ABS

Original Research

Effect of nitrogen and oxygen low-pressure cold plasma and their mixtures on the germination and early seedling growth of Tartary buckwheat

Jure Mravlje [1,2,*]

Abstract

A key challenge of the 21st century is the growing human population and the decline of arable land; therefore, new technologies that could ensure higher yields and minimise losses on existing land without further negative impacts on the environment are being sought. Cold plasma treatment of seeds is a promising green technology in agriculture and food production. This study aimed to investigate the effects of different oxygen and nitrogen gas mixtures in a low-pressure cold plasma system on germination and early seedling growth of Tartary buckwheat (*Fagopyrum tataricum*). Our results indicate that nitrogen cold plasma is less aggressive and thus more suitable for treating Tartary buckwheat grains, as a 45-second treatment did not affect germination or early seedling growth, whereas a 90-second treatment decreased germination by less than 10% compared to the control, without affecting seedling length or biomass. Interestingly, the higher oxygen content in the gas mixture and the longer cold plasma treatment time resulted in a more pronounced negative impact on germination and early seedling growth.

Keywords

low-temperature plasma; plasma agriculture; seeds; alternative crops; plant production

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Učinek nizko-tlačne kisikove in dušikove plazme ter njunih mešanic na kaljivost in rast kalic tatarske ajde

Izvleček

Eden največjih izzivov današnjega stoletja je strmo naraščajoča človeška populacija in zmanjševanje primernih obdelovalnih površin; posledično se iščejo nove tehnologije, ki bi omogočile večji pridelek in zmanjšale izgube na že obstoječih obdelovalnih površinah brez dodatnih negativnih vplivov na okolje. Tehnologija obdelave semen s hladno plazmo velja za obetavno in okolju prijazno tehnologijo na področju kmetijstva in prehrane. Namen te študije je bil raziskati učinke različnih mešanic kisika in dušika v nizkotlačnem sistemu hladne plazme na kalitev in zgodnjo rast kalic tatarske ajde (*Fagopyrum tataricum*). Naši rezultati kažejo, da je dušikova hladna plazma manj agresivna in zato primernejša za obdelavo zrnja tatarske ajde, saj 45-sekundna obdelava ni vplivala na sposobnost kalitve ali zgodnjo rast kalic, medtem ko je 90-sekundna obdelava zmanjšala kalitev za manj kot 10 % v primerjavi s kontrolo, brez vpliva na dolžino ali biomaso kalic. Zanimivo je, da je večji delež kisika v plinski mešanici in daljši čas obdelave s hladno plazmo povzročil bolj škodljiv vpliv tako na kalitev kot na zgodnjo rast kalic.

Ključne besede

Nizko-temperaturna plazma, plazemsko kmetijstvo, semena, alternativne poljščine, rastlinska produkcija

Introduction

Plasma is the so-called fourth fundamental state of matter, fully or partially ionised gas, that can be generated by applying energy (via heat, electricity or electromagnetic field) to a gas (Conrads & Schmidt, 2000). It is composed of free electrons, ions, atoms, molecules, radicals, and other reactive species, as well as photons, including ultraviolet (UV) and vacuum ultraviolet (VUV) photons (D. Popović et al., 2021). All these generated species are responsible for the unique characteristics of plasma, which conducts electricity, although it has a net neutral (zero) charge (Tendero et al., 2006). Plasmas can be classified according to various conditions of their generation based on the relative energy levels of light (electrons) and heavy particles (ions and larger particles). The first group, referred to as the so-called thermal plasmas or local thermodynamic equilibrium plasmas, has a high electron density, and the temperature of electrons is close to that of heavy particles. The second group is so-called low temperature or cold plasmas (CP; also non-local thermodynamic equilibrium plasmas), with much lower electron density and temperature due to the much lower temperature of heavy particles than that of electrons (Moreau et al., 2008; Tendero et al., 2006). CP is already applied in many areas of science and technology, including the processing of textiles and polymers, synthesis of nanoparticles, analytical chemistry (ICP-MS) and some daily life equipment, such as electronics (plasma TV and other display panels), lighting systems (neon lights), etc. (Misra et al., 2016).

CP is also interesting from a biological perspective, as it is suitable for the treatment of thermally unstable or sensitive samples, such as biological materials, as the heating of the samples is minimal, especially in short-term exposure times (Misra et al., 2016; Moreau et al., 2008; Tendero et al., 2006). Therefore, in the last two decades, a new interdisciplinary research field of CP technology for developing applications in many life science areas (such as agriculture, biotechnology and food science) has emerged – called "plasma agriculture" (Puač et al., 2018; Ranieri et al., 2021). It offers new, environmentally friendly approaches in agriculture as an alternative to classical pesticide and other artificial chemical usage with negative environmental impact (Sharma et al., 2019). That is of immense importance as improving efficacy and quality of food production while minimising food losses is crucial and one of the biggest challenges of the 21st century, when the human population is estimated to reach 10 billion by 2050, according to the

Food and Agriculture Organisation of the United Nations (FAO United Nations, 2017). The main areas of research are exploring the promising antimicrobial potential of CP for seed surface decontamination (Mravlje, Regvar, & Vogel-Mikuš, 2021; Scholtz et al., 2021), controlling plant diseases (Adhikari et al., 2020) and enhancing seed germination potential (Ranieri et al., 2021; Šimek & Homola, 2021). It has been reviewed that CP treatment can stimulate seed germination, enhance early seedling growth, improve plants' stress tolerance and overall performance (Randeniya & De Groot, 2015; Starič et al., 2020). This is mainly achieved via plasma-seed surface interactions, as the surface usually becomes more hydrophilic, increasing the wettability of seeds and thus water uptake, and other surface functionalisation changes, changes in DNA, enzymatic and hormone activity, etc. (Starič et al., 2020), all of which can positively affect germination and later growth and devlopments of plants, enhancing their resilience to abiotic and biotic stress.

Nevertheless, despite the promising results of the last two decades, many underexplored areas in CP agriculture remain, as most of the research is focused on cereal crops, legumes and other more widely cultivated crops (Bilea et al., 2024). However, it has already been demonstrated that the response to CP treatment is species-specific and may also be variety (cultivar) dependent (Recek et al., 2023; Starič et al., 2021), as the effect of CP treatment on seed germination depends on many factors such as plant species, size and shape of the seeds, seed coat hardiness, amount of endosperm, etc. (Zahoranová et al., 2016).

Buckwheat is a pseudocereal grain crop often referred to as an alternative crop, traditionally grown in Europe and Asia. It is used for flour and groat products and has many beneficial effects on human health due to its high nutritional value, as it is especially rich in phenolics (flavonoids) compared to other traditional cereal crops, therefore gaining much attention as interest in so-called "functional foods" is rapidly growing nowadays (Bonafaccia, Gambelli, et al., 2003; Bonafaccia, Marocchini, et al., 2003; Bonafaccia & Fabjan, 2003; Kreft et al., 2006). It is also gluten-free and hence safe for consumption by patients with celiac disease (Skerritt, 1986), and due to its modest growing demands, making it suitable for organic farming, its production is increasing in Europe and worldwide (V. Popović et al., 2014). Tartary buckwheat (*Fagopyrum tataricum* Gaertn.; TB) is less well-known compared to its more cultivated relative, common buckwheat (*Fagopyrum esculentum* Moench; CB), as it is mainly grown in mountainous regions of China, India, Bhutan and Nepal, where the growing conditions are harsh (Bonafaccia, Gambelli, et al., 2003; Bonafaccia & Fabjan, 2003). It is more tolerant of cold weather, drought, and generally a very low-input plant (Luthar et al., 2021). It is also very rich in flavonoids and other phenolic substances, especially rutin, as it can have up to more than 100 times higher % of rutin per dry weight of grain compared to common buckwheat (Bonafaccia & Fabjan, 2003). Due to its high content of flavonoids and other phenolics, TB is also more resistant to plant diseases, pests, and UV-B radiation, making it feasible to grow as an organic and ecological crop with little need for artificial fertilisers or other chemicals (Luthar et al., 2021).

There have been some papers published on the effect of CP treatment on CB grain, with little or no positive effect, depending on the CP source and treatment conditions (Mravlje et al., 2022; Mravlje et al., 2021; Šerá et al., 2012) on germination of CB in vitro; however, it was reported that although the percentage of emerged seedlings was lower in field conditions, the growth and yield of CP pre-treated CB was strongly improved (Ivankov et al., 2021). It was also demonstrated that CP treatment can increase the phenolic and flavonoid content and antioxidant activity of CB groat and flour (Amiri et al., 2023) and effectively reduce surface fungal colonisation (Mravlje et al., 2022; Mravlje et al., 2021). The effects of CP treatment on TB have been less studied. The potential of plasma-activated water for improving the growth and performance of TB sprouts has been demonstrated (Wang et al., 2022), and effective fungal decontamination of TB grains with a negative impact on germination at longer exposures to low-pressure CP was reported (Mravlje et al., 2022; Mravlje et al., 2021). It was suggested that TB grains may be more sensitive to powerful CP treatments due to their smaller size and lower biomass compared to CB grains (Mravlje et al., 2022).

This study aimed to examine the effects of low-pressure CP treatment generated by different gas admixtures (nitrogen, oxygen and their combinations) on the germination ability of TB grain, which has not been reported before, as finding optimal conditions for CP treatment of specific plant species is crucial for further development of CP technology and its implications in agriculture. We hypothesise that there will be different responses in the TB grain germination ability to either pure nitrogen or pure oxygen plasma treatments and their different admixtures.

Materials and Methods

Tartary buckwheat grain material

The TB grains were obtained from Rangus Mill (Šentjernej, Dolenjska, southeastern Slovenia), from the 2021 harvest and stored under suitable conditions (dry and dark environment at room temperature) until the experiments were performed in spring 2022.

Cold plasma treatment

The TB grains were treated in a custom-made, large-scale, radiofrequency (RF) low-pressure plasma reactor previously described in detail (Holc et al., 2019; Primc & Mozetič, 2019) as shown in Figure 1. In short, it consists of a 2 m long reactor with a 0.2 m diameter (inner diameter 0.18 m and a volume of approximately 50 L) powered by inductively coupled discharge. Plasma is generated inside an excitation coil (wrapped around a glass vacuum discharge) connected to a 27.12 MHz RF generator (Induktio UHFG-8). The vacuum chamber was pumped with a two-stage rotary pump (Leybold Trivac D65B). TB grains, approximately 200 in number, were evenly distributed on a metallic mesh and placed inside the tube in the coil area. Plasma was generated at a pressure of 50 Pa (working pressure around 10 Pa), under a 2.5 kV voltage and around 1.5 kW working power. TB grains were treated for 45 or 90 s with either pure (>99,99% purity) oxygen or nitrogen plasma, or their mixtures in different ratios: 25% oxygen and 75% nitrogen (1/4 oxygen), 50% oxygen and 50% nitrogen (1/2 oxygen), 75% oxygen and 25% nitrogen (3/4 oxygen). The gas (or gas admixture) was leaked into the vacuum chamber on the opposite side of the pumping duct using the mass flow controller (Aera FC7700 Advanced Energy). Immediately after CP treatment, grains were packed into sterile PVC bags, and further experiments were performed the same day. Control TB grains were not treated with CP, while the vacuum control was only exposed to vacuum conditions for the maximum time (90 s).

Germination tests

Germination tests were performed to evaluate the effect of CP treatment on the germination ability of TB grains. In short, 20 grains of each CP treatment (10 different treatments), control and vacuum control grains were evenly distributed on plastic petri dishes (75 mm in diameter) with two layers of filter paper and moistened with 4 mL of bidestilled water. For each group of grains, a germination test was performed in six replicates. In total, 120 grains per treatment (1200 per whole experiment) were analysed. The Petri dishes were incubated in plant growth chambers at

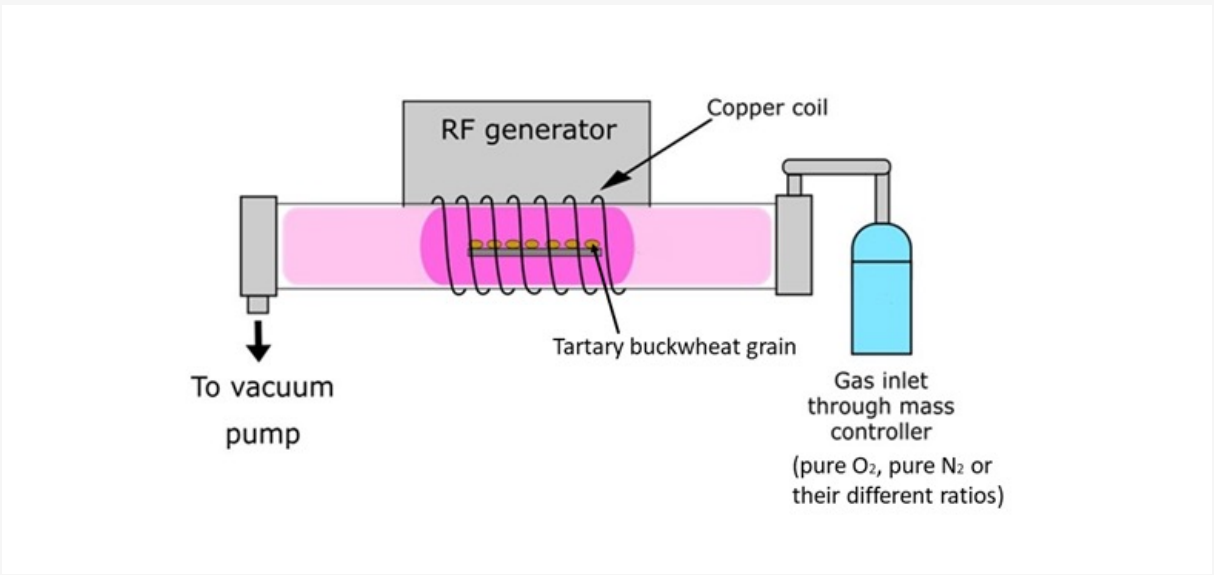


Figure 1. Scheme of the custom-made low-pressure cold plasma system. Adapted from Mravlje et al. (2021).

Slika 1. Shema po meri izdelanega nizkotlačnega hladnega plazemskega sistema. Povzeto po Mravlje et al. (2021).

22 °C and 60% relative humidity, in the dark. If necessary, sterile distilled water was added to the Petri dishes to maintain constant moisture during germination. The number of germinated grains was counted every 24 h for the first three days and then on the 6th and 8th days. The germination rate (GR%) was calculated according to Equation (1). The visible penetration of the radicle through the seed coat was used as the criterion for germination.

(1) GR (%) = (number of germinated grains) / (total number of grains) × 100

To better evaluate the germination process (germination speed), mean germination time (MGT) was calculated according to Equation (2) found by Ellis and Roberts (1981) (Ellis & Roberts, 1981).

(2) $MGT = \Sigma D \times n / \Sigma n$

Where n is the number of seeds germinated on day D, and D is the number of days counted from the beginning of germination.

Seedling length and biomass were measured in 10 randomly selected seedlings per petri dish (in all six replicates), or if fewer than 10 seedlings emerged per petri dish, all seedlings were measured.

Statistical analysis

The reported results are expressed as mean ± standard error (SE) of six replicates. Statistical significance between groups (treatments) was determined using a one-way analysis of variance (ANOVA) with Duncan's post hoc test (Statistica StatSoft version 7). The significance level was set at a p-value of less than 0.05. The graphs were prepared with MS Excel®.

Results and Discussion

Our findings revealed distinct and varied effects of different CP mixtures on the germination rate of Tartary buckwheat (Figure 2). All CP treatments exhibited a delay in germination, as seen in MGT (Figure 3). This was previously reported in the CP treatment of buckwheat grain using different low-pressure oxygen CP setups (Mravlje et al., 2022; Mravlje et al., 2021). Up to this point, there has been no evidence of a significant positive effect of CP treatment on buckwheat germination in either low-pressure or atmospheric pressure CP devices (Ivankov et al., 2021; Mravlje et al., 2022; Mravlje et al., 2021; Šerá et al., 2012; Starič et al., 2023). Most studies report an increasing inhibitory effect on buckwheat germination with increasing treatment time (Mravlje et al., 2021; Šerá et al., 2012; Starič et al., 2023), indicating that buckwheat is very sensitive to CP treatment. In addition to treatment time, the type of plasma reactor (Šerá et al., 2012) and exposure mode (Mravlje et al., 2022) significantly impact the response of buckwheat seeds. The adverse effect is especially profound in the case of powerful low-pressure CP reactors already at shorter treatment times (Mravlje et al., 2022; Starič et al., 2023), as low-pressure CP reactors are known to be a source of intense radiation, especially in the vacuum UV range (D. Popović et al., 2021). This adverse effect could probably be attributed to higher electron density, more UV radiation and heat with longer CP exposure times, which is believed to be responsible for the adverse effect on seed germination (Bol'shakov et al., 2004), as high temperatures (exceeding 45 °C) are considered lethal at physiological level due to protein denaturation and disrupture of membrane lipids, leading to the death of seed cells and tissues (Jemaa Essemine et al., 2010).

The only significant positive effect on germination rate and early sprout growth was observed after treating TB grain with plasma-activated water (PAW), with around 10% increase in germination rate (and 16% in sprout biomass) documented in the PAW-treated group (Wang et al., 2022). One thing to mention is that buckwheat grain usually exhibits relatively high germination rates in the control group (generally well over 60 or even 80%), as shown in the above-mentioned papers; therefore, improving the germination rate after CP treatment significantly is challenging. Nevertheless, to this date, all published data on the CP treatment of buckwheat used either air or pure oxygen as the feeding gas. However, evidence from the CP treatment of other seed species suggests a crucial role of different feeding gases (Pet'ková et al., 2021; Zhou et al., 2016) or their ratios (Szőke et al., 2018) in determining seed germination rate or germination time (Dufour et al., 2021). Our results confirm this and suggest that nitrogen CP treatment is more suitable for TB grain treatment, as no adverse effects were observed after shorter (45 s) CP treatment with pure nitrogen either in final germination rate (Figure 2) or MGT (Figure 3). Longer (90 s) CP treatment with pure nitrogen also did not significantly affect MGT compared to the control; however, a 10% lower final germination rate than the control group was observed. Vacuum conditions themselves did not influence germination in TB, although

a slight increase in germination (around 15%) compared to the control group was observed on the 2nd day. Pure oxygen CP treatment had a more adverse effect on TB germination, reducing the final germination rate by more than 20% or even 80% after 45 or 90 s of CP treatment, respectively, compared to the control group. This finding aligns with our previously reported results, shich showed an adverse effect of pure oxygen low-pressure CP was observed with increased treatment time in both CB and TB grain (Mravlje et al., 2021).

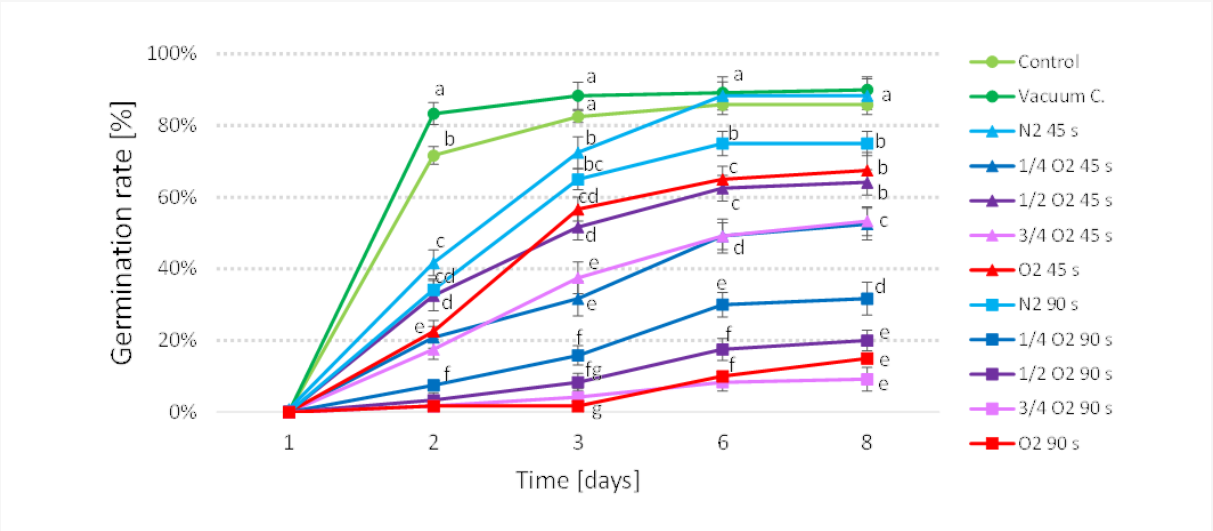


Figure 2. Germination rate (in %) in each treatment (mean ± SE, N=6). Different letters (superscripts) represent statistically significant differences between the treatments (one-way ANOVA, Duncan post hoc test, $p < 0,05$).

Slika 2. Stopnja kalivosti (v %) pri posameznih obdelavah (povprečje ± SE, N=6). Različne črke (nadpisane) označujejo statistično pomembne razlike med obdelavami (enostranska ANOVA, Duncanov post hoc test, $p < 0,05$).

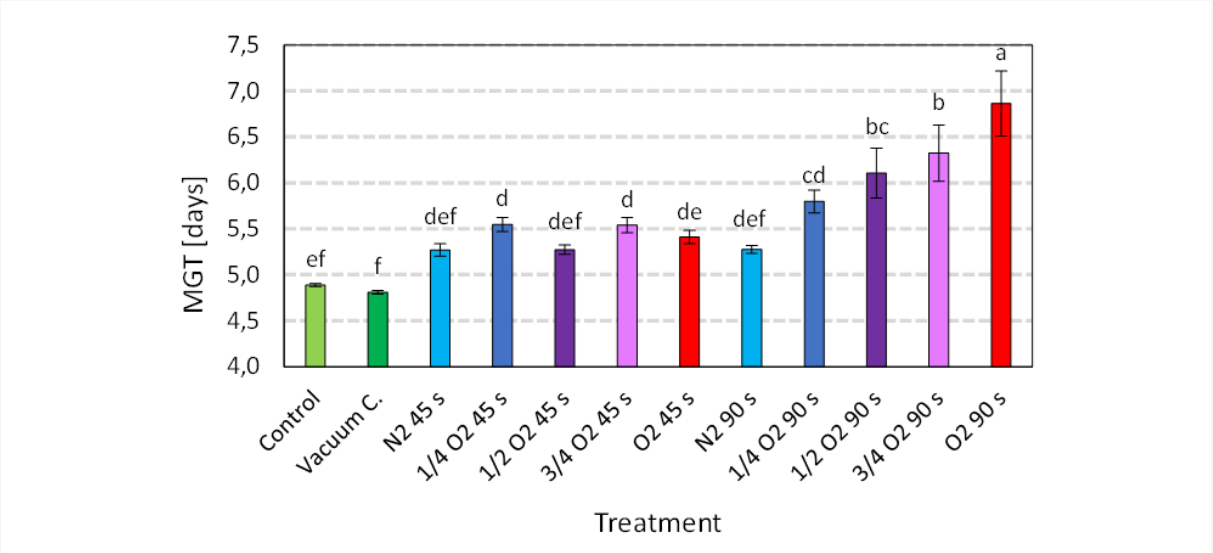


Figure 3. Mean germination time (MGT) in days calculated for each treatment (mean ± SE, N=6). Different letters (superscripts) represent statistically significant differences between the treatments (one-way ANOVA, Duncan post hoc test, $p < 0,05$).

Slika 3. Povprečni čas kalitve (MGT) v dneh, izračunan za vsako obdelavo (povprečje ± SE, N=6). Različne črke (nadpiski) predstavljajo statistično pomembne razlike med obdelavami (enostranska ANOVA, Duncanov post hoc test, $p < 0,05$).

Interestingly, we observed a notable pattern: as the oxygen-to-nitrogen ratio increased, the effect on germination became more negative, especially in the MGT at 90 s CP treatments. This can be attributed to the fact that in low-pressure plasma systems, increasing the oxygen percentage in the mixture results in greater surface erosion of the material (Philip et al., 2002). Oxygen plasma systems (or mixtures containing higher % of oxygen) have already proved to be more efficient in the microbial inactivation process, as it was demonstrated that reactive oxygen species (ROS), including the O_3, metastable state O_2, and atom O, play a crucial role in the microbial inactivation (Philip et al., 2002; Szőke et al., 2018). Plasma mixtures containing more oxygen also proved more efficient at inactivating cells, cell leakage and DNA damage in different bacterial species (Lu et al., 2014). A more adverse effect of oxygen plasma compared to nitrogen on the DNA damage of barley seeds was reported, suggesting ROS generated by the CP treatment could have a more negative impact on seeds than reactive nitrogen species (RNS) (Pet'ková et al., 2021). It has already been demonstrated that ROS and RNS species generated by plasma play a crucial role in germination and plant growth (Zhou et al., 2016). They demonstrated that interactions between plasma-generated species, such as H_2O_2 and N species, and seeds led to accelerated germination and improved seedling length in mung beans. Adding nitrogen to the helium plasma decreased germination time and an increase in the vigour of lentil seeds, while adding oxygen to the mixture had no positive effect and even showed an adverse effect on lentil seeds (Dufour et al., 2021). Using optical emission spectroscopy, they showed that N_2^*, N_2^+, NO and OH promoted seed vigour, while O species had limited beneficial effects, especially O_3, which clearly showed an inhibitory effect on seed vigour.

Our results are in line with these findings, as increasing the oxygen content of the mixture led to a more adverse effect on germination and early seedling growth (Table 1). Treatment with pure nitrogen CP, regardless of treatment time, had no inhibitory effect on seedling length and biomass. In contrast, treatment with pure oxygen CP led to decreased seedling length and biomass after 45 s, and the effect was even more adverse after 90 s. Different ratios of oxygen compared to nitrogen decreased seedling length and biomass, comparable to pure oxygen plasma after 45 s. Interestingly, the most negative effect on seedling growth was observed after 90 s of CP treatment in a gas mixture containing 75% oxygen and 25% nitrogen. This suggests that the combination of CP-generated ROS and RNS may have adverse effects, potentially exceeding those of ROS alone. This calls for further research, as it has already been noted that the role of plasma-generated ROS and RNS in seed response is still far from understood (Dufour et al., 2021). And since it is known that the response of seeds to CP treatment is not only species but also cultivar (variety) dependent (Recek et al., 2023; Starič et al., 2021), it is essential to characterise and optimise CP treatments for specific seeds to make this promising technology a feasible and effective solution for the challenges of modern agriculture and food industry.

Table 1. Average sprout length per treatment (mean ± SE, N=6). Different letters (superscripts) represent statistically significant differences between the treatments (one-way ANOVA, Duncan post hoc test, $p < 0,05$).

Tabela 1. Povprečna dolžina poganjkov po posameznih obdelavah (povprečje ± SE, N=6). Različne črke (nadpisane) označujejo statistično pomembne razlike med posameznimi obdelavami (enostranska ANOVA, Duncanov post hoc test, $p < 0,05$).

Treatment	Seedling length [mm]	Seedling biomass [mg]
Control	$83,8 \pm 1,6^{a}$	$78,6 \pm 3,5^{a}$
Vacuum control	$84,9 \pm 1,4^{a}$	$82,1 \pm 4,1^{a}$
N_2 45 s	$76,1 \pm 1,2^{a}$	$76,9 \pm 3,6^{a}$
¼ O_2 45 s	$51,0 \pm 1,7^{bc}$	$44,8 \pm 4,2^{c}$
½ O_2 45 s	$61,6 \pm 1,5^{b}$	$57,6 \pm 3,1^{b}$
¾ O_2 45 s	$56,7 \pm 2,7^{bc}$	$44,8 \pm 4,2^{c}$
O_2 45 s	$61,3 \pm 2,6^{b}$	$52,6 \pm 2,5^{bc}$
N_2 90 s	$79,8 \pm 1,6^{a}$	$75,3 \pm 5,2^{a}$
¼ O_2 90 s	$59,6 \pm 2,7^{b}$	$42,4 \pm 3,4^{c}$
½ O_2 90 s	$61,7 \pm 8,1^{b}$	$44,2 \pm 5,8^{c}$
¾ O_2 90 s	$23,3 \pm 8,2^{d}$	$24,6 \pm 3,7^{d}$
O_2 90 s	$46,4 \pm 4,7^{c}$	$30,2 \pm 2,8^{d}$

Conclusions

There is an urgent need for innovative, environmentally friendly, and economically sustainable technologies in the food and agriculture sector to reduce reliance on pesticides and other xenobiotics in plant production. The research field of cold plasma (CP) agriculture appears to offer a promising, environmentally friendly alternative, leaving no harmful residues for either humans or the environment. The results published in this paper reported the effect of different gases (nitrogen, oxygen and their mixtures in various ratios) in a low-pressure CP system on germination and early seedling growth of Tartary buckwheat for the first time. We found that treatment with pure nitrogen did not affect germination or early seedling growth after shorter (45-second) exposure, with a decrease in germination of less than 10% compared to the control after 90-second exposure, and no significant effect on seedling growth or biomass. On the other hand, pure oxygen CP treatment decreased the germination ability of Tartary buckwheat grains by more than 20% compared to the control group, already after 45-second exposure, while longer (90-second) exposure caused an 80% decrease in germination rate compared to the control group. Both exposures also adversely affected early seedling growth, and the effect intensified with increased CP treatment time. This trend was also confirmed across different ratios of oxygen and nitrogen gas mixtures, as it was found that an increased % of oxygen in the gas mixture has a more adverse effect on both germination and early seedling growth, which is particularly visible in the MGT, especially after longer CP exposure time. Therefore, we suggest that nitrogen CP (or a mixture containing a lower % of oxygen) is more suitable for treating Tartary buckwheat grains. However, further research should also focus on how different gas mixtures affect not only germination ability and early seedling growth, but also decontamination efficacy and the growth of treated grains on a longer timescale (e.g., field experiments).

Author Contributions

Conceptualisation, J.M.; methodology, J.M.; formal analysis & investigation, J.M.; resources, J.M.; data curation, J.M.; writing—original draft preparation, review and editing, J.M.; visualisation, J.M. All authors have read and agreed to the published version of the manuscript.

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Data Availability

Research data are available on Zenodo (https://doi.org/10.5281/zenodo.18246860)

Conflicts of Interest

The authors declare no conflict of interest.

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