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Effects of Atmospheric Pressure Air Plasma Pretreatment on the Seed Germination and Early Growth of *Andrographis paniculata**

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Abstract The objective of this paper is to demonstrate whether air plasma can change the seed germination characteristics, seedling emergence, as well as biochemical reactivity, in *Andrographis paniculata* (*A. paniculata*) seedlings by modifying the seed coat and finding a beneficial treatment dose. Eight treatment doses and one control were used to conduct electrical conductivity determination, a germination test, a seedling emergence test and a biochemical assay. The results showed that after being treated with air plasma excited at 5950 V for 10 s, the permeability of the seeds was improved significantly, resulting in the acceleration of seed germination and seedling emergence. In the meantime, the catalase activity and catalase isoenzyme expression were also improved, while the malondialdehyde content in the seedlings was decreased (which means greater counteraction with environmental stress). After being treated with 4250 V for 10 s and 5950 V for 20 s, the seed germination was enhanced, but without an obvious change in seedling emergence. However, after treatment with 3400 V for 20 s and 5100 V for 10 s, the permeability of the seeds was decreased, resulting in a delay in seedling emergence. These results indicate that air plasma can change the physiological and biochemical characteristics of *Andrographis paniculata* seeds by modifying the seed coat, combined with the effects of the active plasma species, and that different treating doses have different effects.

Keywords: DBD, air plasma, *Andrographis paniculata*, seed germination, early growth

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(Some figures may appear in colour only in the online journal)

1 Introduction

Andrographis paniculata (Burm.f.) Nees, commonly called the 'King of Bitters', is a widely used medicinal plant in southern and eastern Asian countries due to its extensive bioactivities [1]. The pharmacological activities of this herb include being antibacterial, antivirus, antimalarial, anti-inflammatory, antipyretic, antidiarrhoeal, antihepatotoxic, etc [1,2]. It has been used for a long time in the clinical treatment of the common cold, bronchitis, mouth ulcers, pharyngotonsillitis, acute diarrhoea, dyspepsia, colitis, dysentery, infectious hepatitis, etc [1,2]. Recent research has also found that the herb extract has anti-human immunodeficiency virus (HIV) activity [3,4]. The plant is native to India and southeast Asia, and has been widely cultivated in India and south China [1]. However, worsening environmental conditions and climate change resulting from human activities have affected the growth of the plants. So it is important to think about a change in agriculture production, such as how to utilize the modern technologies of seed sowing to improve the quality of the sowing materials in changing environmental conditions, and consequently improve the stress resistance and reduce the application of chemicals. Therefore, a seed pretreatment method for *A. paniculata* is considered to achieve this aim.

An ideal seed pretreatment should be harmless to the seed (no phytotoxicity), with a relatively stable, long lasting effect before planting, and it must adhere sufficiently and uniformly throughout the coating without leaving hazardous non-target residues [5]. Although they possess many more advantages in improving the vigor of seeds and enhancing plant growth compared with chemical methods, many physical seed treatment methods have limitations. Plasma technology, which

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presents unique characteristics and has been widely used in the surface modification of biomaterials [6], has also been studied for its applications in seed disinfection [7−10], germination improvement [11−15], and the alteration of the germination speed via modification of the seed coat [16−18]. However, the effects depend on the plasma generation technique [11], the gas source of the plasma [9], and the seed feature of the specific plants [9,10,19,20].

The current study aims to prove that the seed germination and early growth of *A. paniculata* change after seed pretreatment by atmospheric pressure dielectric barrier discharge air plasma, and seeks a beneficial treatment dosage for the seeds.

2 Materials and methods

2.1 Seed materials

Pure seeds of *A. paniculata*, obtained by private collection from the Zhangpu, Fujian province of China on October 25th, 2009 (harvest date), were used for processing. The initial moisture content was 10.76%, the thousand seed weight (THW) was 1.1589 g, and ripe (yellow-brown) seeds were selected for their uniform size.

2.2 Apparatus and seed treatment

The atmospheric pressure dielectric barrier discharge (DBD) air plasma generating system was equipped with two parallel cylindrical quartz containers filled with potassium chloride (KCl) solution as discharge electrodes (Fig. 1). Both copper loops were immerged into the solution and connected to high-voltage AC power. The thickness, length and width of each quartz medium between the two containers were 0.001 m, 0.15 m, and 0.05 m, respectively, and the distance between the two mediums was 0.01 m. The system was also equipped with a seed broadcaster and conveyor belt. The plasma seed treatment was performed at the Institute of Physics, Chinese Academy of Sciences, and by using the seed broadcaster, the seeds were spread as a single layer on the uniform-speed conveyor belt. Combinations of four generating voltages (3400 V, 4250 V, 5100 V, and 5950 V) and two treatment times (10 s and 20 s) were achieved by modulating the voltage and speed of the conveyor belt (i.e. the treatment time). The treatment conditions for the eight treated groups and one control group without plasma pretreatment are shown in Table 1.

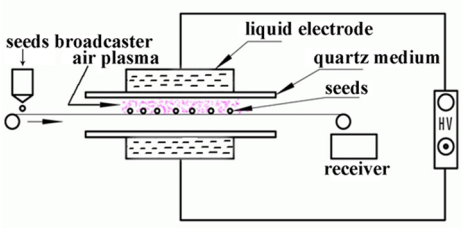


Fig.1 Schematic diagram of the atmospheric pressure DBD air plasma apparatus

Table 1. Seed treatment conditions

Groups	Treatments	
	Voltage (V)	Exposure time (s)
1	3400	10
2	4250	10
3	5100	10
4	5950	10
5	3400	20
6	4250	20
7	5100	20
8	5950	20
ctrl	0	0

2.3 Electric conductivity determination

The relative ion (electrolyte) leakage from the apoplastic or protoplast space of the seeds was assessed by determining the electric conductivity (EC) according to Ref. [21,22]. After being weighed at a precision of 0.1 mg and washed thoroughly in distilled water, following deionized water, sample seeds without visible damage were immersed in 10 mL of deionized water in an Erlenmeyer flask for 6 h at 20 °C [23]. Then, the conductivity of the leachate was measured using a conductivity meter (HORIBA, Ltd. Mode B-173). There were 50 seeds for each replicate, and four replicates for each group, with deionized water as the blank control. A higher electric conductivity means greater ion leakage from the seeds.

$EC = (C_{\text{sample}} - C_{\text{blank}})/\text{seeds weight}[\mu\text{S}\cdot\text{cm}^{-1}\cdot\text{g}^{-1}]$;
EC = electric conductivity,
$C_{\text{sample}}[\mu\text{S}\cdot\text{cm}^{-1}]$ = conductivity of sample,
$C_{\text{blank}}[\mu\text{S}\cdot\text{cm}^{-1}]$ = conductivity of blank control.

2.4 Scanning electron microscopy

After a standard drying protocol was conducted, the scanning electron microscope (manufacturer: LEO, model: 1430 VP) system for image analysis was used to take digital photographs of the seed coat.

2.5 Laboratory germination test

Germination tests were conducted according to the TP (top of paper) method in ISTA rules [24]. After being soaked in distilled water for 20 h, the seeds were disinfected with 0.3% H_2O_2 for 5 min, washed thoroughly with distilled water, and then spread on moist filter paper (filtration rate less than 70 s, percentage of ash max 0.15%) in Petri dishes (90 mm in diameter). Each dish had 100 seeds for one replicate, and four replicates for one group. All the samples were cultured in laboratory conditions of 27.5±1 °C under light/dark (12 h/12 h) [25]. The numbers of germinated seeds were counted every day, and radical protrusions of 1 mm or more were registered as the criterion for germination. The germination percentages (GPs) in 2 days, 3 days, and 7 days were calculated, and the root lengths (RLs) of 20 seedlings randomly selected from each replicate were measured after 7 days, with the germination index (GI) and vigor index (VI) calculated according to ISTA rules [24].

GP (%) = (germinated seeds number/total seeds in one dish) × 100%;

GI=$\sum(G_t/D_t)$, (G_t = germinated seeds number in t day, $D_t = t$ days after sowing);

$VI = GI \times RL$, (RL= root length).

2.6 Seedling emergence test

The seeds of *A. paniculata* were soaked in distilled water for 20 h, and then the surface was disinfected with 0.3% H_2O_2 for 5 min and thoroughly washed with distilled water. Afterwards, they were sown in an aperture disk with homogeneous garden soil. About 0.2-0.5 cm of thick soil was laid over the seeds. In this investigation, each disk had 100 seeds for one replicate, and a triplicate for one group. All the samples were cultured in a common garden, with a temperature of 25 °C-32 °C. Adequate water was kept in the soil, and the seedling numbers were counted every day. The green cotyledons extruded the seed coat, and the soil surface was registered as the criterion for individual emergence. Seedling emergences were calculated after 7 days, 15 days and 30 days from the first emergence. The number of strong seedlings that had more than three pairs of euphylla (real leaf) was registered after 45 days from the first sprout.

2.7 Biochemical test

The SOD (Superoxide Dismutase, EC.1.15.1.1), POD (Peroxidase, EC.1.11.1.7) and CAT (catalase, EC.1.11.1.6) activities were analyzed according to the methods of Prochazkova et al. [26] and Wang et al. [27] with slight modification.

The first pair of leaves from 12 seedlings of the same replicate that had emerged for 50 days weighing approximately 0.5 g was homogenized in 5 mL, 50 mmol/L PBS, pH 7.8, containing 1 g/L PVPP and 1 mmol/L EDTA, and then centrifuged at 11000 g for 15 min at 4 °C. The resulting supernatants were directly used for SOD, POD, and CAT assays. All operations were carried out at 4 °C and all the enzymatic activities were measured in fresh extracts at a certain temperature. The malondialdehyde (MDA) content was determined by the thiobarbituric acid (TBA) reaction, as described by Hodges [28]. The total soluble protein content was determined by the Bradford method [29] with a BioRad Protein Assay Kit. The SOD, POD and CAT activities were expressed as a $U \cdot mg^{-1}$ protein, and the MDA content was expressed as an $nmol \cdot mg^{-1}$ protein.

2.8 CAT native-PAGE analyses

The protein samples portioned out from the former extraction of the biochemical test were stored at −80 °C before use. A 4% stacking gel and a 6% separating gel were used to separate the CAT isoenzymes, and the electrophoresis protocol was referred to Richard [30]. After loading 15 μL of sample solution with a 5 μL 4×loading buffer, the samples were separated at 20 mA for 3 h at 4 °C, and the gel was stained for CAT activity [31].

2.9 Statistical analysis

Statistical analysis was conducted using SPSS for Windows (version 13.0). After testing for the assumption of the normal distribution, the obtained data were analyzed using one-way analysis of variance (ANOVA). The series of Duncan's post-hoc tests for equal numbers were used for multiple comparisons. The results were shown as mean±SD for the replicates in each group. The correlation coefficients between the groups were analyzed using Pearson's test, and the differences were considered significant at the P<0.05 level.

3 Results

3.1 Change in water permeability by seed surface modification

Electrical conductivity (EC) determination showed that air plasma pretreatment changed the water permeability of the seed coat of *A. paniculata* (Fig. 2). After air plasma treatment for 10 s, which was excited at 5950 V (group 4), the EC of the seeds was increased to 190.45±11.85 $\mu S \cdot cm^{-1} \cdot g^{-1}$, 22.87% higher than that of the control sample (P<0.05). After air plasma treatment for 20 s excited at 3400 V (group 5), the EC was decreased to 116.65±20.61 $\mu S \cdot cm^{-1} \cdot g^{-1}$, which was 24.74% lower than that of the control sample (P<0.05). This was consistent with the electron microscopic observation of the seed surface, which showed that many tiny holes appeared on the surface of the seeds treated by air plasma excited at 5950 V for 10 s (Fig. 3(c)), in comparison with the control (Fig. 3(b)). However, fewer holes appeared on the coats of the seeds that were treated at 3400 V for 20 s (Fig. 3(d)).

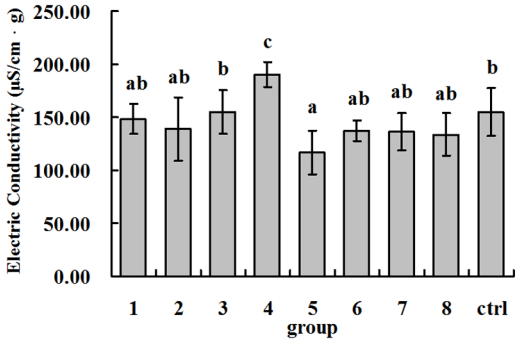
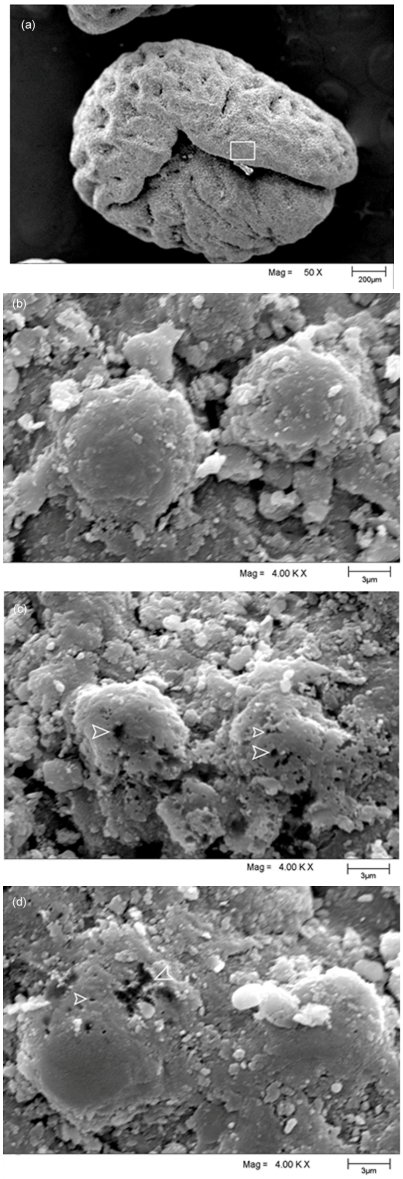


Fig.2 The influence of air plasma on the electrical conductivity of the leaching solution of *A. paniculata* seeds. Mean±SD are given. The means followed by the same letter column-wise were not significantly different in one-way ANOVA (Duncan's test, P<0.05). Groups 1-4: pretreated by air plasma excited at 3400 V, 4250 V, 5100 V, and 5950 V for 10 s; groups 5-8: pretreated by air plasma excited at 3400 V, 4250 V, 5100 V, and 5950 V for 20 s



(a) bar=200 μm, the white pane shows the magnified region, (b)-(d) bar=3 μm, (b) the surface of the untreated seed, (c) the surface of the seed treated by air plasma excited at 5950 V for 10 s, (d) the surface of the seed treated by air plasma excited at 3400 V for 20 s, (c)-(d) the white arrows indicate tiny holes etched by air plasma

Fig.3 The ultrastructure characteristics of *A. paniculata* seeds treated by air plasma

3.2 Germination characteristics after air plasma pretreatment

It was shown that air plasma pretreatment changed the germination percentage of *A. paniculata* seeds in the early stage, and also the germination index and vigor index (Table 2).

Although none of the samples had germination within 24 h, the germination percentages in the second day of all the plasma-treated groups were higher than the control, except for group 7. However, the differences in germination between the groups were diminished after the third day, and the total germination percentages in 7 days had no significant difference between the groups. That is to say, the plasma pretreatment did not increase, but accelerated the germination. Secondly, according to the germination index (GI), the germination speeds of groups 2, 4, and 8 were significantly higher than that of the control group (P<0.05). Thirdly, the growth of the primary root was not uniform, which means that the root lengths of groups 2, 5, and 8 were slightly longer than that of the control group (P>0.05). Finally, due to the GI and root length, the germination vigor indexes of groups 2, 5, and 8 were significantly higher than that of the control group (P<0.05). Thus, appropriate air plasma treatment can enhance seed germination vigor in laboratory conditions.

3.3 Seedling emergence alteration after air plasma pretreatment

The seedling emergence test showed that air plasma pretreatment changed the sprouting speed of *A. paniculata* seeds, resulting in alteration of the strong seedling percentage (Table 3). The first sprout occurred from the sixth day after sowing. After sprouting occurred for 7 days, the seedling emergence rates of group 4 was 33.3% higher than that of the control group (P<0.01), which means accelerated emergence. And 15 days after the first sprout, the seedling emergence of groups 3 and 5 were 15.5% and 16.4% lower than that of the control group, respectively (P<0.05), which means decelerated emergence. In seedling emergence 30 days after the first sprout, there was no significant difference between the groups.

Table 2. Germination of *Andrographis paniculata* after air plasma pretreatment

Group	GP in 2d (%)	GP in 3d (%)	GP in 7d (%)	GI	RL (mm)	VI
1	62.5±6.0 cd	73.0±4.2 a	79.8±5.0 a	36.2±2.4 abc	17.6±1.1 a	634.6±45.7 a
2	65.5±4.4 d	80.3±3.8 b	85.8±2.6 a	38.9±1.4 c	21.7±3.1 c	842.7±102.4 c
3	59.3±6.8 cd	72.3±6.7 a	80.8±4.0 a	35.8±2.7 abc	18.1±1.3 a	647.7±54.2 a
4	59.8±2.8 cd	76.5±1.0 ab	82.8±1.3 a	36.9±0.5 bc	18.1±0.4 a	666.1±16.7 ab
5	52.5±7.6 bc	76.5±2.9 ab	84.0±3.2 a	36.0±1.5 abc	21.1±2.7 bc	758.3±80.9 bc
6	55.3±3.1 bcd	76.0±5.1 ab	81.8±5.3 a	35.9±2.2 abc	17.9±0.5 a	642.0±43.9 a
7	49.0±7.0 ab	75.0±7.4 ab	81.3±5.7 a	34.5±3.1 ab	18.6±1.4 ab	642.1±91.0 a
8	60.5±7.2 cd	78.0±4.1 ab	86.0±2.6 a	37.9±2.1 c	20.1±2.5 abc	757.0±58.2 bc
ctrl	42.6±8.5 a	73.9±3.0 ab	81.6±4.0 a	33.5±1.9 a	19.2±1.2 abc	642.8±71.6 a

Mean±SD are given. The means followed by the same letter column-wise were not significantly different in one-way ANOVA (Duncan's test, P<0.05). GP, germination percentage; GI, germination index; RL, root length; VI, vigor index

Table 3. Seedling emergence of *Andrographis paniculata* after air plasma pretreatment

Group	Seedling emergence in 7 d (%)	Seedling emergence in 15 d (%)	Seedling emergence in 30 d (%)	Strong seedling percentage (%)
1	39.7±7.1 a	63.0±7.0 abc	74.0±6.1 a	41.7±5.1 ab
2	43.0±2.6 a	61.3±0.6 abc	72.3±1.5 a	42.7±4.5 ab
3	39.0±8.0 a	58.3±6.4 ab	71.7±3.1 a	39.0±6.1 a
4	53.7±2.1 b	66.0±2.6 abcd	74.7±1.2 a	50.0±3.6 c
5	38.3±2.1 a	57.7±4.5 a	73.3±2.5 a	35.7±1.5 a
6	43.3±2.1 a	66.0±7.9 abcd	74.3±3.5 a	43.0±4.4 ab
7	46.3±3.2 ab	73.7±5.7 d	79.3±3.8 a	47.3±2.1 bc
8	41.7±3.8 a	68.0±4.0 bcd	75.7±2.1 a	41.3±2.3 ab
ctrl	40.3±3.2 a	69.0±1.7 cd	75.3±2.5 a	41.6±2.1 ab

Mean±SD are given. The means followed by the same letter column-wise were not significantly different in one-way ANOVA (Duncan's test, P<0.05). Seedling emergence in 7 days, group 4 vs. ctrl P<0.01. Seedling emergence in 30 days, shows no significant difference between the groups. Strong seedling means a seedling that has more than three pairs of leaves

The strong seedling percentage of group 4 was 20.2% higher than that of the control group (P<0.05). The Pearson correlation coefficient between strong seedling percentages and seedling emergences in the first 7 days was 0.877 (P<0.001), and greater than the Pearson correlation coefficient between strong seedling percentages and seedling emergences in 15 days (0.579, P=0.002). This means that seeds sprouting in the first 7 days seem to have more of a chance of forming a strong seedling.

In a word, air plasma excited by 5950 V treatment for 10 s (group 4) is propitious to promoting seedling emergence ahead of time and increasing the chances of strong seedlings. 5100 V treatment for 20 s (group 7) seems to have similar effects, but showed no significance.

3.4 Biochemical characteristics of seedlings altered by air plasma pretreatment

According to the results of the biochemical test (Table 4), the SOD activities of groups 1, 3, 6, and 7 were lower than that of the control group (P<0.05). As for POD activity, there was no significant difference between the groups. However, the CAT activity of group 4 was significantly greater than that of the control group (P<0.05). The MDA contents of groups 2 and 4 were lower than that of the control group (P<0.05), which means that cell oxidative damage under environmental stress is maintained at a lower level. Thus, air plasma seed pretreatment for 10 s excited at 5950 V (group 4) can stimulate CAT activity and decrease MDA content in *A. paniculata* seedlings, which means that it is beneficial in resisting environmental stress.

Native-PAGE analyses showed that the catalase isoenzyme expression of the plasma pretreated seedlings was changed (Fig. 4). The CAT-2 bands of the treated groups were all clearer than that of the control group, and there was an additional band of CAT-1 after air plasma pretreatment, except for group 3. In particular, the CAT-1 band of groups 4 and 5 (5950 V for 10 s and 3400 V for 20 s) were clearer than that of the control group. Obviously, an appropriate dose of air plasma pretreatment can promote the CAT expression of the *A. paniculata* seedlings.

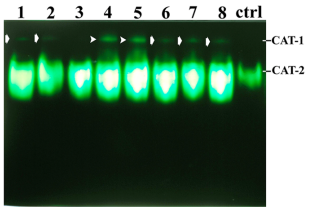


Fig.4 The CAT native-PAGE of *A. paniculata* seedlings pretreated by air plasma. (White arrows indicate the additional bands of the treated groups compared with the control group. The band of the CAT-1 of groups 4 and 5 was clear, while the control group did not have this band, and the CAT-2 of the control group was not as clear as the treated groups)

Table 4. The biochemical parameters of *Andrographis paniculata* after air plasma pretreatment

Group	SOD ($U \cdot mg^{-1}$)	POD ($U \cdot mg^{-1}$)	CAT ($U \cdot mg^{-1}$)	MDA ($n \cdot mol \cdot mg^{-1}$)
1	189.449±28.193 a	21.357±2.440 b	16.011±3.303 abc	3.991±0.681 d
2	212.183±36.349 abc	20.289±0.774 ab	17.204±3.494 bc	1.970±0.295 a
3	190.846±23.344 a	17.042±0.894 a	18.790±3.486 bc	2.868±0.808 bc
4	207.890±14.338 ab	20.228±1.195 ab	21.291±1.160 c	2.358±0.269 ab
5	209.228±15.254 abc	21.011±3.286 b	16.729±3.855 bc	2.672±0.471 abc
6	179.094±20.382 a	19.401±0.834 ab	19.499±4.377 bc	2.819±0.363 abc
7	201.136±15.985 a	20.043±2.089 ab	17.909±0.984 bc	2.976±0.422 bc
8	255.568±35.138 c	20.468±2.790 ab	10.789±3.100 a	3.477±0.365 cd
ctrl	253.469±26.248 bc	19.931±1.265 ab	13.480±2.992 ab	3.346±0.183 cd

Mean±SD are given. The means followed by the same letter column-wise were not significantly different in one-way ANOVA (Duncan's test, P <0.05)

4 Discussion

Seed germination, seedling emergence and the biochemical characteristics in seedlings of *A. paniculata* were changed after pretreatment by air plasma. The fourth group treated by air plasma excited at 5950 V for 10 s showed positive effects, which seedling emergence accelerated, the number of strong seedlings increased, the activity and expression of the protecting enzymes (i.e. catalase, CAT) improved, and the MDA (toxic material) content decreased, after the seed coat etching and water permeability increased.

The results also showed that the effects depended on the treatment dosage. As for groups 2 and 8, the germination energy (i.e. germination percentage in 2 days), germination index, and vigor index were improved. However, the emergence of seedlings in groups 3 and 5 was suppressed significantly. In this paper, the effect of laboratorial germination using the same dosage was not consistent with seedling emergence, which is similar to former research [20]. In addition, the effects of the enzymatic activities in the different groups had no linear efficiency with treatment dosage. Besides, a previous study found that over-dosage (a high power density or long treatment time) can lead to oppression of the seeds [19,32−34]. In our previous research, the seed vigor can be oppressed with a longer treatment time or higher driving voltage of air plasma [35]. Thus, selecting a favorable dosage for the specific seed in agricultural production is extremely important.

How does the air plasma influence the seed vigor? Since previous studies have proved that the seed coat can be eroded by plasma [13,14,20], we supposed that the erosion of the hydrophobic wax layer on the *A. paniculata* seed coat by air plasma caused the change in water permeability. In this paper, the results showed that the *EC* of seeds treated by plasma excited at 5950 V for 10 s (group 4) was significantly increased, which can be explained as the etching effect of air plasma produced tiny holes on the seed coat observable by SEM, resulting in imbibition improvement and in turn acceleration of seedling emergence. On the contrary, the permeability of the seeds treated by 3400 V for 20 s (group 5) was decreased, leading to deceleration of imbibition and delay of seedling emergence. The presumed reason for this was the predominant re-position effect by air plasma. As to the problem of how the air plasma influenced the biochemical reactivity, former researchers supposed that plasma-induced degradation of the seed surface would lead to a deeper penetration of plasma-induced UV-B radiation and reactive species (including ions, reactive oxygen species and reactive nitrogen species), which could favor further biochemical reactions [36]. The results in this paper support the supposition of the former researchers, however, more evidence is needed to prove the complicated underlying mechanism, which is a challenging issue. The mechanisms of plasma interaction with living tissues and cells can be quite complex, in view of the complexity of both the plasma and the tissue [37].

Additionally, the plasma has advantages in uniform treatment [18]. It requires short exposure times which is technically attractive. Secondly, the plasma process does not require chemicals, and hence no environmental pollutants remain after the treatment, which means that it is environmentally friendly [18]. There is also no destruction of the seeds or supporting medium after plasma treatment [8], and the seeds can be stored again if they are not used for germination [18]. Furthermore, the atmospheric pressure air plasma apparatus do not need a rare gas source or complicated vacuum system, which provides practical convenience and low cost [8]. A seed pre-sowing treatment approach based on plasma methods would therefore offer a more effective and environmentally friendly alternative besides traditional seed processing technologies.

Previous investigations have shown that atmospheric pressure air plasma can induce many favorable mutations of coxcombs [38]. Application of this technology to plant breeding would therefore be promising. Further studies will focus on whether plasma pretreatment could change the final yield and the secondary metabolic content (e.g. andrographolide) of *A. paniculata*, and investigate the variation.

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