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Effect of Seed Size and Ultrasound Treatments on Nodulation Ability in Bird's-Foot-Trefoil (*Lotus corniculatus* L.)

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***Abstract* – Bird's-foot-trefoil (*Lotus corniculatus* L.) has a wide variability of seed size due to both genetic features and to natural conditions and production and harvesting technologies of seed crops. To this feature we also add the share of hard seeds, which, together, influence growth and development stages in the bird's-foot-trefoil plants: seed germination capacity, plant vigour, and nodule-formation ability. This paper presents the effect of interaction between sowing seed size and ultrasound treatments under experimental conditions. Applying ultrasound treatments differentially depending on seed size (from very small seed size to very large seed size) influences greatly the number and weight of nodosities and their size share: from 41% small nodosities to 53% large nodosities in the control variant; from 32% medium size nodosities to 27% medium size nodosities in the control variant; from 27% large nodosities to 20% large nodosities in the control variant.**

***Keywords* – *Lotus corniculatus* L., Seed Size, Ultrasounds, Nodosities.**

I. INTRODUCTION

Bird's-foot-trefoil (*Lotus corniculatus* L.) has the widest ecological plasticity among perennial legume species among permanent grassland floristic structure.

Physical factors, such as ultrasound treatment were used to improve the germination and development of several plant species [1]-[4]. Ultrasound treatment and low temperature exposure were found to enhance the germination of bird's-foot-trefoil seeds [5].

The growth and development of bird's-foot-trefoil plants depends on the size of the seeds used to seed or self-seed by scattering upon maturity. The wide variability of seed size has been an important goal in the improvement of bird's-foot-trefoil and in the sowing technology in this species (selection and conditioning of the seeds per size groups).

The seeds of bird's-foot-trefoil (*Lotus corniculatus* L.) have a considerable influence on plant growth and development, i.e. on both seed quality (energy and germination ability) and plant vigour during vegetation [5], [6]. Studies show that seed size also influences nodule-formation ability, i.e. the number, weight and size of the root nodules (nodosities) [7], [8].

In this paper, we present the influence of seed size and ultrasound treatments (of different ultrasound intensities and durations of exposure) on the nodule-formation and nodosity size share in bird's-foot-trefoil plants.

II. MATERIALS AND METHODS

Our research was carried out at the Research Centre in Grassland and Fodder Crops of Banat's University of Agricultural Science and Veterinary Medicine " King Michael I of Romania" from Timisoara, Romania.

The research was conducted under laboratory conditions at a temperature of 22-25 °C, at constant humidity and natural light.

We performed a bifactorial experiment with the following factors: a – seed size, b – ultrasound treatments.

The biological material we used was the *Lotus corniculatus* L. Nico cultivar from seeds produced in the year 2013. Before sowing, the seeds were grouped into five size groups (depending on seed diameter): a1 – control (unsieved) seeds, a2 – very small seeds (of diameter Ø = 0.50-1.00 mm), a3 – small seeds (Ø = 1.01-1.25 mm), a4 – medium-size seeds (Ø = 1.26-1.40 mm), a5 – large seeds (Ø = 1.41-1.50 mm), and a6 – very large seeds (Ø > 1.51 mm).

These seed groups were treated with low frequency ultrasounds of various intensities, for various durations. The device producing the ultrasound was built at the Politehnica University of Timisoara, consisting of a generator (Fig. 1, right) with continuously adjustable power (0-60 W) and frequency (18-25 kHz), and a magnetostrictive ultrasonic transducer (Fig. 1, left).

Fig.1. The experimental device for producing ultrasounds

The ultrasonic treatment variants, denoted as b1 to b6, correspond to the following parameters (intensity of ultrasounds (W/cm^2), and duration of ultrasound exposure (s)):

b1 - (0,11W/cm^2+60s);
b2 - (0,98W/ cm^2+60s);
b3 - (2,72W/ cm^2+60s);
b4 - (1,74W/ cm^2+20s);
b5 - (1,74W/ cm^2+40s);
b6 - (1,74W/ cm^2+80s).

From each seed group, we sowed ten seeds in germination vases and, after sprouting, we studied each plant individually. During vegetation, we measured each plant quantitatively and qualitatively from the point of view of their height, ramifications, sprouting, and weight.

The nodule-formation capacity was measured at the beginning of blooming in ten plants in each seed size group. Twelve hours after the plants were immersed in water, we removed the soil with a gentle flow of water and we sampled roots from the root system that we collected in Petri dishes on porous paper to remove the water. Finally, we weighed the roots with a digital scale of 0.1 mg measuring precision BP 221 S (Sartorius, Germany).

To facilitate the counting of nodosities and the measurement of their size, nodosities were photographed with a digital camera and the images were processed using Image J [9]. To this end, we used the following menu items: File – Open; Process – Enhance contrast – Subtract Background; Image – Adjust – Colour Threshold; Analyze – Analyze Particles.

The nodosities collected from the root of each plant were grouped into three size groups: small, medium, and large nodosities. The classification was done using Microsoft Excel (menu: Home – Sort&Filter).

The resulting data (number and weight of nodosities per plant, as well as the size of nodosities) were processed statistically via variance analysis and the Duncan test using the MSTAT-C program (Michigan State University, USA). Student's t-test was performed using the Analysis ToolPak add-in program of Microsoft Excel. To point out the existence of a relationship between seed size and nodule-formation ability, we also studied the correlations between the sizes of seeds and nodosities.

III. RESULTS AND DISCUSSION

Influence of seed size on nodule-formation ability in Lotus corniculatus L.

The number of nodosities formed on the roots of bird's-foot trefoil was strongly influenced by seed size. From this point of view, there was a wide variation of the feature, with limits ranging between 59 and 274 nodosities/plant. In the variants sowed with very small seeds, there was the largest number of nodosities (274 nodosities/plant), followed by the variant sowed with medium seeds (200 nodosities/plant) and, far behind, by the variant sowed with small seeds (91 nodosities/plant). Large and very large seeds had no effect on the number of nodosities, i.e. 59 nodosities/plant in the variant sowed with large seeds and 83 nodosities/plant in the variant sowed with very large seeds (Table 1). Differences with respect to the control variant (a1) are listed in the third column of Table 1 (Δ1). These differences were found very significant, with $p < 0.001$ (denoted by ***). We adopt the notation * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$.

Table 1 Number of nodosities per plant depending on seed size in *Lotus corniculatus* L.

Seed size variants	Number of nodosities	Δ1	Δ2
a1 – control	61	-	–213***
a2 – very small	274	213***	-
a3 – small seeds	91	30***	–183***
a4 – medium	200	139***	–74***
a5 – large	59	-2	–215***
a6 – very large	83	22***	–191***

DL 5% = 7 DL 1% = 9 DL 0,1% = 12

Δ1-Difference and significance compared to a1

Δ2-Difference and significance compared to a2

Data presented in Table 2 show that there is no direct relationship between the number of nodosities and their weight. Thus, if in the variant sowed with very small seeds there was the largest number of nodosities (200 nodosities/plant), they weighed only 103.11 mg/plant compared to the variant sowed with large seeds that produced the smallest number of nodosities (59 nodosities/plant) weighing the most (127.8 mg/plant). In the variant sowed with very large seeds, there was a lower weight of nodosities (70.1 mg/plant), but larger than in the variants sowed with small and medium size seeds. The number of nodosities was lowest in the variant sowed with large seeds (Table 1), whereas the weight of nodosities was lowest the variant sowed with small seeds (Table2).

Table 2: Weight of nodosities depending on seed size in *Lotus corniculatus* L.

Seed size variants	Mass of nodosites per plant (mg)	Δ1	Δ2
a1 – control	45.2	-	–57.93***
a2 – very small	103.1	57.93***	-
a3 – small	43.3	–1.90***	–59.83***
a4 – medium	63.5	18.28***	–39.65***
a5 – large	127.8	82.59***	24.66***
a6 – very large	70.1	24.92***	–96.10***

DL 5% = 4.18 DL 1% = 5.57 DL 0.1%=7.28

Δ1-Difference and significance compared to a1

Δ2-Difference and significance compared to a2

The share of nodosity size also showed significant differences between size variants. From this perspective, the share of small nodosities had the highest values with limits ranging between 29% and 60%, depending on seed size. The highest share of small nodosities (60%) was in the variant sowed with small seeds while the lowest share of small nodosities (29%) was in the variant sowed with very large seeds. The differences calculated compared to the control variant or with the variant sowed with very small seeds are very significant statistically (Table 3).

Table 3: Share of small nodosities depending on seed size in *Lotus corniculatus* L.

Seed size variants	Share of small nodosities (%)	Δ1	Δ2
a1 – control	58	-	15***
a2 – very small	42	–15***	-
a3 – small seeds	60	7***	22***
a4 – medium	40	–18***	–3***
a5 – large seeds	36	–20***	–5***
a6 – very large	29	–22***	–7***

DL 5% = 1.3 DL 1% = 1.8 DL 0.1% = 2.3
Δ1-Difference and significance compared to a1
Δ2-Difference and significance compared to a2

The share of large nodosities of the total formed nodosities was lower, with limits ranging between 13% (in the variant sowed with small seeds) and 30% (in the variants sowed with large and very large seeds). In the variants sowed with control seeds or with very small and small seeds, the share of large nodosities was lower, i.e. between 13% and 21%, compared to the variants sowed with larger seeds (Table 4).

Table 4: Share of large nodosities depending on seed size in *Lotus corniculatus* L.

Seed size variants	Share of large nodosities (%)	Δ1	Δ2
a1 – control	19	-	–2**
a2 – very small	21	2**	-
a3 – small	13	–6***	–8***
a4 – medium	26	5***	3***
a5 – large	30	11***	9***
a6 – very large	30	11***	9***

DL 5% = 1.4 DL 1% = 1.9 DL 0.1% = 2.3
Δ1-Difference and significance compared to a1
Δ2-Difference and significance compared to a2

Combined influence of seed size and ultrasound treatments on nodule-formation ability in Lotus corniculatus L.

The effect of the ultrasound treatments on germination ability particularly in hard seeds has been pointed out by several researchers [2],[3], [10]-[15]. Depending on the intensity of ultrasounds and treatment duration, and on seed physical features (moisture and storage temperature), we could see a positive influence on seed germination due to the destruction of peripheral "hard" cell layer of the seeds followed by the penetration of water and oxygen and embryo activation [5].

Our results show that ultrasound treatment of seeds before sowing influences nodule-formation ability in bird's-foot trefoil plants in a differentiated manner, depending on seed size. Thus, there was a stronger effect of ultrasound treatment in the variants sowed with very small seeds (a2) and with medium seeds (a4). In this case, the maximum number of nodosities (428 nodosities/plant) was in the variants sowed with very small seeds treated with ultrasounds whose intensity was 1.74 W/cm^2 and lasting for 40 seconds (b5). In the same variant, but with ultrasounds whose intensity was 2.72 W/cm^2 for 60 seconds, there were 307 nodosities/plant. Depending on the 42 interactions studied, the ultrasound treatments increased the number of nodosities 1.2-2.7 times compared to the control variant, i.e. from 25-347 nodosities/plant to 68-428 nodosities/plant (Fig.2).

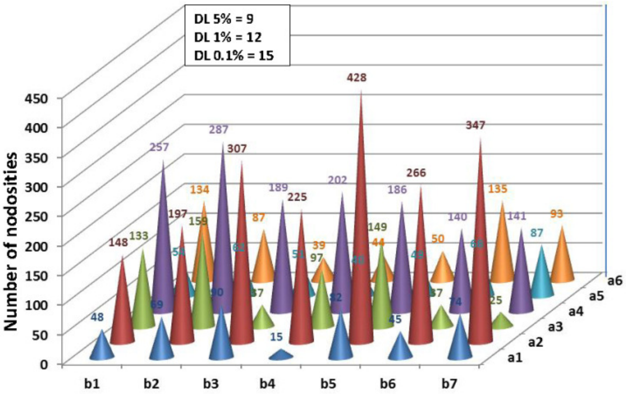

Fig.2. Number of nodosities per plant as a function of seed size (a1-a6) and ultrasound treatment (b1-b7).

The effect of ultrasound treatments was pointed out in nodosity weight within very large limits depending on seed size and on the size of ultrasound parameters (Fig. 3):

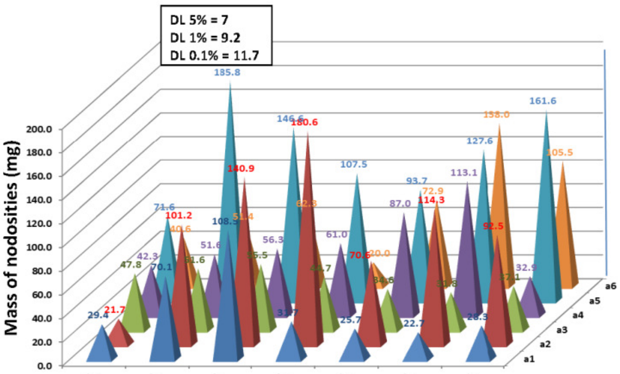

Fig.3. Mass of nodosities per plant as a function of seed size (a1-a6) and ultrasound treatment (b1-b7).

between 22.65 and 108.5 mg/plant in control seeds (a1), between 21.68 and 180.64 mg/ plant in very small seeds (a2), between 31.75 and 55.47 mg/plant in small seeds (a3), between 32.94 and 113.08 mg/plant in medium seeds (a4), between 71.60 and 185.80 mg/plant in large seeds (a5) and between 20.04 and 137.98 mg/plant in very large seeds (a6). Maximum nodosity weight was when treating large seeds with ultrasounds of 0.98 W/cm^2 for 60 seconds (a5).

Ultrasound treatments also group nodosity share depending on seed size. Thus, in lower nodosity shares, their highest share (70%) was in the variant sowed with small seeds (a3) treated with ultrasounds of 1.74 W/cm² for 40 seconds, while the lowest share (39%) was in the variant a4 (sowed with medium size seeds) treated with ultrasounds of 1,74 W/cm² for 80 seconds (Fig. 4a).

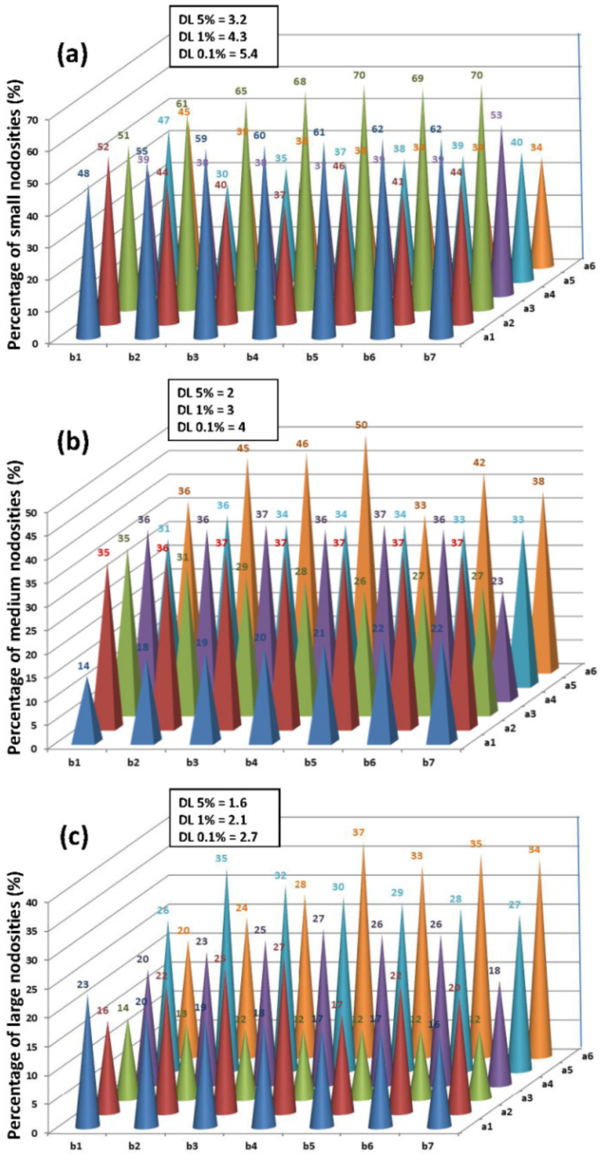

Fig.4. Percentage of small nodosities (a), medium nodosities (b) and large nodosities (c) as a function of seed size (a1-a6) and ultrasound treatment (b1-b7).

In medium size nodosities, the effect of ultrasound treatments was stronger in the variants sowed with very large seeds, with variation limits ranging between 45% and 50% depending on ultrasound parameters. In all studied interactions, the share of medium size nodosities ranged between 22% and 38% in the control variants and between 4% and 50% in the variants treated with ultrasounds (Fig. 4b).

The share of large nodosities under the influence of seed size and ultrasound treatments ranged between 16% and 37%. The highest share (26% - 37%) was in the variants sowed with large and very large seeds (Fig. 4c).

On the average, applying ultrasound treatments on bird's-foot trefoil seeds increased the share of nodosities per size groups: 41% to 53% small nodosities in the control variant; 32% to 27% medium nodosities in the control variant; 27% to 20% large nodosities in the control variant.

IV. CONCLUSION

The results reported in this article point out the long-run effect of seed size combined with ultrasound treatments on nodule-formation ability in bird's-foot trefoil (*Lotus corniculatus* L.). The larger size of seeds, as well as ultrasound treatments have a positive influence on the number, weight and share of large nodosities in bird's-foot trefoil compared to control seeds or very small seeds used in sowing.

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Ilie Toth

was born in Tormac, Timiş County, Romania on November 25, 1964. He obtained a degree in mechanical engineering in 1989 from the Politehnica University of Timisoara, Romania. He further pursued training in technological and informatics education at the same university, and obtained a teaching position at the Public School of Tormac. In 2013 he obtained his PhD degree in biophysics at the Victor Babes University of Medicine and Pharmacy Timisoara, Romania.

His major field of interest is the use of physical factors for improving plant growth and development. More specifically, he is interested in the uses of ultrasounds for enhancing hard seed germination, and in exploiting the symbiosis between plants and risobial bacteria.

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was born in Nehoiu, Buzău County, Romania on November 26, 1949. He graduated as agronomic engineer from the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania, in 1972. He also obtained a degree in agricultural accounting from the Babeş-Bolyai University Cluj-Napoca, Romania, in 1977. In 1979, for three months, he studied biotechnological methods for plant improvement at the Uppsala University, Sweeden; then, in 1981, for 6 months, he worked in France (Lussignan, Clermot-Ferrand, and Clermont-Theix) on genetic techniques for fodder plant improvement. In 1981 he obtained his PhD degree in agronomic sciences at the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca.

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