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Screening of domestic common bean cultivar for salt tolerance during *in vitro* cell cultivation

Abstract: One of the principal limitations in application of cell and tissue culture techniques for improving crop plants resistance to adverse environmental stresses, such as high salinity, is insufficient knowledge of cellular and molecular-genetic basics of this type of tolerance. This approach will provide opportunity to identify new salt-tolerant cultivars, which arisen from somatic mutations increasing the pool of salt-tolerant breeding lines. It is well known that salinity causes sharp reduction in bean productivity and thus significant losses in quality and quantity of products derived from it. Choosing salt-tolerant plant genotypes for cultivation may solve this problem. The aim of this study is to trace tolerance and accompanying changes in lectin accumulation in calli of common bean (*Phaseolus vulgaris L.*) grown under the conditions of tissue culture. In our experiment, induction of certain common bean cultivars by *in vitro* cell cultures have been optimized. The most appropriate composition of the nutrient media for the induction of callusogenesis have been established together with the cultivars possessing high callus-forming ability. We have not observed strict correlation between callus-forming propensity and morphogenic calli generation. However, we noticed that under-passaging in selective conditions calli leads to gradual growth decline and browning, as well as slow growth and in some cases death of cell cultures. Nevertheless, we have identified common bean cultivars with average salt tolerance and high propensity to callusogenesis for use as a starting material for breeding. Differences in lectin content between morphogenic and non-morphogenic calli let us suggest that lectin content depends on hormonal composition of the nutrition medium since morphogenic type of callus was formed when the media contained NAA and low concentrations of 2,4-D. The cultivars used in this study have demonstrated moderate salt tolerance and high callusogenesis efficiency thus regarded as suitable material to breeding for salt tolerance.

Key words: common bean, cultivar samples, *in vitro* culture, calli, salt tolerance, lectins.

Introduction

Salinity is one of the most critical environmental factors leading to decrease in crops productivity under growing salt concentrations in the soil, and this issue keeps on growing [1]. Principal reason for increasing soil salinity is the use of irrigation. This matter may be solved using efficient agricultural practices, improved irrigation methods, and completely or partly desalted water for sprinkle irrigation.

Common bean is a pulse vegetable crop with high activity of lections [2]. Study of the protein composition of the common bean seed is particularly relevant nowadays because there is a need to develop new cultivars, which may serve as the source of protein. This can be achieved by deploying biotechnological approaches towards their identification, selection and their uses in different sectors of agriculture.

Within the last decades, there is a growing need in new sources for extraction of nutritional and anti-nutritional protein components to study their action on different cell models, advanced methods for their isolation, and further use to obtain new plant protection reagents as well as drugs and diagnostics [3; 4].

In the cultivation and use of common bean, lectins (including intracellular) are of particular concern, since they are involved in a number of undesirable reactions, like wounding, cold, drought, osmotic shock and salinity of the growth medium, increasing their hemagglutinating activities [5]. It has been reported that lectin gene expression is induced by low humidity and high salinity stress [6]. Consequently,

development of salt-tolerant cultivars received much attention in common bean breeding lately.

Salt tolerance is thought to be genetically controlled trait in beans [7]. Although it is shown to be monogenic in soybean, the consensus is that it is a polygenic trait. Variability for salt tolerance within the plant species is shown by various surveys [4]. In contrast, there are species with no such type of variability [5].

One of the principal limitations in application of cell and tissue culture techniques for improving crop plants resistance to adverse environmental stresses, high salinity, in particular, is insufficient knowledge of cellular and molecular-genetic basics of such tolerance.

Selection at the cellular level is used in the majority of cases to obtain plant forms that are tolerant or resistant to stress factors, such as salt (including ion stress), diseases, chemical stress caused by herbicides, etc. The aim of this study was to investigate salt tolerance and accompanying changes in lectin accumulation in calli of common bean cultivars (cvs.) grown under tissue culture conditions

Materials and methods

Seeds of 10 domestic and international cvs. of common bean were used for this study. They were: Aktatti, Nazym, Talgat (Kazakhstan), Katka, Luna, Zuzka (Czech Republic), Bijchanka, Ufimskaya (Russian Federation), Camelia, and Red Goya (USA).

Cell and tissue culture work was carried out under strictly aseptic conditions, and thus, sterility was maintained throughout the whole study, i.e., from preparation of glassware and instruments to preparation of seed stocks and nutritional media. In order to obtain aseptic seedlings of common bean, seeds were sterilized sequentially in 60% sulphuric acid for 3 min, 5% chloramine for 5 min, 0.1% mercuric chloride for 10-15 min and 70% ethanol for 1-2 min.

Seeds were then washed thrice in sterile distilled water and planted on the agaric Murashige-Skoog (MS) medium containing half the concentration of macro- and microelements and no growth regulators [8]. After germination, seedlings were grown at 20-25 °C and 16-hour photoperiod.

Then, epicotyls and hypocotyls of 10-15 day-old sterile seedlings were used as explants. Epicotyls were rooted using MS medium without growth regulators to obtain sufficient amount of explants preserving their initial genotype. All nutrition media were autoclaved at 120 °C for 25 min. Analysis of calli formation and morphogenic capacity were performed on Uchimiya-Murashige nutritional medium [9]. 2,4-Dichlorophenoxyacetic acid (2,4-D), kinetin, 1-naphthylacetic acid (NAA), 6-benzylaminopurine (BAP) and yeast extract were used as phytohormone and organic additives.

Sodium chloride (NaCl) concentrations of 0.17 M, and 0.26 M were used as selective agent for salinity tolerance, and the normal medium (no added NaCl) served as control.

Identification and selection of salt-tolerant cell lines were carried out by direct stair-step selection in callus cultures on MS medium containing the above-mentioned NaCl concentrations. Tissues were cultivated at each concentration of NaCl for 8 weeks, and then on the media inducing callusogenesis (4 weeks) and media inducing somatic embryogenesis (4 weeks), respectively. Growth characteristics of calli and biomass accumulation were estimated on all of selective media. Biomass of calli was measured under sterile conditions of laminar box using the torsion scales, whereas the growth activity of calli was assessed microscopically using an MBS-10 device (Levenhuk, Russia).

Biomass of morphogenic calli was proliferated every fortnight by 3-4 times by transferring on fresh modified Uchimiya-Murashige medium, containing 2 mg/L 2,4-D and 0.25 mg/L kinetin. Increased biomass of calli was measured every 28 days.

Basic method of lectin isolation from cell biomass of common bean was developed for seeds and described by Alexidze et al. [10]. We applied it to calli cultures using more gentle homogenization of plant tissue and reduced extraction time.

Statistical analysis of the data was performed using standard methods of statistical research, methods of correlation and variation analyses [11].

Results and discussion

The main objective of this study was to identify and select salt-resistant common bean cultivars by screening domestic cultivar samples within *in vitro* culture. To meet this objective, it was necessary to develop approaches that will allow obtaining highly morphogenic common bean cell cultures.

Callus-forming and somatic embryogenic capacity of common bean. At the earlier stage of this investigation, effective methods of seed and explant sterilization were tested. It was found that duration of seed sterilization differed prior to the time of exposure to chloramine and ethanol solutions, when chloramine concentration was kept at 5% and ethanol concentration achieved 70%. These tests showed that optimal

time for sterilization in chloramine and ethanol would be 5 and 1-2 min, respectively. In subsequent experiments, we carried out extensive screening of Kazakh common bean cultivars to determine their ability to generate calli and promote somatic embryogenesis in tissue culture so that genotypes with high morphogenetic potential can be identified. In this study, 10 cultivars of domestic and foreign origin were examined. Screening was carried out under the following conditions: callusogenesis induction on Uchimiya and Murashige medium (UM) (2,4-D – 2 mg/L, kinetin – 0.25 mg/L) and somatic embryogenesis induction on the same UM medium with other additives (NAA – 0.05 mg/l, BAP – 0.5 mg/L). Epicotyls and hypocotyls of 7-10 day-old sterile seedlings were used as explants. Results showed that both types of explants demonstrated callus formation in 7 to 10 days after the onset of cultivation. However, there were differences respectively to the callus proliferation and the amount of callusogenesis, depending on genotypes and explant sources.

Callus formation started on all nutrition media on 7 to 10 days after cultivation. Comparative analysis of the frequency of callusogenesis dependent on the explant type indicated that the epicotyl and the hypocotyl were capable of forming calli, but the epicotyl was more suitable than the hypocotyl for the induction of callus formation. There was substantially greater mass of the cotyledonary leaves on the epicotyl, increasing the final yield of the callus much higher than that on hypocotyls. Moreover, the proportion of explants forming callus varied from 75 to 95% in most cultivar samples, as shown in the Table 1.

Table 1 – Frequency and amount of callus formation and production of somatic embryoids from the epicotyls of domestic and foreign common bean cultivars

Genotype	Frequency of callusogenesis, %	Intensity of callusogenesis, points	Frequency of morphogenic calli,%	Average number of embryoids per callus
Aktatti	81±0.54	1.9 ±0.14	20±0.31	0±0.21
Bijchanka	84±0.41	1.6±0.21.	14±0.22	7±0.20
Camelia*	93±0.42	3.6±0.32	35±0.11	11±0.17
Katka	89±0.67	3.3±0.17	0	0
Luna	75±0.31	2.0±0.11	18±0.14	9±0.14
Nazym	79±0.42	2.2±0.21	19±0.17	7±0.15
Red Goya*	95±0.71	2.9±0.31	33±0.21	10±0.9
Talgat	82±0.56	2.1±0.26	15±0.17	7±0.11
Ufimskaya	87±0.31	2.7±0.11	13±0.19	8±0.16
Zuzka	80±0.42	2.1±0.12	11±0.23	5±0.12

As can be seen from the Table 1, cvs. Ufimskaya, Camelia, Katka and Red Goya showed the highest callus yields, achieving 87%, 93%, 89% and 95%, respectively. Intensity of the callus formation was assessed by the five-point scale. The highest percentage of the callus formation (more than 2 points) was also observed in the same cultivar samples. Callus tissues differed within and between genotypes with respect to morphology, color and density, and they were subdivided into the following types: 1) friable, granular, weakly watery, heterogenous, white yellow; 2) dense, moisture-deficient, homogeneous, light green; and 3) extensively hydrated, homogeneous, white. Of these calli, their morphology, color, density the first type turned out to be embryogenic.

Within a given common bean genotype, morphology of calli showed less variability than that between the genotypes. It should be noted that we could not experimentally confirm the presence, if any, of a strong correlation between the frequency of callusogenesis and the propensity to produce morphogenic calli.

According to the results, which were obtained using Student-test method, the difference between cvs. Red Goya and Camelia is equal to 2.44 ($p<0.01$), between Katka and Ufimskaya is 2.70 ($p<0.01$), between Talgat and Biichanka is 2.88, and between Luna and Nazym is 7.66. Thanks to this data, which might be considered as statistically significant, we can prove, that all of the cultivars, listed in Table 1, belong to the different lines.

Different investigators emphasize the importance of the relationships between calli morphology and the ability to regenerate plants. Specific features of morphogenic calli may vary among plant species [12]. In this study, we observed that dense, compact callus had reduced morphogenic ability. In our study, formation of morphogenic structures was observed only in cvs. Red Goya and Camelia (Figure 1), whereas cvs. Katka and Ufimskaya produced friable, globular calli. Cv. Katka appeared to have high frequency of callusogenesis (3 points and higher) by its growth. However, it showed only further proliferation of callus, but no somatic embryoid formation or morphogenic calli.

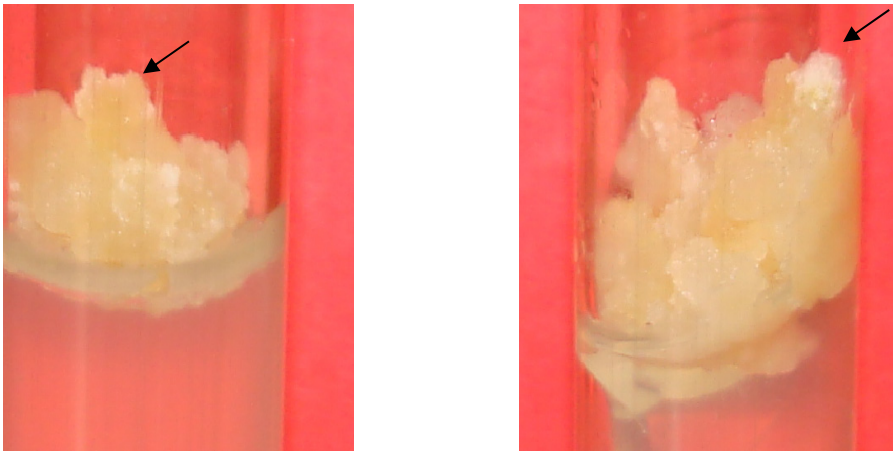

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Figure 1 – Induction of morphogenic calli in tissue culture of common bean cvs. Red Goya (a) and Camelia (b). Note: Morphogenicity is determined by the form, color and density of the callus and the presence of morphogenic tubercules are shown by arrows

Thus, sterilization conditions for the different types of explants were determined along with the optimal cultivation media composition, as well as the relationship between the callusogenesis and morphogenesis *in vitro*. Epicotyls from cvs. Katka, Camelia and Red Goya exhibited maximal callus-generating ability when cultivated in modified Uchimiya-Murashige medium.

As for the relationship between the intensity of callusogenesis and morphological structures of the calli, tight, compact calli had lower morphogenic potency. Morphogenic structures were observed only in cvs. Camelia and Red Goya, which formed loose, globular calli. In callus-producing cultures, the induction of calli was observed upon two-three weeks of cultivation in Murashige-Skoog medium containing NAA (0.05 mg/L) and BAP (0.5 mg/L) (Figure 2).

Before the emergence of green meristematic foci on the light surface of the calli, bud formation was detected. Frequency of morphogenesis in epicotyls was substantially higher than that in hypocotyls, the value varying from 11.1% to 19.3% (Figure 3), whereas the morphogenic capacity in hypocotyls was in the range of 6.5-14.2%.

Next stage of the present study was focused on cellular level selection for salt tolerance in callus cultures of common bean. Subsequent to cultivation under light conditions for a month, part of the calli obtained was transferred on to a selective medium containing varying (0.17 M to 0.26 M) concentrations of NaCl to investigate the accumulation of the callus biomass. The results showed that calli proliferated at 0.17 M/L NaCl concentration but their biomass was decreased by 14 to 48%, depending on the genotype when compared to salt-free control. At 0.26 M NaCl concentration, calli of all cultivars completely stopped accumulating the biomass (Table 2).

Under the increasing salt (NaCl) concentration in the growth medium up to 0.26 M, the calli developed necrotic areas, especially in those genotypes that are more susceptible to salinity. These genotypes turned necrotic already after the first passage of cultivation on medium containing 0.17 NaCl (Figure 4).

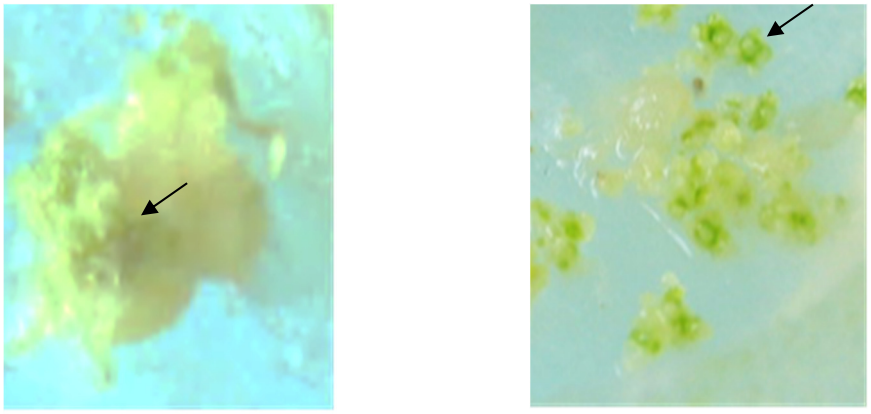

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Figure 2 – Induction of meristematic foci on the surface of the calli in common bean cvs. Red Goya (A), Camelia (B). Note: Shoots are shown by arrows

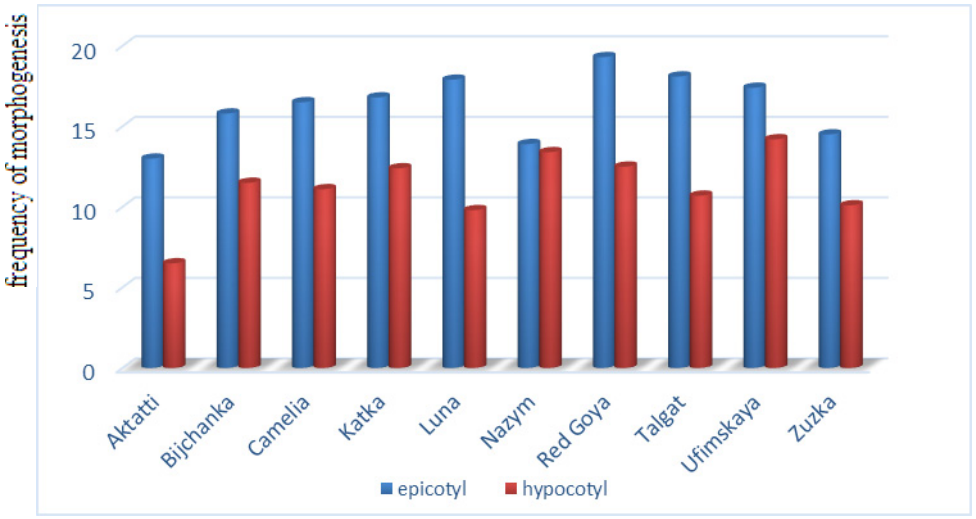

Figure 3 – Dependence of the frequency of morphogenesis on the type of explant in different cultivars of common bean

Table 2 – Accumulation of the biomass by common bean calli on selective medium simulating soil salinity

Genotype	Initial average mass, mg	Mass of callus in the absence of salt, mg (blank)	Mass of callus on stressor-containing medium, mg
Aktatti	101.67 ± 1.41	118.71 ± 1.23	102.92 ± 1.37
Bijchanks	86.42 ± 1.82	106.71 ± 1.98	86.45 ± 3.24
Camelia	120.66 ± 2.34	152.72 ± 1.45	136.58 ± 1.79
Katka	132.75 ± 1.71	166.81 ± 2.46	130.18 ± 1.63
Luna	85.32 ± 1.83	163.71 ± 1.56	85.74 ± 1.36
Nazym	84.59 ± 1.10	110.83 ± 1.53	84.69 ± 2.74
Red Goya	112.66 ± 2.93	158.62 ± 2.45	135.93± 1.84
Talgat	92.79 ± 1.89	126.61 ± 1.34	93.92 ± 1.49
Ufimskaya	119.55 ± 1.85	145.73 ± 1.47	125.73 ± 1.55
Zuzka	94.79 ± 1.95	125.83 ± 0.67	94.5 ± 1.38

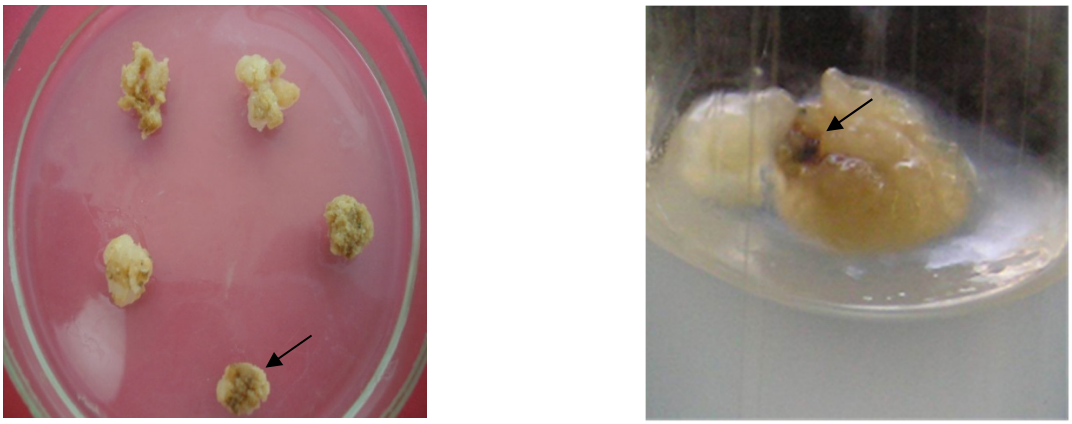

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Figure 4 – Change in the callus tissue morphology (shown by the arrows) from genotypes Nazym and Talgat after 30-day cultivation in the medium containing 0.17 M/L NaCl.
Note: a – growth and development inhibition in the callus tissue of cultivar Nazym;
b – necrosis of the callus tissue from cultivar Talgat

Our results indicate that the reduction of growth under salinity conditions in comparison to control was especially remarkable in the calli of such highly salt-susceptible genotypes as Aktatti, Bijchanka, Zuzka, Luna, Katka, Nazym, and Talgat. Although all genotypes have displayed susceptibility to salinity. American cvs. Red Goya and Camelia were less sensitive to salinity than other cultivars tested. In addition, we observed substantial differences among cultivars with respect to calli morhology in that the calli of less susceptible genotypes under the salinity stress (during the first passage) were more dense, and they retained their original color and consistency. In contrast, the calli of strongly salt-susceptible genotypes began to darken and become necrotic even under minimal salt concentration.

Selection of resistant cell clones under salt stress was done on the basis of two criteria: growth and maintenance of morphogenetic capability. However, morphogenetic potency weighted more heavily than growth characteristics during the selection. Cv. Red Goya was a standout candidate for selection because it was able to grow and form tolerant morphogenic calli for a long period.

Performing cell selection in two stages allowed us to estimate the degree of resistance of common bean genotypes to salinity, including those that maintain callus morphogenicity.

As a rule, *in vitro* growth and accumulation of biomass tend to decline as concentration of the stressor (i.e., NaCl) in nutrient medium increases. This is evident from the data (presented in Table 2) that growth decreases under the saline stress compared to control in all of the calli of the following genotypes: Aktatti, Bijchanka, Zuzka, Luna, Katka, Nazym and Talgat. Thus, we conclude that only American cvs. Red Goya and Camelia are relatively salt-resistant. Therefore, out of the 10 cultivars used in this study, only these two cultivars may be used as source material to breed salt resistant cultivars in further studies. All in all 1016 explants and 926 calli of common bean were planted on the NaCl-containing selective media, and of this total, 201 callus lines were passed for regeneration yielding 21 regenerated plants (the yield of 7.6%).

The other focus of our study was on lection accumulation in callus tissues of common bean and its relationship to salinity. The first evidence of protective properties of lectins under salinity was reported in conjunction with the description of a protein encoded by Sal T gene in rice, *Oryza sativa* L. [13]. This protein was a cytosolic mannose-specific lectin [14; 15], and it was not detectable under normal conditions but was induced and detectable in roots subjected to salt stress or drought [16; 17]. Nonetheless, even after induction by abiotic stress factors, it was not highly expressed.

The generic functions attributed to lectin are binding to carbohydrates with high degree of specificity, and their postulated role in cell division [18; 19]. Hence, lectins may play essential roles in morphological and physiological processes and intracellular interactions, which are crucial for cellular and tissue differentiations. Lectins may also be involved in induction of somatic embryogenesis in tissue cultures [20; 21]. With these

consideration in mind, we performed , comparative analysis of lectin content in callus tissues of common bean cultivars. Calli of these cultivars differing by morphogenetic potency were obtained under optimized conditions. The analysis indicated significant differences in lectin content among callus tissues of common bean cultivars, namely, high lectin concentration being a characteristic of the morphogenic type callus. This consistent pattern was not genotype-dependent. Although the analysis of extracts from callus cultures showed variability with respect to lectin content, maximal lectin levels were always observed in calli extracts from morphogenic callus tissue (Figure 5).

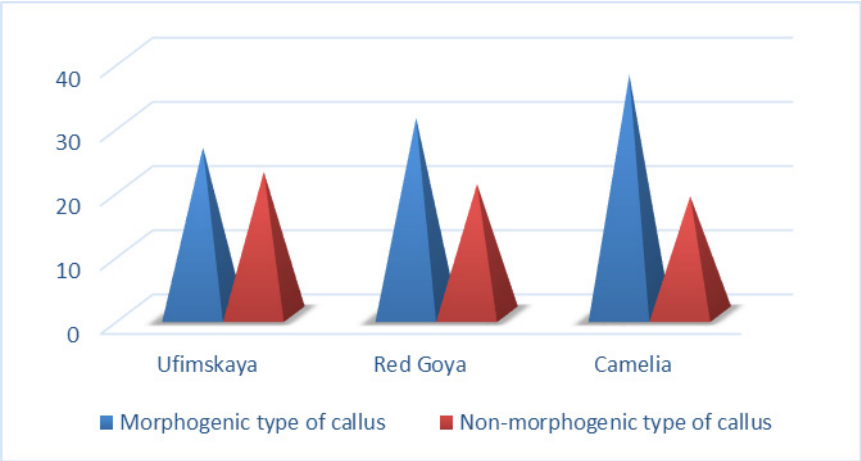

Figure 5 – Lectin content in common bean calli (mg/ 100g of fresh weight)

According to the results presented in Figure 5, lectin concentration in the morphogenic calli ranges between 26.0 mg/100 g of fresh weight in cv. Ufimskaya and 37.3 mg/100 g of fresh weight in cv. Camelia. Callus tissues from non-morphogenic cultivars contained low lectin concentrations, which ranged from 18.4 to 25.2 mg/100 g of fresh weight.

Conclusion

Salt tolerance is not necessarily manifested at the level of the whole plant as the mechanisms of cellular and organismic adaptations may differ. That is why screening and selection for salt tolerance *in vitro* should not be used without prior establishment of substantial correlation between the whole plant and cell culture responses to stress by performing appropriate experiments [22].

Tissue culture technology may facilitate investigations on salt tolerance at cellular level in order to identify germplasm to be used as source material in breeding for salinity tolerance [23]. In addition, this approach will provide opportunity to identify new salt-tolerant variants arising from somatic mutations increasing the pool of salt-tolerant breeding lines.

The correlation linkage between biomass accumulation in calli under osmotic stress and plant drought resistance traits *in vivo* is shown [24; 25]. Consequently, the ability to grow and accumulate biomass under stress conditions may be critical indicators of genotype resistance to stress.

Our data shows that by using NaCl-containing media, it is possible to conduct a primary evaluation of salt tolerance among genotypes based on morphological characteristics of callus tissues and growth inhibition as measured by the degree of reduction in biomass accumulation. Such preliminary evaluation will pave the way to further studies using *in vitro* cultures for selecting salt-tolerant and salt-sensitive common bean cultivars.

Differences in lectin content between morphogenic and non-morphogenic calli suggest that lectin content is dependent on hormonal composition of the nutrition medium since morphogenic type of callus was formed when the media contained NAA and low concentrations of 2,4-D. It is known from the literature [26; 27] that the synthesis of lectins is triggered by abscisic acid (ABA), and high concentrations of 2,4-D reduce the ABA content.

It was discovered that the common bean cultivars used in this study possess average salt tolerance and high callusogenesis potency; and they are suitable to be used as a source material for breeding salt tolerant lines.

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