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THE DORMANCY OVERCOMING AND AFFECTION OF EARLY GROWTH OF ALFALFA (*MEDICAGO SATIVA* L.) SEEDS BY NON-THERMAL PLASMA AND PLASMA ACTIVATED WATER

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Abstract. The effect of non-thermal plasma (NTP) and plasma activated water (PAW) on the dormancy and early growth of commercial and wild-type seeds of alfalfa (*Medicago sativa*) were studied. The seeds were exposed to NTP and irrigated by PAW, and the factorial ANOVA statistics confirmed that factors of plant species plasma treatment and treatment time had high importance for early growth of seedlings. The germination of wild seeds increased from 21 % to 30–34 % after plasma treatment together with PAW irrigation NTP technology having a high potential in break down dormancy in seeds in comparison with mechanical type of dormancy.

Key words: non-thermal plasma (NTP), seed germination, seedling, seed stimulation.

1. INTRODUCTION

Alfalfa is a genus of vascular perennial plants (*Medicago* spp., *Fabaceae* family). It is agricultural forage cultivated crop in the whole world [1] and it is grown for hay, silage and grazing as a valuable crop characterized by a high nutritional quality, abundant biomass production and broad adaptability to a wide range of cultivation conditions. It plays an important role in a farming system and contributes to stabilization of the ecosystem by improving soil physical, chemical, and biological properties [2]. Alfalfa is widely adaptable to diverse environmental conditions partly because of its deep root system and the ability to fix atmospheric nitrogen in a bacterial symbiosis.

The seeds of the *Fabaceae* family are often dormant, it means, seeds do not germinate immediately after the fall from the mother plant into environment normally favourable for germination but they often need an additional stimulus *e.g.* cold period [3, 4]. Under natural conditions, these seeds germinate after long periods (within weeks to months), when coats become permeable due to opening of a water gap in response to environmental factors, especially temperature [5–7]. These seeds germinate well after the disruption of their hard impermeable seed coats [8].

In recent years, many scientific studies are dealing with the bio-applications of non-thermal plasma (NTP) - the partially ionized gas often generated by electrical discharges. The studies are mainly focused to medicine [9–11], agriculture [12–14], forest and woody industries [15–17], or food processing [14, 18, 19]. Many of extensive reviews have been published, *e.g.* Bourke *et al.*, Ehlbeck *et al.*, Graves, von Woedtke *et al.*, and Scholtz *et al.* [20–24]. Recently, it was found out that NTP treatment of seeds can have a positive effect on seed germination and early growth of seedlings. There are even studies proving the deactivation of the surfaces of infected seeds with various phytopathogenic organisms [25, 26]. Some papers document the possibility of seed dormancy overcoming after NTP treatment [27] and provide possible explanations [28–31].

Moreover, the benefit effect of NTP may also persist in exposed medium, most often water. The effect of water exposed to plasma, so called plasma activated water (PAW), is well documented in many papers, *e.g.* Julák *et al.* and Thirumdas *et al.* [32, 33].

Advancing global warming is changing farmers' attitudes towards crop species composition. Alfalfa is a relatively adaptable drought-tolerant plant that is used all over the world. This article contributes to the knowledge of the wider use of hard dormant alfalfa seeds, which may score better germination and growth parameters after NTP or PAW applications. However, despite many papers dealing with bioapplication of NTP and PAW, those dealing with application for dormancy overcoming are only rare.

The aim of this paper was to test variable cultivars of alfalfa (wild and commercial) and to find the way to increase seed germination and positive affect overcoming seed dormancy and early growth of seedlings. The objectives were (a) does it exist different reactions of wild and commercial cultivars of alfalfa seeds after NTP treatment, (b) to examine what is the difference in use of NTP and NTP+PAW, (c) to explore what time of NTP treatment had a positive effect on seed germination and early growth of alfalfa seed.

2. MATERIALS AND METHODS

2.1. PLANT MATERIAL

Three sets of alfalfa (*Medicago sativa* L.) seeds were used in our experiment: two cultivars Vlasta and Zuzana (Oseva uni, Choceň, Czech Republic) and one wild-type set.

Zuzana is a medium-early cultivar that has a very high resistance to winter, spring frosts and a high resistance to disease of alfalfa mosaic virus [34, 35]. Vlasta is a very early cultivar. Its spring growth is moderately fast to fast. This cultivar has a very high resistance to winter and spring frosts [34, 35]. Wild seeds of purple-flowered alfalfa were collected on uncultivated meadow in one location in Prague in September 2019.

All the used seeds were stored in the dark at 23 °C. Only healthy seeds without obvious defect with a uniform size (2 g/1000 seeds) were selected for the experiment.

2.2. APPARATUS DESCRIPTION

The NTP was generated by point-to-plane corona discharge in regime of transient spark (TS). Discharge burns on the point electrode realized by the intramuscular injection needle Medoject 0.6×30 mm (Chirana T. Injecta, Stará Turá, Slovakia), connected to the high voltage source of 4.6 kV. The ground electrode was realized by the surface of water of 1 ml grounded with immersed platinum. The distance between the tip of the needle and plate electrode was 3 mm, adjusted by a micrometric screw to obtain the average current of 350 μA. Apparatus is depicted in Fig. 1, for more detailed description and characteristics see [36].

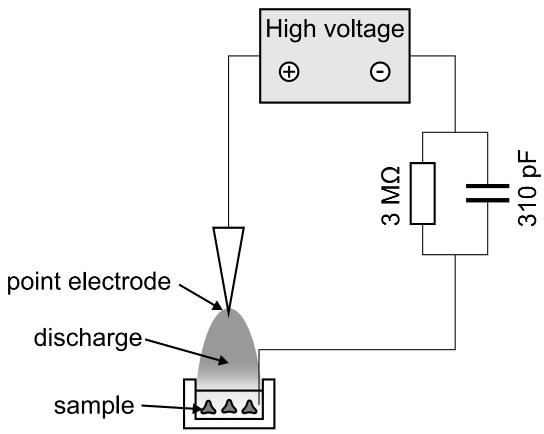

Fig. 1 – The schematic draw of plasma generating apparatus.

The PAW was generated in the same apparatus by the same way. The volume of 1 ml of deionized water was treated and used for the irrigation of seeds and plants. PAW was evaluated semi quantitatively by following indicating papers: pH by indicator papers 0–12 (Lach-Ner, Neratovice, Czech Republic); H_2O_2 by peroxides test sticks Quantofix Peroxide 100 (Macherey-Nagel, Düren, Germany); NO_{3^-} and NO_{2^-} by Quantofix Nitrate Nitrite (Macherey-Nagel, Düren, Germany). Determined values are: pH ≈ 3–4; c(H_2O_2) ≈ 100 mg/l; c(NO_{3^-}) ≈ 500 mg/l; c(NO_{2^-}) ≈ 1 mg/l. For details about the PAW generation, chemical processes and precise measurements see our previous paper [37].

2.3. PLASMA TREATMENT OF SEED

The sample of 30 seeds were immersed into the 1 ml of water and treated for 5, 10, 20 min. Untreated sample was used as the control one. Immediately after the exposure, the seeds were put on three layers of filter paper in a 9 cm diameter Petri dish. To determine the possible effect of PAW, seeds were divided into two groups, the first one denominated as TS (Transient Spark) was after NTP exposition irrigated by pure water and the second one denominated as TS+PAW was irrigated by freshly prepared PAW. All experiments were done in five repetitions.

2.4. GERMINATION AND EARLY GROWTH TESTS

The germination and early-growth tests were run at 23 °C. The data about the number of germinating seeds were collected every day, the length of seedling and fresh seedling weights were measured on the 7^{th} day of cultivation. The seedlings were then dried at 60 °C for 24 hours and the dry seedling weights were measured.

Following characteristics of seed germination and seedlings were determined according to Šerá *et al.* [38]: number of germinated seeds (1), seed germination (%), and initial growth of seedlings: mean length of seedling (mm), fresh seedling weights (mg), dry seedling weights (mg).

2.5. STATISTICAL ANALYSIS

All data were analyzed using the STATISTICA package at the significance level of 0.05. Logarithmic transformation (y=log(x)) of the basal data was used for normalization before statistical analyses.

The factorial analyses of variance (ANOVA) with fixed factors was used to evaluate the influence of the alfalfa cultivars (factor Medicago), type of NTP treatment (factor Treatment) and time of plasma treatment (factor Time) on seed germination and growth reaction. The factorial structure: Medicago included wild seed and seeds of the two cultivars, Treatment included three possibilities (control, TS, TS+PAW) and Time with four possibilities of NTP treatment (0 min, 5 min, 10 min, 20 min). The dependent variances were the data obtained during seed cultivation: seed germination, length of seedling, and weight of seedling (fresh and dried biomass). The detailed testing of experimental variances among each other was done using the one-way ANOVA test followed by the Tukey HSD test for multiple comparisons.

3. RESULTS

ANOVA shows the proportions of variance of each calculated factor in the experimental structure (Table 1). All investigated factors of Medicago, Treatment and Time was important, because they observed three statistically significant differences in four measured characteristics. From the possible factor combinations, the most important was Medicago*Treatment, on the other hand no differences was found in the combinations of Treatment*Time and Medicago*Treatment*Time. The highest variability was found in characteristic of fresh seedling weights (Table 1).

Significant differences among characteristics in type of plant species was observed in length of Vlasta seedling, in weight of fresh wild and Vlasta seedlings (Table 2).

The highest values of the measured characteristics were found after TS+PAW treatment with 10 minutes duration in alfalfa Vlasta in seed germination 97.33±1.25% (control 94.67±2.00%) and in mean length of seedling 62.17±2.33 mm (control 55.78±2.09 mm); in wild alfalfa after TS+PAW with 5 minutes duration in fresh seedling weights of 30.88±1.39 mg (control 28.37±2.51 mg) and with 20 minutes duration in dry seedling weights of 1.85±0.04 mg (control 1.81±0.08 mg) (Table 2).

The highest difference in seed germination was observed in wild alfalfa, where the control set had germination of 21.33±2.00% and the seed set treated with TS had seed germination of 30.00±3.33% (Table 2). The course of germination dynamics is presented in Fig. 2, where the differences in trends between wild seeds and cultivar seeds are evident.

The highest difference in mean length of seedling was in Vlasta cultivar after of the both applied treatments; it was 59.76±1.47 mm (control 51.44±1.04 mm) after TS treatment and 62.17±2.33 mm after TS+PAW treatment (control 55.78±2.09 mm); see Table 2.

The highest differences in fresh and dry seedling weights were observed in Vlasta cultivar, both after TS+PAW treatment.

Fresh seedling weight was 23.45±0.93 mg and control was 20.84±0.94 mg, whereas dry seedling weight was 1.43±0.01 mg and control was 1.32±0.07 mg; see

Table 2.

Table 1

Results of factorial analyses. Relationship among the type the alfalfa cultivars (factor Medicago), type of NTP treatment (factor Treatment) and time of plasma treatment (factor Time) on seed germination and characteristics of early growth.

Factor	**DF**	**Seed germination**			**Mean length of seedling**		
		SS	F	*p*	SS	F	*p*
Medicago	2	10013.62	1593.68	**0.000**	52.87	1.56	0.216
Treatment	1	37.41	11.91	**0.001**	1.61	0.10	0.759
Time	3	7.09	0.75	0.524	448.33	8.81	**0.000**
Medicago*Treatment	2	14.32	2.28	0.108	386.99	11.41	**0.000**
Medicago*Time	6	29.18	1.55	0.171	223.01	2.19	0.050
Treatment*Time	3	3.89	0.41	0.744	52.50	1.03	0.382
Medicago*Treatment*Time	6	18.48	0.98	0.443	79.51	0.78	0.587
Factor	**DF**	**Fresh seedling weights**			**Dry seedling weights**		
		SS	F	*p*	SS	F	*p*
Medicago	2	58834.57	10924.07	**0.000**	270.02	31787.39	**0.000**
Treatment	1	1446.27	134.27	**0.000**	5.65	332.62	**0.000**
Time	3	685.54	127.29	**0.000**	0.00	0.01	0.940
Medicago*Treatment	2	60.64	3.75	**0.013**	0.04	1.39	0.250
Medicago*Time	6	90.02	8.36	**0.000**	0.02	1.07	0.347
Treatment*Time	3	19.60	0.61	0.724	0.03	0.56	0.764
Medicago*Treatment*Time	6	6.42	0.40	0.755	0.01	0.53	0.665

Table 2

Seed germination and characteristics of early growth of alfalfa cultivars after NTP treatment with two methods of irrigation, TS irrigation by pure water, TS+PAW by freshly prepared PAW. Detail see in Plasma treatment of seed.

Medicago	**Treatment**	**Time (min)**	**Seed germination (%)**			**Mean length of seedling (mm)**			**Fresh seedling weights (mg)**			**Dry seedling weights (mg)**		
			Mean	SE	HSD	Mean	SE	HSD	Mean	SE	HSD	Mean	SE	HSD
Wild	TS	0	21.33	2.00	a	53.56	2.18	a	22.97	0.52	a	1.75	0.07	a
		5	29.33	3.06	a	61.08	3.28	a	25.28	1.31	acd	1.83	0.04	a
		10	22.00	3.74	a	59.20	0.58	a	24.39	1.16	acd	1.84	0.07	a
		20	30.00	3.33	a	60.26	2.01	a	25.05	1.05	abc	1.84	0.04	a
	TS + PAW	0	32.00	5.33	a	52.10	2.53	a	28.37	2.51	abc	1.81	0.08	a
		5	32.67	3.86	a	55.79	1.75	a	30.88	1.39	b	1.82	0.04	a
		10	34.00	5.31	a	56.39	2.57	a	28.81	1.02	bc	1.70	0.02	a
		20	31.33	2.26	a	54.90	3.20	a	30.18	1.89	bd	1.85	0.04	a
Zuzana	TS	0	91.33	2.00	a	55.19	1.65	a	18.29	0.46	a	1.31	0.01	a
		5	88.00	2.49	a	56.32	1.42	a	19.23	0.71	a	1.32	0.04	a
		10	96.00	1.25	a	61.53	1.44	a	18.38	0.91	a	1.34	0.02	a
		20	94.00	1.63	a	59.04	1.21	a	20.62	0.94	a	1.34	0.02	a
	TS + PAW	0	94.67	1.33	a	57.89	1.31	a	21.32	0.33	a	1.28	0.02	a
		5	90.00	2.36	a	55.34	0.78	a	21.42	0.64	a	1.29	0.02	a
		10	94.67	1.33	a	55.42	1.11	a	21.98	0.73	a	1.33	0.03	a
		20	95.33	0.82	a	56.75	0.63	a	21.80	0.41	a	1.33	0.03	a
Vlasta	TS	0	93.33	1.83	a	51.44	1.04	a	16.31	0.44	ad	1.42	0.01	a
		5	92.67	2.87	a	54.07	1.27	ac	16.98	0.56	acd	1.36	0.03	a
		10	94.00	2.21	a	58.34	1.66	abc	13.46	0.90	d	1.32	0.03	a
		20	91.33	1.70	a	59.76	1.47	abc	16.07	0.84	ad	1.35	0.04	a
	TS + PAW	0	94.67	2.00	a	55.78	2.09	abc	20.84	0.94	a	1.32	0.07	a
		5	96.00	1.25	a	59.85	2.11	abc	23.25	0.67	b	1.43	0.01	a
		10	97.33	1.25	a	62.17	2.33	bc	22.08	0.81	bc	1.40	0.04	a
		20	95.33	1.33	a	64.63	1.07	b	23.45	0.93	b	1.43	0.02	a

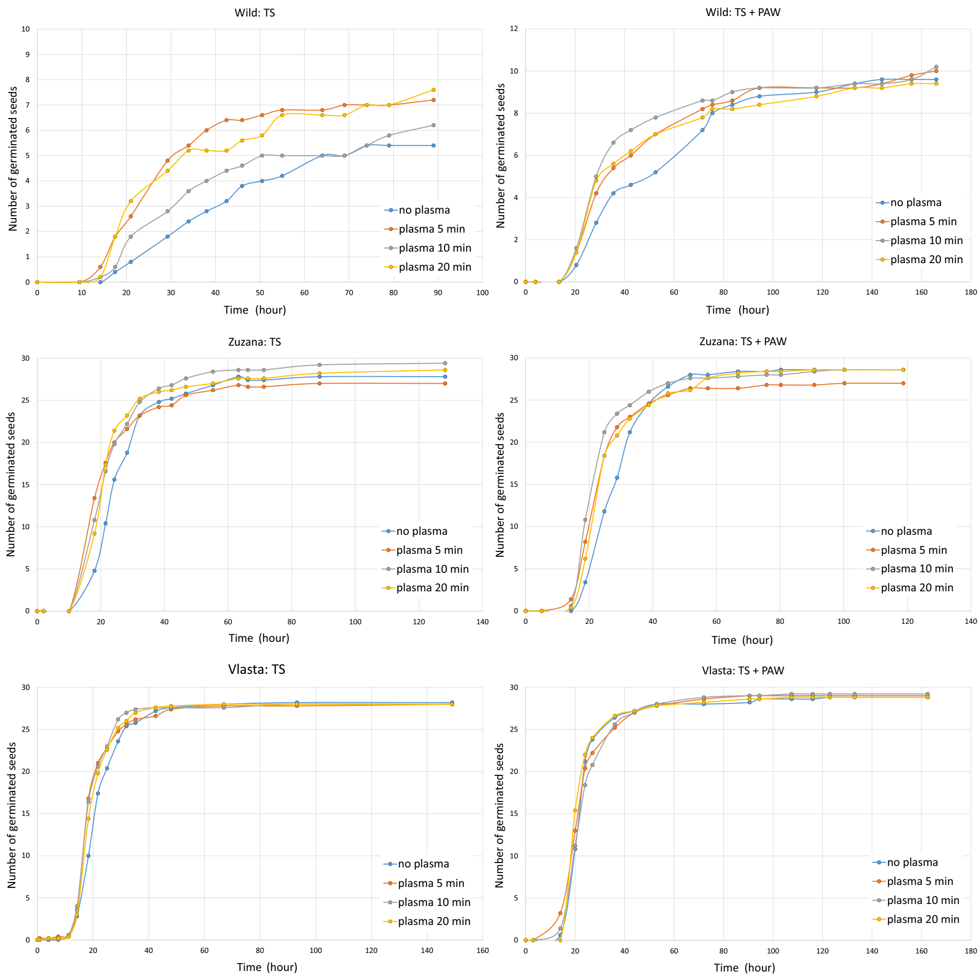

Fig. 2 – Germination dynamics presented by the number of germinating alfalfa seeds over time. Curves in two different regimens of NTP treatment. See details in Plasma treatment of seed.

4. DISCUSSION

The impermeable hard coat of alfalfa seed can reduce germination to an extent unacceptable for commercial use [39]. It has been found that also storage temperature and germination temperature can significantly accelerate alfalfa germination [39, 40]. Hard seeds of alfalfa break down the seed dormancy (increase seed germination) by scarification, it is the method when seed coat is mechanically weakened or breached [8]. Disruption of the surface of hard seeds is a certain way to eliminate dormancy, which is manifested by the seed germination increasing.

The seed sets monitored in the presented experiment were biologically diverse and their germination and initial growth were also different. Seed sets of both cultivars are bred for frost resistance, soil desiccation and good seed germination [34, 35]. The response of alfalfa to NTP was found in the germination process (Fig. 2). In the presented experiment, the seed germination was about 91% and 93%, Zuzana and Vlasta respectively (Table 2). In contrast, wild-type alfalfa seeds had seed germination of about 21% and thanks to the use of TS seed treatment their germination increased to about 30% (20 minutes of duration) and to the use of TS+PAW increased to about 34% (10 minutes of duration) (Table 2). The response of alfalfa to NTP was found in the germination process (Fig. 2). The recorded results are not statistically significant, yet finding the trend has possible potential. The course of germination over time shows that the wild-type seeds were obviously dormant and this course changed after TS and TS+PAW applications (Fig. 2).

It may be assumed that the presented effects are caused mainly by the increasing of seeds wettability allowing faster water transfer into the seeds and initializing the germination process. Similar results were reported in Ref. [41], where the plasma treatment promoted rapid sinking of pepper (*Capsicum annuum* L.) seeds.

Positive and negative reactions of germination and initial growth of seedlings after NTP application in different cultivars of the same plant species in different species of the same botanical genus are known. Different responses to NTP have been found in different cultivars of poppy (*Papaver somniferum* L.), hemp (*Cannabis sativa* L.), sugar beet (*Beta vulgaris* subsp. *vulgaris* convar. *vulgaris* var. *altissima*) taxa [38, 42, 43], and in different species of pines (*Pinus* sp.) [26]. The results of this experiment showed that the cultivated alfalfa seeds respond to NTP (specifically TS and TS+PAW) differently than wild-type seeds.

5. SUMMARY

The possibility of NTP and PAW treatment of alfalfa seeds germination and early growth were studied. It was shown that the main effect was observed for wild-type seed of alfalfa where the seed germination increased from 21% to 30–34%. For commercial cultivars (Zuzana, Vlasta) seed germination increased from approximately 91% to 94%. We assume an eroded seed surface and increased wettability as the main causes: better seed germination and effect to seed dormancy breaking. NTP treatment together with PAW irrigation also affected some characteristics of initial growth, namely length of seedling and weight of fresh seedling.

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