Increase the ecological safety of the soil biogrouting using plant urease

Viktor Stabnikov, Dmytro Stabnikov, Viktor Udymovych

National University of Food Technologies, Kyiv, Ukraine

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Corresponding author:

Viktor Stabnikov
E-mail: vstabnikov1@gmail.com

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Abstract

Introduction. Biocement is a new building material based on the use of bacterial urease. The application of plant urease is the way for a wide and environmentally friendly application of this biotechnology for soil biogrouting.

Materials and methods. Urease activity of germinated soy seeds was determined by changing the electrical conductivity of the urea solution, μS/cm, due to its hydrolysis under the action of the urease enzyme. Calcium concentration was determined by titration with ethylenediaminetetraacetate using Eriochrome black T indicator. Assessment of sand biocementation was provided by the change of its water permeability.

Results and discussion. The disadvantages of the biocementation process are the potential biohazard from the used urease-producing bacteria and the unpredictable effect of introducing a significant amount of live bacterial biomass into the environment in the case of soil biogrouting. A possible replacement for bacterial urease may be plant-derived urease. Screening of seeds of agricultural crops grown in Ukraine showed that soybean seeds can be used as a source of urease for biocementation in the form of a crude aqueous extract from the crushed mass of the seeds themselves or germinated within 24-48 hours.

The urease activity of the homogenized mass of soy seeds was higher than the activity of pure extracts, and the specific activity – activity per unit of plant material – of both the homogenized mass and the extract was higher when using seeds that were germinated for 24–48 hours and specific activity of seeds germinated for 96 hours decreased.

The use of a crude extract from soybean seeds showed its effectiveness for the precipitation of calcium carbonate from a solution of calcium chloride and urea. Application of plant urease for sand biocementation made it possible to reduce water permeability by 600 times and obtain values of water seepage $1 \cdot 10^{-6}$ m/s that allowed the use of plant urease instead of urease-producing bacteria for soil biogrouting.

Conclusions. The possibility to replace in biocementation bacterial urease with plant-derived urease, particularly extract from soybean seeds, was shown. Plant urease effectively precipitated calcium carbonate from a mixture of solutions of calcium chloride and urea, and its use in the biocementation of sand reduced its water permeability to values corresponding to the seepage of sand biocemented with urease-producing bacteria.

Introduction

A new area of biotechnology – the use of microorganisms for the needs of construction has been successfully developing in the world over the past 15 years. A special place among new building materials is occupied by the production of biomement and biogrouts based on the application of urease-producing bacteria, which, in the presence of urea and calcium ions in an alkaline medium, catalyze the process of the so-called microbial induced calcium carbonate precipitation (MICP) and form insoluble calcite crystals (Ivanov and Stabnikov, 2017; Rosquoet et al., 2002).

Bacteria-induced mineral precipitation is a well known phenomena in Nature, which plays a significant role in Earth's mineral deposits (Hoffmann et al., 2021). The formation of calcium carbonate $CaCO_3$ is one of the most researched and studied biomineralization processes (Bang et al., 2010; Fernandez et al., 2018; Ronholm et al., 2014). Biocement is considered an ecological material inspired by nature itself (Achal, 2015).

An interesting example of natural biocementation in Ukraine can be a rock in the village of Podkamen, Brody district, Lviv region, Ukraine. This is a huge rock 16 m high and about 11 million years old (Figure 1a). In places where the outer hard crust is broken, clean sand is visible. An example of the use of MICP, the transformation of 1 m^3 of sand into stone using urease-producing bacteria, is shown in Figure 1b (Ivanov and Stabnikov, 2017).

a ***b***

Figure 1. Object of natural biocementation: the rock in the village of Pidkamin, Ukraine (*a*) and the result of artificial biocementation using MICP of sand (*b*)

To obtain biocement, most researchers use bacterial biomass with urease activity, since the use of the urease enzyme is expensive. Urease-producing bacteria *Sporosarcina pasteurii* (formerly *Bacillus pasteurii*) that is the most commonly used for biocementation (Bang et al., 2010; Keykha et al., 2018; Mortensen and DeJong, 2011; Whiffin et al., 2007) are belonging to the RG1 group of microorganisms (biologically safe) according to the European Union Directive (Directive 2000/54/EC). At the same time, there are many opportunistic and pathogenic species, halotolerant and alkaliphilic, with significant urease activity, which are considered in some studies as possible biological agents for biocementation (Dosier, 2014; Han et al., 2013; Maheswaran et al., 2014; Varalakshmi, 2014; Zaghloul et al., 2021). Some authors have isolated active urease-producing bacteria for further use in biocementation processes from activated sludge of sewage treatment plants (Al-Thawadi, 2012; Varalakshmi and Devi, 2014; Xu et al., 2017), while the risk of isolation of opportunistic pathogens is high. The use of biosafe and relatively cheap sources of urease is indicated as one of the essential requirements for the widespread practical use of soil biogrouting, when a large number of living cells of urease-producing bacteria are introduced into the environment (Ivanov et al., 2019).

A possible replacement for bacterial urease could be an environmentally friendly plant-derived urease (Dilruksh and Kawasaki, 2016; Ivanov et al., 2019). The main role of urease in plants is to allow the organism to use urea as a source of nitrogen. Several families of common plants are very rich in urease, including certain varieties of beans, melons and pumpkins, and even the pine family (Das et al., 2002). It is known to use Jack beans (*Canavalia ensiformis* and *Canavalia adans*) for the industrial production of plant urease (Kakimoto et al., 1992). There are studies where plant leaves, rather than seeds, are studied as a source material with urease activity, for example, mulberry leaves (*Morus alba*) (Hirayama et al., 2000), wheat and soybean leaves (Hogan et al., 1983).

The authors usually propose the use of local plant materials to obtain extracts with urease activity for their further application as a source of urease in biocementation. For example, crude extracts from watermelon (*Citrullus lanatus*) seeds, which are considered food waste (Al Imran et al., 2021; Dilruksh et al., 2018), from crushed outer leaves of cabbage and soy pulp (Baiq et al., 2020), and jack beans (*Canavalia gladiata*) (Tirkolaei et al., 2020) for biocementation.

The aim of the present study was to increase the ecological safety of the process of soil biograiting using urease of agricultural plants.

Materials and methods

Plant seeds

To determine the possibility of using the seeds of agricultural crops as a source of urease, the seeds of green lentils, peas, white beans, black beans, soybeans, pumpkins, watermelons and melons were germinated by water-air method. Seeds were soaked for 48 hours: the period of exposure to water was 6 hours, followed by 6 hours of exposure to air), and then self-germination was carried out for 96 hours with periodical spraying with water to prevent the seeds from drying out (Stabnikova et al., 2021; 2023).

Sand

For biocementation, river sand sifted through a metal sieve with holes (diameter of 0.5 mm) was used. Particle size distribution and mean size were measured using a Bettersizer S3 Plus particle size analyser (Bettersize Instruments, Dandong, China). For each sample, 3 measurements were done to determine the average particle size. The maximum size of 10% (D10), 50% (D50) and 90% (D90) of all particles was also determined.

Determination of urease activity

Urease activity was determined using a TDS-3 portable conductometer: the amount of released ammonium was determined according to the calibration graph by changing the electrical conductivity of the solution, μS/cm, due to hydrolysis of urea under the action of the urease enzyme. The molar concentration of NH_4^+ (Y) correlated linearly ($R^2 = 0.999$) with the change in the electrical conductivity of the solution (ΔX) in μS/cm over 5 minutes. Urease activity (UA) was defined as the amount of ammonium formed in 1M urea solution per minute.

To determine the urease activity of plant raw materials, seeds together with sprouts or sprouts separately were ground in a mortar, 0.5 g of grounded material were weighed, and mixed with 10 ml of distilled water in a glass beaker (the concentration of the obtained extract was 50 g/l) and urease activity of this extract was determined.

Determination of calcium concentration

The amount of calcium carbonate was measured by filtration and drying at 60°C.

Calcium concentration was determined by the standard APHA 2340C method with ethylenediaminetetraacetate (EDTA) titration (APHA, 1999). A liquid sample, 50 cm^3, was placed in a conical flask, 1 cm^3 of buffer solution to maintain pH 10.0 and a few drops of Eriochrome black T indicator were added. The sample was titrated with 0.01 M EDTA solution until the colour changed from purple to blue.

Determination of water permeability of biocemented sand

To determine the water permeability of the treated sand samples, 0.1–0.2 dm^3 of tap water was supplied by gravity from 1 dm^3 of a container with water at a practically constant hydraulic pressure of 0.5 m of water. This measurement was close to ASTM D2434-68 (2006) "Standard Test Method for Permeability of Granular Soils". The hydraulic permeability of sand, P, in a sand core was calculated according to equation:

$$P = \frac{V}{t} \cdot A \text{ , m/s,}$$

where – V is the volume of water supplied to a sand column; t is the time for which water passes through the sand; A is the cross-sectional area of the column.

Statistical analysis

The experiments were carried out in triplicates. Statistical processing of the experimental results was carried out using special computer programs for personal computers. Data are presented as arithmetic mean standard deviation.

Results and discussion

Selection of a plant whose seeds can be used as a safe source of urease for biocementation

Seeds of agricultural crops were selected for the study. The urease activity was measured in water extracts of germinated seeds with sprouts, as well as in sprouts separated from seeds. The urease activity of some plant materials is shown in Table 1.

Table 1

Urease activity of germinated seeds and sprouts of agricultural plants

Plant	Urease activity, mM hydrolyzed urea/min/g	
	Germinated seeds	Sprouts
Green lentils	0.29	0.31
Pumpkin	0.23	N/D*
White beans	0.26	024
Black beans	0.29	0.20
Soya	0.84	0.55
Watermelon	0.54	0.43
Melon	0.56	0.39

* N/D –not determine.

The urease activity of the germinated seeds with sprouts was a little bit higher than that of the sprouts only. According to the level of urease synthesis, the germinated seeds of the studied plants were arranged as follows: seeds of soybean, melon, watermelon, green lentil, black bean, white bean and pumpkin. Germinated soybean seeds showed higher urease activity. The urease activity of germinated seeds of melon, green lentil, black bean, white bean and pumpkin was 66, 64, 35, 35, 31 and 27% of the urease activity of germinated soybean seeds, respectively. Compared with the literature data on the urease activity of plants, urease activity of soybean seeds can be respected as relatively high. For example, the urease activity of the original extract from the leaves of mulberry (*Morus alba* L.), which was used to obtain purified plant urease, was 0.064 mM/min (Hirayama et al., 2000), and the activity of the pumpkin seed extract used in the work (Al Imran et al., 2021) was 0.01 mM/min. Soybean seeds were chosen as the most promising plant source of urease.

Choice of the form, which will be advisable to use plant urease for biocementation

Urease activity of germinated soybean seeds, 0.84 mM hydrolyzed urea/min, is lower than the activity of urease-producing bacteria commonly used in biocementation processes, but it is sufficient to use for soil biogrouting, since it has been shown that surface treatment of contaminated soil with small doses of biocement is effective in controlling its wind and water erosions and significantly reduces the release of dust, as well as bacterial and chemical pollutants into the environment (Hao et al., 2021; Ivanov and Stabnikov, 2020; Namdar-Khojasteh et al., 2022; Stabnikov et al., 2013).

To characterize the accumulation of urease in plant biomass, soybeans were germinated and samples of seeds were taken at 24, 72, and 96 hours. The value of urease activity was determined both in the homogenized mass of crushed seeds together with sprouts, and in the aqueous extract, which was obtained after removal of the suspended plant mass by centrifugation. For the preparation of extracts, different amounts of the homogenizing mass of seeds with sprouts were used, namely in proportions, g of mass to 10 ml of distilled water: 1 (10%); 2 (20%) and 4 (40%). The results on the urease activity of the homogenized mass of germinated soybean seeds and the extract obtained after removal of the plant mass by centrifugation are shown in Figure 2.

Urease activities of the homogenizing mass of seeds with sprouts were higher than the activities of water extracts and the activities of both were higher at the beginning of seeds germination. So, there was no significant change in urease activity during germination for 24–72 hours, but it decreased by 30–80% at 96 hours of germination. During the germination of peas (*Cicer arietinum* L.), another pattern was found: urease activity increased in germinated seeds and was maximum in the extract at 192 hours of germination, 0.03 mM/ml, and then decreased (Pervin et al., 2009).

To select a method for preparing plant materials to be used as a source of urease, the specific urease activity of the homogenizing mass of soybean seeds with sprouts and water extracts obtained by removing the suspended plant mass were compared. The seeds were germinated for 3 days, a homogenized mass was prepared from seeds and sprouts at 24, 48 and 96 hours of germination by treatment in a blender for 2 minutes to destroy plant cells and tissues, and then mixed thoroughly. Mixtures with different content of homogenized mass were prepared (10, 20 and 40% w/v), and to make extracts plant biomass was separated by centrifugation, and specific urease activities were determined in homogenized mass and extracts. Specific urease activity remained almost constant during 24–72 hours of germination and decreased by 96 hours. According to the data obtained, the homogenized mass of germinated seeds had a higher urease activity than the extracts, but the introduction of a large mass of plant material during biocementation can interfere with the process, so the possibility of using a homogenized mass, hereinafter a crude extract, had to be checked experimentally (Table 2).

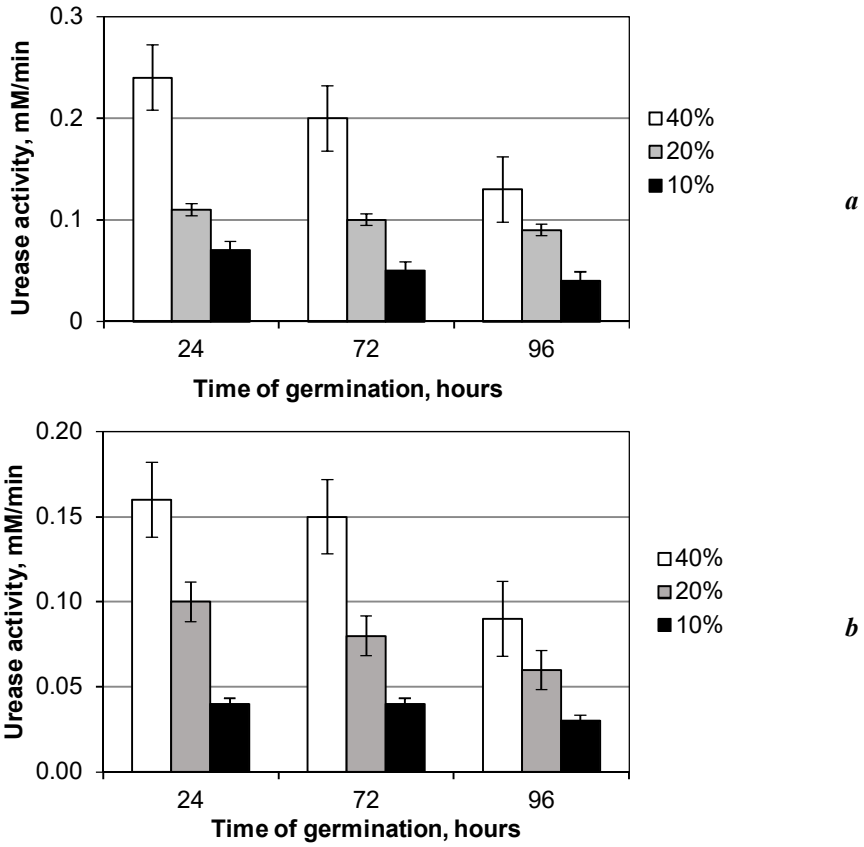

Figure 2. Urease activity of the homogenized mass with different amounts of crushed seeds with sprouts (10, 20, and 40%) (a) and the extracts obtained after removal of the plant mass by centrifugation (b).

Table 2

Specific urease activity of homogenized mass of germinated soybean seeds and extracts depending on germination time

Homogenized mass, %	Specific urease activity, mM/min·g at time of generation, h		
	24	72	96
homogenized mass (crude extract)			
40	0.6±0.05	0.5±0.03	0.3±0.02
20	0.6±0.03	0.5±0.02	0.5±0.02
10	0.7±0.05	0.5±0.05	0.4±0.03
extract			
40	0.4±0.02	0.4±0.02	0.2±0.01
20	0.5±0.05	0.4±0.04	0.3±0.01
10	0.4±0.01	0.4±0.04	0.3±0.02

Using crude extracts from soybean seeds as a source of urease for the precipitation of calcium carbonate

To confirm the possibility of using urease of soybean seed for biocementation, precipitation of $CaCO_3$ was done from a solution of chemical compounds used for microbially initiated precipitation of calcium carbonate, namely, a mixture of equimolar solutions of $CaCl_2$ and urea, with the addition of crude extracts from soybean seeds or germinated seeds. 20 ml of a mixture of equimolar solutions of $CaCl_2$ and urea with different molar concentrations were added to the propylene tubes. Prepared 10% crude extracts of soy seeds and germinated soy seeds without centrifugation, were added in the amount of 5 ml per tube and incubated at 30 °C for 48 hours. Part of the tubes were placed on a laboratory shaker at 150 rpm and stirred for 4 h every day. Precipitation of $CaCO_3$ was determined by titration with the indicator Eriochrome black (Tables 3 and 4).

Table 3

Calcium precipitation using soy seed crude extracts (no agitation)

$CaCl_2$: urea	Initial concentration of Ca^{2+}, g/l	Precipitated Ca^{2+} using crude extract of			
		Seeds		Germinated seeds	
		g/l	%	g/l	%
0.3 M:0.3 M	12	11.87	98.92	11.78	98.17
0.5 M:0.5 M	20	16.04	80.20	17.10	85.5
0.7 M:0.7 M	28	17.35	61.96	19.23	68.68
1.0 M:1.0 M	40	21.18	52.95	22.32	55.80
1.5 M:1.5 M	60	23.47	39.12	24.79	41.32

Table 4

Calcium precipitation using soy seed crude extracts (with agitation)

$CaCl_2$: urea	Initial concentration of Ca^{2+}, g/l	Precipitated Ca^{2+} using crude extract of			
		Seeds		Germinated seeds	
		g/l	%	g/l	%
0.3 M:0.3 M	12	10.63	88.58	11.92	99.30
0.5 M:0.5 M	20	10.35	51.75	17.67	88.35
0.7 M:0.7 M	28	13.69	48.89	29.19	72.11
1.0 M:1.0 M	40	22.12	55.30	23.73	59.33
1.5 M:1.5 M	60	25.81	41.68	25.21	42.02

The concentration of dissolved calcium in the liquid under precipitate was determined by titration with the indicator Eriochrome black. The amount of precipitated calcium was determined from the difference between the initial content of calcium and that remaining in the solution, and the percentage of precipitation was calculated. As can be seen, the precipitation of calcium occurred better with the use of crude extracts from germinated seeds and with periodic mixing of the solutions, but this difference was insignificant and amounted to no more than a few percent. The amount of precipitated calcium increased with an increase in its initial value, but the percentage of removal, on the contrary, decreased. Therefore, when

carrying out soil biogrouting or biocementation, an equimolar ratio of calcium chloride and urea above 1.5 should not be used, and application of seeds without germination as a source of urease resulted in formation of calcium carbonate.

Biocementation of sand using plant urease

Screened river sand was used for biocementation. Particle size distribution and mean size were measured using a Bettersizer S3 Plus particle size analyser (Bettersize Instruments, Dandong, China) (Figure 3, Table 5).

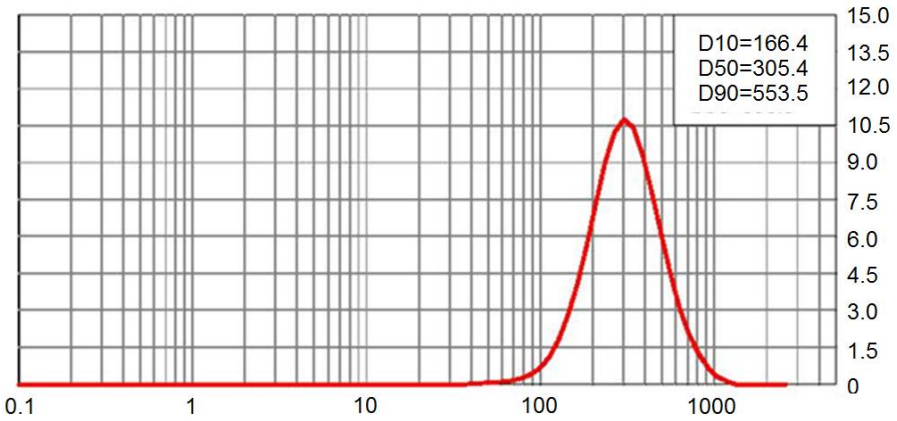

Figure 3. Particle size distribution of screened sand

Table 5

Particle size distribution of screened sand

d, µm	**0-200**	**200-300**	**300-400**	**400-500**	**500-600**	**600-700**	**700-800**	**800-900**	**900-1000**	**1000-1500**
%	18.12	30.31	23.74	13.47	7.22	3.44	1.86	0.99	0.50	0.35

Thus, the main sand fractions contained particles ranging in size from 200 to 300 µm (30.31%) and in size from 300.0 to 400 µm (23.74). D_{10}=166.4 µm; D_{50}=305.4 µm; D_{90}=553.5 µm, i.e. 10% of the sand had particles with diameter less than 166.4 µm, 50% less than 330 µm and 90% less than 1600 µm. Based on the grain size of the particle, sand is classified as fine sand (75 to 425 µm), medium sand (425 to 2000 µm), and coarse sand (2000 to 4750 µm). That is, the sand that was used for biocementation was fine. For comparison, biocementation studies typically use: ASTM (American Society for Assaying and Materials) sorted sand with an average grain size of 400 µm; standard round sand (Societe Nouvelle du Littoral, France) with an average grain size of 420 µm; standard Ottawa sand with an average size of 300 µm with a particle size variation from 150 to 1180 µm.

Biocementation of screened sand was carried out in syringes (3 cm diameter and 10 cm in length). Sand was placed into each syringe in an amount of 60 g. Suspension of homogenized mass of seeds or germinated seeds (seeds with sprouts) were used as a source of urease. The efficiency of biocementation was evaluated by the change in the permeability of the treated sand. The zero point was the rate of water passage in the sand before the start of biocementation.

Caputo (2004) defines soil permeability as the amount of water passing through the pores between particles at different speeds over a certain period of time. Estimating the rate of water passage is relevant because the water content of any soil zone is related to the relationship between soil tension and pressure, which depends on the increased amount of water infiltrating. The coefficient of water permeability (the amount of passing water) is usually advisable to use for porous media. There were 8 cycles of biocementation, and they consisted of the following steps: slow feed of the crude extract, 10 ml; exposure for 2 hours; supply of solutions of calcium chloride and urea with different ratio $CaCl_2$: urea, namely: 0.5 M : 0.5 M; 0.7 M: 0.7 M; 1.5 M: 1.0 M. After each biocementation, the sand was allowed to stand for 24 hours for calcium carbonate crystals to form, and the water permeability of the sand was determined. The results are presented in Figure 4.

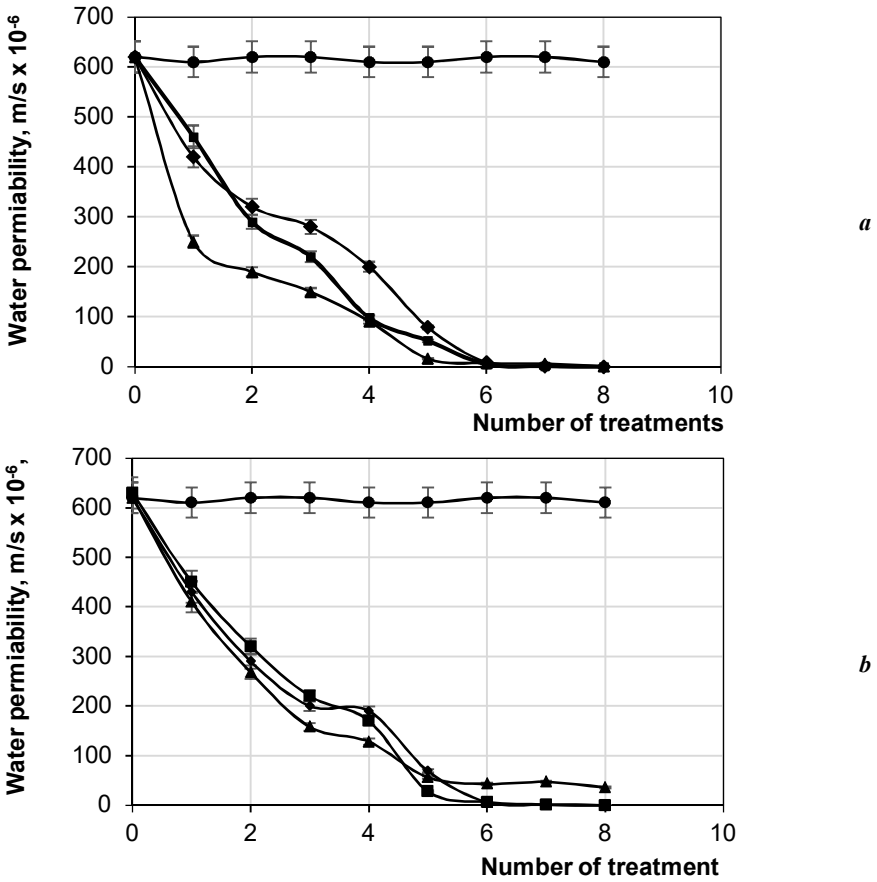

Figure 4. Changes in the water permeability of biocemented sand when using an crude extract from germinated seeds (a) and seeds (b):

● control;
▲- sand, which was treated with a 0.5 M solution of urea and calcium chloride;
■ – sand that was treated with a 1.0 M solution of urea and calcium chloride;
◆ – sand, which was treated with a 1.5 M solution of urea and a 1 M solution of calcium chloride.

In general, during the biocementation of sand using plant urease, the water permeability decreased from $6 \cdot 10^{-4}$ m/s to $1 \cdot 10^{-6}$ m/s, that is, it decreased by 600 times. The values obtained correspond to the seepage rates of sand biocemented with conventionally used urease-producing bacteria (Chu et al., 2013; Ivanov and Stabnikov, 2017), and seepage rates from actual aquaculture ponds (Teichert-Coddington et al., 1988; Weisburd and Laws, 1990). Thus, due to the action of plant urease, because of the formation of calcium carbonate $CaCO_3$, the pores in loose sand are filled, the sand particles are bound, the water permeability is reduced and, as a result, their strength is increased. Similar results were obtained in the work of Japanese scientists (Al Imran et al., 2021), who used crushed and mixed watermelon seeds (both dry and germinated) as a source of the urease enzyme for sand biocementation at neutral pH ~7 and temperature 30 °C. Biocementation assessed with decrease of water permeability was going faster when solutions with low concentration of calcium chloride and urea were used and the use of 1M solutions should be considered optimal, which provided a lower final water permeability of the sand samples. Photos of biocemented sand are shown in Figure 5.

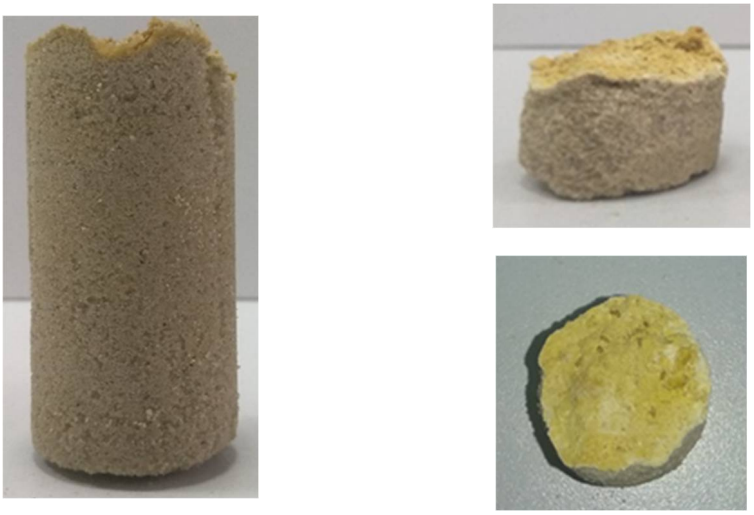

Figure 5. Sand after biocementation using soybean seed urease

The advantages of this method of biocementation are evident: (a) there is no need to grow a microbial producer of urease, that is, the technology is greatly simplified; (b) the crude extract can be prepared just prior to its use; (c) the cost of the biocementation process is significantly reduced, since there is no need for a nutrient medium for growing bacteria, electricity consumption for aeration in the growing process, and the use of highly qualified personnel to obtain microbial biomass with urease activity; (d) one of the important problems of biocementation is completely solved – cells of the microbial urease producer do not enter the environment, and an aqueous solution of homogenized plant biomass does not need additional determinations of its biosafety. This biocementation method can be used as an environmentally friendly and sustainable method for solving numerous geotechnical problems, such as soil stabilization, elimination of the consequences of soil liquefaction during earthquakes, soil protection from erosion, the atmosphere from dust, and chemical pollutants.

Conclusions

1. Screening of seeds of agricultural crops grown in Ukraine showed that soybeans can be used as a source of urease for biocementation in the form of a crude aqueous extract from the seed mass itself or germinated within 24–48 hours.
2. The use of a crude soybean seed extract has been shown to be effective in precipitating calcium carbonate from a mixture of calcium chloride and urea solutions.
3. The use of a crude soybean extract in the biocementation of sand made it possible to reduce its water permeability and obtain values corresponding to the seepage rates of sand biocemented with traditionally used urease-producing bacteria.

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