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Research article

Effects on phenotypic and genotypic modifications of low-pressure radiofrequency capacitively coupled plasma treatment of Thai purple glutinous rice seeds

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Abstract

Importance of the work: Low-pressure radiofrequency capacitively coupled plasma (RF-CCP) affects biological material modifications. However, the effective parameters with the more pivotal roles have not yet been clearly identified based on demonstration.

Objectives: To investigate the effects of RF-CCP treatment of rice (*Oryza sativa 'Leum Pua'*) seeds on phenotypic and genotypic modifications.

Materials & Methods: The effects of the gas type on the seed texture were explored using nitrogen (N), argon (Ar), helium (He) and oxygen (O) as source gases in the plasma treatment of the seeds, with a fixed treatment time of 5 min for each experiment. Subsequently, the treatment time duration (15–30 min) effect on the seed germination was studied using O-plasma. High annealing temperature, random-amplified polymorphic DNA (HAT-RAPD) was used to analyze genetic variation between the mutant and control rice samples.

Results: Only O-plasma caused cracks on the rice seed surface; however, a fixing treatment time of 5 min produced no phenotypic change in the plasma of the treated rice. Increasing the treatment time using O-plasma at 15 min, 25 min and 30 min decreased the germination rates by approximately 50%, 23% and 20%, respectively. With the treatment time of 30 min, one rice seedling had phenotypic changes (short stem and green color in the leaf and pale in the rice husk). The HAT-RAPD investigation showed that 3 of the 10 arbitrary primers revealed genetic modifications in the rice mutant induced by the RF-CCP treatment.

Main finding: RF-CCP could act as a promising tool for inducing mutations in rice with its advantages being a relatively simple system, low cost and convenient operation and maintenance.

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Introduction

Nonthermal (or cold) plasma (NTP) as a type of popularly applied plasma, has recently received considerable attention due to its applications in a broad range of fields, particularly in biology-related areas, such as the decontamination and sterilization of food surfaces (Niemira, 2012; Niemira et al., 2014), improvement in wound healing (Haertel et al., 2014), induction of DNA transfer (Sangwijit et al., 2015), induction of DNA modification and bacterial mutation (Sarapirom et al., 2010), improvement in bacterial enzyme activity (Polsa et al., 2020), increased seed germination (Chen et al., 2016; Ji et al., 2016; Gómez-Ramírez et al., 2017) and promotion of resistance to certain abiotic stresses (Ling et al., 2015). In addition to other biology-related areas, agriculture is becoming increasingly popular as an NTP application objective due to agriculture providing humans with the most basic food staples (Misra et al., 2016; Attri et al., 2020; Holubová et al., 2020). Rice is one of the most important food plant species and hence, it has been involved in frequent treatments using NTP, such as to improve seed germination (Khamsen et al., 2016) and growth (Attri et al., 2020) and to control pathogens on the seed surface (Nobuya et al., 2014; Kang et al., 2015; Ochi et al., 2017). In Thailand, rice (*Oryza sativa* L.) is the most important food and export product (Amnuaysin et al., 2018), providing the basic economic foundation for the nation; consequently, Thai scientists have also put great effort into improving rice using the NTP technology. Besides the work on rice seed sterilization and germination enhancement (Khamsen et al., 2016), atmospheric pressure NTP has been was applied to study its influence on germination and growth improvements of various types of Thai rice seed (Wongpanom, 2016; Amnuaysin et al., 2018; Tanakaran and Matra, 2021) and on plant disease control (Adhikari et al., 2020). However, induction mutation of rice using NTP has not yet been internationally reported, though rice mutation inductions using either high-energy (orders of 1–100 MeV) or low-energy (orders of 10–100 keV) ion beams have been extensively and intensively studied and reported; among numerous publications, see for example, using high-energy ion beams (Amano, 2006; Yamaguchi et al., 2009) and using low-energy ion beams (Zengliang et al., 1991; Zengliang, 2006). Using ion beams to induce crop mutation has a shortcoming in that it requires accelerators (either high-energy ion accelerators or low-energy ion implanters), which are relatively costly and technically complex. In contrast, NTP can generate ions but at a relatively low cost and in a simpler manner. However, the key problem with NTP in inducing materials modification, particularly genetic mutation, is that its low energy of ions is incomparable to the ion energy in ion beams. It remains questionable whether NTP can induce mutation of biological systems. Taking the low-energy ion character of NTP into account, scientists have achieved successes in NTP induction of mutations for small and vulnerable biological targets, such as microorganisms, including bacteria (Wang et al., 2010; Ren et al., 2012; Zhang et al., 2015; Ottenheim et al., 2018; Polsa et al., 2020), fungi (Zhu et al., 2019), fish eggs and sperm (Ji-Lun et al., 2019), simply naked DNA (Sarapirom et al., 2010; Yaopromsiri et al., 2015) and even DNA blocks (Wang et al., 2020). Applying NTP to treat relatively large biological targets and induce mutation, such as in plant seeds, has rarely been demonstrated, due to the challenge in managing the ultra-low energy ions in the plasma to interact with the genetic substance deep inside the cells. Therefore, the current pioneer study initiated a trial in response to this challenge.

Capacitively coupled plasmas (CCPs) are widely used as low temperature plasmas and are widespread in many applications, such as etching, sputtering, deposition, microelectronics, aerospace and biology (Becker et al., 2005; Valderrama et al., 2010; Shihab, 2018).Their property of energetic ions in chemically active species, radicals and energetic neutral species, has made CCPs very popular in the semiconductor industry because of their low cost and robust uniformity over a large area (Bora et al., 2011). However, there is no evidence on the interaction of CCPs on plant DNA modification. The current study addressed this by introducing plasma ions to interact with rice seeds under low-pressure conditions. In the plasma treatments of biological materials for biological purposes, the reactive species in the plasma play critical roles in the modification of the material properties. Most of the reported plasma treatments have occurred under atmospheric pressure with a mixture of various species. However, whether the species play equal roles or perhaps certain species play more pivotal roles has not yet been clearly demonstrated. Therefore, the current study applied low-pressure plasma using various gases (light and heavy, inert and active) and different types of activity, to demonstrate which active species are more important in inducing biological material modifications.

Materials and Methods

Plant materials

Seeds of Thai purple glutinous rice (*Oryza sativa* L. 'Leum Pua') were obtained from a registered local farm in Chun district, Phayao, a northern province in Thailand. Fresh harvested seeds were heated at 49°C in an oven system for 3 d to break dormancy and reduce the moisture content to 11%. Then, the rough rice seeds were carefully hand-husked to avoid any damage to the embryo tissue.

Radio frequency capacitively coupled plasma system

A capacitively coupled plasma reactor with radio frequency (RF) excitation used to sustain discharge of gas is illustrated schematically in Fig. 1. The RF capacitively coupled plasma (RF-CCP) reactor was a cylindrical chamber made from stainless-steel, with an inner diameter of approximately 295 mm and a height of approximately 362 mm. A pair of parallel, circular plates with a diameter of 190 mm spaced 120 mm apart were used as the power electrodes. The RF power was coupled using a matching network (AT-10; Seren IPS Inc.) to the lower electrode of the reactor, whereas the upper electrode was connected to a bipolar pulse power supply (AE Pinnacle PLUS+; Advanced Energy) and the chamber wall was grounded. The RF generator used for this source (R1001; Seren IPS Inc.) was able to provide RF power up to 1,000 W at a fixed frequency of 13.56 MHz. An internal impedance (50 W) of the generator was matched with the matching network to give minimum reflected power. The pressure inside the reactor chamber was maintained and controlled using a turbo pump for plasma operation. Applying two power supplies separately to the upper and lower electrodes, respectively, provided a special advantage compared to conventional single-electrode plasma reactors. Using one electrode powered by RF in a normal reactor adjusts both the plasma density and ion energy simultaneously. However, in the plasma treatment of living plant seeds, the ion energy should not be too high as that may result in overheating the seeds and subsequently affecting the survival and germination rates. Thus, separately adjusting the plasma density and ion energy would be desirable for treating the seeds. In the tested reactor, the bipolar pulse power supply was used to the upper electrode to increase the plasma density with a low frequency of 50 kHz for excitation. The RF power supply was used to the lower electrode to increase the active plasma species and it was operated in pulsed mode to reduce overheating of the seeds.

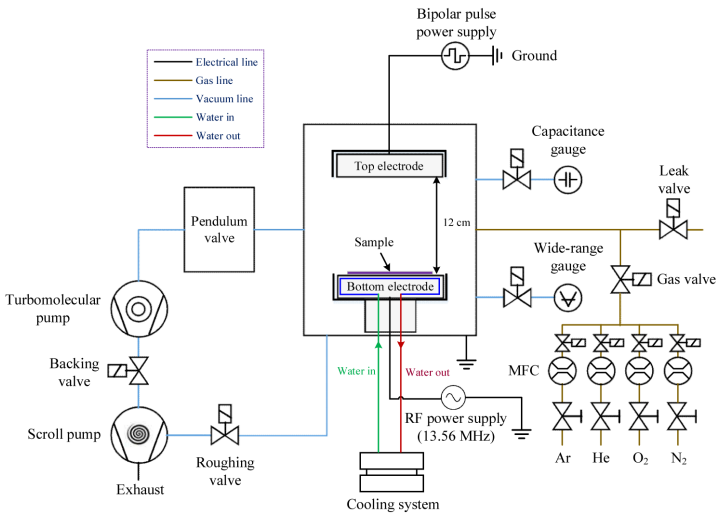

Fig. 1 Schematic diagram of the radiofrequency capacitively coupled plasma system (sideview) and not to scale

Plasma treatment of seeds

The prepared rice seeds were horizontally placed on carbon tape which was then placed on aluminum foil as a holder. This was then placed directly on the lower electrode in the reactor chamber. Approximately 250 rice seeds were used for each treatment and 1,000 seeds were treated for each condition. The vacuum system for the chamber consisted of two pumps: a scroll pump (nXDS10i; Edwards) for pressures above 1×10^{-3} Torr and a turbomolecular pump (TC600; Pfeiffer vacuum) for pumping to a background pressure of 1×10^{-6} Torr. Oxygen gas was supplied and controlled using a mass flow controller with a flow rate of 20 standard cubic centimeters per minute. The pumping speed for the chamber was adjusted via a pendulum valve to maintain the operating pressure at 2×10^{-1} Torr. To generate plasma, the top electrode was connected to the bipolar pulse power supply (100 W and a frequency of 50 kHz). To increase the plasma density and the energy of ions, the lower electrode was biased using 200 W RF power, corresponding to a direct current (DC) self-bias of -500 V. To reduce heating of the seeds treated, the RF power supply was run in pulse mode with a frequency of 500 Hz. The effect of gas type (nitrogen, argon, helium or oxygen) on the seed germination was studied by treating the seeds for 5 min. In the inert gas (Ar or He) plasmas, besides electrons and Ar or He atom/molecule neutrals, there were simple ions of Ar^+, or He^+ and even He^{2+}, while in the active gas (O_2 or N_2) plasmas, there were not only electrons and

atomic and molecular ions of oxygen and nitrogen (such as O^+, O_2^+, or N^+ and N_2^+), but also a number of reactive species (as listed in the section of Discussion). All these ions and reactive species could possibly have effects on DNA changes. The effect of the exposure time on the seed germination was studied for 15 min, 25 min and 30 min using oxygen plasma. Rice seeds not subjected to the vacuum and plasma environment were used as the natural control, while vacuum controlled seeds were placed in the chamber and covered by carbon tape so that they could not be exposed to the plasma.

Observation of seed surface morphology

After the plasma treatment, the surfaces of the treated and un-treated seeds were investigated using scanning electron microscopy (SEM). The seeds were fixed on stubs and coated with gold using a sputter coater. The sample surfaces were observed using a scanning electron microscope (FEI Quanta 250).

Seed germination and modification screening

Seeds with and without plasma treatment were placed on tissue paper in Petri dishes and 10 mL of distilled water was added. The number of germinated seeds was recorded after 7 d. The germination percentage was calculated based on Equation 1:

$$\text{Germination percentage (\%)} = \frac{\text{Number of germinated seeds}}{\text{Total number of seeds tested}} \times 100 \quad (1)$$

Means of germination percentage were subjected to analysis of variance and then analyzed for the differences among variables ($p \leq 0.05$) using Duncan's multiple range test. The SPSS for Windows software was used for the statistical analysis.

The seedlings were transferred to grow as transplanted rice in soil in plastic pots with 1 seedling/pot. Phenotypic and genomic variations were recorded and analyzed at age 1 mth.

DNA analysis

For DNA variation analysis, samples of leaf tissue at 30 d were ground in liquid nitrogen to a fine powder and DNA extraction was performed using a DNA extraction kit (Thermo Scientific). Amplification of the DNA was carried out using the high annealing temperature-random amplified polymorphic DNA (HAT-RAPD) technique, which increased the annealing temperature in the polymerase chain reaction (PCR) to 46°C (Anuntalabhochai et al., 2000). Distinct banding patterns of DNA fragments from the PCR amplification were used to indicate genetic modification resulting from the plasma treatment. Ten arbitrary primers were selected for genetic variation studies, as shown in Table 1. PCR was performed using a 20 µL mixture, containing sample DNA (10–25 ng), 1 × Qiagen PCR buffer, 100 µM of each dNTP, 40 ng of each primer and 0.5 U of *Taq* DNA polymerase. For DNA amplification, a PCR thermocycler (Gene Amp System 2400; Perkin-Elmer) was programmed for incubation at 94°C for 5 min, 30 cycles at 94°C for 45s, 48°C for 45 s and 72°C for 1 min, followed by one final extension cycle of 7 min at 72°C. The genetic variation was recognized using electrophoresis. To confirm the DNA polymorphisms between the control and its mutant, all experiments were carried out using three replicates.

Table 1 Primer sequences applied in DNA analysis

Primer name	Sequence
OPAP09	GTGGTCCAGA
OPAG02	CTGAGGTCCT
OPAW03	CCATGCGGAG
OPBH10	GTGTGCCTGG
OPBH15	GAGAACGCTG
OPAC09	AGAGCGTACC
OPAR11	GGGAAGACGG
OPAR17	CCACCACGAC
OPAI12	GACGCGAACC
OPAT19	ACCAAGGCAC

Results

Effect on seed surface

For the plasma treatment, the sample was placed on the powered electrode whose area was smaller than that of the grounded electrode; hence, this configuration acted as an asymmetric reactive ion etching system. The impinging of electrons on the powered electrode allowed a buildup of a negative DC field, in addition to the AC field, to form a DC self-bias voltage (V_{bias}). As V_{bias} was comparatively higher than the plasma potential, V_p, the equipotential across the bulk plasma, V_{bias} is conventionally used to describe the ion energy that played a role in the plasma acceleration of ions to bombard the rice seed surface (Rafalskyi and Aanesland, 2015) leading to a higher rate of ion etching of the surface. The SEM

microphotographs of the rice seed surface after nitrogen, argon, helium and oxygen plasma treatments for 5 min are shown in Fig. 2, with the seed surface of the natural control having complete natural morphology, including some rough original structures on the top surface, while for the plasma treatments, no matter what working gas was used, there was sputtering or etching of the original rough structures from the top surface to expose the embryo cell envelope. Although the DC self-bias voltage was kept at -500 V for all treatment conditions, it was also noticed that different gas plasmas had different effects on the seed embryo cell envelope modification. While the He plasma and the N_2 plasma both had a mild effect, the Ar plasma and the O_2 plasma produced a stronger modification, especially the O_2 plasma which created some larger cracks than with the Ar plasma. However, after cultivation, there were no observed phenotypic changes in the rice in any of seeds treated for 5 min. Large cracks were observed only on the seed surface treated with the O_2 plasma, indicating this was more effective in inducing surface damage. The plasma-modified seed surface structure, especially in terms of crack creation, might be an important indicator of some channels formed for energetic ions from the plasma to be able to pass through the embryo cell envelope and interact with the substance inside the cell. Therefore, the O_2 plasma treatment was focused on in the subsequent investigation extending the treatment time to 15 min, 25 min and 30 min. The results of this extension of treatment time indicated that 30 min treatment with the O_2 plasma seeds produced noticeable large cracks on the surface structure, as shown in Fig. 2F, as well as phenotypic changes, described later.

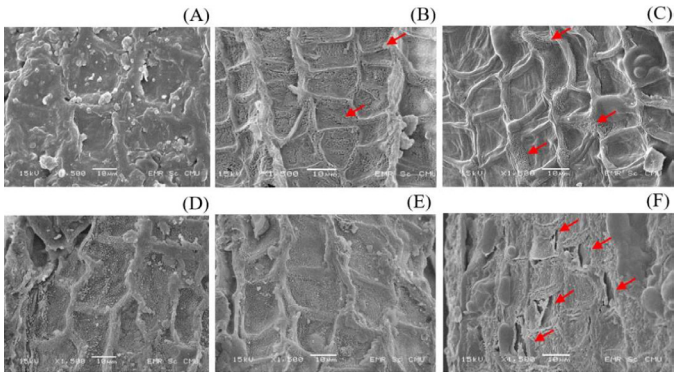

Fig. 2 SEM micrographs of rice seed surface morphology of: (A) control; (B) 5 min O_2 plasma treatment; (C) 5 min Ar plasma treatment; (D) 5 min He plasma treatment; (E) 5 min N_2 plasma treatment; (F) 30 min O_2 plasma treatment, where red arrows indicate rice surface structure modifications and all scale bars = 10 μm.

Effects on seed germination

The effect of the O_2 plasma treatment on the rice seeds using varied treatment times on germination ability is shown in Fig. 3, with an exponential decrease in the germination percentage with increased plasma treatment time, as described in Equation 2:

$$Gp(t) \text{ (Germination percentage as a function of treatment time)} = A \exp(-\alpha t), \quad (2)$$

where A is a constant depending on the control or initial $Gp(t = 0)$, t is the plasma treatment time in minutes and α is the germination coefficient per minute, representing a germination decrease rate per minute of the treatment time. The 30 min plasma treatment resulted in the minimum percentage of germination (20%) that was significantly lower than that of the control group (85%) by more than four times. As shown in Fig. 3, the best fit of the measured data produced a germination coefficient of 1/20 or 0.05/min.

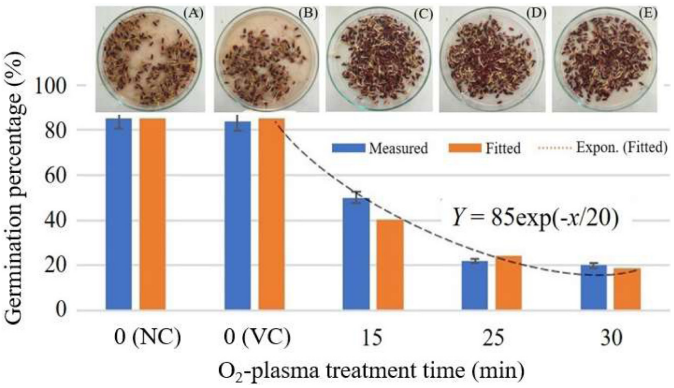

Fig. 3 Effect of oxygen radiofrequency capacitively coupled plasma treatment on germination percentage of rice seeds as function of treatment time, where NC = natural control, VC = vacuum control, Expon. = exponentially fitted curve with best fit equation for Germination Percentage (%) = $85e^{-t/20}$, where t is the plasma treatment time in minutes, above bar graphs are photos of experimentally observed germination of seeds of (A) NC; (B) VC; (C) treated for 15 min; (D) treated for 25 min; (E) treated for 30 min

Phenotypic changes

On one plant grown from all of the seeds treated for 30 min with O-plasma, some phenotypic changes were observed, such as the short stem and green color in the leaf, as shown in Fig. 4. In addition, the harvested rough rice seeds of this mutant (M1) had pale rice husks and darker husked grains, as well as relatively larger grains, compared to the control grains, as shown in Fig. 5.

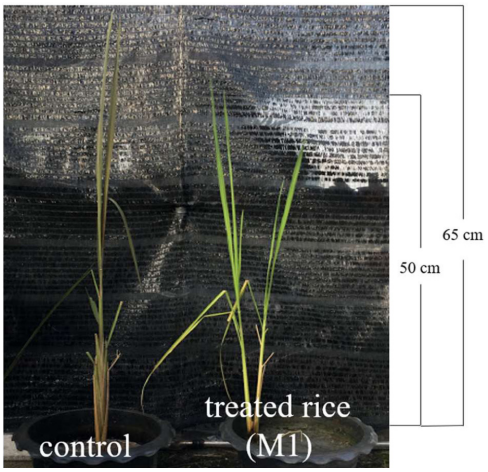

Fig. 4 Phenotypic changes observed in one M1 mutant at age 60 d, showing shorter stem and green color in leaves, compared with taller stem and purple color in leaves of the control

Fig. 5 Phenotypic changes observed in grains harvested from the M1 mutant showing pale grain husk, compared with purple color in husk of control, where upper row shows rough rice seeds and lower row shows husked seeds

Genomic variation

The HAT-RAPD technique has been used for taxonomic and phylogenetic studies in several plant species, such as banana (Ruangsuttapha et al., 2007), *Ficus* spp. (Anuntalabhochai et al., 2008; Phromthep, 2012), *Musa* (Thomsopa et al., 2013), *Paphiopedilum* (Siritheptawee et al., 2018), Zingiberaceae (Sangvirotjanapat et al., 2019) and cassava (Rangsiruji et al., 2019), as well as being used for the detection of genotypic changes in mutant organisms, because it could provide reproducible polymorphism. It has been used successfully to detect mutation in Thai jasmine rice (Phanchaisri et al., 2007), Pathumthani 1 rice (Sangwijit et al., 2012) and mung bean (Sumrith et al., 2013). In the current experiment, this technique was applied to detect DNA alteration in a rice mutant induced by O_2 plasma. In addition, the annealing temperature was increased to 48°C, resulting in a highly reproducible degree of polymorphism and detection of DNA alteration in the mutant. Among the 10 primers investigated, 3 primers (OPBH15, OPAR17 and OPAI12) distinguished the DNA modification of the plasma-treated rice based on a variety of polymorphic bands in the range 200–800 bp that were observed only in the mutant, as shown in Fig. 6.

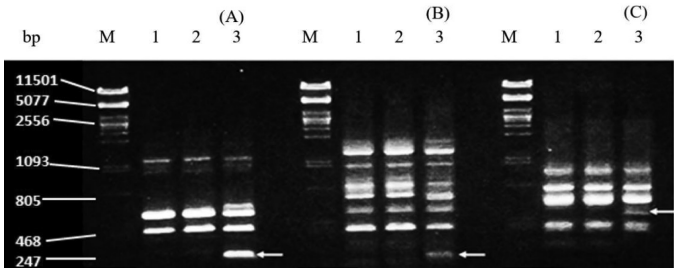

Fig. 6 high annealing temperature-random amplified polymorphic DNA products generated using the primers: (A) OPBH15; (B) OPAR17; (C) OPAI12, using purple glutinous rice genomic DNA as template from (1) natural control, (2) vacuum control and (3) M1 rice, where M is the molecular weight marker lane and white arrows point to additional bands in mutant

Discussion

To the best of the authors' current knowledge, the current study was the first on applying cold (non-thermal or low-temperature) plasma for rice mutation induction, though there have been a number of reports on rice mutations successfully induced using ion beams. However, the mechanisms involved in the ion beam and cold plasma inductions of crop mutations are quite different. With ion beams, direct interaction between highly energetic ions and biological materials dominates the mutation induction, represented by the ion impact causing DNA strand breaks, especially double strand breaks (DSBs). In contrast, with plasma, indirect interactions between reactive species and radicals and biological molecules completely dominate the mutation induction processes. In cold plasmas, there are not only the basic components of plasma, namely ions, electrons and neutral particles as well as ultraviolet (UV) (Bruggeman et al., 2022), but also rich reactive species, especially in the atmospheric or gas-fed plasmas, such as hydroxyl (OH), atomic oxygen (O), singlet delta oxygen ($O_2(^1\Delta)$), superoxide (O_2^-), hydrogen peroxide (H_2O_2), and nitric oxide (NO) (Pizzino et al., 2017; Laroussi, 2018), most of which are reactive oxygen species (ROS). These reactive species are the critical agents in mutagenesis through a series of complex indirect interactions with the cellular DNA. Since the current experiment showed that the 30 min O plasma treatment of rice seeds induced mutation expressed by certain phenotypic changes, the discussion on the mechanism responsible for the mutation induction focused on the O plasma case. Fig. 7

schematically illustrates the entire process of the mutagenesis for the O plasma treatment of rice seeds.

A body of O-dominated cold plasma is formed between the upper and bottom electrodes after the electrodes are triggered and controlled oxygen gas is fed into the reactor chamber. As mentioned above, the plasma is composed of charged particles, such as ions and electrons, neutrals and ROS, as well as ultraviolet light (which also assists in the ROS production), as diagonalized in various experiments (such as Steinbruchel et al., 1986, as a very early one), including those by the members of the current authros (Boonyawan, 2017, Kosumsupamala et al., 2022). As the bottom electrode is biased with -500 V, the ions have a higher concentration near the bottom electrode (above which the seeds are placed) than near the upper electrode (while electrons have the opposite concentration distribution), as illustrated in Fig.7. These ions act as low-energy ion bombardment of seed embryo cells. During this ion bombardment process, the energetic ions tailor and erode (Amnuaysin et al., 2018) the cell envelope materials resulting in sputtering of the envelope and consequently, the envelope is thinned and some parts are even removed (as shown in Figs. 2 and 7) depending on the treatment time, which corresponds to the ion fluence and determines the sputtered material quantity. Simultaneously, the ion charge deposition in the envelope (which is a mixture of non-conducting polymeric materials) possibly causes breaking up of the built electric field among the charges; meanwhile, ion mass deposition applies stress to the envelope. Both effects may induce cracking of the cell envelope (as shown in Figs. 2 and 7) and the formed cracks can act as channels for the penetration of the ROS. Furthermore, the plasma ROS can directly modify polymeric materials, such as those of the cell envelope (Song et al., 2016; Kim et al., 2002). This results in the thinned cell envelope or even the cell cytoplasm being directly exposed to ROS that can actually penetrate biological tissues up to depths of more than 1 mm and therefore interact not only with the cells on the surface but with those underneath (as shown by many studies in the review by Laroussi, 2018). In addition, plasma can induce free radicals inside the cells (Ji et al., 2019). O_2 combines with an electron to form a superoxide as well as water, with the water subsequently becoming the source of more free radical production in the cells (Mesa et al., 2012) (Fig. 7). Various additional free radicals join with the ROS to attack the DNA, which is located in either the nucleus (nuclear DNA) or the mitochondria (mtDNA). Compared to the nuclear DNA, mtDNA is more vulnerable to ROS attacks—especially the hydroxyl radical (Juan et al., 2021)—following oxidative stress with more extensive and durable damage (Yakes and Van Houten, 1997). Therefore, mtDNA mutation is possible through a series of chemical pathways (Juan et al., 2021) in which the ROS not only oxidizes DNA bases, but also breaks DNA strands in the backbone (Han and Chen, 2013; Juan et al., 2021). Although the DNA changes in the mutant were confirmed based on the HAT-RAPD analysis that revealed the plasma treatment effect on the DNA level, the specific genes responsible for the phenotypic changes of the mutant require further analysis in the 5th generation of the mutant for genetic stability.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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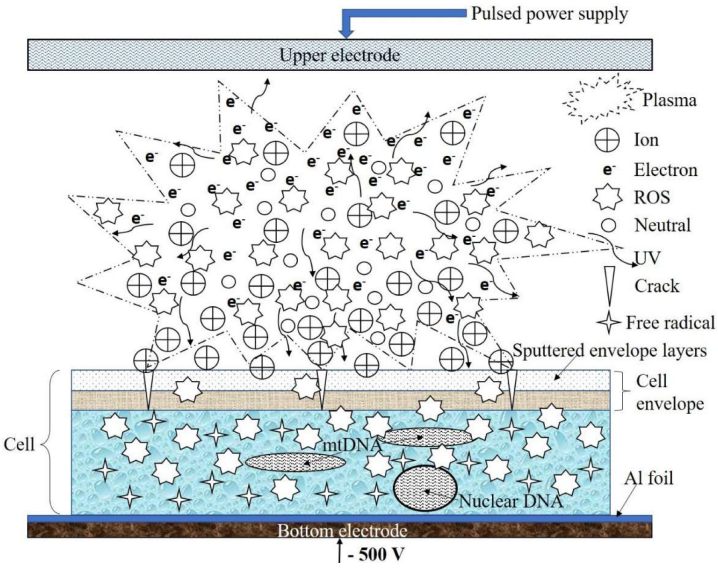

Fig. 7 Schematic illustration (not to scale) of experimental plasma treatment of rice seed to induce mutation, where notably, since the rice seed is dehusked to expose the embryo, the cell is actually the embryo

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