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ARTICLE

Effects of nitrogen-fixing bacteria on seabuckthorn growth *(Hippophae rhamnoides. L)*

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Abstract: Mongolia's fertilizer industry produces 2,000 tons of fertilizer in 10 years, while imports amount to 6-8 tons a year. One of the main biological features of the root system of sea buckthorn is the ability to absorb nitrogen from the air with the help of its root nodules. The main goal of the research is to identify the native nitrogen-absorbing bacteria of sea buckthorn and to increase their activity by preparing bacterial fertilizers and determining their effects on plants. In our study, we obtained 2 types of nitrogen-fixing bacteria using common microbiological methods. Sea buckthorn mongolica subspecies was used in this study. The study of root nodules of sea buckthorn (*Hippophae rhamnoides L.*) aims to identify symbiotic nitrogen-fixing bacteria. Sea buckthorn root nodules contain *Frankia* actinorrhizal microorganisms. Sea buckthorn seedlings, including those fertilized with nitrogen-fixing bacterial cultures, showed a positive correlation with plant growth. From June to September, when air temperatures are high and photosynthesis active in the field, the number of bacterial cells and nitrogenase activity were found to elevate in the root nodules of sea buckthorn plants. During this period, the roots extended 1.4 to 2.0 meters in length. The first- and second-order small roots, along with the main root, began forming small nodules filled with microorganisms. These microorganisms play a crucial role in capturing nitrogen in its molecular form from the air and converting it into a form usable by plants. However, studies on the diversity and distribution of *Frankia* strains have been hindered by challenges in isolating them from field-collected root nodules. In the field, nitrogenase activity in root nodules remained high from May to September, corresponding with the periods of high air temperatures and active photosynthesis.

Keywords: *Nodules, biological fertilizers, mineral fertilizers, microorganisms;*

INTRODUCTION

Article 3/e of a resolution passed by the National Assembly of Mongolia on 17 June 2022 I concerning measures to be taken within the framework of the

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national campaign "Food Supply and Security" stipulates the development of domestic fertilizer production in order to compensate for the loss of soil nutrients and to increase the quality and quantity of crops.

Nutrient availability is one of the limiting factors for plant growth and development, and nutrients on the Earth are unevenly distributed. Plants adapt to this challenge by modifying their morphology and physiology, especially that of the root system. It performs symbiotic N2 fixation and has high adaptability to environmental constraints. Sea buckthorn is drought- and cold-tolerant and is a symbiotic fixer of nitrogen [1].

Our country has been using fertilizers in agriculture at a dose 10 times higher than the world average (3-5 kg/ha). This shows that there is a need to carry out extensive research and analysis to determine the use of fertilizers in agriculture in Mongolia, including the creation of biologically-derived fertilizers that do not have negative ecological effects, and further, to take appropriate measures to protect fruits and berries [2].

Lateral roots begin to development when the main root of sea buckthorn reaches a length of 5-7 cm. After 1.5-2 months of rapid growth, the main root reaches 14 to 20 cm in length, and small nodules filled with microorganisms start to form. Nitrogen absorption is activated following the formation of these nodules. There is an urgent need to isolate nitrogen-fixing bacterial strains from the root nodules of sea buckthorn and prepare them as biofertilizers for plants. *Frankia*, an actinomycetic, nitrogen-fixing, nodule-forming endophyte, is commonly associated with woody trees and shrubs.

The sea buckthorn subspecies *mongolica* used in this study is widely cultivated in Mongolia and Siberia.

In this study, we investigated the capacity of symbiotic nitrogen fixation in sea buckthorn root nodules and the effects of nitrate on nitrogenase activity.

The *Frankia* is a symbiotic nitrogen fixer, living in root nodules of many non-leguminous plants. Atypical characteristic of this endophytic organism is the formation of specialized swollen cell structures called vesicles. *Frankia* vesicles have been brought in relation to nitrogen fixing activity, but many questions about their formation and their function still had to be answered.

Symbiotic plants infected with Frankia are termed actinorhizal plants and are classified into eight families and 25 genera comprising more than 220 species [3].

MATERIALS AND METHODS

Origin of samples

In order to determine the nitrogen-absorbing activity and capacity of the bacteria contained in the nodules of sea buckthorn, the effects on the formation of the nodules, and the viability of the plants, an experiment was conducted on plant growth by way of growing a pure colony of bacteria cultured on agar medium and preparing the following versions of the mother culture ($3x10^8$ cells/ml-$15x10^8$ cells/ml) for fertilization.

Preparation of bacterial liquid culture was carried out using laboratory methods with bacterial root cultures. The seedlings were fertilized with bacterial root cultures. Seed fertilization was carried out using the following method.

The plants were then watered to 30 per cent of the soil's full moisture capacity. The results of the experiment included measurements of plant height, wet weight, the number of nitrogen-

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absorbing bacterial cells in the soil, plant rhizosphere, and rhizophland, as well as the plant's nitrogen-absorbing activity.

Bacterial Fertilizer Stock Culture*:* *Frankia alni*, a nitrogen-fixing bacterial strain was used.

1. *Nutrient medium*: An agar medium with FDM+0.5% sea buckthorn juice **DPM** (Frankia Defined Minimal Medium) **MEDIUM** ingredient:

KH_2PO_4 1.0 g, $MgSO_4$ x 7 H_2O 0.1 g, $CaCl_2$ $_2$x $2H_2O$ 0.01 g, Na-propionate 1.2 g, *Chelated iron solution* (see below) 1.8 ml, Trace element solution (see below) 1.0 ml, Distilled water 1000 ml. Agar 20.0g

Chelated iron solution: $FeSO_4$ x $7H_2O$ 5.54 g,Na_2-EDTA 7.56 g, Distilled water1000 ml. *Trace element solution:* $CoCl_2$ 0.025 g, $CuSO_4$ x 5 H_2O 0.08 g, H_3BO_3 2.86 g, $MnCl_2$ x 4 H_2O 1.81 g,Na_2MoO_4 x 2 H_2O 0.025 g, $ZnSO_4$ x 7 H_2O 0.22 g, Distilled water 1000 ml. The pH of the final solution is adjusted to 6.8 [4].

2.*Preparation of stock culture*: Inoculation with pure culture of *F.alni*bacteria on liquid medium.

3. *Inoculated:* The pure culture contained 10^9 cells in 1 ml. Prepared 100ml liquid medium.

4. *Cultivation:* Incubate t° at 28° for 48 hours on a shaker with cells up to 15 x 10^8 cells/ml in 1 m.

5. *Packaging*: The source culture of the fertilizer used in the experiment was prepared by packaging it in liquid form of 10ml-100ml [5].

In the experiment, 1 ml, 2 ml, 4 ml, 6 ml, and 8 ml doses are mixed with 100 ml of irrigation water and irrigated to the seedlings planted in pots.

Trial version: 1 ml or $3x10^8$cells/ml, 2 ml or $6x10^8$cells/ml, 4 ml or 9 $x10^8$cells/ml, 6 ml or 12 $x10^8$ cells/ml, 8 ml or $15x10^8$ cells/ml. Each variety was tested with 4 replicates.Tested on 40 plants in 1 version.

Methodology for testing seedlings planted in pots with a liquid stock culture of bacteria

In the study, soil collected from Udleg's sea buckthorn field was used. One-year-old seedlings of the *Chuiskaya* and *Elizabeta* varieties were planted in individual containers with varying concentrations of bacterial inoculum: 1 mL (3×10^8 cells/mL), 2 mL (6×10^8 cells/mL), 4 mL (9×10^8 cells/mL), 6 mL (1.2×10^9 cells/mL), and 8 mL (1.5×10^9 cells/mL).

Seedlings were fertilized around the roots with bacterial root culture. They were then watered to 60 per cent of the full soil moisture capacity. The results of the experiment were calculated as plant height, wet weight, the amount of nitrogen-absorbing bacterial cells in soil, plant rhizosphere and rhizophland, and plant nitrogen-absorbing activity. The bacterial liquid culture was prepared and fertilized to test the seedlings planted in a total pot. 840 ml + 840 = 1680 ml.

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Table 1. Methods of application seedlings planted of liquid biofertilizers

Variable (dozes)	Duration (in months)				total	liquid biofertilizers ml
	VI	VII	VIII	IX		
Chuiskaya varieties						
Control	**10**	**10**	**10**	**10**	**40**	--
1 ml or 3x10⁸ cells/ml	10	10	10	10	40	40
2 ml or 6x10⁸ cells/ml	10	10	10	10	40	80
4 ml or 9 x10⁸ cells/ml	10	10	10	10	40	160
6 ml or 12 x10⁸ cells/ml	10	10	10	10	40	240
8 ml or 15x10⁸ cells/ml	10	10	10	10	40	320
Total	60	60	60	60	**240**	**840**
Elizabeta varieties						
Control	**10**	**10**	**10**	**10**	**40**	--
1 ml or 3x10⁸ cells/ml	10	10	10	10	40	40
2 ml or 6x10⁸cells/ml	10	10	10	10	40	80
4 ml or 9 x10⁸cells/ml	10	10	10	10	40	160
6 ml or 12 x10⁸cells/ml	10	10	10	10	40	240
8 ml or 15x10⁸ cells/ml	10	10	10	10	40	320
Total	60	60	60	60	**240**	**840**

Statistical analyses

A one-way analysis of variance (ANOVA) was conducted to determine whether there were differences between the treatments (fertilizer doses) in terms of sea buckthorn seedling growth and root nodule bacterial cell numbers.

RESULTS AND DISCUSSION

One-year-old seedlings grown in the sea buckthorn garden were planted in pots containing 1 kg of soil.

They were treated with varying concentrations of bacterial inoculum: 1 mL (3×10^8 cells/mL), 2 mL (6×10^8 cells/mL), 4 mL (9×10^8 cells/mL), 6 mL (1.2×10^9 cells/mL), and 8 mL (1.5×10^9 cells/mL). Each concentration was prepared in 100 mL of water, and measurements were taken after fertilizing the potted plants. Seedlings planted in unfertilized, sterile soil were used as controls.

Figure 1. Experiments on seedlings planted in pots
A. Seedlings planted in May; B. Seedlings were planted in August

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The height of the plants grown in June-September was calculated, the number of nitrogen-fixation bacterial cells in the soil, and the number of plant rhizosphere and rhizophland were analyzed.

Table 2. Results of biometric measurements of plants with liquid bacterial fertilizer in potted seedlings

Variable (dozes)	Plant height cm (20 May)	Plant height cm (20 August)	Growth cm	Plant height cm (20 May)	Plant height cm (20 August)	Growth cm
	Chuiskaya varieties			Elizabeta varieties		
Control	57,5	95,56	38,06	64	98,8	34,8
1 ml or $3x10^8$cells/ml	63,5	103,5	39,17	76,75	122,6	45,87
2 ml or $6x10^8$ cells/ml	62,1	106,6	41,17	65	108,3	43,3
4 ml or 9 $x10^8$ cells/ml	62,88	120	45,38	61,1	119,1	58
6ml or 12 $x10^8$ cells/ml	66,6	122,6	47,54	62	117	55
8 ml or $15x10^8$ cells/ml	70,89	116,67	47,25	67,4	123,4	56

According to the results of the experiments on potted seedlings fertilized with liquid bacterial fertilizer, *Chuiskaya* seedlings grew 1.1-9.5 cm more than the control seedlings, and the *Elizabetha* seedlings grew 8.5-23.2 cm more than the control ones.

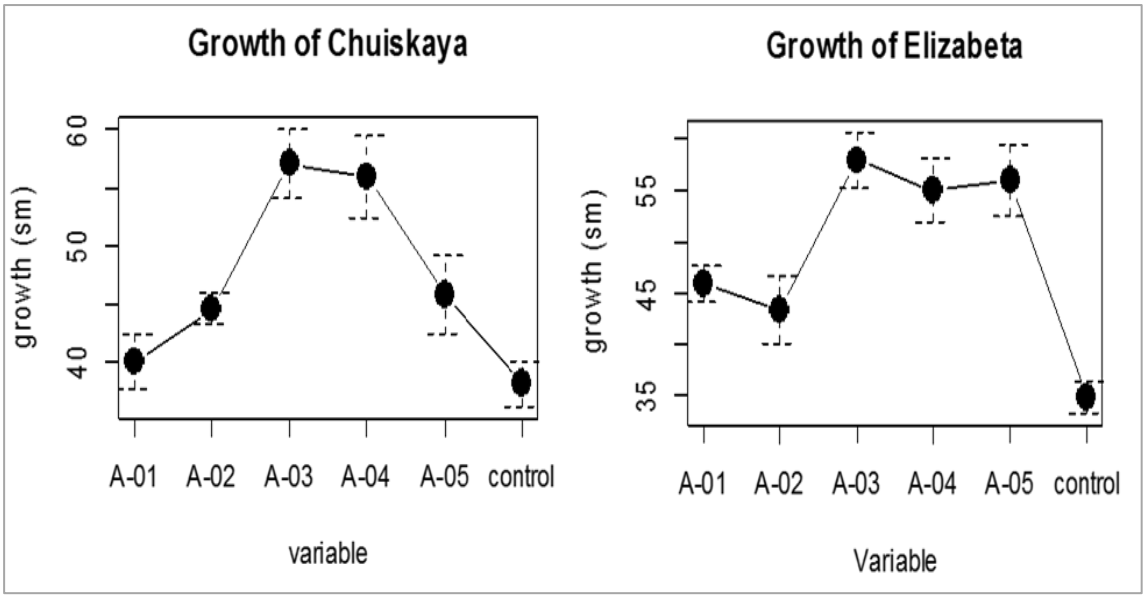

Figure 2. Differences in the growth of 2 varieties of sea buckthorn fertilized with liquid bacterial fertilizers

The *Elizabeta* variety growth was 7.4 cm - 13.7 cm taller than that of the *Chuiskaya* variety. Varieties differ in growth.

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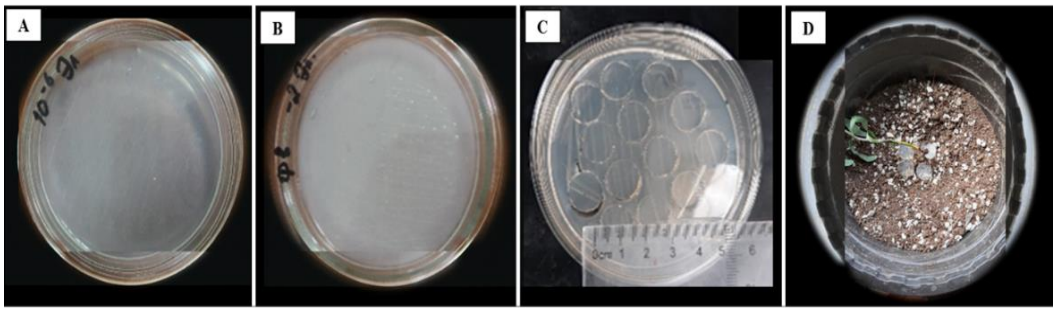

Figure 3. Bacterial culture preparation
A. F. *alni* bacterial cultured in FM medium on agar, B. A. *chrooccocum* bacterial culture cultured on Ashby medium on agar medium, C. Culture prepared on agar medium, and, D. Fertilizer prepared in agar medium

The deviation was calculated by comparing the number of nitrogen-fixing bacterial cells in nodules of 1-year-old sea buckthorn seedlings of *Chuiskaya* and *Elisabeta* varieties used in the experiment with samples from June to September compared to the unfertilized control

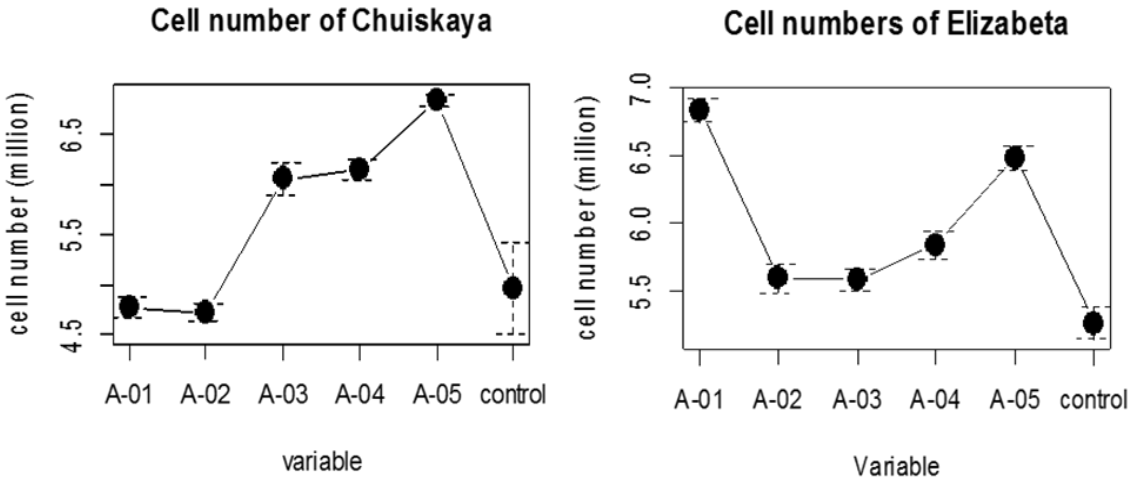

Figure 4. Changes in the number of plant cells during the experiment in potted seedlings (millions per milliliter)

Based on the above, it can be concluded that the cell numbers in the 1 mL (3×10^8 cells/mL) and 2 mL (6×10^8 cells/mL) treatments were lower than in the control. Additionally, the number of cells increased in the other treatments. The correlation between the number of cells in the fertilized and unfertilized versions was r = 0.22–0.45, indicating a low to medium correlation.

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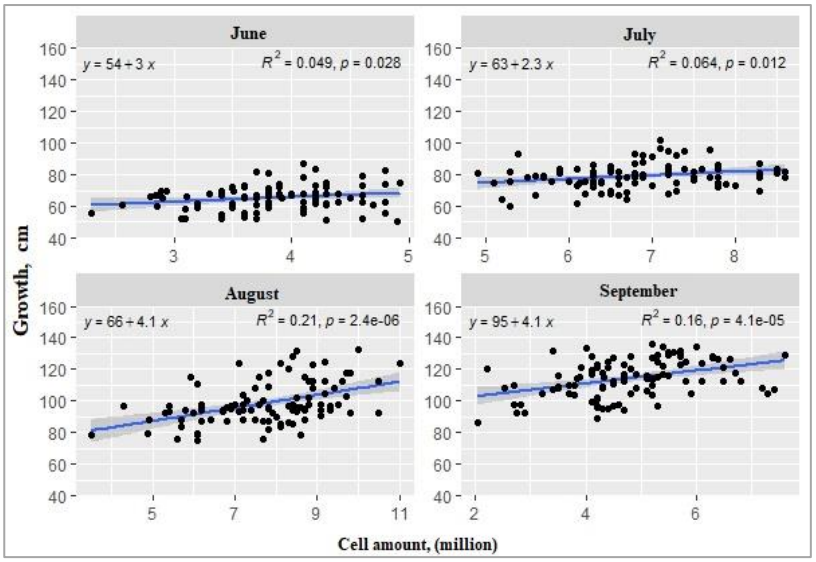

Figure 5.Cell amount (million)

The *Chuiskaya* variety showed 3.07 cm more growth than the control in the fertilized treatments. Growth increased in the 1-4 mL fertilized treatments and further increased in the 5 mL fertilized treatment. In the treatments with the *Elizabeta* variety, there was 3.38 cm more growth, and in the 1-4 mL fertilized treatments, the height of the *Elizabeta* variety bushes increased by 0.31 cm as compared to the control version.

In the absence of an organic source of nitrogen, cells of *Frankia* bacteria can assimilate nitrogen, whereas most rhizobia cannot. However, vesicle formation of F.alni under N-deficiency is different in nodules. In brief, the nodule-accumulating bacteria are more numerous and have a significantly different shape and uptake than other N-fixation conditions, nodule-forming bacteria are vesicular and have more cells [6].

In the absence of an organic source of nitrogen, cells of *Frankia* bacteria can assimilate nitrogen, whereas most rhizobia cannot. However, vesicle formation by F. alni under N-deficiency is different in nodules. Briefly, absorptive, nodule-forming bacteria are spherical and more truncated [7].

All Frankia strains are slow-growing heterotrophic aerobes, doubling conciseness in cell number in 15 hours or more. Some strains grow better than others in less aerated environments and do poorly in strongly aerated environments, especially when using sparse soil. Because the growth medium often produces large sporangia in liquid culture, the growth kinetics of *Frankia* strains are complex, and growth is generally active after purification to homogenization, followed by a short exponential phase, followed by slow growth. The growth process and stages affects the plants' biomass.

This growth pattern affects the cell density of the culture and can be increased depending on the agitation during the culture. Thus, progress has been made in the growth of nitrogen-absorbing *Frankia* cells using semi-continuous agitation during culture [8].

Cell growth can be enhanced from agar mediums containing suitable carbon sources to selective mediums containing basal salts, yeast extract, meat albumin, malt extract, meat extract, NZ Amine A, Casamino Acids, vitamin supplements Tweens [8]. Almost all isolated Frankia strains are

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prototrophs that do not require specific growth factors and therefore, grow well even on simple media. For *Frankia*, DPM (defined as propionate minimal) by Baker and O'Keefe is a low propionate medium [9].

Frankia strains are grown and maintained in a liquid medium and often grow slowly in agar medium. Agar medium is often used to clean cultures, it promote spore growth, or identifies strains. Colonies formed from spores or mycelia are visible to the naked eye for 7-10 days more under optimal conditions [10].

The *Frankia* spp. are among the very few soil microbes able to modify the plant hormonal balance, induce tissue differentiation, and establish nitrogen-fixing structures on the roots of higher plants belonging to eight dicotyledonous families collectively called actinorhizals[11].

Despite marked morphological differences in nodules between actinorhizal and leguminous plants, the molecular phylogeny of these two groups of plants that engage in root symbioses showed they all belong to a single lineage, the *Rosid I clade* (Soltis et al. 1995), suggesting the existence of conserved determinants, such as the SymRK kinase that is essential to establish nodulation with *Frankia* spp[11].

Schematic representation of actinorhizal root infection by *Frankia*. *Frankia* penetrates via a root hair infection process in host plants from the families *Betulaceae, Casuarinaceae* and *Myricaceae,* and intercellularly in Eleagnaceae, Rosaceae and Rhamnaceae. Prenodule formation resulting from mitotic activity in the root cortical cells is observed only during the intracellular infection process. Nodule primordia arise from divisions in root pericycle cells, located opposite a protoxylem pole, and near the site of infection.

Frankia hyphae progresses either from cell to cell in the intracellular mode of infection, or apoplastically in a matrix secreted into the intercellular spaces. *Frankia* hyphae progresses towards the nodule primordium where they will penetrate developing cortical cells intracellularly. Mature nodules consist of multiple lobes[12].

CONCLUSIONS

-Fertilized seedlings planted in pots with laboratory-prepared bacterial agar cultures showed a 9.2 per cent increase in plant height, a 23.2 per cent increase in branching, a 40.7 per cent increase in root branching, and a 10.7 per cent increase in root length. As a result, the number of leaves increased by 32 per cent.

-Two strains of nitrogen-fixing bacteria were isolated from the root of sea buckthorn. *A. chroococcum* MN and *F. alni* bacteria in the native nodules of sea buckthorn *(Hippophae rhamnoides. L)*, which affected the DNA nucleotide sequence according to in-culture support analysis, were similar to this species in terms of genetic characteristics.

-When fertilized with laboratory-prepared bacterial liquid fertilizer, high-dose fertilized *Chuiskaya* seedlings grew 1.1-9.5 cm more than the non-fertilized control, while *Elizabeta* seedlings grew 8.5-23.2 cm more than the control.

A comparison of the growth of the fertilized bushes of these two varieties showed that the *Elizabeta* variety was 7.4-13.7 cm taller than the *Chuiskaya* variety.

-The number of bacterial cells in the root tuber during the test period of 1-year-old sea buckthorn seedlings of *Chuiskaya* and *Elisabeta* cultivars showed a lower number of cells in the versions with a low dose of fertilizer, and a higher number of cells in the

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versions with 4 ml or 9 x 10^8 cells/ml and 6 ml or 12 x 10^8 cells/ml.
-Cell number of 1ml or 3x10^8, 2ml or 6x10^8cells/ml versions have less cells than the control. Additionally, the number of cells increased in the other versions and the number of cells in the fertilized and unfertilized versions are R^2 = 0.049-0.21. r = 0.22-0.45, which shows a low and medium correlation.

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