Effects of non-thermal atmospheric plasma on physiological characteristics of black cumin

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Abstract

The objective of the present study was to determine the effects of non-thermal atmospheric plasma (NTAP) on physiological features of black cumin seeds. Black cumin seeds were divided into 4 groups, one control group and three experimental groups. The experimental groups were exposed to NTAP for 5, 10, and 20 minutes, respectively. Then the seeds of the experimental and control groups were grown for 21 days. Subsequently, total sugar levels, total flavonoids, malondialdehyde (MDA) levels, and the activities of superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) enzymes were evaluated. Results showed that the concentration of sugar, total flavonoids, and total proteins in the experimental groups increased significantly compared to the control group. SOD, CAT, and APX activity decreased in all NTAP- treated plants significantly compared to the control group. It can be concluded that black cumin seeds treated with non-thermal atmospheric plasma may increase plant resistance by changing the enzymatic antioxidant system.

Keywords: *Non-thermal atmospheric plasma; black cumin; Nigella sativa;* plant resistance; plant growth

Yasamani Masouleh, F., G. Barzin, M. Entezari, T. Davari Mahabadi, and L. Pishkar. 2020. 'Effects of non-thermal atmospheric plasma on physiological characteristics of black cumin'. *Iranian Journal of Plant Physiology* ,11(1): 3473- 3480.

Introduction

The harmful effects of chemical drugs are noted by the general public in recent years. On the other hand, more than 6,000 species of medicinal plants are recognized in Iran which are the subject of numerous research studies. Black cumin is a medicinal plant with antibacterial, antiviral, antiepileptic, antiparasitic, analgesic, and many other benefits (Al-Jassir, 1992). It is a dicotyledonous, herbaceous, and annual plant that belongs to the Ranunculaceae family. Seeds form the medicinal part of the plant (Bourgou et al., 2010).

In recent years, the effect of plasma in agricultural science has been proposed as an alternative to the traditional pre-crop seed treatment such as scarification, heat treatment,

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Received: April, 2020
Accepted: August, 2020

chemical treatment, etc. (Dhayal et al., 2006). In physical scraping, the seed coat is scraped using an abrasive mixer. However, this mechanical method increases the probability of the number of seeds being destroyed as well as the non-uniform treatment. In the case of heat treatment, hot water or a hot surface is usually used while chemical treatment involves the treatment of seeds with commercial sulfuric acid for 5-10 minutes, and this method leads to environmental pollution (Dhayal et al., 2006). Plasma has the advantage of uniform treatment, no seed degradation, no use of harmful chemicals and therefore, it is not harmful for the environment and is environment friendly (Selcuk et al., 2008, Volin et al., 2000).

Studies have been conducted to investigate the effect of plasma treatment on seed germination and on plant growth (Sera et al., 2008; Kitazaki et al., 2012). Increases in germination rate of plasma-treated seeds compared to controls have been reported in several articles (Sera et al., 2008; Lynikiene et al., 2006). For example, Dial et al. (2006) reported 50% increase in the germination rate of sunflower seeds (*Carthamus tinctorium* L.) exposed to plasma for 130 minutes. Sera et al. (2008) obtained a nearly threefold increase in the germination rate of Chenopodium album plant after treatment with plasma for 48 minutes. On the other hand, there are also studies that show plasma does not affect the germination rate of wheat (Selcuk et al., 2008), bean (Selcuk et al., 2008), barley (Sera et al., 2010), and radish (Kitazaki et al., 2012). In some cases, improved germination results compared to controls for plasma treatment duration have been studied and optimization has been reported (Filatova et al., 2013; Henselová et al., 2012).

Improvement of seedling-related growth parameters has been reported after seed exposure to plasma (Henselová et al., 2012; Kitazaki et al., 2012). In a study by Kitazaki et al. (2012), a 60% increase in radish plant shoots exposed to plasma was reported. Also, 21% increase in root length and 10% increase in root fresh weight, as well as a 14% increase in dry weight of maize roots exposed to plasma were reported (Henselová et al., 2012). In another study, doubling the length of sunflower roots by plasma treatment was reported (Dhayal et al., 2006).

The NTAP action mechanism on seed parameters has not been fully understood. As the seeds have a very complicated system, the seeds of each plant can behave differently under NTAP treatment (Goyoaga et al., 2011). Thus, miscellaneous parameters including plasma treatment, plasma properties, plasma strength, plasma state, and pressure can affect seeds' response, germination, and growth (Misra et al., 2016).

According to the literature reviewed, there is no report about the effects of non-thermal atmospheric plasma on black cumin seeds. Therefore, the aim of this study was to investigate the effects of non-thermal atmospheric plasma application on seed parameters and physiological characteristics of black cumin plant.

Materials and Methods

Plant materials and experimental design

Pure seeds of black cumin in this study were prepared from Pakan Seed Research Center in Isfahan. Seed purity, viability, and 1000-seed weight were 70%, 80%, and 2 g, respectively.

The seeds were divided into four groups and each group had three repetitions. Group 1 included seeds exposed to NTAP for 5 minutes, group 2 involved seeds exposed to NTAP for 10 minutes, group 3 contained seeds that were exposed to NTAP for 20 minutes, and group 4 was the control group. The seeds were soaked in distilled water for 24 hours and then transferred to a petri dish containing wet filter paper for germination. After germination and reaching a height of 3 cm, the seedlings were transferred to pots containing nutrient solution. Seedlings grew below the average daily and night temperatures of 25 and 17 ℃, photoperiod 16 hours, and light intensity of 10000 lux. After one week of pretreatment of seedlings with 25% nutrient solution, 50% nutrient solution was used and after one week, 100% nutrient solution was used. After one week of seedling growth in the 100% nutrient solution, treatments were started. In each group, 21 days after cultivation and germination,

physiological characteristics of seedlings were evaluated.

The number of germinated seeds was recorded every day for 14 days. Germinated cumin seeds were considered germinated when the radicle was 2 mm long (Milivojević et al., 2018).

The experiment was performed on a commercial PC-HD 2N plasma processing machine, which included a vacuum device, a plasma generator, a transmission, and an input/output channel. Seeds were uniformly selected and exposed to helium-induced plasma discs under the following parameters: Plasma frequency 13.56 MHz, power 60 (T1), 80 (T2), 100 (T3) and 120 W (T4), pressure 150 Pa and the volume of the evacuation chamber was 1200 mm×180 mm×20 mm.

Physiological parameters

To measure the concentration of total flavonoids, aluminum chloride colorimetric method was used. For this purpose, 0.2 g of plant sample was extracted in 3 ml of PBS buffer with a pH of 1.6. The resulting suspension was then centrifuged at 15000 g for 4 min at 4 ℃. Then, 1ml of supernatant, 6 ml of distilled water, 0.3 ml of 5% sodium nitrite were mixed. Next, 0.3ml of 10% aluminum chloride and 2 ml of 1 mM sodium hydroxide were added to the solution and the sample absorbance was read by a spectrophotometer at 510 nm wavelength. To calculate the concentration of flavonoid compounds, the standard quercetin curve manufactured by Merck Company with concentrations of 20 to 80 mg/l was used (Toor and Savage, 2005).

Soluble sugars content

Colorimetry was used to determine the amount of soluble sugars. For this purpose, 0.1 g of samples were rubbed with 0.2 M PBS buffer. The 0.5 ml of the sample was then mixed with 0.5 ml of phenol 5% (w/w) and 2.5 ml of sulfuric acid (95.5%). Then the solution was shaken and incubated for 20 minutes in a hot water bath at 30 ℃. The absorption samples at 480, 485, and 490 nm were then read for glucose, mannose, and xylose, respectively by spectrophotometer. After drawing the standard curve, the glucose content was considered as total sugar and the sugar contents of mannose and xylose were calculated from the standard curve (Scroccarello et al., 2019).

Malondialdehyde (MDA)

First, 0.2 g of plant tissue was thoroughly rubbed with 0.5 ml of 0.1% trichloroacetic acid (TCA) solution in a Chinese mortar. The resulting extract was then centrifuged at 15000 g for 4 minutes at 4 ℃. Then, 1ml of supernatant with 4 ml of 20% chloroacetic acid solution containing 5% thiobarbituric acid (TBA) were added. The resulting mixture was incubated at 95 ℃ for 30 minutes. The samples were then immediately frozen and the mixture was centrifuged at 200 g again for 10 min. The absorption intensity of the solution was read at 532 nm, which is the wavelength of red complex absorption TBA-MDA (Davey et al., 2005).

Superoxide dismutase

The NBT (Nitroblo Tetrazolium) optical reduction method was used to measure the activity of the superoxide dismutase enzyme. For this purpose, a mixture of 13 mM methionine, 75 µM NBT, 0.1 mM EDTA, and 2 µM riboflavin was mixed and the final volume was 150 cc. Then, 3 ml solution was then added to 100 µl of cumin extract and the samples were placed in 5000 LUX light for 15 minutes. After that, the light was turned off and the absorption of the samples was read by a spectrophotometer at a wavelength of 560 nm (Elavarthi and Martin, 2010).

Catalase

Pereira et al. (2002) method was used to measure the activity of catalase enzyme. For this purpose, 0.2 g of the plant sample was extracted in 3 ml PBS buffer at pH=6.8. The resulting suspension was then centrifuged at 15000 g for 15 min at 4 ℃. The supernatant was used to measure catalase activity and 2.5 ml of Tris/HCl buffer and 6 µl of plant extract were mixed in an ice bath. Then, 50 µl of 1% hydrogen peroxide was added to it and the absorbance was immediately read at 240 nm wavelength. The rate of catalase activity in the samples was calculated according to the

enzyme activity per minute per milligram of protein (Pereira et al., 2002).

Ascorbate Peroxidase

To measure the activity of the peroxidase enzyme, 0.2 g of the plant sample was extracted in 3 ml of PBS buffer with a pH of 6.1. The resulting suspension was then centrifuged at 15000 g for 4 min at 4 ℃. The supernatant was used to measure the activity of ascorbate peroxidase. Two (2) ml of acetate buffer, 200 µl of benzidine solution, and 200 µl of hydrogen peroxide were mixed in an ice bath, and immediately after adding 100 µl of plant extract to it, the absorbance of the solution was read using a spectrophotometer at 530 nm. Enzyme activity in samples was calculated based on enzyme activity per minute per milligram of protein (Elavarthi and Martin, 2010).

In order to investigate the effect of different plasma treatments on the levels of antioxidant enzymes and biochemical parameters, one-way and two-way ANOVA analyses were used. Tukey test was performed for multiple comparisons.

Results

Total Soluble Sugars

The results of changes in the amount of soluble sugars due to non-thermal atmospheric plasma treatment are given in (Fig. I). The results of ANOVA showed that the treatment of plants with NTAP caused an increase in the amount of total soluble sugar, which was statistically significant in 20 minutes.

Total Flavonoids

The trend of total flavonoids changes due to NTAP treatment is given in (Fig. II). The results of ANOVA showed that the treatment of plants with NTAP increased total flavonoids, which was a significant increase in all times used.

Malondialdehyde (MDA)

The results of changes in the amount of MDA due to NTAP treatment are given in (Fig. III). The results of ANOVA showed that the treatment of plants with non-thermal atmospheric plasma caused a slight decrease in the amount of MDA, which was not statistically significant.

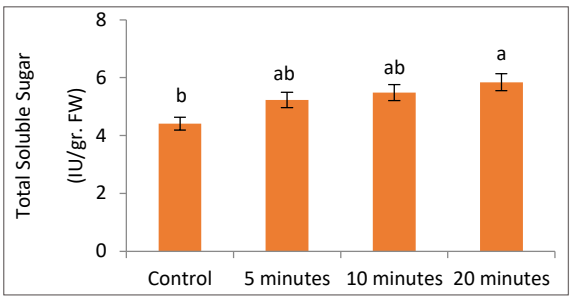

Fig. I. the effect of NTAP on total soluble sugars in black cumin; different letters show statistically significant differences ($p < 0.05$).

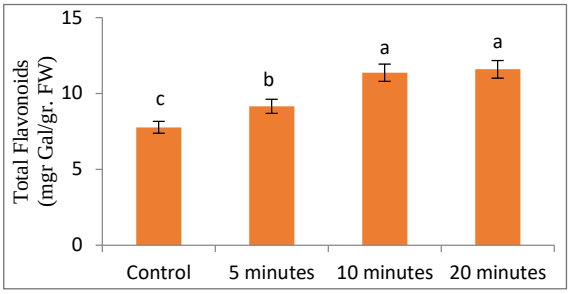

Fig. II. the effect of NTAP on total flavonoids in black cumin; different letters show statistically significant differences ($p < 0.05$).

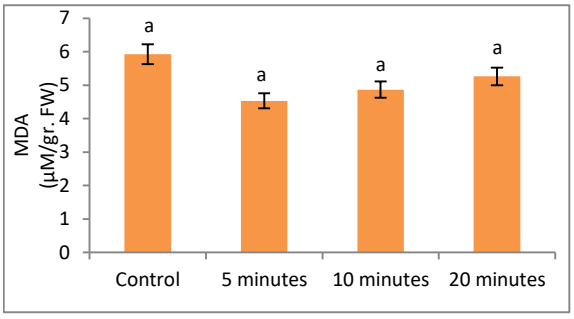

Fig. III. the effect of NTAP on MDA in black cumin; different letters show statistically significant differences ($p < 0.05$).

Antioxidant Enzymatic Activity

Peroxidase

NTAP treatment reduced peroxidase activity in black cumin seedlings and this decrease became more severe with increasing the duration of non-thermal atmospheric plasma treatment and the lowest activity was achieved in 20 min NTAP treatment (Fig. IV).

Catalase

The results of ANOVA showed that NTAP treatment of black cumin seedling led to the reduction of catalase activity, which was not statistically significant in 5-minute treatment while it was significant in 10- and 20-minute treatments. The greatest reduction was observed in 20-minute treatment (Fig. V).

Superoxide dismutase (SOD)

Results showed that NTAP-treated black cumin plants showed the low activity of SOD enzyme, which was statistically significant in the 20-minute treatment and the highest reduction was observed in the 20-minute NTAP treatment (Fig. VI)

Discussion

In the present study, cold plasma treatment caused an increase in the germination process. Consistent with the results of this study, an improvement in the germination process in plants has been observed following the NTAP (Dhayal et al., 2006; Kitazaki et al., 2012; Henselová et al., 2012). In one study, it was found that the germination rate of safflower increased by 50% due to the effect of plasma (Dhayal et al., 2006), which is consistent with the results of the current study. In a study on Chenopodium album (Sera et al., 2008), the germination rate increased almost threefold, which was consistent with the results of this study.

In this study, NTAP treatment significantly reduced the activity of antioxidant enzymes. The activity of peroxidase, catalase, and superoxide dismutase enzymes decreased with the use of non-thermal atmospheric plasma and this reduction was significantly higher than the control ($p \leq 0.05$) compared to the control. Plants, which are immobile organisms, face many challenges, including biological and non-biological stresses, and in response to these stresses they have developed an adaptive defense system that changes the level of activities of antioxidant enzymes. Changes in the activity of antioxidant enzymes under different environmental conditions have been reported in many plants (Verma and Dubey, 2003; Zhang et al., 2017).

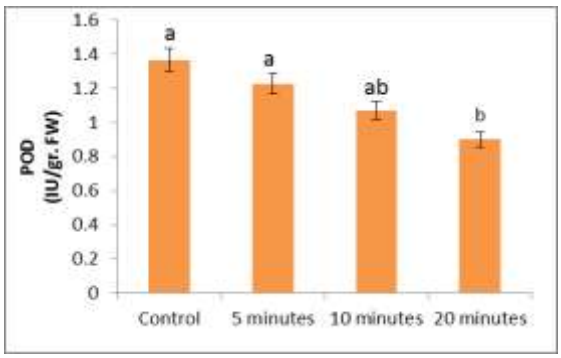

Fig. IV. the effect of NTAP on peroxidase activity in black cumin; different letters show statistically significant differences ($p<0.05$).

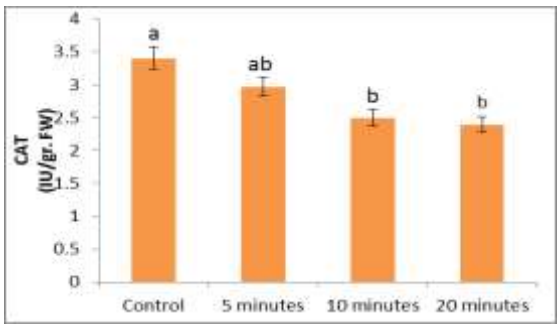

Fig. V. the effect of NTAP on catalase activity in black cumin; different letters show statistically significant differences ($p<0.05$).

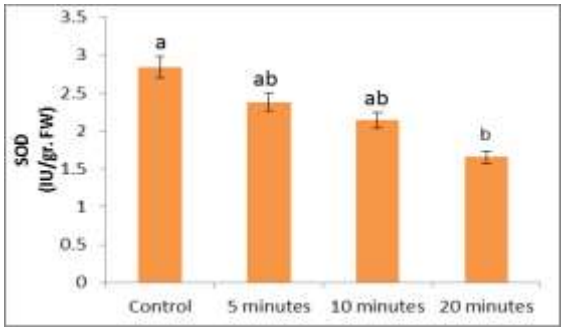

Fig. VI. the effect of NTAP on SOD activity in black cumin; different letters show statistically significant differences ($p < 0.05$).

Consistent with this research, in a study on the poplar plant, NTAP reduced peroxidase activity (Pauzaite et al., 2018). Similar results have been reported for plants including Arabidopsis (Michalak, 2006), Malva sylvestris (Julák et al., 2018), and Rapeseed (Ling et al., 2015b), in response to NTAP. Previous studies have shown that in poplar, rice, and wheat plants, NTAP treatment significantly reduced the activities of antioxidant enzymes, which is consistent with the results of the current study (Pauzaite et al., 2018;

Khamsen et al., 2016). In an experiment on *Fogopyrum esculentom*, it was observed that the activity of these enzymes increases following NTAP treatment, which is not consistent with our study because part of the various antioxidant enzymes and related proteins interact with each other and have opposite synergies with each other, so the use of cold plasma increased the amount of enzymes in this plant (Jiang et al., 2017). In another study on wheat, non-thermal atmospheric plasma was found to reduce catalase activity, which is consistent with the results of this study (Mildažienė et al., 2019). In another study, higher concentrations of H_2O_2 and antioxidant enzymes including peroxidase and polyphenol oxidase were reported in tomato seedlings treated with NTAP (Jiang et al., 2014). Similarly, other researchers reported that seed treatment with NTAP increased proline and soluble sugars while decreasing MDA content in seedlings, which is consistent with the results obtained from the present study (Li et al., 2018, Guo et al., 2017). Previous studies have shown that increased metabolic activity in NTAP-treated seeds occurred during germination along with the production of reactive oxygen species (ROS) and other free radicals (Laroussi, 2005). By increasing the ROS in the plant and creating oxidative stress, the activities of antioxidant enzymes increase (Sharma et al., 2012). The results of the present study showed an increase in the activity of the superoxide dismutase enzyme in black cumin seedlings, the seeds of which were exposed to NTAP for 5 minutes, compared to the control group.

The results of the present study showed that the treatment of black cumin seeds with NTAP significantly increased soluble sugars compared to control seedlings. Also, as treatment time increased, the amount of soluble sugars also increased. Similar to the results of the present study, Wu et al. (2007) reported that treatment of seeds with NTAP increased the amount of soluble sugars in corn seedlings compared to control (Wu et al., 2007). In a study by Sadhu et al. (2017) treatment of seeds of bean plants (Vigna radiate) with NTAP increased the amount of soluble sugars in seedlings (Sadhu et al., 2017). It has been suggested that degradation and conversion of fats during seed germination is associated with increased levels of soluble sugars in the plant (Goyoaga et al., 2011). Therefore, it is likely that the degradation and conversion of fats in seeds after NTAP treatment is to some extent responsible for increasing the amount of soluble sugars in seedlings (Ling et al., 2014; Singh et al., 2019).

In the current study, 5 and 10 minute NTAP-treatments of black cumin seeds reduced the levels of MDA compared to controlled seedlings. In confirmation of the results of the present study, Ling et al. (2015) reported a reduction in MDA production in the flowering plant after treating its seeds with NTAP and increasing the plant's resistance to drought stress (Ling et al., 2015a). Lipid peroxidation increases free radicals to excess cell tolerance thresholds and impairs the function of cells and cellular organs. Therefore, by increasing the activity of antioxidant enzymes and antioxidant compounds such as flavonoids in black cumin seedlings due to NTAP treatment, damage to membrane lipids is reduced and as a result, MDA levels are reduced.

Conclusion

In general, NTAP treatment had a positive effect on black cumin physiological parameters. Therefore, priming of black cumin seeds with NTAP may be a new and optimal strategy to protect black cumin from environmental stress.

Acknowledgment

The authors would like to thank the officials of the Islamic Azad University, Islamshahr Branch for financing this project.

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