Plasma Medicine, 6(3–4): 349–359 (2016)

Response of Silkworm Larvae to Atmospheric Pressure Nonthermal Plasma Irradiation

Thapanut Sarinont,* Yosuke Wada, Kazunori Koga, & Masaharu Shiratani

Department of Electronics, Kyushu University, Fukuoka-shi, Fukuoka 819-0395, Japan

*Address all correspondence to: Thapanut Sarinont, Department of Electronics, Kyushu University, 744 Motooka, Fukuoka-shi, Fukuoka 819-0395, Japan; Tel./Fax: +81-92-802-3723, E-mail: t.sarinont@plasma.ed.kyushu-u.ac.jp

ABSTRACT: Responses of fifth-instar silkworm (*Bombyx mori* L.) larvae to plasma irradiation were studied using atmospheric pressure dielectric barrier discharge plasma. The growth rate decreased sharply until the day after plasma irradiation and was dependent on the duration of plasma irradiation. Growth rates were similar after irradiation for 60 s and 300 s, but after 600 s, the rate was lower than that after 60 s. The total weight of silkworm larvae decreased as the number of plasma irradiations was increased from one to three. The weight of silk cocoons produced after a single plasma irradiation was 11% heavier than for non-irradiated larvae, whereas the cocoons were 6% and 13% lighter after three and five periods of plasma irradiation, respectively. We conclude that plasma irradiation to fifth-instar silkworm larvae had little effect on silk protein and silk surface morphology.

KEY WORDS: silkworm larvae, silk, cocoon, dielectric-barrier discharge, plasma

I. INTRODUCTION

Atmospheric pressure non-thermal plasma is being used extensively in research aimed at novel medical applications, including hemostasis and in cancer therapy as an alternative to surgery, chemotherapy, and radiotherapy.[1–4] A potential advantage is that plasma supplies reactive oxygen species, reactive nitrogen species, charged species, UV light, and electric fields without causing thermal damage to living tissues.[5–17] More recently, atmospheric pressure non-thermal plasma has been applied to agricultural fields.[18–26] Plasma could offer a potential solution to the global food crisis by boosting crop yield, reducing cultivation time, and improving agricultural productivity.[27] We have found that plasma irradiation of plant seeds induces continuous growth enhancement of the plants after their germination.[28,29] Furthermore, compared with non-treated seeds, plasma irradiation of the seeds led to an 11% shorter harvest time, a 56% increase in total seed weight, and a 39% increase in the number of seeds harvested from the grown plants.[30] An important agricultural application of plasma is as an insecticide. However, although there are reports on the insecticidal effects of atmospheric pressure non-thermal plasma irradiation,[31–32] there are few reports on the effects of plasma irradiation on beneficial insects. This study opens a new area of agricultural applications of plasma by examining the effects of plasma irradiation on silkworms (*Bombyx mori* L.), one of the most important beneficial insects.

Silkworms are domesticated insects that produce silk thread in the form of a cocoon. During the larval period, silkworms consume leaves. Their growth and development is greatly influenced by environmental conditions, including temperature, humidity, gases, and light. The silkworm was chosen as a model insect for studying responses of living organisms to plasma irradiation for the following reasons: (1) silkworms produce a valuable resource, silk[33]; (2) comprehensive genomic information is available[34]; (3) transgenic silkworms have been used to develop cheaper drugs,[35] targeted proteins,[36] and high-strength silk[37]; and (4) silkworm larvae provide an animal model of bacterial infections pathogenic to humans.[38]

The aim of this research was to elucidate the responses of silkworms to atmospheric pressure non-thermal plasma irradiation. For this purpose, we measured weight growth rates and respiration rates of fifth-instar larvae of silkworms and examined sericin protein extracted from cocoons and the surface morphology of silk.

II. MATERIALS AND METHODS

A. Materials

Silkworm cocoons were supplied by Satoyama. The cocoons were fresh and had not undergone any thermal, mechanical, or chemical treatments. Alkaline protease, trypsin, hexane, ethyl alcohol, and ethyl acetate were supplied by Sigma-Aldrich.

B. Dielectric Barrier Discharge Plasma

Experiments were carried out using a scalable dielectric-barrier discharge (DBD) device.[19,30] The device consisted of 20 stainless steel electrodes 1 mm in outer diameter and 60 mm long, each covered with a ceramic tube 2 mm in outer diameter, as shown in Fig. 1. The electrodes were arranged parallel with each other and separated by 0.2 mm gaps. The discharge voltage and the discharge current were measured with a high-voltage probe (Tektronix, P6015A) and a Rogowski coil (URD, CTL-28-S90-05Z-1R1).[39,40] The peak-to-peak amplitude and the frequency of the applied voltage were 9.2 kV and 10 kHz, respectively. The discharge current was 0.2 A. The corresponding discharge power density was 1.49 W/cm^2, which was deduced from a voltage/charge Lissajous plot.

C. Plasma Irradiation Conditions

Fifth-instar silkworm larvae were used in this experiment. Ten larvae were placed 3 mm below the electrodes. Plasma was irradiated to the larvae once (day 1), three times (days 1, 3, and 5), or five times (days 1–5). Plasma irradiation durations were 60, 300, and 600 s. The temperature and relative humidity of air were 24–26°C and 57–65%, respectively. The silkworms were weighed daily until the start of the cocoon stage on the seventh day from the beginning of the fifth instar.

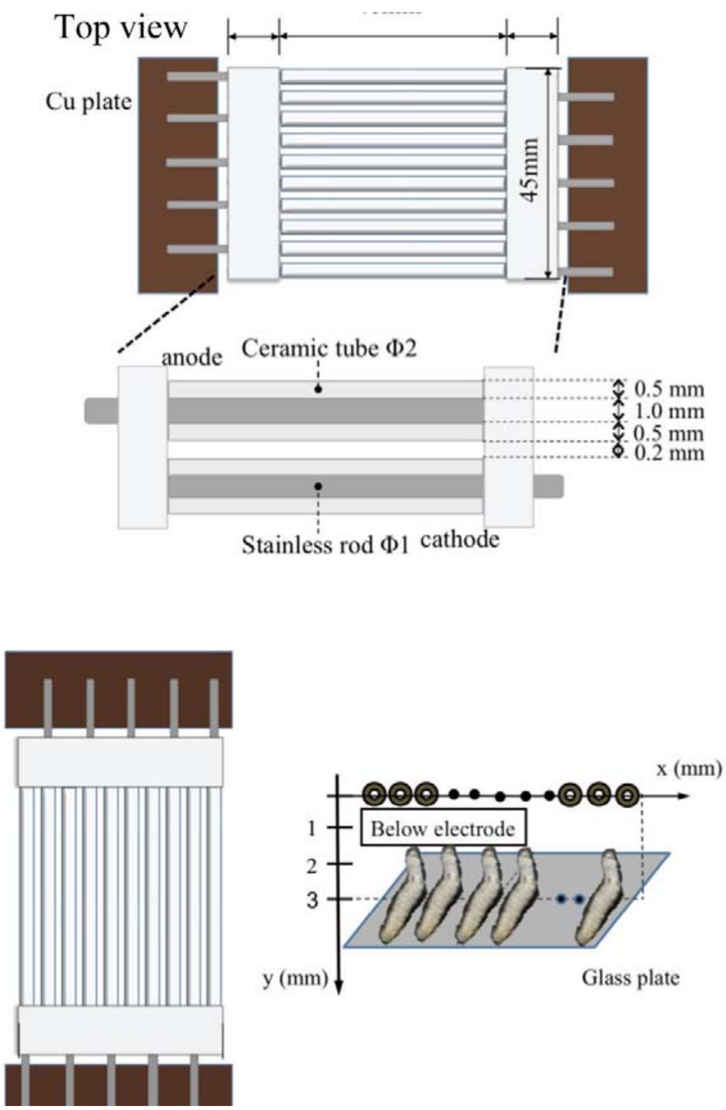

FIG. 1: Top view of electrode configuration of scalable DBD device and schematic image of plasma irradiation to silkworms

D. Respiration Rate of Larvae

Ten silkworm larvae were placed in a closed glass vessel (1000 mL Schott DURAN® bottle) filled with air. To determine the respiration rate of the larvae, O_2 and CO_2 in the vessel were measured with a mass spectrometer (Stanford Research Systems, QMS 100) 1 h after plasma irradiation.

E. Extraction of Sericin from Silk

Extraction of sericin from silk was carried out using 2–2.5 g/L alkaline protease at 60°C for 90 min at pH 10.[41] Hydrolysis with 1% trypsin solution was complete in 10 h at 37°C or 32 h at 20°C. The 280 nm absorbance of the sericin-containing solution was measured with a microwell plate reader (BioTek Synergy HTX multi-mode reader).

III. RESULTS AND DISCUSSION

A. Effects of the Dose of Plasma Irradiation

We carried out plasma irradiation of fifth-instar silkworm larvae situated 3 mm below the electrodes. Figure 2 shows the time course of the rate of increase in their weights (g/d) for plasma irradiation durations of 60, 300, and 600 s. The average weight of the silkworms was measured until the cocoon stage on the seventh day of the fifth instar. The rate of increase in weight of the controls decreased monotonically with time and became negative on the sixth day as the larvae prepared to enter the cocoon stage. In the plasma-irradiated larvae, the rate of growth in weight varied with the duration of the plasma irradiation. Compared with controls, the weight growth rate decreased sharply over the day after plasma irradiation and then increased during the period until the next plasma irradiation. The decreases in growth rate after plasma irradiation for 60 and 300 s were quite similar, but after 600 s of irradiation, the decrease was smaller than that for 60 s. There was a threshold in the plasma dose required to elicit a response in silkworms.

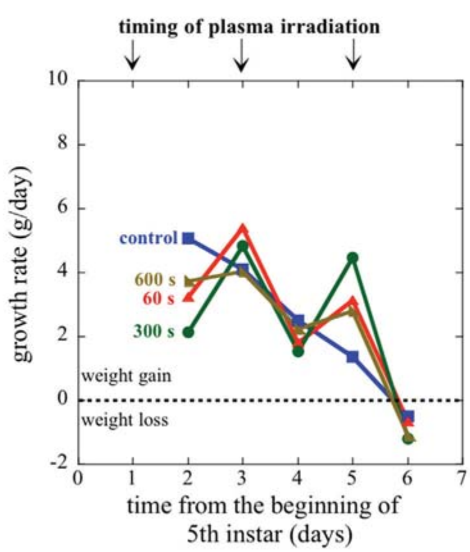

FIG. 2: Weight growth rate of fifth-instar larvae of silkworm for 60, 300, and 600 s plasma irradiation sessions together with control

In short, plasma irradiation to silkworms had a strong effect on their growth, although the mechanism remains to be clarified.

B. Effect of the Number of Plasma Irradiation Periods

Periods of 60 s of plasma irradiation were applied one, three, or five times, as shown in Fig. 3a. There was a significant difference among the total weights of 10 larvae measured after 6 d ($p < 0.05$). Plasma irradiation reduced the weight of larvae compared with control larvae. Single and triple irradiations were not significantly different, but after five irradiations, the larvae were significantly lighter.

Figure 4 shows a photograph of fifth-instar silkworm larvae after a single 60 s plasma irradiation together with control larvae. No difference was apparent between these two groups of silkworms.

Silkworm larvae metamorphosed into cocoons after 1 week. The total weight of 10 silk cocoons is shown in Fig. 5. The weight of silk cocoons after a single plasma irradiation was 11% heavier ($p < 0.05$) than that without irradiation, whereas after three and five

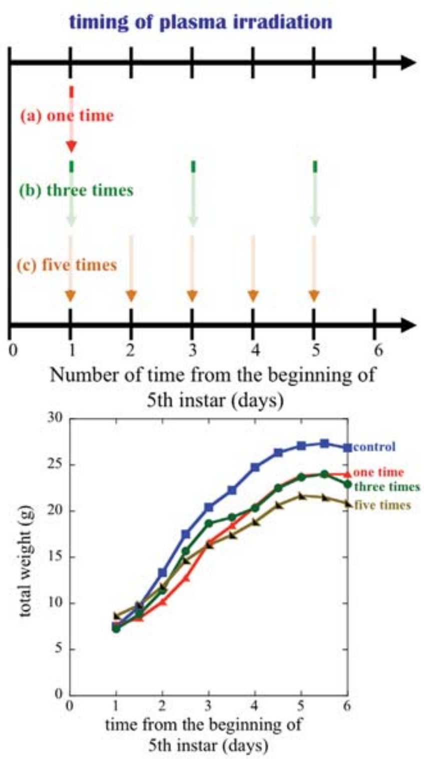

FIG. 3: Time evolution of total weight growth rate of fifth-instar larvae of 10 silkworms for one, three, and five 60 s plasma irradiation sessions together with control

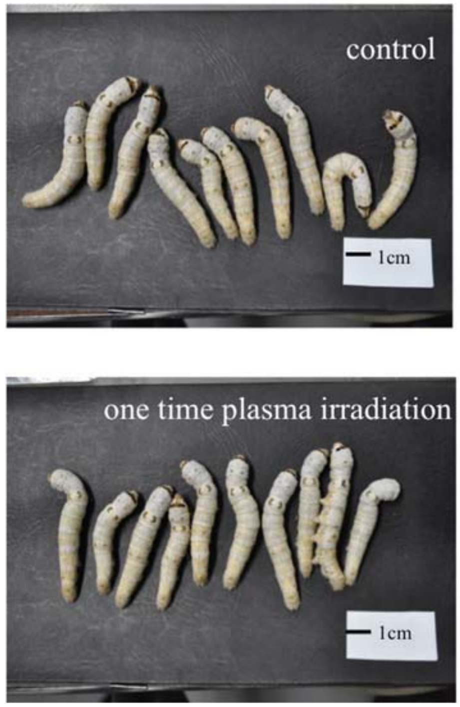

FIG. 4: Photo image of fifth-instar larvae of silkworms for one 60 s plasma irradiation session together with control

periods of plasma irradiation, the cocoons were 6% and 13% lighter, respectively ($p <$ 0.05). Therefore, one-time plasma irradiation enhanced the production of silk cocoons.

C. Respiration of Silkworm

The concentrations of O_2 and CO_2 were measured by mass spectrometry. Figure 6 shows the respiration rates of larvae after three-fold plasma irradiation over 6 d. The rates of O_2 consumption and CO_2 output after irradiation tended to be lower than those values in the control. Possibly, O_3 produced by plasma[42] was responsible for this reduction because O_3 is known to affect the respiratory system.[43] Moreover, the decrease in the respiration rate may have led to the suppression of weight growth shown in Figs. 2 and 3.

D. Properties of Silk

Silk, which forms the cocoons of *B. mori*, consists of a continuous strand of two proteinaceous filaments cemented together by a sticky substance called sericin or silk gum. We studied the effects of plasma irradiation of fifth-instar larvae on the sericin concentration of the cocoons. Cocoons were degummed enzymatically with alkaline protease and

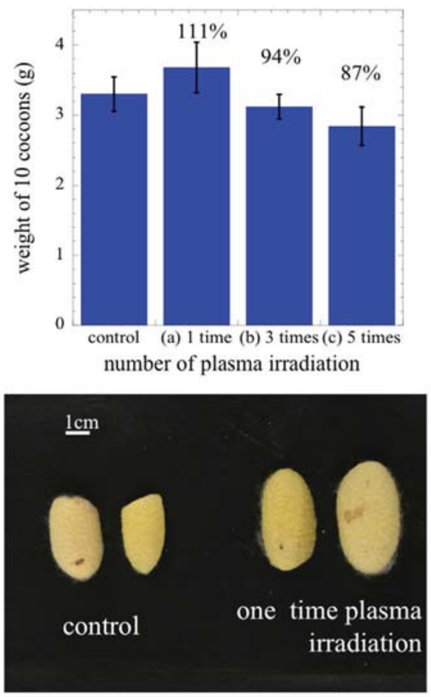

FIG. 5: Total weight of 10 cocoons for one, three, and five 60 s plasma irradiation sessions together with control. Images of cocoons are also shown for one 60 s plasma irradiation session together with control

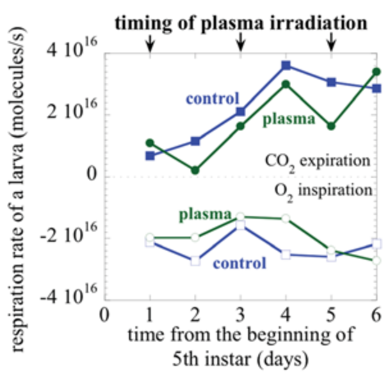

FIG. 6: Respiration rate of fifth-instar larvae of silkworms with and without plasma irradiation for 6 days

the sericin protein was extracted from cocoons into a solution. Figure 7 compares the absorption spectra of the solution extracted from cocoons produced by plasma-irradiated larvae with that of controls. There was little difference between these two spectra.

Therefore, plasma irradiation of fifth-instar larvae of silkworms did not influence the sericin concentration in cocoons significantly.

The surface morphology of silk was investigated by scanning electron microscopy (SEM). Figure 8 shows SEM images of the surface morphology of silk without plasma irradiation and with 60 s of plasma irradiation to the fifth-instar silkworm larvae. Plasma irradiation did not affect the surface morphology of silk cocoons significantly.

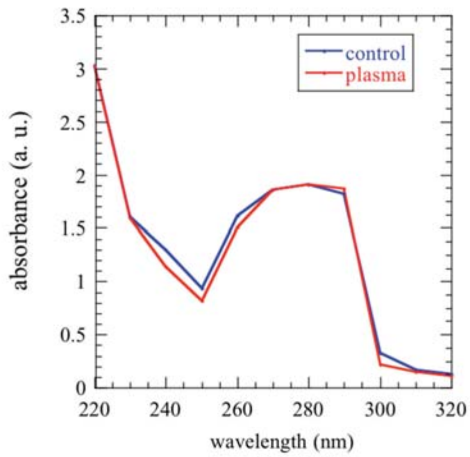

FIG. 7: Absorbance of sericin-containing solution for silkworms with and without plasma irradiation for 6 days

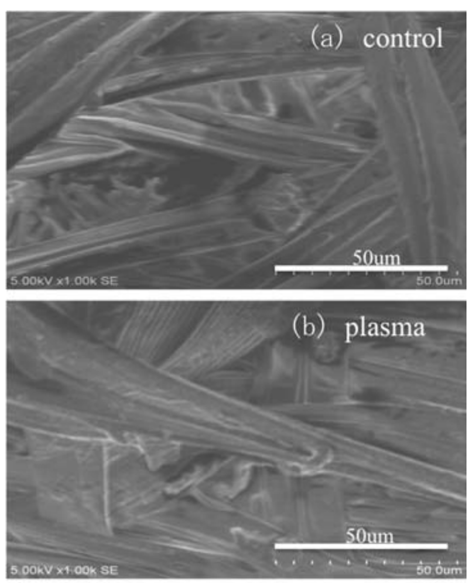

FIG. 8: SEM image of surface morphology of cocoons of silkworms for one 60 s plasma irradiation session together with control

IV. CONCLUSIONS

Here, we investigated responses of silkworm larvae to atmospheric pressure non-thermal plasma irradiation and found the following.

First, the weight growth rate of control larvae decreased monotonically with time. The rate became negative on the sixth day of the fifth instar as the larvae prepared to pupate to the cocoon stage. In plasma-irradiated larvae, the weight growth rate depended on the duration of irradiation. Compared with controls, the weight growth rate decreased sharply during the day after irradiation and then increased during the intervening period until the next plasma irradiation. Decreases in growth rate after plasma irradiations for 60 and 300 s were similar, but after 600 s of irradiation, the rate of decrease was smaller than that after 60 s.

Second, the total weight of silkworm larvae decreased as the number of plasma irradiation periods was increased from one to three. Third, the weight of silk cocoons produced after a single plasma irradiation was 11% heavier than without irradiation, but after three and five periods of irradiation, cocoons were 6% and 13% lighter, respectively. We conclude that plasma irradiation of fifth-instar silkworms has little effect on silk protein and silk surface morphology, but further study is needed to elucidate the mechanism underlying these effects.

ACKNOWLEDGMENTS

This work was partly supported by the Ministry of Education, Culture, Sports, Science and Technology (MEXT KAKENHI Grant Number 24108009) and the Japan Society for the Promotion of Science (JSPS KAKENHI Grant Number JP16H03895). T.S. would like to express his sincere gratitude to the Japanese government for the Japanese Government Scholarship.

REFERENCES

1. Hajime S, Yuzuru I. Irradiation experiments on a mouse using a mild-plasma generation for medical applications. Plasma and Fusion Research. 2010;5:S2117.
2. Vargo JJ. Clinical applications of the argon plasma coagulator. Gastrointest Endosc. 2004;59: 81–88.
3. Havanond C, Havanond P. Argon plasma coagulation therapy for acute non-variceal upper gastrointestinal bleeding. Cochrane Database Syst Rev. 2005;2:CD003791.
4. Ginsberg GG, Barkun AN, Bosco JJ, Burdick JS, Isenberg GA, Nakao NL, Petersen BT, Silverman WB, Slivka A, Kelsey PB. The argon plasma coagulator: February 2002. Gastrointest Endosc. 2002;55:807–810.
5. Volin JC, Denes FS, Young RA, Park SMT. Modification of seed germination performance through cold plasma chemistry technology. Crop Sci. 2000;40:1706–1718.
6. Šerá B, Stranák V, Šerý M, Tichy M, Špatenka P. Germination of Chenopodium album in response to microwave plasma treatment. Plasma Sci Technol 2008;10:506–511.
7. Chen HH, Chen YK, Chang HC. Evaluation of physicochemical properties of plasma treated brown rice. Food Chem. 2012;135:74–79.

8. Šerá B, Špatenka P, Šerý M, Vrchotová N, Hrušková I. Influence of plasma treatment on wheat and oat germination and early growth. IEEE Trans Plasma Scie. 2010;38:2963–2968.
9. Huang J, Chen W, Li H, Wang XQ Lv GH, Khohsa ML, Guo M, Feng KC, Wang PY, Yang SZ. Deactivation of A549 cancer cells in vitro by a dielectric barrier discharge plasma needle. J Appl Phys. 2011; 109:053305.
10. Kalghatgi U, Kelly CM, Cerchar E, Torabi B, Alekseev O, Fridman A, Friedman G. Effects of non thermal plasma on mammalian cells. PLoS One. 2011;6:e16270.
11. Nakamura WM, Miyahara H, Sato H, Matsuzaki H, Koga K, Shiratani M. Two dimensional spatial profile of volume fraction of nanoparticles incorporated into a-Si:H films deposited by plasma CVD. IEEE Trans Plasma Sci. 2008;36:888–889.
12. Nakamura WM, Miyahara H, Sato H, Matsuzaki H, Koga K, Shiratani M. Cluster incorporation control for a-Si:H film deposition. J Phys Conference Series. 2008;100:082018.
13. Victor J, Thannickal V, Fanberg B. Reactive oxygen species in cell signaling. Am J Physiol Lung Cell Mol Physiol. 2000;279:L1005–L1028.
14. Laroussi M, Leipold F. Evaluation of the roles of reactive species heat and UV radiation in the inactivation of bacterial cells by air plasmas at atmospheric pressure. Int J Mass Spectrom. 2004;233:81–86.
15. Stoffels E, Keift IE, Sladek RE. Superficial treatment of mammalian cells using plasma needle. J Phys D: Appl Phys. 2003;36:2908–2913.
16. Dobrynin D, Fridman G, Friedman G, Fridman A. Physical and biological mechanisms of direct plasma interaction with living tissue. New J Phys. 2009;11:115020 (1–26).
17. Pozeliene A, Lynikiene S. The treatment of rape seeds with the help of electrical field. Agron Res. 2009;7:39–46.
18. Kitazaki S, Koga K, Shiratani M, Hayashi N. Rapid growth of radish sprouts using low pressure O_2 radio frequency plasma irradiation. Materials Research Society. 2012: mrss12-1469-ww02-08.
19. Kitazaki S, Sarinont T, Koga K, Hayashi N, Shiratani M. Plasma induced long-term growth enhancement of Raphanus sativus L. using combinatorial atmospheric air dielectric barrier discharge plasmas. Curr Appl Phys. 2014;14:S149.
20. Fridman A, Chirokov A, Gutsol A. Non-thermal atmospheric pressure discharge. Jpn J Appl Phys. 2005;38:R1.
21. Chirokov A, Gutsol A, Fridman A. Atmospheric pressure plasma of dielectric barrier discharges. Pure Appl Chem. 2005;77:487–495.
22. Ito M, Ohta T, Hori M. Plasma agriculture. J Korean Phys Soc. 2012;60:937–943.
23. Zhou Z, Huang Y, Yang S, Chen W. Introduction of a new atmospheric pressure plasma device and application on tomato seeds. Agric Sci. 2011;2:23–27.
24. Kong MG, Kroesen G, Nosenko T, Shimizu T, Van Dijk JK, Zimmermann J. Plasma medicine: an introductory review. New J Phys. 2009;11:115012–1/35.
25. Nastuta AV, Topala I, Grigoras C, Pohoata V, Popa G. Stimulation of wound healing by helium atmospheric pressure plasma treatment. J Phys D: Appl Phys. 2011;44:105204 (1–9).
26. Lee J, Shon CH, Kim YS, Kim S, Kim GC, Kong MG. Degradation of adhesion molecules of G361 melanoma cells by a non-thermal atmospheric pressure microplasma. New J Phys. 2009;11:115026 (1–13).
27. Pimentel D, Hurd LE, Bellotti AC, Forster MJ, Oka IN, Shores OD, Whitman WJ. Food production and the energy crisis. Science. 1973;182:443–491.
28. Hayashi N, Tsutsui S, Tomari T, Guan W. Sterilization of medical equipment using oxygen radicals produced by water vapor RF plasma. IEEE Trans Plasma Sci. 2008;36:1302–1303.
29. Hayashi N, Yagyu Y. Treatment of protein using oxygen plasma produced by RF discharge. J Plasma and Fusion Res, 2009;8:552–555.
30. Koga K, Sarinont T, Amano T, Seo H, Itagaki N, Hayashi N, Shiratani M. Simple method of improving harvest by nonthemal air plasma irradiation of seeds of Arabidopsis thaliana (L.). Appl Phys Express. 2016;9:012601.

31. Mitsugi F, Abiru T, Ikegami T, Ebihara E, Aoqui SI, Nagahama K. Influence of ozone generated by surface barrier discharge on nematode and plant growth. IEEE Trans Plasma Sci. 2016;99:1–6.
32. Ebihara K, Mitsugi F, Ikegami T, Yamashita Y, Hashimoto Y, Yamashita T, Hashimoto Y, Yamashita T, Kanazawa S, Stryczewska HD, Pawlat J, Teii S, Sung TL. Sterilization characteristics of ozone-mist spray for chemical free agriculture. Int J Plasma Environ Sci Technol. 2016;10:11–15.
33. Weldeyohannes AA. Silkworm production and constraints in eastern Tingray, northern Ethiopia. Int J Inn Sci Res. 2014;10:517–521.
34. Mita K, Morimyo M, Okano K, Koike Y, Nohata J, Kawasaki H, Kadono-Okuda K, Yamamoto K, Suzuki MG, Shimada T, Goldsmith MR, Maeda S. The construction of an ET database for Bombyx mori and its application. PNAS. 2002;100:14121–14126.
35. Itoh K, Kobatashi I, Nishioka H, Machii H, Tamura T. Recent progress in development of transgenic silkworms overexpressing recombinant human proteins with therapeutic in silk glands. Drug Discov Ther. 2016;10:34–39.
36. Hong SM, Yamashita J, Mitsunobu H, Uchino K, Kobayashi I, Sezutsu H, Tamura T, Nakajima T, Miyagawa Y, Lee JM, Mon H, Miyata Y, Kawaguchi Y, Kusakabe T. Effeicient soluble protein production on transgenic silkworms expressing cytoplasmic chaperones. Appl Microbiol Biotechnol. 2010;87:2147–2156.
37. Kuwana Y, Sezutsu H, Nakajima KI, Tamada Y, Kojima K. High-toughness silk produced by a transgenic silkworm expressing spider (Araneus ventricosus) dragline silk protein. PLoS One. 2014;9:e105325.
38. Kaito C, Kurakawa K, Matsumoto Y, Terao Y, Kawabata S, Hamada S, Sakimizu K. Silkworm pathogenic bacteria infection model for identification of novel virulence genes. Mol Microbiol. 2005;56: 934–944.
39. Kitazaki S, Hayashi N. Sterilization characteristics of tube inner surface using oxygen plasma produced by AC HV discharge. IEEE Trans Plasma Sci. 2008;36:1304–1305.
40. Takaki K, Ihara S. Agricultural and food processing applications of pulsed power technology. IEEJ Trans Fundamentals Materials. 2009;129:439–445.
41. Padamwar MN, Pawar AP. Silk sericin and its applications: A review. J Sci Ind Res. 2004;63:323–329.
42. Sarinont T, Amano T, Attri P, Koga K, Hayashi N, Shiratani M. Effects of plasma irradiation using various feeding gases on growth of Raphanus sativus L. Arch Biochem Biophys. 2016;605:129–140.
43. Liu SK, Cai S, Chen Y, Xiao B, Chen P, Xiang XD. The effect of pollutional haze on pulmonary function. J Thorac Dis. 2016;8:E41–E56.