations varies depending on the processes. The soil samples are tested in 15 accredited Regional Laboratories of the EEA.

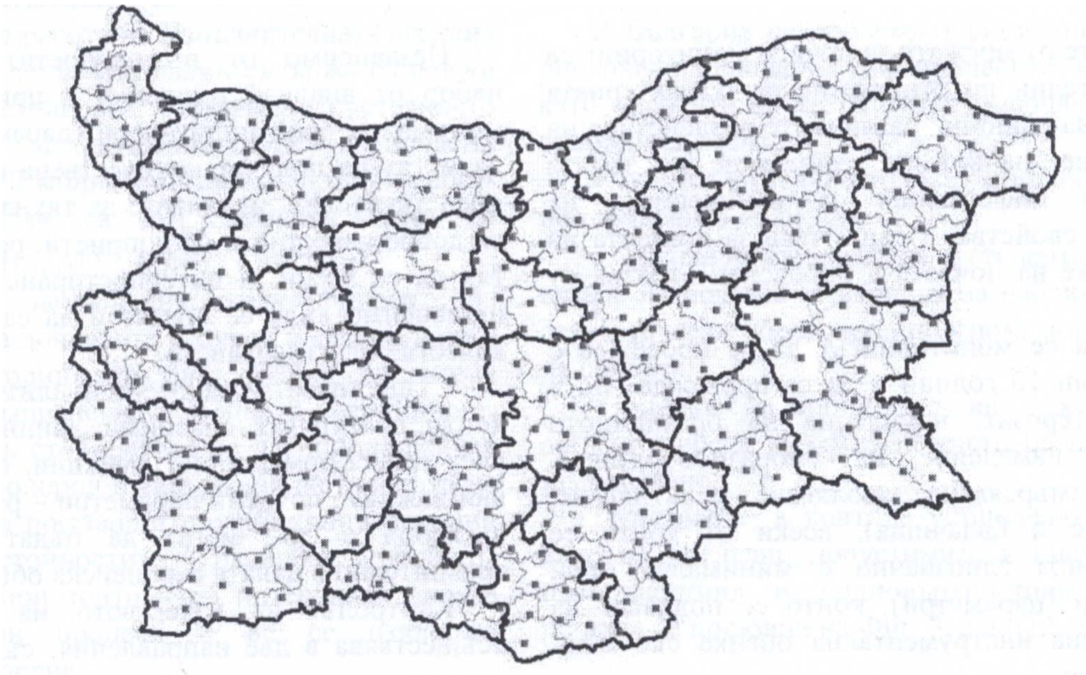

Figure 1. *National soil monitoring network***.**

Ordinance № 3 (2008) determines the limit values of harmful substances in the soil and the requirements for soil sampling and testing to determine the content of harmful substances. According to the published soil monitoring data in Bulgaria (http://eea.government.bg/bg/soer/2016/land-use/politiki-i-merki-za-opazvane-na-pochvite), in the recent years there has been a tendency for reducing soil contamination and absence of exceedances of the permissible limits of pollutants. This concerns mainly agricultural land and is due to a reduction in arable land and more limited use of pesticides and fertilizers.

Bioassays with one of the most widely used test object *Lepidium sativum* L. are applied for determining the effects of soil heavy metal on its growth and germination (Pavel et al., 2013). This test object can also be used for soil monitoring as for example Gyekye (2013) recommends the use of *L. sativum* L. as a test organism for biomonitoring and detecting toxic urban soils or for monitoring the removal of PAH pollutants from soil (Maila and Cloete, 2002). Other authors (Alvarenga et al., 2008) propose the use of battery of tests for assessing the quality of soils polluted from mine activities.

The main purpose of the study is to conduct bioassays with a widely used test object in the ecotoxicology - *Lepidium sativum* L. and to compare the impact of soil samples from three monitoring sites, diagnosed as unpolluted, on physiological parameters such as: germination energy and length of the embryonic root. The other aim of the study is to investigate the relationship between the reported differences in these indicators and the established concentrations of the chemical indicators monitored in the soil. This will give additional information on the ability of *Lepidium sativum* L. to be used for soil monitoring and for diagnosing the functional capacity of the soil to provide material and support services.

Materials and Methods

Soil samples are taken according to Appendix 3 to Art.5, Paragraph 3 of Ordinance № 3 on the limit values of harmful substances in the soil (Table 1).

Table 1. *Depth of soil sampling according to the types of land use.*

Type of land use	Depth of sampling (cm)
Settlements Parks Playgrounds	0 - 10 10 - 40
Industrial / production sites	0 - 10 10 - 40
Arable land	0 - 20 20 - 40
Permanent lawns	0 - 10 10 - 40

To determine the content of harmful substances in the soils, samples shall be taken and tested according to the Bulgarian or international standards and, if there are none, by validated intradepartmental methods. Sampling and testing of soil shall be carried out by accredited laboratories. The test methods used shall guarantee a detection limit for the relevant harmful substance with an order of magnitude lower than the standards under Art. 2 (Ordinance № 3, 2008).

Characteristics of the soil in the sampling areas

Soil samples from three monitoring sites in Blagoevgrad District, which are part of the first level NEMS, are tested. (Table 2).

Table 2. *Characteristics of sampling sites, soil monitoring level I.*

Settlement	Municipality	EKNM	Geographical coordinates		Sampling date	Type of land use - year 2015	Sampling depth, cm
Beslen (BE)	Hadzhidimovo	3976	41°28,112′	23°58,028′	7/10/2015	Corn field	0-20; 20-40
Breznitsa (BR)9	Gotse Delchev	6306	41°40,175′′	23°39,017′	7/09/2015	Meadows	0-10; 10-40
Satovcha (SA)	Satovcha	65440	41°37,399′	24°01,096′	9/09/2015	Pasture land	0-10; 10-40

Samples taken from the territory of the village of Satovcha are Albic CAMBISOLS (FAO, 1988). They are formed on sandy clay eluvium, deluvius or prolivius, in humid climate and with the wide participation of broadleaved (beech), coniferous and mixed forests. They are distinguished by shallow soil horizon (40-80 cm), insignificant humus horizons and insignificant humus content (3-9%). The soils on the territory of Beslen village and Breznitsa village are CHROMIC CAMBISOLS (FAO, 1988). They are the most widespread soil type in Bulgaria, mainly in agricultural areas. They are characterized by a 75-120 cm soil profile and a humus horizon up to 35 cm. By mechanical composition, they have higher clay content than the typical cinnamon soils, but with less humus (2-3%). 500 ml of distilled water was added to a 50 g soil sample. Flasks are placed on a shaker apparatus for 24 hours at 160 rpm, and then the suspension is filtered and stored in a refrigerator.

Kres salad is an easy object for conducting bio-test because the seed germination and energy of germination is high and the seeds are sensitive to influences. The biotest with *L. sativum* L. is applied for toxicological and functional soil assessment. The seeds are pre-chilled for 24 hours and set for determining the energy of germination or put in distilled water for germination and growth assay. 50 seeds or sprouts (with the same size) are put at equal distances in pre-sterilized petri dishes with 18 cm diameter. The bottoms of the petri dishes are covered with 2 layers sterile filter paper (autoclaved at 120°C for 20 min) and 1 layer for the lid. The paper is carefully moistened (without air bubbles) with 10 ml (bottom) and 5 ml (lid) respectively distilled water (for the control, K) or extract from the soils from the respective depths of the three test sites (variants of the experiment). During the experiment, the petri dishes are moistured by adding the same amounts distilled water or soil extraction (BSS601-85, 2009). The germination energy (Ek) is determined on the 4th day by counting the germinated seeds in each dish and calculating in percent relative to the control. After 48 hours, the root lengths were measured in the growth test (Lyubenova et al., 2009). The results are averaged for each variant and calculated in percent relative to the control, the values of which are assumed to be 100% (ISO 7346, 1996; Lyubenova et al., 2009). Each variant and the control are placed in 6 replicates.

Log-transformations of the soil variables and response variable standardization (sprouts variables) were performed prior to the multivariate analysis to obtain normality of variance in data. Parametric (Pearson’s correlation) and nonparametric (Spearman’s rank order correlation) analyses were used (Statistica 7.0) to assess the traits relationships and to evaluate if the selected soil factors significantly ($p<0.05$) explained the variation in trait values. An Analysis of Variance (ANOVA) was used to test species differences in trait means.

Results and Discussion

Most of the reported Ek values are close or exceed those of the control, i.e. soil extracts stimulate seed germination. The lowest value of Ek is recorded for 10-40 cm soil depth from the samples of Breznitsa (96.5% of the control) and the highest for the village of Satovcha in the same soil layer (104.3% of the control). For two of the sampling points the stimulative effect on Ek is better expressed in the surface soil layer: 0-20 cm for Beslen village and 0-10 cm for village of Breznitsa. Samples from Satovcha village (10-40 cm) have higher values for Ek, probably related to the organic pasture pollution (Figure 2).

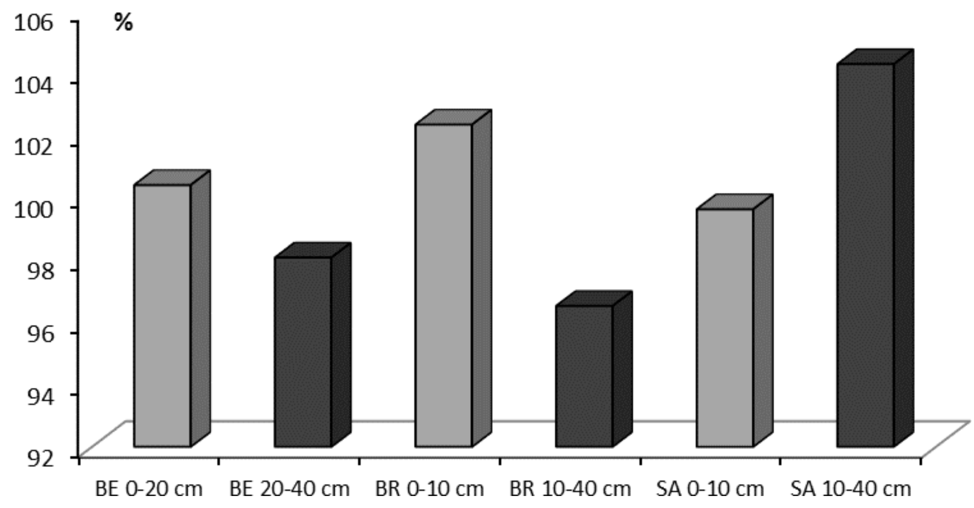

Figure 2. *Number of germinated seeds, Ek (% of the control).*

Samples from the Beslen village have the greatest length of the roots and at the same time exceeding that of the control, being 0.63 ± 0.46 cm for 0-10 cm depth and 0.65 ± 0.5 cm for 10-40 cm depth, i. e. the extract from deeper soil layer plays a more stimulating impact on the development. This is related to the type of land use and the agro-technological treatment, which on the one hand leads to faster oxidation of humus and washing of nutrients and contaminants from the surface layer. The smallest root length (0.49 ± 0.39 cm) is recorded at the Satovcha sample site for a depth of 0-10 cm. Extracts from deeper soil samples from Beslen (20-40 cm) and Satovcha village (10-40 cm) have stimulating effect on the root lenghts. This effect of the extracts from the deeper soil layer is probably due to the soil treatment in the first case and organic contamination in the second. For the tested soil from the site in the village of Breznitsa, which is covered by grass vegetation, the trend is opposite (Figure 3).

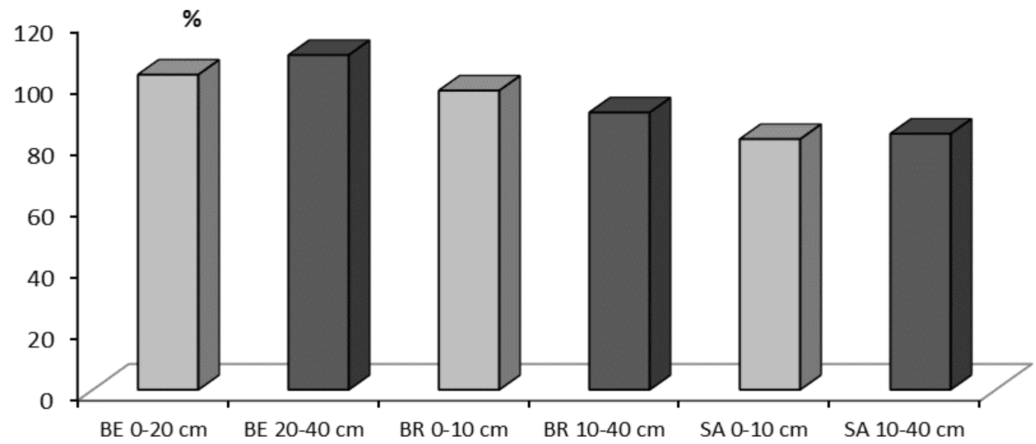

Figure 3. *Length of roots of biotest in different variants (% of control).*

The results of the ANOVA analysis show differences in the mean values of Ek and the root lengths of the sprouts between the sampling stations (p = 0.0005, ANOVA, F-test, Fig 4A) and soil layers (Figure 4B) are significant.

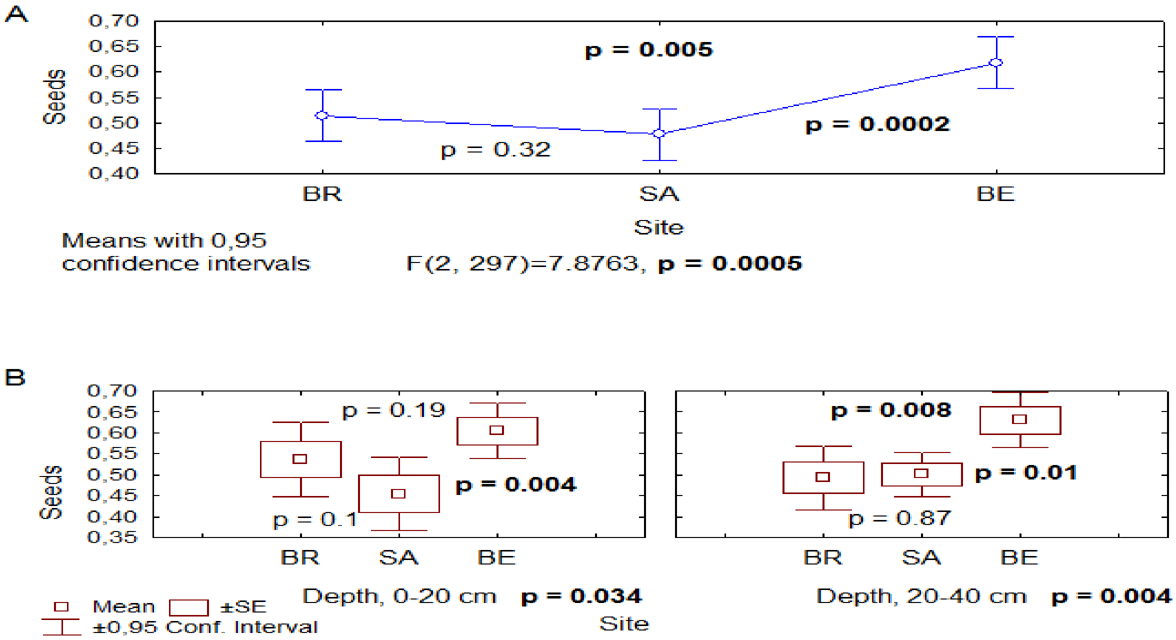

Figure 4. *ANOVA F-tests (with given p-values) showing the differences of the seeds development between 3 sites (villages Brestnitsa- BR, Satovcha - SA and Beslen - BE) (A) and for each of the two sampling depths, 0 -10 (20) cm and 10 (20) - 40 cm, in the same sites (B).*

The soil chemical analysis from the three sampling points in 2016 do not exceed the limit values of the indicators, which is in accordance to Ordinance № 3 (2008).

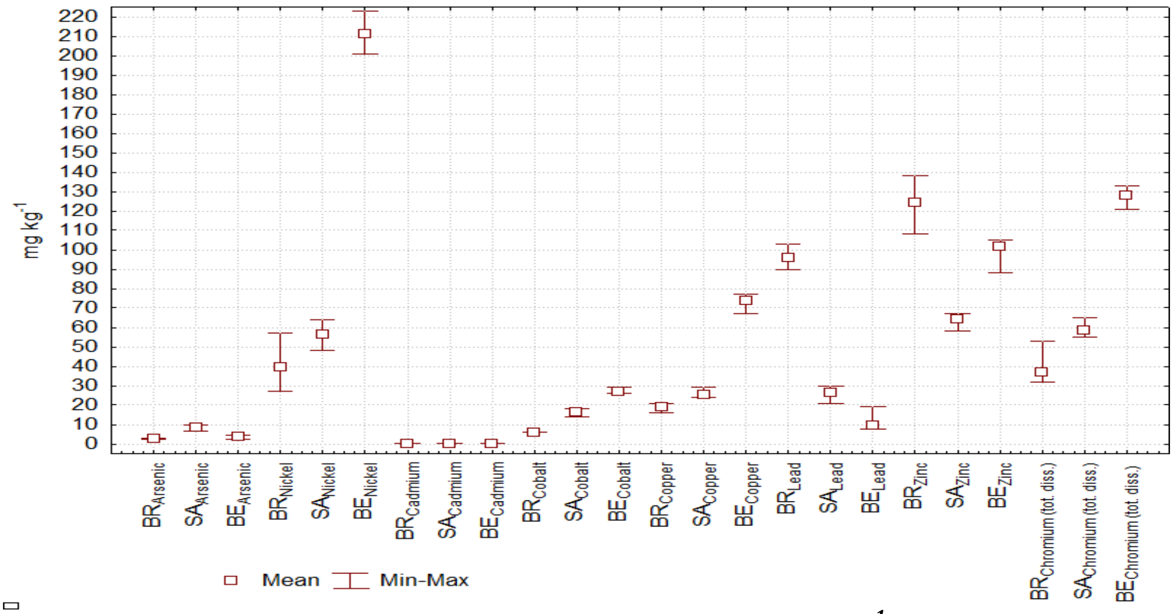

Figure 4. *Average values of metals concentration (mg.kg^{-1}) with their variance (Executive Environmental Agency, 2016)*

According to the Spearman's nonparametric and Pearson's parametric tests for the three studied sites and the soil layers, there is a positive correlation between the Ek and the length of the root of the biotest and Kjeldahl nitrogen; to the level of confidence is the negative correlation with the total phosphorus in the soils from the village of Satovcha and

TOC for $BR_{0\text{-}10cm}$ (Table 3). Studied parameters of the seeds treated with soil extraction from the surface layer at the site in Beslen village and the concentrations of cobalt have positive correlation, and those from the surface layer at the point in the village of Satovcha and the concentrations of cobalt and copper are close to confidence. The positive correlations between the studied parameters of the seeds treated with soil extracts from the surface layer at the sampling points in the villages of Breznik and Satovcha and the concentrations of nickel and zinc are significant or to the level of significance. The negative correlation between the studied parameters and conductivity for point BE, as well as with pH in $CaCl_2$ for $BE_{20\text{-}40\ cm}$ are also close to the confidence level. Correlation coefficients vary from 0.99 to 1.00. The determined very strong correlations show that the reported change for the seed test and shoot development is related to the variation in nitrogen content and the concentration of cobalt, nickel, zinc and copper in the soil.

Table. 3. *Significant or to the level of significance correlations between the parameters studied and soil characteristics*

Sampling Site / Soil layer	**Soil parameter**	**Corelation coefficient, r**	**P values**
$BE_{0\text{-}20\ cm}$	Cobalt	1.0000	0.0000 perfect positive correlation
	Kjeldahl nitrogen	1.0000	0.0000 perfect positive correlation
$BE_{20\text{-}40\ cm}$	pH in $CaCl_2$	-0.9946	0.0664
	Kjeldahl nitrogen	1.0000	0.0000 perfect positive correlation
$BR_{0\text{-}10\ cm}$	Nickel	0.9995	0.0203
	Total organic carbon (TOC)	-0.9885	0.0965
	Kjeldahl nitrogen	1.0000	0.0000 perfect positive correlation
$BR_{10\text{-}40\ cm}$	Kjeldahl nitrogen	1.0000	0.0000 perfect positive correlation
$SA_{0\text{-}10\ cm}$	Cobalt	0.9930	0.0752
	Zinc	0.9986	0.0334
	Kjeldahl nitrogen	1.0000	0.0000 perfect positive correlation
$SA_{10\text{-}40\ cm}$	Kjeldahl nitrogen	1.0000	0.0000 perfect positive correlation

Conclusion

Based on the obtained results, we could conclude that soil extracts stimulate seed germination in most cases. Ek was better expressed in the surface soil layer for Beslen and Breznitsa sampling stations probably due to soil treatment in the first case and organic contamination in the second. Samples from Satovcha have higher values for Ek in deeper soil layers, probably related to the organic pasture pollution.

Although there is no exceedance of the soil indicators limit values, the variations in energy germination and root length by sites, soil depth and nutrients concentrations are obtained. The strong correlations between the seed germination and sprouts development and the variation in nitrogen content and the concentration of cobalt, nickel, zinc and copper in the soil are expressed. The conducted experiments present some of the possibilities for the application of biotests in the soil monitoring, which could complement monitoring with functional soil diagnostics for determining soil functional capacity and ability to provide material and support ecosystem services.

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