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GENETIC VARIABILITY OF RED CLOVER SEEDLINGS IN RELATION TO SALT STRESS

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Abstract: Red clover is highly salt-sensitive plant, especially during germination and early seedling growth stages. The aim of this investigation was to estimate the effects of different saline conditions (0, 50, 100, 150, and 200mM NaCl) on germination and early seedling growth in four red clover varieties (Kolubara, K-32, K-17 and K-39). Germination test was conducted in the laboratory conditions using sterile plastic vessels on filter paper moistened with 10ml of the appropriate salt test solutions. It was observed that the germination energy (GE), germination (G), percentage of dead or infected seeds (DIS), normal seedlings (NS), root length (RL), shoot length (ShL), fresh weight (FW) and dry weight of seedling (DW) and seedling vigor index (SVI) were significantly decreased with increasing concentrations of NaCl in the growing medium. The tested varieties of red clover showed different NaCl tolerance at the seedling stage. Generally, studied red clover varieties are very sensitive to salt, especially K-32 which has the lowest values for GE, G, NS and SVI and highest for DIS. Variety K-17 proved to be a variety that the best tolerates conditions of salt stress because the values for GE, G, NS, RL and SVI were highest. Testing of varieties of red clover in the early seedling growth at different concentrations of NaCl in the growing medium could be helpful in the identification and selection of varieties for cultivation on saline soils.

Key words: variety, germination, early seedling growth, red clover, salinity

Introduction

Red clover is the second most important perennial forage legume in Republic of Serbia. Red clover is planted on area of about 120.000 ha in Republic of Serbia with average production on 4 t ha^{-1}, especially in mountainous regions

(Mandić et al., 2011). The soil salinity is one of the important abiotic factors affecting crop production. Estimates are that about 7% of the world's total land area is affected by salt *(Munns et al., 2002)*. There is 5.112.000 ha of total agricultural land in Serbia, of which are 233,000 ha of saline and alkaline soils *(Ličina et al., 2011)*. Legume species are significant genotypic differences with respect to salt tolerance *(Asgharipour and Rafiei, 2011)*. Legumes are generally more sensitive to salinity, especially red clover *(Asci, 2011)*. *Shereen and Ansari (2001)* reported that the salt salinity might affect legume growth and development independently. *Asci (2011)* reported that germination is an important stage in the life cycle of crop plants, particularly in saline soils as it determines the degree of crop establishment. *Kara and Kara (2010)* concluded that salinity has toxic effect on germinating seeds, and excessive salt hinders seed from water uptake during germination. *Machado et al. (2004)* pointed out that the main negative effect of salinity on seed water uptake difficult. Also, *Khan et al. (2001)* reported that salinity is a major environmental stress factor that affects seed germination. Salinity resistance of germination seeds of forage rape, berseem clover, alfalfa, and red clover has been shown to be a heritable trait which could be used as an efficient criterion for the selection of salt-resistant populations *(Ashraf et al., 1987)*. *Rogers et al. (1995)* found that germination significantly decreased with increasing NaCI concentrations between 60 to 200 mol m^{-3} NaCl in three populations of white clover. Many researchers reported that the highest concentration of NaCl strongly affected germination and growth of seedlings in several species of leguminous fodder crops: Egyptian, red and Persian clovers *(Gravandi, 2013)*, Persian clover *(Ates and Tekeli, 2007)*, strawberry clover *(Can et al., 2013)*, white clover and Egyptian clover *(Saberi et al., 2013)*, alfalfa *(Zhanwu et al., 2011)*, *Medicago ruthenica (Guan et al., 2009)*, yellow sweet clover *(Ghaderi-Far et al., 2010)* and sainfoin *(Majidi et al., 2010)*.

The aim of this paper was to estimate the effects of various NaCl concentrations (0, 50, 100, 150, and 200mM NaCl) on germination and early seedling growth in four Serbian red clover varieties (Kolubara, K-32, K-17 and K-39).

Materials and methods

The experiment was carried in March 2011 in the laboratory conditions at the Institute for Animal Husbandry in Belgrade. The research included the seeds of four varieties of red clover: Kolubara, K-32, K-17 and K-39. Seeds were taken from the second growth in 2010. The seeds were stored in paper bags in laboratory room. Seeds were sterilized in 6% sodium hypochlorite solution during 5 min and washed 3 times in sterile distilled water. Before sowing the seeds scarified in a ceramic mortar with fine quartz sand. Germination tests were carried out at 20 ± 1°C, in darkness in sterile plastic vessels (15 cm wide, 21 cm long and 4 cm high) on filter paper moistened with 10ml of the appropriate salt test solutions (0, 50,

100, 150, and 200mM of NaCl), using four replicates of 100 seeds. The experimental design was arranged in a Randomized Complete Block Design (RCBD).

According to *ISTA (1999)* seeds germinate when root elongation of about 2 mm. Germination energy (GE) and germination (G) were evaluated after 4 and 7 days after sowing, respectively *(ISTA, 2008)*. Percentage of dead or infected seeds (DIS), percentage of hard seed (HS), normal (NS) and abnormal seedlings (AS), root length (RL), shot length (ShL), fresh weight (FW) and dry weight (DW) of seedling were evaluated after 14 days. Values for DW were obtained after drying NS at 80 °C for 24 hours. Seedling vigor index (SVI) was calculated as per formula *(ISTA, 1999)*: Vigor Index = (Root length + Shoot length) x Germination percentage.

Data were subjected to an ANOVA using Statistica version 10, a Randomized Complete Block Design and Duncan's Multiple Range Test was used to compare differences among treatment means ($P < 0.05$).

Results and discussion

Seed traits. Results of ANOVA indicated that variety had significant effect on GE, G, DIS, and HS (Table 1). The salt had highly significant effect on GE, G and DIS. The interaction of salinity and varieties had significant effect on GE, G and DIS.

GE and G of studied red clover varieties were affected by salt treatment. Varieties Kolubara and K-17 have higher GE (53.6% and 49.2%) and G (63.6% and 62.6%) than varieties K-32 (24.4% and 34.3%, respectively) and K-39 (34.3% and 46.6%, respectively). GE and G were decreased with increasing salinity. Maximal GE (86.1%) and G (88.6%) were at 0 mM NaCl, and minimal at 200 mM NaCl (5% and 17.8%, respectively). This can be explained due to compression of membranes at high osmotic potential under salt stress conditions. Varieties Kolubara and K-17 have tolerance to low salt stress (50mM NaCl). Generally, seeds of red clover were as sensitive to high levels of NaCl concentrations. *Asci (2011)* and *Atis et al. (2011)* also concluded that increasing the salinity decreases the G of red clover. Salinity affects germination of seeds either by creating osmotic potential which prevent water uptake, or by toxic effects of Na^+ and Cl^- on embryo viability *(Lianes, 2005)*. *Ates and Tekeli (2007)* have found that G of different *Trifolium resipinatum* sp. was 5% in dose of 150 mM salt. In our study red clover germinated in a dose of 200 mM salt. Higher salt doses were not used since *Nichols et al. (2009)* have concluded that the G in pasture legumes did not germination on higher NaCl levels.

NaCl concentrations and varieties significantly effect on DIS. The highest DIS values were determined in K-32 (60.7%) and lowest in K-39 (24.4%). Minimal DIS was recorded at control (3.6%) and maximal at 200mM NaCl (72.2%). *Mandić et al. (2011)* found that variety and pH did not significantly affect on DIS in two Serbian red clover varieties (Kolubara and K-17). Also, in our

studies there were not differences for DIS between these Serbian red clover varieties, indicating of similar their behavior in the environment conditions.

HS were affected by variety. The number of seed who not germinate within 10 days after placement on germination is the number of HS. The HS ranged from 0.2% (Kolubara) to 29% (K-39). Results indicate that the hard seed characteristic is under genetic control. This result is consistent with the researches *Mandić et al. (2011)*. The NaCl concentrations did not affect the HS. *Elçi (2005)* concluded that the genetic and environmental factors during plant growth determine the maximum proportion of hard seeds in *Fabaceae* family.

Table 1. The effects of variety and different NaCl concentration level on seed properties of red clover

Parameters	Variety (A)	NaCl concentration effects mM NaCl (B)					Means
		0	50	100	150	200	
Germination energy (GE), %	Kolubara	94.8	84.0	60.0	22.0	7.0	53.6[a]
	K-32	87.0	21.0	10.0	3.0	1.0	24.4[c]
	K-17	94.0	81.0	44.0	20.0	7.0	49.2[a]
	K-39	68.5	54.0	32.0	12.0	5.0	34.3[b]
	Means	86.1[a]	60.0[b]	36.5[c]	14.2[d]	5.0[e]	40.4
Germination, %	Kolubara	98.0	91.0	72.0	40.0	17.0	63.6[a]
	K-32	88.5	30.0	21.0	21.0	11.0	34.3[c]
	K-17	98.0	91.0	62.0	34.0	28.0	62.6[a]
	K-39	70.0	67.0	53.0	28.0	15.0	46.6[b]
	Means	88.6[a]	69.8[b]	52.0[c]	30.8[d]	17.8[e]	51.8
Dead or infected seeds (DIS), %	Kolubara	2.0	9.0	28.0	60.0	82.0	36.2[b]
	K-32	6.5	65.0	74.0	74.0	84.0	60.7[a]
	K-17	2.0	7.0	35.0	63.0	69.0	35.2[b]
	K-39	4.0	3.0	18.0	43.0	54.0	24.4[c]
	Means	3.6[a]	21.0[b]	38.8[c]	60.0[d]	72.2[e]	39.1
Hard seed (HS), %	Kolubara	0	0	0	0	1.0	0.2[c]
	K-32	5.0	5.0	5.0	5.0	5.0	5.0[b]
	K-17	0	2.0	3.0	3.0	3.0	2.2[c]
	K-39	26.0	30.0	29.0	29.0	31.0	29.0[a]
	Means	7.8[a]	9.2[a]	9.2[a]	9.2[a]	10.0[a]	9.1
F test	GE	G	DIS	HS			
A	**	**	**	**			
B	**	**	**	ns			
AB	**	**	**	ns			

Legend: [(1)] Means followed by the same letter within a column are not significantly different by Duncan's Multiple Range Test at the 5% level ($p \leq 0.05$), ** - significant at 1% level of probability, * - significant at 5% level of probability and ns - not significant

Table 2. The effects of variety and different NaCl concentration level on seedling traits of red clover

Parameters	Variety (A)	NaCl concentration effects mM NaCl (B)					Means
		0	50	100	150	200	
Normal seedling (NS), %	Kolubara	88.0	79.0	58.0	27.0	10.0	52.4[a]
	K-32	74.5	15.0	9.0	9.0	1.0	21.7[c]
	K-17	88.0	83.0	53.0	25.0	9.0	51.6[a]
	K-39	62.0	58.0	43.0	23.0	5.0	38.2[b]
	Means	78.1[a]	58.8[b]	40.8[c]	21.0[d]	6.2[e]	41.0
Abnormal seedling (AS), %	Kolubara	10.0	12.0	14.0	13.0	7.0	11.2
	K-32	14.0	15.0	12.0	12.0	10.0	12.6
	K-17	10.0	8.0	9.0	9.0	19.0	11.0
	K-39	8.0	9.0	10.0	5.0	10.0	8.4
	Means	10.5	11.0	11.2	9.8	11.5	10.8
Root length (RL), cm	Kolubara	2.0	2.1	2.0	0.9	0.4	1.5[b]
	K-32	2.4	2.0	1.7	1.5	1.0	1.7[ab]
	K-17	2.6	2.5	2.2	1.5	0.93	1.9[a]
	K-39	2.7	2.6	2.1	1.2	0.6	1.8[a]
	Means	2.4[a]	2.3[ab]	2.0[b]	1.3[c]	0.7[d]	1.7
Shoot length (ShL), cm	Kolubara	7.4	6.2	5.2	2.9	2.1	4.8
	K-32	7.2	6.9	5.7	4.0	2.4	5.2
	K-17	7.9	7.4	5.5	3.5	2.4	5.4
	K-39	8.1	7.0	5.1	3.2	1.6	5.0
	Means	7,6[a]	6,9[b]	5,4[c]	3,4[d]	2,1[e]	5.1
Fresh weight (FW), g	Kolubara	17.03	15.85	6.84	5.07	0.95	9.15
	K-32	17.48	17.10	10.70	6.56	0.26	10.42
	K-17	14.65	14.54	11.95	5.54	2.32	9.80
	K-39	15.91	13.95	9.71	5.27	2.04	9.38
	Means	16.27[a]	15.36[a]	9.80[b]	5.61[c]	1.39[d]	9.69
Dry weight (DW), g	Kolubara	1.57	1.60	1.06	1.07	0.29	1.12
	K-32	2.02	1.79	1.62	1.05	0.70	1.44
	K-17	1.67	1.59	1.31	1.08	0.99	1.33
	K-39	1.60	1.40	1.38	1.28	0.48	1.22
	Means	1.71[a]	1.59[a]	1.34[ab]	1.12[b]	0.61[c]	1.28
Seedling vigor index (SVI)	Kolubara	916.5	753.3	510.6	140.4	43.6	472.9[b]
	K-32	853.3	260.5	157.8	118.2	38.9	285.8[d]
	K-17	1026.0	899.4	481.4	161.1	84.9	530.6[a]
	K-39	758.6	641.8	379.6	124.5	35.2	388.0[c]
	Means	888.6[a]	638.7[b]	382.3[c]	136.1[d]	50.7[e]	419.3
F test	NS	AS	RL	ShL	FW	DW	SVI
A	**	ns	*	ns	ns	ns	**
B	**	ns	**	**	**	**	**
AB	**	ns	ns	ns	ns	ns	**

Legend: [(1)] Means followed by the same letter within a column are not significantly different by Duncan's Multiple Range Test at the 5% level ($p \leq 0.05$), ** - significant at 1% level of probability, * - significant at 5% level of probability and ns - not significant

Seedling traits. Results of ANOVA indicated that variety had significant effect on NS, RL and SVI (Table 2). The salt had highly significant effect on NS, RL, ShL, FW, DW and SVI. The interaction of salinity and varieties had significant effect on NS and SVI.

Varieties Kolubara and K-17 have statistically significant higher NS (52.4% and 51.6%) than K-32 (21.7%) and K-39 (38.2%). The NS was significantly decreased when seeds were subjected to higher salinity levels. Result shows that at 0 mM NaCl the NS is 78.1%, at 50 mM NaCl is 58.8%, at 100 mM NaCl is 40.8%, at 150 mM NaCl is 21.0% and at 200 mM NaCl is 6.2%. *Atis et al. (2011)* concluded that 240 mM salinity level had the most negative influence on NS of red clover all studied seeds lots.

According to *ISTA (2009)* the AS are those which do not show capacity for continued development into normal plants when grown in good quality soil, under favorable condition of heat, light ad water supply. Variety and NaCl concentrations did not significantly affect on AS. The AS was higher when seeds were germinated at higher levels of salinity. However, the difference is not significant. AS are incapable of normal growth and are therefore incapable of developing into healthy seedlings in the field. Previous research indicates that the initial phase of seed deterioration is seed degradation in which there is a reduction in ATP synthesis, respiration and biosynthesis rates, resulting in reduced emergence and development of AS *(Dornbos, 1995)*.

Varieties K-17 and K-39 have significantly higher RL (1.9 cm and 1.8, respectively) than Kolubara (1.5 cm). Salt concentrations significantly decreased RL. The highest RL (2.4 cm) was obtained at 0 mM NaCl and lowest (0.7 cm) at 200 mM NaCl.

Varieties of red clover did not have significant differences in ShL. Salt concentrations significantly decreased ShL. The highest ShL was obtained at 0 mM NaCl (7.6 cm) and lowest at 200 mM NaCl (2.1 cm). *Asci (2011)* reported that populations of red clover have not significant effect on RL and ShL, and that RL and ShL decreased with increasing levels of salinity and the lowest value for both traits was obtained at 180 mM NaCl. Reduced seedling growth under salt stress conditions are also reported *Ates and Tekeli (2007)* on persian clover and *Zhanwu et al. (2011)* on alfalfa. High salinity levels led to a decrease in these parameters due to retardation in water and essential mineral nutrients absorption from soil by plant.

The varieties did not have significant differences in FW and DW. The NaCl concentrations had significant effect on the FW and DW. Minimal FW (1.39 mg) and DW (0.61 mg) were at 200 mM NaCl and maximal FW (16.27 mg) and DW (1.71 mg) at 0 mM NaCl. The reduction in DW could be due to the high concentration of Na^+ and Cl^-.

Varieties and NaCl concentrations have significant effect on SVI. K-17 had significantly higher SVI (530.6) than Kolubara (472.9), K-32 (285.8) and K-39

(388.0). Maximal SVI (888.6) was at 0 mM NaCl and minimal SVI (50.7) at 200 mM NaCl. This indicates that increased NaCl concentration caused a harmful effect in the seed. Seed vigor is the ability of a seed to germinate and grow rapidly to establish a normal seedling. Good seed vigor means rapid and uniform emergence and development of normal seedlings under a wide range of field conditions. *Ferguson (1995)* concluded that seed vigor is concept describing the interaction of several characteristics (the rate and uniformity of germination and growth, tolerance of environmental stresses after sowing, and retention of performance capacity after storage). *Vieira and Carvalho (1994)* reported that the vigor comprises a set of characteristics that determine seed vigor and is influenced by environmental conditions and handling during the stages of pre-and post-harvest. In addition to the above, vigor index determines the longevity of seed, without adverse consequences *(ISTA, 2009)*.

Conclusion

The properties GE, G, DIS, NS, RL and SVI of red clover were significantly affected by variety and NaCl concentration. Also, the variety had significant effect on HS and the NaCl concentration on ShL, FW and DW. Results indicate genetic variability existing among Serbian red clover varieties for salinity tolerance. Generally, studied commercial varieties of red clover are sensitive to salt stress conditions during seed germination and early seedling growth although variety K-17 had best salt tolerance performance. Results indicate on ability growth of seedling of red clover of different NaCl concentration. Also, results indicate that testing of genotypes of red clover in the early seedling growth at different NaCl levels would be helpful in the identification and selection of genotypes for cultivation on saline soils.

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Genetička varijabilnost klijanaca crvene deteline u odnosu na soni stres

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Rezime

Crvena detelina je vrlo osetljiva biljka na veću koncentarciju soli, posebno tokom klijanja i rane faze porasta klijanaca. Cilj ovog istraživanja bio je da se proceni uticaj različite zaslanjenosti (0, 50, 100, 150 i 200mM NaCl) na klijanje i rani porast klijanaca četiri sorte crvene deteline (Kolubara, K-32, K-17 и K-39). Test klijavosti sproveden je u laboratorijskim uslovima u sterilnim plastičnim posudama na filter papiru natopljenom sa 10 ml odgovarajuće koncentracije soli. Uočeno je da energija klijanja (EK), klijavost (K), neklijala i bolesna semena (NB), normalni klijanci (NK), dužina korena (DK), dužina hipokotila (DH), sveža (SvMK) i suva masa klijanaca (SuMK) i vigor indeks klijanaca (VIK) se značajno smanjuju sa povećanjem koncentracije NaCl u podlogama za naklijavanje. Ispitivane sorte imale su različitu toleranciju na soni stres u fazi klijanaca. Generalno, proučavane sorte su veoma osetljive na veću koncentraciju soli, posebno K-32 koja je imala najniže vrednosti za EK, K, NK i VIK, kao i najveći broj NB. Sorta K-17 se pokazala kao sorta koja najbolje toleriše soni stres jer su vrednosti za EK, K, NK, DK i VIK bile najviše. Testiranje sorti crvene deteline u ranom porastu klijanaca na podlogama za naklijavanje sa različitom koncentracijom NaCl može pomoći u indentifikaciji i izboru sorti za gajenje na zaslanjenim zemljištima.

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