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Temperature and water solution pH effects on crimson clover (*Trifolium incarnatum* L.) imbibition and seedling traits

Utjecaj temperature i pH otopine na upijanje sjemena i svojstva klijanaca inkarnatke (*Trifolium incarnatum* L.)

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ABSTRACT

Crimson clover (*Trifolium incarnatum* L. convar. *Inkara*) seeds were imbibed with water solution of various pH values (4, 5, 6 and 7) at various ambiental temperatures (10, 15 and 22 °C) with the aim to reveal treatments effects on seed (imbibition, germination energy, germination and electric conductivity) and seedling traits (stem, root and total length). Seeds were imbibed in a laboratory by the standard method for seed germination. When averaged over the inquired pH values, ambient temperature affected all the investigated traits except stem length. The highest seed imbibition (58.8%) and electric conductivity (82 µS/cm per gram) were achieved at 22 °C. The highest values for germination energy (70%), germination (68%), root length (31 mm) and total seedling length (77 mm) were achieved at 10 °C. When averaged over the inquired ambiental temperatures, the highest germination energy (71%), germination (73%), root length (34 mm) and total seedling length (78 mm) were achieved at pH 4.

Keywords: *Trifolium incarnatum* L., germination, pH, electrical conductivity

SAŽETAK

U laboratorijskim uvjetima ispitivan je utjecaj upijanja sjemena inkarnatke (cv. „Inkara“) pri različitim temperaturama (10, 15 i 22 °C) i pH otopina (4, 5, 6 i 7) na svojstva sjemena (upijanje, energiju klijanja i klijavost, električni konduktivitiet) i klijanaca (dužinu stabljike, korjena te ukupnu dužinu klijanaca). Sjeme izloženo upijanju na navedenim temperaturama i pH vrijednostima otopina naklijavano je standardnom metodom. Temperatura upijanja u prosjeku za pH vrijednosti otopine upijanja utjecala je na sva ispitivana svojstva sjemena i klijanaca osim na dužinu stabljike klijanaca. Najveće vrijednosti na 22 °C dobivene su za upijanje sjemena (58,8%) i električni konduktivitet (82 µS/cm per gram). Dobivene vrijednosti za energiju (70%) i klijavost sjemena (68%) kao i dužinu korjena (31 mm) i ukupna dužina klijanaca (77 mm) bile su najveće na 10°C. U prosjeku za temperature, na pH 4 vrijednost otopine za upijanje dobivene su najveće vrijednosti za energiju klijanja (71%) i klijavost sjemena (73%), dužinu korjena (34 mm) i ukupnu dužinu klijanaca (78 mm).

Ključne riječi: *Trifolium incarnatum* L., klijavost, pH, električni konduktivitet

INTRODUCTION

Crimson clover is an annual forage legume which can be grown on a broad range of soil types but is limited to regions with a long period of mild and moist weather. At lower environmental temperatures it produces more biomass than a majority of other legumes (Young-Mathews, 2013). In favourable agroecological conditions, it can achieve a fresh herbage yield over 100 t/ha (Ban et al., 2008). It tolerates a broad range of soil pH values (pH 5 to 8), but the optimum for growth and nodulation is between pH 5.5 and 7.5 (Hoveland and Evers, 1995). In 3 year trial in Spain on soil with pH value 5.4 Lloveras and Iglesias (2001) obtained an average annual dry matter yield of 4.2 t/ha.

Although the agroecological conditions greatly affect the crop yield, seed quality for the crop establishment is of special importance. There are many traditional tests for the determination of seed quality (McDonald, 1998), of which the standard method for seed germination ISTA (2006) is the most often in use. Though, this method is conducted in ideal conditions, so the results are valid only for optimal field conditions (Siddique and Wright, 2004), and standard germination often exceeds the field emergence (Hamman et al., 2002). On the contrary, researches of Kendall et al. (1994) and Voigt et al. (1997) have revealed the relationship between laboratory and field germination at various environmental temperatures, water availability and soil acidity.

Salinas et al. (2010) stated that the seed electric conductivity test is a more reliable determinant for field emergence than the standard germination test for the majority of arable crops, which is also in accord with the results of Milošević et al. (2010) research.

After sowing, the first phase in plant growth and development is imbibition. For seed imbibition and germination, ambient temperature is of primary importance and it affects the soil water regime and biological activity of seed, with temperature range specific for plant species (Hadas, 2005). For seeds of three bushy species from grasslands, Booth and Bai (1999) have revealed greater imbibition at lower temperatures (5 and 15 °C) than for seeds of field crops (cucumber, sunflower, tomato, bean, pea, chicory, spinach and wheat), which absorbed more water from 20 to 30 °C. Also, for water absorption, there are important ratios between water and seed, and seed and substrate. Due to imbibition, seed mass increases 2 to 3 times compared to dry mass (Bewley and Black, 1978).

Seed imbibition occurs with water absorption through the cell wall and protoplasmic macromolecules, i.e. proteins and polysaccharides, and it is a consequence of diffussion (Woodstock, 1988). In Leopold's (1983) seed imbibition research, the greatest seed volume increase was observed for soybean seeds rich in protein, maize followed, while the castor seeds had the lowest volume increase.

The germination process starts with seed rehydration after sowing, and the volume of absorbed water depends on many factors (Bewley and Black, 1978). Temperature, besides moisture, oxygen and light is a key factor affecting germination. Therefore, the environmental temperature is the determinant for the sowing term of field crops.

The aim of this research was to determine the value of seed imbibition and electric conductivity at various ambient temperatures (10, 15 and 22 °C) and water solutions pH (4, 5, 6 and 7), and then to determine the seed traits (germination energy and germination) of imbibed seeds by standard method for germination, with afterwards measurement of seedling traits (stem length, root length, and whole seedling length).

MATERIALS AND METHODS

The research was conducted in practicum for plant physiology and ecophysiology at the Faculty of Agrobiotechnical Sciences Osijek, Croatia, with crimson clover (cv. Inkara) seeds. For seeds imbibition treatment (IM) there were used water solutions of pH values 4, 5, 6, and 7. Solution pH was adjusted by the addition of HCl or NaOH into distilled water, and pH was measured by pH-meter Mettler Toledo. Seeds were soaked into the prepared solution for imbibition for 3 hours at various temperatures: 10, 15 and 22 °C. Seeds were afterwards drained and weighed to determine the water absorption.

Imbibed and drained seeds were submitted to germination and measurement of electrical conductivity (EC).

For germination assay there were used 100 previously imbibed seeds in 4 replicates for each water solution pH and temperature combination. Seeds were sowed into filter paper, put into PVC bags and stored in the clima-chamber at 20 °C. Further germination was conducted in line with standard procedure (ISTA, 2006). There were determined the seed traits germination energy (GE) after 4 days, and germination (G) on 10th day after sowing, and afterwards there were measured the seedling (RL) and stem length (SL). Total seedling length (TSL) was obtained by adding the stem length to the root length.

For determination of EC there were 50 imbibed seeds soaked into 100 mL of deionized water for 24 hours, in 4 replicates for each treatment. EC was measured by a universal conductometer in µS/cm per gram of initial seed mass. The ANOVA was performed using SAS 9.4 Software. LSD test was used to calculate the differences between the means at $P<0.05$ and $P<0.01$, and the correlation was used to calculate the relatioship between two variables.

RESULTS

Crimson clover seed imbibition during initial treatments was significantly affected ($P<0.01$) by the applied temperatures, and it rose with the temperature increase (Table 1). At a temperature of 10 °C imbibition was greatest at pH 7 and pH 6, and the lowest was at pH 4 and pH 5. At 15 °C imbibitions was greatest at pH 5 and lowest at pH 6. At 22 °C the lowest imbibition value was at pH 5. Among the others there were no significant differences.

Germination energy (Table 1) was significantly affected by both treatments and their interaction ($P<0.01$). When averaged over all pH values, the highest GE was observed at 10 °C, and the lowest at 15 °C. When averaged over the applied temperatures, the greatest GE was observed at pH 4, with no significant differences among the rest pHs. At each temperature treatment, the greatest GE was observed at pH 4. At 10 and 15 °C the lowest GE values were observed at pH 7, while at 22 °C for pH 5. A significant positive correlation was detected between GE and G, RL, and TSL (Table 3).

Seed germination was significantly affected by the applied temperature ($P<0.05$), water solution pH ($P<0.01$) and their interaction ($P<0.01$). When averaged over pH values, the highest germination was obtained at 10 °C, and significantly greater only from the value at 15 °C (Table 1). When averaged over temperatures, G was highest after treatment at pH 4, while the averages among the rest pHs didn't differ significantly. At 10 °C, the greatest G was associated with seed soaking in pH 6, while for the rest temperatures with soaking in pH 4. Seed germination was positively correlated with seedling RL and TSL (Table 3).

Seeds electrical conductivity (Table 1) was significantly affected by the applied temperature ($P<0.01$) and the interaction of treatments ($P<0.05$). When averaged over applied pH values of water solutions, the highest EC was obtained at a temperature of 22 °C, and the lowest at 10 °C. At 10 °C, the greatest EC was obtained with treatment with a water solution of pH 4, while at higher temperatures with pH 6. EC values were negatively correlated with seedling RL and TSL (Table 3).

Temperature and water solution pH treatments didn't significantly affect the seedling SL (Table 2), but there was observed interaction of the applied treatments ($P<0.05$). The greatest seedling SL at 10 °C was achieved with pH 5, at 15 °C with pH 6 and 7, and at 22 °C with pH 6.

Seedling RL was significantly affected by the temperature treatment ($P<0.05$), water solution pH treatment, and their interaction ($P<0.01$). When averaged over applied pH values, seedling RL was the greatest at 10 °C treatment and the lowest at 22 °C. The highest values at all temperatures were with pH 4, while the overall maximum was achieved at a combination of 15 °C and pH 4.

Table 1. Influence of temperature and solution pH value on properties of seed

Temperature (A)	pH (B)				Average (A)
	4	5	6	7	
			Imbibition (%)		
10 °C	54.3	53.8	55.4	56.5	55
15 °C	54.1	60.6	52.8	56.9	56.1
22 °C	59.8	55.8	59.5	60.2	58.8
Average (B)	56.1	56.7	55.9	57.9	56.6
	LSD $_{0.05}$ (A) = 1.7		LSD $_{0.05}$ (B) = ns		LSD $_{0.05}$ (AB) = 1.4
	LSD $_{0.01}$ (A) = 2.2		LSD $_{0.01}$ (B) = ns		LSD $_{0.01}$ (AB) = 1.9
			Germination energy (%)		
10 °C	73	71	69	65	70
15 °C	68	64	59	58	62
22 °C	71	60	63	68	65
Average (B)	71	65	64	64	66
	LSD $_{0.05}$ (A) = 3.1		LSD $_{0.05}$ (B) = 3.7		LSD $_{0.05}$ (AB) = 2.8
	LSD $_{0.01}$ (A) = 4.1		LSD $_{0.01}$ (B) = 4.9		LSD $_{0.01}$ (AB) = 3.8
			Germination (%)		
10 °C	70	69	71	63	68
15 °C	72	63	59	59	63
22 °C	77	61	59	65	65
Average (B)	73	64	63	62	65
	LSD $_{0.05}$ (A) = 4.2		LSD $_{0.05}$ (B) = 3.7		LSD $_{0.05}$ (AB) = 3.3
	LSD $_{0.01}$ (A) = ns		LSD $_{0.01}$ (B) = 5.0		LSD $_{0.01}$ (AB) = 4.4
			Electrical conductivity (µS/cm per gram)		
10 °C	76	73	68	70	72
15 °C	75	72	82	73	76
22 °C	74	85	86	81	82
Average (B)	75	77	75	76	76
	LSD $_{0.05}$ (A) = 5.8		LSD $_{0.05}$ (B) = ns		LSD $_{0.05}$ (AB) = 11.3
	LSD $_{0.01}$ (A) = 7.8		LSD $_{0.01}$ (B) = ns		LSD $_{0.01}$ (AB) = ns

*ns – not significant

Table 2. Influence of temperature and solution pH during germination of seed on seedling properties (mm)

Temperature (A)	pH (B)				Average (A)
	4	5	6	7	
			Stem seedling length (mm)		
10 °C	43	49	47	48	46
15 °C	44	45	46	46	45
22 °C	46	45	47	44	46
Average (B)	44	47	47	46	46
	$LSD_{0.05}$ (A) = ns		$LSD_{0.05}$ (B) = ns		$LSD_{0.05}$ (AB) = 0.3
	$LSD_{0.01}$ (A) = ns		$LSD_{0.01}$ (B) = ns		$LSD_{0.01}$ (AB) = ns
			Root seedling length (mm)		
10 °C	35	33	29	28	31
15 °C	36	29	24	25	29
22 °C	32	23	26	24	26
Average (B)	34	28	26	29	29
	$LSD_{0.05}$ (A) = 0.4		$LSD_{0.05}$ (B) = 0.4		$LSD_{0.05}$ (AB) = 0.5
	$LSD_{0.01}$ (A) = ns		$LSD_{0.01}$ (B) = 0.5		$LSD_{0.01}$ (AB) = 0.7
			Total seedling length (mm)		
10 °C	78	82	76	76	77
15 °C	80	74	70	71	74
22 °C	77	68	73	68	72
Average (B)	78	75	73	71	74
	$LSD_{0.05}$ (A) = 0.4		$LSD_{0.05}$ (B) = 0.5		$LSD_{0.05}$ (AB) = 0.7
	$LSD_{0.01}$ (A) = ns		$LSD_{0.01}$ (B) = ns		$LSD_{0.01}$ (AB) = 1.0

*ns – not significant

Total seedling length (Table 2) was significantly affected by temperature and solution pH treatments (P<0.05). There was observed significant interaction between treatments (P<0.01). When averaged over all pH values, the greatest TSL was observed at 10 °C, and the lowest at 22 °C. When averaged over all applied temperatures, TSL was the highest for pH 4, and the lowest for pH 7. Upon the 10 °C treatment, seedlings were greatest with pH 5, and upon both higher temperatures with pH 4. The overall greatest value was observed in a combination of 10 °C and pH 5.

Table 3. Correlation coefficients between inquired seed and seedling traits of crimson clover

	GE	G	EC	RL	SL	TSL	IM
GE	1						
G	0.76883**	1					
EC	-0.25039	-0.26737	1				
RL	0.5546**	0.58869**	-0.35116*	1			
SL	-0.08657	-0.01784	-0.15699	0.1293	1		
TSL	0.4322**	0.49339**	-0.36378*	0.90364**	0.53941**	1	
IM	0.00067	-0.08207	0.04506	-0.21577	-0.03631	-0.19084	1

GE germination energy, G germination, EC electrical conductivity, RL root length, SL stem length, TSL Total seedling length, IM imbibition treatment

DISCUSSION

Results of this research have generally shown the greatest imbibition at the highest applied temperature (22 °C).

In temperature conditions of 17-19 °C on different media (distilled water, NaCl, nutrient solution) with red clover seeds (*Trifolium pratense* L.) Costa et al. (2019) determined the end of imbibition after 18 hours, but no differences in imbibition values between obtained media. Therefore, the authors conclude that a few factors influence the imbibition of red clover seeds but act multiplicatively independently of the seed's medium and colour.

Tiryaki et al. (2009) investigated the effect of pretreatment of alfalfa (*Medicago sativa* L.) and white clover (*Trifolium repens* L.) seeds with different solutions (2% NaCl, 2% KNO_3 and 300 g/L polyethylene glycol). The seeds of the mentioned species were treated for 2 hours at a temperature of 20 °C, after which standard germination was carried out at 15 °C. Application of all pretreatments compared to untreated seed increased germination of white clover at 15 °C but produced minimal or negative effects on alfalfa. The authors concluded that by applying the mentioned pretreatments, it is possible to increase the germination rate and the germination rate of the seeds of some legumes under stress conditions such as low temperature. In accordance with the above, in our research, the pretreatment/imbibition of incarnate seeds with solutions of different pH values showed a significant positive effect at the lowest tested temperature (10 °C) for seed energy and germination, root length and total seedling length.

In the research of Cheng et al. (2010), there was observed greater soybean (*Glycine max*) seed imbibition at 22 °C than at 4 °C. In the research of Waggoner and Parlange (1976), the water absorption rate in pea (*Pisum sativum* L.) seeds was greater at 20 °C than at 0 °C. Szczerba et al. (2021) have realized that temperature effects on imbibition are associated with enzyme activity. The authors state that hydrophile components of the cell wall and colloidal compounds in the cytoplasm (mainly proteins and starch) are crucial for the imbibition and that the water absorption rate is greater at higher ambient temperatures due to increased enzymes activity. The greatest dehydrogenase activity in all 4 investigated soybean cultivars have Szczerba et al. (2021) observed at the highest temperature (25 °C), and the lowest at 10 °C. Considering the α-amylase enzyme, the lowest activity was also at 10 °C, while the greater values varied across the investigated cultivars and temperatures.

Contrary to the imbibition values, germination energy and germination values of crimson clover (*Trifolium incarnatum* L.) seeds were generally greatest at the lowest applied temperature (10 °C). Butler et al. (2014)

have revealed that the temperature of 20 °C is optimal for the germination of cool-season legumes, to which the crimson clover belongs. The authors also concluded that the optimum temperature range for germination of cool-season legumes is between 15 and 25 °C, while for crimson clover is somewhat broader: 10 to 25 °C.

In Ching's (1975) research with crimson clover seeds (cv. Dixie) at 10, 20 and 30 °C temperatures, 20 °C was found optimal. At that temperature maximum germination rate was achieved after 36 hours of germination. At 10 °C maximum germination was achieved after 60 hours. At 30 °C only 20% of the seeds germinated. The cited author has linked the temperature effect with the activity of a number of enzymes.

In Baxter et al. (2019) research with 3 clover species and 3 cultivars, tested seeds exhibited germination in a broad temperature range from 4.9 to 28.2 °C. Maximum germination of clovers was observed between 10.9 and 17.2 °C, after 34.2 to 52.8 hours.

In this research, electrical conductivity generally rose with an increase of temperature during initial imbibition treatment, which was in accord with Plenzler and Cieśla (2003) findings on pea seeds.

Considering the water solution pH effects during the initial imbibition, this research has revealed the highest germination energy and germination upon the initial treatment with pH 4. This was in accord with previous researches on white clover (*Trifolium repens* L.) cultivars (Bukivć et al., 2008a), lucerne (*Medicago sativa*) (Bukvić et al., 2008b) and red clover (*Trifolium pratense* L.) (Bukvić et al., 2009) at various pH values of germination medium. According to Leopolod (1983), among the factors that affect the dynamics of seed volume enlargement are the diluted substances in the solution that is being absorbed by seeds (i.e. salts, valence effects, pH, etc.). Agić et al. (2009) research with the germination of red clover seeds on filter paper for 9 days with daily measurements has shown a significant effect of medium pH (pH 4, 5, 6, 7) on α-amylase activity, percentual germination rate and radicule length. There was revealed a significant correlation between investigated traits, and α-amylase activity and radicule length were greatest at pH 5 and 6.

A significant effect of water solution pH on seed and seedling traits was exhibited in large-seeded legumes too, like in pea (Osmanovic et al., 2016) and soybeans (Bukivć et al., 2007). Buranji et al. (2019) have investigated pH effects on flax (*Linum usitatissimum* L.) seeds and seedlings also.

CONCLUSIONS

Based on the presented results, there is concluded that temperature during the initial phase of seed imbibition of crimson clover (first 3 hours) affects seed and seedling traits. Generally, the greatest imbibition and electrical conductivity were obtained upon the highest applied temperature of 22 °C, while the greatest germination energy, germination, seedling root length and total seedling length were greatest upon the lowest applied temperature of 10 °C.

When averaged over the applied temperatures, pH values of water solutions for the initial imbibition treatment affected germination energy, germination, seedling root length and total seedling length. All these traits generally rose with the fall of pH from 7 to 4.

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