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Unrevealing the impact of pulsed electric fields (PEF) on cucumber seed vigour and surface disinfection

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Abstract

Chemicals used for seed treatments help to increase the agricultural production by preventing pests and pathogens but also cause environmental and health problems. Thus, environmentally-friendly technologies need to be developed for a seed treatment that inactivates surface microflora and improves seed vigor. One such pulsed electric field (PEF) treatment applied to cucumber seeds in the range of 1.07-17.28 Joule (J) significantly enhanced a mean germination rate (MGR) by up to 9%, a normal seedling rate by 25.73%, and a resistance to 100 and 200 mM salt stresses by 96% and 91.67%, respectively, with a stronger and faster growth of roots and seedlings. PEF treatment provided 3.34 and 3.22 log-reductions in the surface microflora of total mold and yeast and total aerobic mesophilic bacteria, respectively. The electrical conductivity (EC) values of the control samples increased over time, from 4 to 24 h. Those of the PEF-treated samples after 4, 12, and 24th hours were also more affected by the measurement time not by the PEF treatment.

The joint optimization of 18 responses based on the best-fit Gaussian process model pointed to 19.78 s and 17.28 J as the optimal settings. The PEF treatment appeared to improve seed germination ability and stress resistance with the adequate inactivation of surface microflora.

Keywords: Cucumber seeds; Pulsed electric fields; Germination; Seed vigour; Stress tolerance

Introduction

Chemical seed treatments are usually applied to agricultural products for pest control. However, some of these methods have been costly, while the others have caused adverse environmental and public health impacts. Non-chemical and physical treatments have been on demand since they have reduced the pesticide releases and their residues into the environment. Some of the non-chemical methods include use of steam (13), solarization of soil (18), microwave, electron beam irradiation (58), hot water, and magnetic fields (58). Resistance inducers and plant-derived products such as Bion 50 WG, Chitoplant, salicylic acid, jasmonic acid, Comcat, Milsana flüssig, Kendal, and plant essential oils were also practiced (2) to influence physiological and biochemical processes involved to improve seed vigor and crop stand.

The germination ability of seeds is adversely affected by many factors. For example, increased salinity of soil is a critical factor in agricultural production and one of the major problems in (semi-)arid regions (2,35). Soil salinity is mostly caused by increased sodium chloride concentration (35,56), reduces the rate of germination, and retards the cucumber seed initiation (*Cucumis sativus* L.) (2,16,55,65). Thus, it is important to increase germination and induce mechanisms for salt-stress resistance in cucumber seeds to maximize yield.

Pulsed electric fields (PEF) at high frequencies are applied to biological membrane though specifically designed electrodes (55). Electric field at higher magnitude is lethal, and thus, used for microbial inactivation (34,57). However, electric field at a lower magnitude is sub-lethal and used to improve extraction yield (21,32), increase drying efficacy (21,32), and modify tissue and cell cultures (10) depending on both cell structure

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and treatment parameters. Low-intensity PEF is also used to promote barley germination (24,26), extract oil from papaya seeds (45), investigate antioxidant metabolism of wheatgrass (*Triticum aestivum* L.) seeds (40), determine early germination of *Arabidopsis thaliana* seeds (53), disinfect vegetable seeds and grains (28), growth parameters of wheat and nutritional properties of wheat plantlets juice (4), and inactivate endogenous bacteria of winter wheat, barley, and vegetable seeds (29). However, existing knowledge regarding the impact of PEF on germination of cucumber seeds, their resistance to salt, and inactivation of surface microflora is limited in related literature. Thus, the objectives of the study were to 1) determine PEF processing parameters to treat cucumber seeds; 2) evaluate the effectiveness of the PEF treatment on mean germination rate, normal seedling rate, conductivity, root formation, and resistance to salt as well as inactivation of surface microflora; and 3) optimize the PEF processing parameters and responses. Germination ability of the seedling and root formation was also given to determine the positive effect of PEF on the seedling growth.

Materials and Methods

Seed samples

Beith alpha cucumber *Cucumis sativus* L. (cv. Hokus) seed samples kindly provided by the seed company (Nadide Tohum, Antalya, Turkey) were kept at ambient temperature in air-tight containers until they were treated.

Pulsed electric field treatment

A pilot-scale PEF system constructed by our research team at Bolu Abant Izzet Baysal University (Turkey) was used to treat cucumber seeds in response to 110, 140, 160, and 180 Hz frequencies and 2.47, 7.42, 12.37, and 19.79 sec treatment times with 1.07 to 17.28 Joule (J) energies. Applied treatment times and energy levels were derived from the treatment parameters mentioned above (15).

Germination tests

Control and PEF-treated cucumber seed samples (50 seeds) in three replications were placed on a filter paper moistened with sprayed water. The quantity of water used for irrigation was 2.5 times the substrate weight. All the samples were settled in a germinator at 25 oC for 2-4 days under a constant light, while germination was checked every day. Two mm radicle emergence was the criteria to determine germination expressed in percentage (63). Seedling was checked on a daily basis in terms of good shoot and root developments (normal), curling, and abnormal and glass-like body (not normal) (63).

Electrical conductivity measurement

Electrical conductivity (EC) measurement was performed using a Sension 5 model conductivity meter (HACH, CO, USA). Conductivity was measured at 4, 8, and 24 h (30).

Effect of salt stress on germination

Germination under salt stress was performed at two levels. Conductivity of the water used to irrigate seedling was adjusted to 10.8 and 19.8 mS cm^{-1} EC with addition of NaCl. Fifty cucumber seeds of PEF-treated and control samples were planted in four cm soil, and then, all the samples were placed in a temperature controlled cabinet. Each pot was irrigated with 100 mL of salted water for each level at first day; whereas, 50 mL of salted water was added for the following 13 days. All experiments were repeated in triplicate (30,63).

Inactivation of surface microflora

Number of total mold and yeast (TMY) and total aerobic mesophilic bacteria (TAMB) as a representative of surface flora were quantified. Seed samples diluted with 0.1% peptone water at the ratio of 1:9 (v/v) were surface plated on plate count agar (PCA) (Fluka, Steinheim, Germany) for TAMB and potato dextrose agar (PDA) (Fluka, Steinheim, Germany) plates in triplicate, respectively. PCA and PDA plates were incubated at 35 ± 2 °C for 24-48 h and 22 ± 2 °C for 3-5 days, respectively. Results were calculated as log cfu/g (30).

Statistical analyses

Seed quality and microbial inactivation data were analyzed by one-way analysis of variance (ANOVA) with Tukey's multiple comparison tests (Minitab Statistical Software 17.1.0, MiniTab Inc., PA, USA). Stepwise regression analyses were used to estimate the changes and the interactions in the response variables. Joint optimization was also conducted to determine composite desirability with the most optimal solutions.

The best-fit Gaussian process (GP) model was used to obtain a prediction formula on which the joint optimization was carried out. The objective function of the joint optimization was set to minimize the responses. The parameters are as follows: μ is the Gaussian Process mean, σ^2 is the Gaussian Process variance, theta corresponds to the values of θ_k in the definition of R. This model assumes that Y is normally distributed with mean μ and covariance matrix $\sigma^2 R$. The elements of the R matrix are defined as follows:

$$r_{ij} = \exp\left(-\sum_{k=1}^{K} \theta_k (x_{ik} - x_{jk})^2\right)$$

where K = # of continuous predictors, θ_k = theta parameter for the k^{th} predictor, x_{ik} = the value of the k^{th} predictor for subject i,x_{jk} = the value of the k^{th} predictor for subject j.

Factors with small theta values have little (or no) impact on the prediction formula. Total sensitivity is a measure of the sum of influence and explains % of the variation in the response. Main effect is the ratio of the functional main effect and the total variation for each factor in the model. Effects of interaction are also calculated similar to main effects.

Results

Physical properties of seeds such as moisture content, size, and shape are important parameters to determine the magnitude

electric field energy and duration of treatment time for PEF. Initial experiments were conducted to determine the PEF processing conditions for cucumber seeds. Based on preliminary experiments, 18 kV of output voltage with 110, 140, 160, and 180 Hz frequencies were applied to cucumber seeds. Treatment times and applied energies were calculated as 2.47, 7.42, 12.37, and 19.79 sec and 1.07, 1.36, 1.92, 2.16, 3.21, 4.08, 5.35, 5.76, 6.48, 6.80, 8.55, 9.60, 10.80, 10.89, 15.36, and 17.28 J, respectively.

Compared to the control samples, all the PEF treatments provided a significant difference in the mean germination rate (MGR) on the 2, 3, and 4th days with 8.9% increase on the 2nd and 3rd days, and 6.7% increase on the 4th day. As the germination rate increased from 2nd to 4th day, MGR of the most of the samples significantly increased by the time (Table 1).

Table 1. Germination rate (%) of the control and PEF-treated cucumber seed samples

Energy (J)	Germination rate (%)		
	2.day	3.day	4.day
0.00	90.00 ± 0.00^{bB}	91.11 ± 1.92^{cB}	93.33 ± 0.00^{cA}
1.07	95.56 ± 1.92^{aA}	95.56 ± 1.92^{bA}	96.67 ± 0.00^{bA}
1.36	93.33 ± 1.33^{aB}	96.67 ± 3.33^{abA}	98.89 ± 1.92^{abA}
1.92	95.56 ± 1.92^{Aa}	96.67 ± 3.33^{abA}	98.89 ± 1.92^{abA}
2.16	98.89 ± 1.92^{aA}	98.89 ± 1.92^{abA}	100.00 ± 0.00^{aA}
3.21	95.33 ± 1.33^{aA}	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}
4.08	96.67 ± 1.33^{aB}	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}
5.35	96.67 ± 1.33^{aB}	96.67 ± 0.00^{bB}	100.00 ± 0.00^{aA}
5.76	95.56 ± 4.09^{aA}	98.89 ± 1.92^{abA}	100.00 ± 0.00^{aA}
6.48	93.33 ± 3.33^{aA}	94.44 ± 1.92^{bA}	97.78 ± 1.92^{abA}
6.80	96.67 ± 1.33^{aA}	96.67 ± 1.33^{bA}	98.89 ± 1.92^{abA}
8.55	95.56 ± 1.92^{aB}	95.56 ± 1.92^{bB}	100.00 ± 0.00^{aA}
9.60	93.33 ± 0.00^{aC}	96.67 ± 0.00^{bB}	100.00 ± 0.00^{aA}
10.80	96.67 ± 1.33^{aB}	97.78 ± 1.92^{bB}	100.00 ± 0.00^{aA}
10.89	95.56 ± 1.92^{aB}	96.67 ± 0.00^{bB}	100.00 ± 0.00^{aA}
15.36	96.67 ± 0.00^{ab}	96.67 ± 0.00^{bB}	100.00 ± 0.00^{aA}
17.28	98.89 ± 1.92^{aB}	97.78 ± 1.92^{bB}	100.00 ± 0.00^{aA}

*Means in the same column with lowercase superscript letter and in the same row with uppercase superscript letter are significantly different ($p \leq 0.05$)

Normal seedling rate of control samples significantly increased with all the PEF treatments ($p \leq 0.05$). The lowest and highest normal seedling rates were 75.86 ± 0.00% with 1.07 J and 95.56 ± 1.92% with 17.28 J, respectively. Compared to the control samples, the PEF treatment provided a 25.7% increase in the normal seedling rate (Table 2). Except for those treated with 4.08, 10.80, 10.89, and 17.28 J, all the other PEF treatments provided an earlier and better germination, a stronger body formation, taller seedlings (Figure 1), and a stronger root formation (Figure 2).

The EC values of the control samples increased over time, namely from 4 to 24 h. Those of the PEF-treated samples after 4, 8, and 12th hours were also more affected by the measurement time not by the PEF treatment. The EC values of the PEF-treated samples for 4, 8, and 12th h ranged from 5.43 ± 0.29 µS cm^{-1}g^{-1} with 10.89 J to 7.94 ± 0.79 µS cm^{-1}g^{-1} with 17.28 J, from 8.22 ± 0.17 µS cm^{-1}g^{-1} with 1.07 J to 9.18 ± 0.28 µS cm^{-1}g^{-1} with 17.28 J, and from 8.98 ± 0.47 µS cm^{-1}g^{-1} with 6.80 J to 10.61 ± 0.15 µS cm^{-1}g^{-1} with 17.28 J, respectively (Table 3).

The control samples had no germination until 12th day, whereas some PEF-treated samples started to germinate on the 9th day when exposed to salinity level of 100 mM NaCl. The samples treated with 5.35, 6.48, and 6.80 J on 9th day, 2.16, 3.21, 5.35, 6.48, 6.80, 8.55, 10.80, 10.89, and 17.28 J on 10th day; 2.16, 3.21, 5.35, 6.48, 6.80, 8.55,10.80, 10.89, and 17.28 J on 11th day; 2.16, 3.21, 5.35, 6.48, 6.80, 8.55, 10.80, 10.89, 15.36, and 17.28 J on 12th day; and all the PEF-treated samples on the 13th day presented significantly higher germination rate under salinity level of 100 mM NaCl (Table 4). Except for the samples treated with 17.28 J, the PEF-treated and control samples did not germinate on 8 and 9th days under 200 mM NaCl salt stress. The control samples only showed germination with 3.33 ± 0.30% on 13th day, whereas the PEF-treated samples with 8.55, 10.80, 10.89, 15.36 and 17.28 J on 10th day; 8.55, 9.60, 10.80, 10.89, 15.36 and 17.28 J on 11th day; 1.07, 1.92, 3.21, 4.08, 5.35, 8.55, 9.60, 10.80, 10.89, 15.36 and 17.28 J on 12th day; and all the PEF-treated samples on 13th day showed germination. The samples treated with 17.28 J presented a significantly higher germination rate, and 100.00 ± 0.00% germination was observed on both 12 and

Table 2. Normal seedling rate (%) of the control and PEF-treated cucumber seed samples

Energy (J)	Normal seedling rate (%)
0.00	72.62 ± 2.06^{d}
1.07	75.86 ± 0.00^{c}
1.36	89.81 ± 6.11^{b}
1.92	96.63 ± 0.47^{a}
2.16	95.18 ± 1.92^{a}
3.21	86.67 ± 2.00^{b}
4.08	86.67 ± 6.67^{b}
5.35	85.56 ± 5.09^{b}
5.76	92.22 ± 3.85^{ab}
6.48	94.37 ± 3.78^{ab}
6.80	83.14 ± 6.67^{b}
8.55	85.56 ± 6.94^{b}
9.60	93.33 ± 3.33^{ab}
10.80	92.22 ± 5.09^{ab}
10.89	83.33 ± 5.77^{b}
15.36	91.11 ± 5.09^{ab}
17.28	95.56 ± 1.92^{a}

*Means in the same column with lowercase superscript letter are significantly different ($p \leq 0.05$)

13th days. Samples treated by PEF presented significantly higher germination under salt stress of 200 mM NaCl (Table 5). Germination rate significantly increased over time as the seed samples exhibited higher germination rate closer to the end rather than beginning of germination studies (Tables 4 and 5). Maximum of 100, 75, 89, 89, and 70% increases were observed on 9, 10, 11, 12, and 13th day of germination under 100 mM NaCl stress, whereas 100% increase on 8, 9, 10, 11 and12th day, and 92% increase on 13th day were observed for germination under salinity level of 200 mM NaCl, respectively.

The mean initial TAMB and TMY counts were reported as 6.25 ± 0.26 and 9.38 ± 0.05 log cfu g^{-1}, respectively. Except for 1.07 J, the other PEF treatments significantly reduced the mean initial TAMB count. The lowest number of TAMB and TMY were detected as 3.03 ± 0.10 and 6.04 ± 0.02 log cfu g^{-1} after treated by 17.28 J energy revealing 3.22 and 3.34 log reductions in TAMB and TMY, respectively (Figure 3).

Normal seedling rate was significantly affected by frequency in addition to interaction of the treatment time and frequency with R^2 and R^2_{adj} values of 0.591 and 0.507, respectively. Treatment time, frequency, and interaction of frequency and treatment time significantly affected inactivation of TAMB with R^2 and R^2_{adj} values determined as 0.508 and 0.407. TMY inactivation was significantly affected by treatment time with the interactions of frequency and frequency, and treatment time and frequency with R^2 and R^2_{adj} values of 0.618 and 0.540.

Nonlinear regression modeling revealed the R^2 values higher 90% for 8, 9, 10, 11, 12, and 13th day germination under both 100 and 200 mM NaCI salt stresses, indicating variation of a dependent variable is strongly explained by the independent variable(s) in a regression model (Table 6). The joint optimization of the 18 responses (Table 7) showed that the optimum process parameters were 17.28 J and 19.78 s for TAMB (3.52 log cfu g^{-1}) and TMY (7.29 cfu g^{-1}) counts, respectively (Figure 4).

Discussion

Plants have evolved several stress response mechanisms such as increased and accelerated growth rate, increased biomass production, and diminished adverse effect on the plant tissue. Increased calcium concentration triggered by several external stimuli like ozone, temperature, salinity, and mechanical signals (39,47,51) may lead to changes such as growth, physiology, and development of organisms as well as development of the control mechanisms of stimulus. Effect of PEF on the growth stimulation may be related to the stress response mechanisms of the plants (27). For example, H_2O_2 production as a plant response to the PEF stress and cell wall healing to reduce permeability was revealed for potato cells (33,50). It is possible that PEF may provide the conversion of intracellular calcium stores to free cytosolic calcium in order to compensate stress and induce growth mechanisms (27). This mechanism in seedlings in response to the PEF stress may increase the germination rate and provide an earlier germination and a stronger body and root formation in the cucumber seeds.

Some other physical treatments were also reported to increase the germination of cucumber seeds. For example, the combination of magnetic field (MF) treatment and UV-B irradiation accelerated germination and growth of seedling for the cucumber seeds (61). PEF applied at 5 kV cm^{-1} electric field for 3 min along with hydropriming significantly enhanced the germination percentage for Bingo I cucumber seeds (36). Average leaf area of *A. thaliana* was positively affected by PEF with 10 nanosecond electrical pulses and 5-20 kV cm^{-1} electric field a few days after germination. Significantly positive effect of PEF applied at 10 kV cm^{-1} with 80% increase was observed in the 2nd week after the treatments (53). Positive effect of PEF with 4 kJ kg^{-1} energy level on growth development of *A. thaliana* was clear at 7th day of germination (53). Growth of soy seedlings were also positively affected by static PEF treatment with 50/60 Hz application. Application of 36 V cm^{-1} electric field with 50 Hz provided a 12% increase in soy seedling length (22). Acceleration in tomato seed germination was reported after application of electric field in the range of 4 and 12 kV cm^{-1} (43). Seed yield before sowing was positively influenced by PEF treatment with 4 kV cm^{-1} for 12 min (23). Growth stimulation effect of PEF changed by applied treatment parameters. For example, while barley growth was positively stimulated by 0.5 kV cm^{-1} electrostatic field application for 5-day exposure, 2 kV cm^{-1} electric fields presented no growth promoting action (7). Exposing seeds to electric fields was reported to improve germination performance of soybean (64), tomato (43), and cucum-

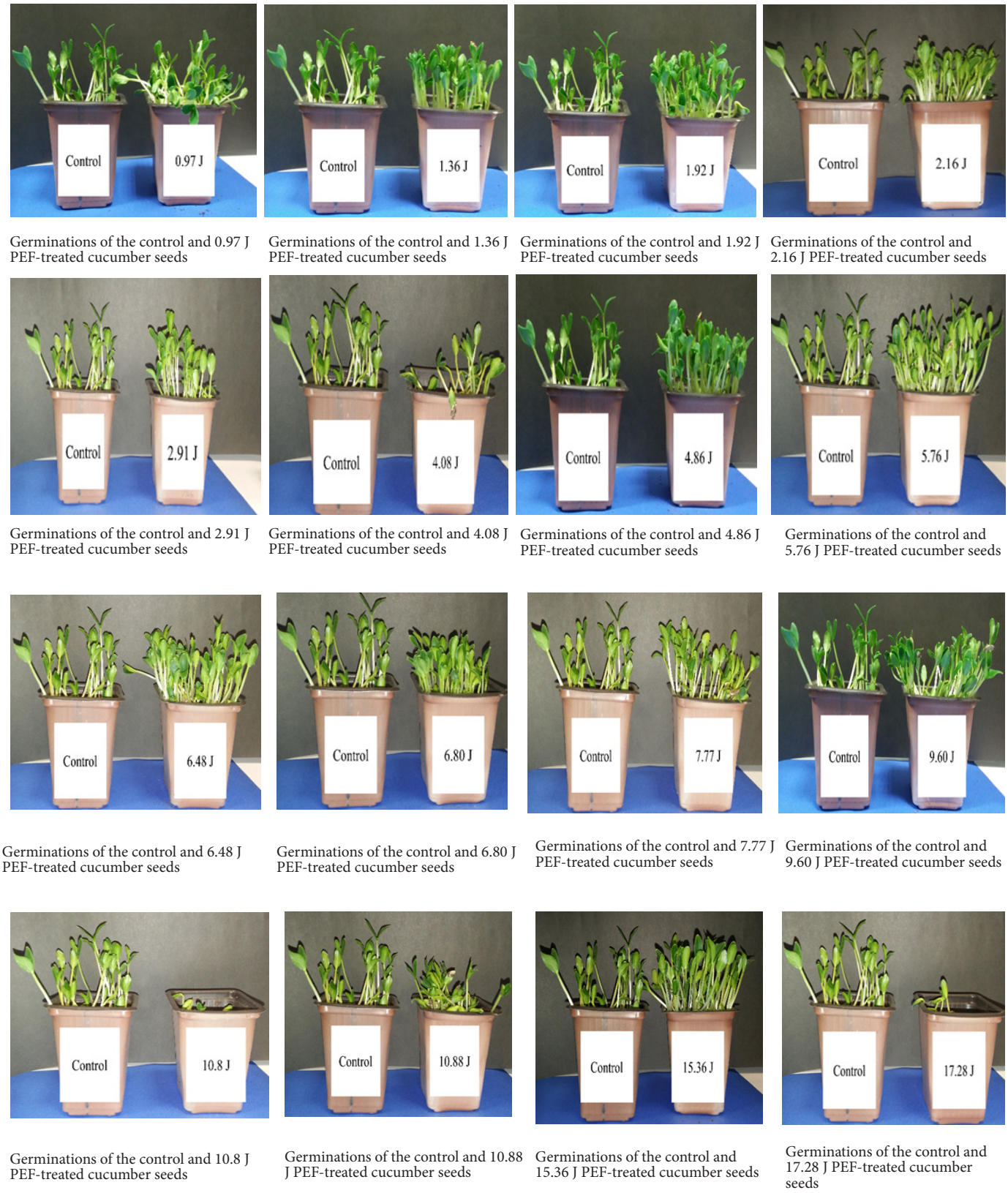

Figure 1. Impact of PEF treatment on germination ability of cucumber seeds.

ber (19) seeds. Similar to the present study, most of the earlier reports indicated that PEF treatment provides 10-20% increase in plant growth and germination rate.

The PEF treatment enhanced the germination performance and altered the membrane permeability of the cucumber seeds. When subjected to electric fields, cellular membrane is the first organelle subjected to electric fields related damage in the cell (46). Effect of PEF on cell membrane is also moisture-depen-

Root formation of the control samples

Root formation of 0.97 J PEF-treated samples

Root formation of 1.36 J PEF-treated samples

Root formation of 1.92 J PEF-treated samples

Root formation of 2.16 J PEF-treated samples

Root formation of 2.91 J PEF-treated samples

Root formation of 4.08 J PEF-treated samples

Root formation of 4.86 J PEF-treated samples

Root formation of 5.76 J PEF-treated samples

Root formation of 6.48 J PEF-treated samples

Root formation of 6.80 J PEF-treated samples

Root formation of 7.77 J PEF-treated samples

Root formation of 9.60 J PEF-treated samples

Root formation of 10.8 J PEF-treated samples

Root formation of 10.88 J PEF-treated samples

Root formation of 15.36 J PEF-treated samples

Root formation of 17.28 J PEF-treated samples

Figure 2. Impact of PEF treatment on root formations of cucumber seeds.

Table 3. Changes in electrical conductivity (μS $cm^{-1}g^{-1}$) of the control and PEF-treated cucumber seed samples

Energy (J)	Electrical conductivity (μS $cm^{-1}g^{-1}$)		
	4 hour	8 hour	24 hour
0.00	7.29 ± 0.79^{abcA}	8.06 ± 0.67^{aAB}	9.65 ± 0.58^{abA}
1.07	7.17 ± 0.76^{abcB}	8.22 ± 0.17^{aB}	9.82 ± 0.14^{abA}
1.36	5.92 ± 1.82^{abcB}	8.65 ± 0.62^{aA}	9.02 ± 0.57^{bA}
1.92	6.48 ± 1.06^{abcC}	8.79 ± 0.56^{aB}	9.74 ± 0.57^{abA}
2.16	7.44 ± 0.18^{abcC}	9.07 ± 0.50^{aB}	10.17 ± 0.05^{abA}
3.21	7.56 ± 0.21^{abcC}	8.75 ± 0.27^{aB}	9.62 ± 0.37^{abA}
4.08	6.91 ± 0.21^{abcB}	8.45 ± 0.84^{aAB}	9.73 ± 0.64^{abA}
5.35	7.24 ± 0.32^{abcB}	8.35 ± 1.34^{aB}	10.19 ± 0.43^{abA}
5.76	6.42 ± 0.07^{abcC}	8.84 ± 0.23^{aB}	10.33 ± 0.08^{abA}
6.48	5.57 ± 0.40^{bcC}	8.56 ± 0.18^{aB}	9.55 ± 0.29^{abA}
6.80	6.17 ± 0.66^{abcC}	7.85 ± 0.39^{aB}	8.98 ± 0.47^{bA}
8.55	6.28 ± 0.69^{abcB}	8.36 ± 0.21^{aA}	9.09 ± 0.56^{bA}
9.60	6.07 ± 1.72^{abcC}	8.93 ± 0.46^{aB}	10.41 ± 0.73^{abA}
10.80	5.87 ± 0.11^{abcC}	7.98 ± 0.49^{aB}	9.47 ± 0.30^{abA}
10.89	5.43 ± 0.29^{cC}	7.65 ± 1.38^{aB}	9.74 ± 1.04^{abA}
15.36	7.75 ± 0.08^{abcC}	8.88 ± 0.07^{aB}	9.85 ± 0.67^{abA}
17.28	7.94 ± 0.79^{abC}	9.18 ± 0.28^{aB}	10.61 ± 0.15^{aA}

*Means in the same column with lowercase superscript letter and in the same row with uppercase superscript letter are significantly different ($p \leq 0.05$)

Table 4. Germination rate (%) of the control and PEF-treated cucumber seed samples under 100 mM NaCl salt stress

Energy (J)	Germination rate (%)					
	8. day	9. day	10. day	11. day	12. day	13. day
0	0.00 ± 0.00^{aB}	0.00 ± 0.00^{cB}	0.00 ± 0.00^{eB}	0.00 ± 0.00^{gB}	0.00 ± 0.00^{gB}	3.33 ± 0.30^{hA}
1.07	0.00 ± 0.00^{aA}	0.00 ± 0.00^{cA}	0.00 ± 0.00^{eA}	0.00 ± 0.00^{gA}	0.00 ± 0.00^{gA}	8.33 ± 0.00^{Ga}
1.36	0.00 ± 0.00^{aB}	0.00 ± 0.00^{cB}	0.00 ± 0.00^{eB}	0.00 ± 0.00^{gB}	0.00 ± 0.00^{gB}	16.67 ± 0.30^{fA}
1.92	0.00 ± 0.00^{aB}	0.00 ± 0.00^{cB}	0.00 ± 0.00^{eB}	0.00 ± 0.00^{gB}	0.00 ± 0.00^{gB}	16.67 ± 0.30^{fA}
2.16	0.00 ± 0.00^{aB}	0.00 ± 0.00^{cB}	8.33 ± 0.00^{dA}	8.33 ± 0.00^{fA}	8.33 ± 0.00^{fA}	8.33 ± 0.00^{gA}
3.21	0.00 ± 0.00aE	0.00 ± 0.00^{cE}	8.33 ± 0.00^{dD}	16.67 ± 0.30^{eC}	25.00 ± 0.00^{dB}	33.33 ± 0.00^{dA}
4.08	0.00 ± 0.00^{aB}	0.00 ± 0.00^{cB}	0.00 ± 0.00^{eB}	0.00 ± 0.00^{gB}	0.00 ± 0.00^{gB}	16.67 ± 0.30^{fA}
5.35	0.00 ± 0.00^{aE}	16.67 ± 0.00^{aD}	25.00 ± 0.30^{bC}	33.33 ± 0.00^{cB}	33.33 ± 0.30^{cB}	41.67 ± 0.40^{cA}
5.76	0.00 ± 0.00^{aB}	0.00 ± 0.00^{cB}	0.00 ± 0.00^{eB}	0.00 ± 0.00^{gB}	0.00 ± 0.00^{gB}	8.33 ± 0.00^{gA}
6.48	0.00 ± 0.00^{aD}	16.67 ± 0.00^{aC}	16.67 ± 0.30^{cC}	25.00 ± 0.00^{dB}	33.33 ± 0.30^{cA}	33.33 ± 0.00^{dA}
6.80	0.00 ± 0.00^{aD}	8.33 ± 0.00^{bC}	8.33 ± 0.30^{dC}	16.67 ± 0.00^{eB}	16.67 ± 0.00^{eB}	25.00 ± 0.00^{eA}
8.55	0.00 ± 0.00^{aD}	0.00 ± 0.00^{cD}	8.33 ± 0.00^{dC}	25.00 ± 0.00^{dB}	25.00 ± 0.00^{dB}	33.33 ± 0.00^{dA}
9.60	0.00 ± 0.00^{aB}	0.00 ± 0.00^{cB}	0.00 ± 0.00^{eB}	0.00 ± 0.00^{gB}	0.00 ± 0.00^{gB}	16.67 ± 0.30^{fA}
10.80	0.00 ± 0.00^{aA}	0.00 ± 0.00^{cA}	16.67 ± 0.00^{cD}	41.67 ± 0.40^{bC}	58.33 ± 0.00^{bB}	75.00 ± 0.30^{bA}
10.89	0.00 ± 0.00^{aC}	0.00 ± 0.00^{cC}	8.33 ± 0.00^{dB}	8.33 ± 0.00^{fB}	16.67 ± 0.20^{eA}	16.67 ± 0.00^{fA}
15.36	0.00 ± 0.00^{aC}	0.00 ± 0.00^{cC}	0.00 ± 0.00^{eC}	0.00 ± 0.00^{gC}	8.33 ± 0.00^{fB}	16.67 ± 0.30^{fA}
17.28	0.00 ± 0.00^{aD}	0.00 ± 0.00^{cD}	33.33 ± 0.00^{aC}	75.00 ± 0.00^{aB}	75.00 ± 0.00^{aB}	83.33 ± 0.00^{aA}

Means in the same column with lowercase superscript letter and in the same row with uppercase superscript letter are significantly different ($p \leq 0.05$)

Table 5. Germination rate (%) of the control and PEF-treated cucumber seed samples under 200 mM NaCl salt stress

Energy (J)	Germination rate (%)					
	8. day	9. day	10. day	11. day	12. day	13. day
0	0.00 ± 0.00^{bB}	0.00 ± 0.00^{bB}	0.00 ± 0.00^{dB}	0.00 ± 0.00^{dB}	0.00 ± 0.00^{eB}	8.33 ± 0.60^{eA}
1.07	0.00 ± 0.00^{bB}	0.00 ± 0.00^{bB}	0.00 ± 0.00^{dB}	0.00 ± 0.00^{dB}	8.33 ± 0.60^{dA}	8.33 ± 0.60^{eA}
1.36	0.00 ± 0.00^{bB}	0.00 ± 0.00^{bB}	0.00 ± 0.00^{dB}	0.00 ± 0.00^{dB}	0.00 ± 0.00^{eB}	8.33 ± 0.60^{eA}
1.92	0.00 ± 0.00^{bC}	0.00 ± 0.00^{bC}	0.00 ± 0.00^{dC}	0.00 ± 0.00^{dC}	16.67 ± 0.00^{cB}	25.00 ± 0.00^{cA}
2.16	0.00 ± 0.00^{bB}	0.00 ± 0.00^{bB}	0.00 ± 0.00^{dB}	0.00 ± 0.00^{dB}	0.00 ± 0.00^{eB}	8.33 ± 0.60^{eA}
3.21	0.00 ± 0.00^{bB}	0.00 ± 0.00^{bB}	0.00 ± 0.00^{dB}	0.00 ± 0.00^{dB}	8.33 ± 0.60^{dA}	8.33 ± 0.60^{eA}
4.08	0.00 ± 0.00^{bB}	0.00 ± 0.00^{bB}	0.00 ± 0.00^{dB}	0.00 ± 0.00^{dB}	8.33 ± 0.60^{dA}	8.33 ± 0.60^{eA}
5.35	0.00 ± 0.00^{bC}	0.00 ± 0.00^{bC}	0.00 ± 0.00^{dC}	0.00 ± 0.00^{dC}	8.33 ± 0.60^{dB}	16.67 ± 0.00^{dA}
5.76	0.00 ± 0.00^{bB}	0.00 ± 0.00^{bB}	0.00 ± 0.00^{dB}	0.00 ± 0.00^{dB}	0.00 ± 0.00^{eB}	8.33 ± 0.60^{eA}
6.48	0.00 ± 0.00^{bB}	0.00 ± 0.00^{bB}	0.00 ± 0.00^{dB}	0.00 ± 0.00^{dB}	0.00 ± 0.00^{eB}	8.33 ± 0.60^{eA}
6.80	0.00 ± 0.00^{bA}	0.00 ± 0.00^{bA}	0.00 ± 0.00^{dA}	0.00 ± 0.00^{dA}	0.00 ± 0.00^{eA}	8.33 ± 0.60^{eA}
8.55	0.00 ± 0.00^{bD}	0.00 ± 0.00^{bD}	8.33 ± 0.60^{cC}	8.33 ± 0.00^{cB}	8.33 ± 0.60^{dB}	16.67 ± 0.00^{dA}
9.60	0.00 ± 0.00^{bD}	0.00 ± 0.00^{bD}	0.00 ± 0.00^{dD}	8.33 ± 0.00^{cC}	16.67 ± 0.00^{cB}	66.67 ± 0.00^{bA}
10.80	0.00 ± 0.00^{bE}	0.00 ± 0.00^{bE}	25.00 ± 0.50^{bD}	41.67 ± 0.00^{bC}	50.00 ± 0.60^{bB}	66.67 ± 0.00^{bA}
10.89	0.00 ± 0.00^{bB}	0.00 ± 0.00^{bB}	8.33 ± 0.00^{cA}	8.33 ± 0.00^{cA}	8.33 ± 0.60^{dA}	8.33 ± 0.00^{eA}
15.36	0.00 ± 0.00^{bD}	0.00 ± 0.00^{bD}	8.33 ± 0.00^{cC}	8.33 ± 0.00^{cC}	16.67 ± 0.00^{cB}	66.67 ± 0.00^{bA}
17.28	8.33 ± 0.00^{aD}	8.33 ± 0.00^{aD}	41.67 ± 0.00^{aC}	83.33 ± 0.70^{aB}	100.00 ± 0.00^{aA}	100.00 ± 0.90^{aA}

*Means in the same column with lowercase superscript letter and in the same row with uppercase superscript letter are significantly different ($p \leq 0.05$)

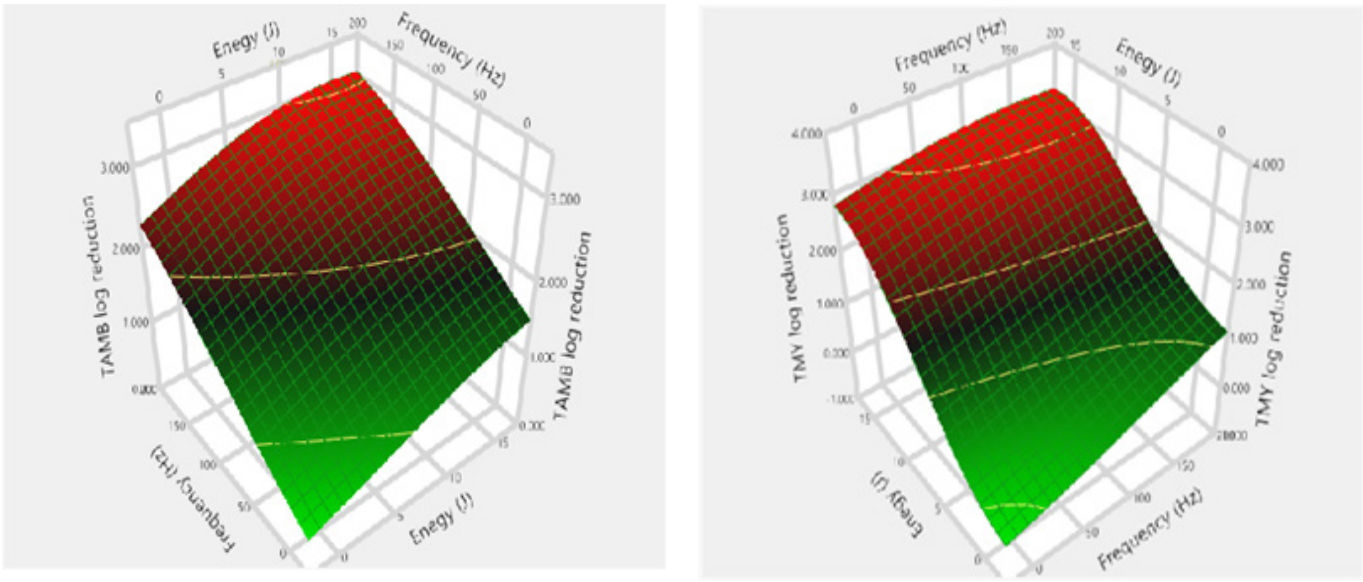

Figure 3. Inactivation of cucumber seed endogenous microflora treated by PEF a) total aerobic mesophilic bacteria (TAMB) and b) total mold and yeast (TMY).

dent as it is important in transmission of applied electric fields to cell membrane. If moisture content is higher than 20%, the cell membrane remains fully hydrated. With the lower water content, on the other hand, the fluid phase could transit to a more compressed state like the gel phase in a dry seed and hydration of the seeds force it back to the fluid phase. During fluid-gel phase transition, this reorientation of membrane components could take place (41), and such reorientation of the membrane components may induce the damage repair and preserve the membrane integrity.

Electrolyte leakage of plant tissue indicating increased tissue permeability and membrane damage is utilized in seed

Table 6. Best-fit non-linear regression models for the response variables of cucumber seed samples.

Response variable	Goal	Model	R^2 (%)	R^2_{pred} (%)	SE	*p*	DW
2nd day germination (%)	Max	$90.64+0.03362X_1$	21.26	16.20	2.94	0.001	1.949
3rd day germination (%)	Max	$91.15+0.0876X_1-0.000303\ X_1*X_1$	31.14	22.38	2.22	0.008	1.578
4rd day germination (%)	Max	$93.379+0.0735X_1+0.1887X_2-0.000246\ X_1*\ X_1$	65.63	60.94	1.14	0.005	1.499
Normal seedling rate (%)	Max	$75.53+0.000623X_1*X_1$	58.89	55.83	5.05	0.000	1.596
Cold test 24 °C- 5 day (%)	Max	$68.89+0.1715X_1-0.000000X_1*X_1*X_1*X_1+\ 0.000007X_1*X_3*X_3*X_3$	43.72	33.18	4.96	0.047	1.215
EC- 4 h (µS $cm^{-1}g^{-1}$)	Target (7.30)	$7.335+0.000054X_3*X_3*X_3*X_3-0.000000X_1*X_1*X_1*X_2$	35.59	28.36	0.88	0.000	1.754
TAMB (log cfu mL^{-1})	Min	$6.026-0.01320X_1-0.000031X_3*X_3*X_3*X_3+0.000000X_1*\ X_1*X_1*X_3$	55.42	48.86	0.52	0.001	1.233
TMY (log cfu mL^{-1})	Min	$9.4140-0.01034X_1+0.000000\ X_1*X_1*X_1*X_1-0.000005\ X_3*X_3*X_3*X_3$	71.93	68.17	0.17	0.000	1.578
8th day (200mM NaCI)(%)	Max	$-0.000000+0.000043X_1-0.000001X_1*X_1*X_1+\ 0.1300X_3*X_3*X_3+\ 0.000093X_1*X_3*X_3-0.005486X_1*X_3*X_3+0.000063X_1*X_2*X_2-0.003200\ X_3*X_2*X_2+0.000000X_1*X_1*X_1*X_1+\ 0.02446X_3*X_3*X_3*X_3-0.000001X_1*X_1*X_1*X_3+0.000034X_1*X_1*X_3*X_3+0.000003X_1*X_2*X_3*X_3-0.000783X_1*X_3*X_3*X_3-0._1035X_2*X_3*X_3*X_3+0.02328X_3*X_3*X_2*X_2-0.004283X_3*X_2*X_2*X_2$	100	100	0.00	*	*
9th day (200mM NaCI)(%)	Max	$-0.000000+0.000043X_1-0.000001X_1*X_1*X_1+\ 0.1300X_3*X_3*X_3+0.000093X_1*X_1*X_3-0.005486X_1*X_3*X_3+0.00006_3X_1*X_3*X_3-0.003200X_3*X_2*X_2+\ 0.00000022X_1*X_1*X_1*X_1+0.02446X_3*X_3*X_3*X_3-0.00000_1X_1*X_1*X_1*X_3+0.000034X_1*X_1*X_3*X_3+0.000003X_1*X_2*X_3*X_3-0.000783X_1*X_3*X_3*X_3-0.1035X_2*X_3*X_3*X_3+0.02328X_3*X_3*X_2*X_2-0.004283\ X_3*X_2*X_2*X_2$	100	100	0.00	*	*
10th day (100mM NaCI) (%)	Max	$8.316+0.10445X_1-_16.635X_2-0.24945X_1*X_3+\ 0.01730X_1*X_2+\ 19.458X_2*X_3+0.6244X_2*X_2-0.024510X_1*X_3*X_2-2.570X_2*X_2*X_3-0.16956X_2*X_3*X_3-0.04402X_2*X_2*X_2*X_2+0.000006X_1*X_1*X_1*X_1+0.002509X_1*X_2*X_3*X_2+0.13530\ X_2*X_2*X_2*X_3$	99.97	99.95	0.18	0.000	1.122
10th day (200mM NaCI)(%)	Max	$0.000-3.313X_2-0.000397X_1*X_1+\ 0.2139X_1*X_3\ +0.3253X_3*X_3*X_3-0.000749X_1*X_1*X_3+\ 0.004663X_2*X_2*X_2-0.05417X_1*X_3*X_3-0.00806_2X_3*X_3*X_3*X_3+\ 0.000196X_1*X_1*X_3*X_3-0.00494X_2*X_3*X_3*X_3$	99.53	99.30	0.86	0.000	1.039
11th day (100mM NaCI) (%)	Max	$8.310-0.04121X_1+3.9408X_2*X_2-0.001377X_1*X_1*X_3-0.013595X_1*X_3*X_3-\ 0.06285X_2*X_2*X_2*X_2+0.000008X_1*X_1*X_1*X_3+0.000050X_1*X_1*X_3*X_3+0.012250\ X_2*X_2*X_3*X_2$	99.52	99.41	1.50	0.000	1.099
11th day (200mM NaCI)(%)	Max	$-1.310+0.009838X_1*X_2*X_2-0.019665X_1*X_3*X_3+0.000106\ X_1*X_1*X_3*X_3$	97.06	96.76	3.78	0.000	1.000
12th day (100mM NaCI) (%)	Max	$8.55-0.0908X_1+0.1832X_1*X_2-0.002519X_1*X_1*X_3-0.00435X_2*X_2*X_2*X_2+0.000012X_1*X_1*X_1*X_3$	94.54	93.18	5.28	0.000	0.673
12th day (200mM NaCI)(%)	Max	$-0.39+0.1158\ X_1+0.009501X_1*X_2*X_2-0.02382X_1*X_3*X_3-0.001925X_3*X_3*X_3*X_3-0.000002X_1*X_1*X_1*X_3+0.000169\ X_1*X_1*X_3*X_3$	96.54	95.66	4.96	0.000	0.869
13th day (100mM NaCI) (%)	Max	$23.61-0.1593X_1+1.699X_2*X_2+0.1132X_1*X_2-0.002365X_1*X_1*X_3-0.01960\ X_2*X_2*X_2*X_2+0.000012\ X_1*X_1*X_1*X_3$	91.66	88.83	7.60	0.021	0.905
13th day (200mM NaCI) (%)	Max	$5.32+0.615X_2*X_2-0.1553X_3*X_3*X_3+\ 0.000913X_1*X_3*X_3*X_3$	57.29	53.13	17.93	0.011	0.790

*X_1: frequency X_2: treatment time X_3: energy

Table 7. Three best solutions for the joint optimization of the 18 responses (R) as a function of the PEF treatments for cucumber seeds with the composite desirabilities of 0.868, 0.432 and 0.416.

Solution	Response variable	The three best solutions		
		1	2	3
1	2^{nd} day germination (%)	96.692	95.795	95.752
2	3^{rd} day germination (%)	97.104	97.461	97.468
3	4^{th} day germination (%)	100.136	99.879	99.957
4	Normal seedling rate (%)	95.724	90.181	89.940
5	Cold test 24 °C-5 day (%)	86.611	85.060	85.174
6	Electrical conductivity-4 h ($\mu S\ cm^{-1}g^{-1}$)	8.050	4.788	4.850
7	TAMB (log cfu/g)	3.335	4.002	4.020
8	TMY (log cfu/g)	8.168	8.374	8.369
9	8^{th} day (200mM NaCI) (%)	8.333	3.705	2.995
10	9^{th} day (200mM NaCI) (%)	8.333	3.705	2.995
11	10^{th} day (100mM NaCI)(%)	33.322	15.469	0.743
12	10^{th} day (200mM NaCI)(%)	41.715	1.849	7.273
13	11^{th} day (100mM NaCI)(%)	74.977	63.537	63.512
14	11^{th} day (200mM NaCI)(%)	85.361	43.076	49.439
15	12^{th} day (100mM NaCI)(%)	78.818	143.255	151.728
16	12^{th} day (200mM NaCI)(%)	101.526	60.239	66.096
17	13^{th} day (100mM NaCI)(%)	89.275	126.314	134.443
18	13^{th} day (200mM NaCI)(%)	91.636	23.407	26.173

vigor tests to estimate emergence of some seeds in fields. The increased EC resulted in higher leaching of solutes as well as water and nutrient uptake from soil but decreased the seed quality (41,42,54). Changes in EC by the PEF treatments and time are correlated with the changes in both membrane fluidity and membrane permeability in the cucumber seeds (3,6,48).

Both percentage and rate of germination were reduced by the increased salinity level (1,31,37). Salt tolerance in some crops was linked with antioxidant systems (AOS) (12) as it acted as the control mechanisms to reactive oxygen species (ROS) (5) lethally damaging the cell membrane in plants (62). Even though PEF-induced resistance to salt tolerance mechanism is not explained and not fully understood, antioxidant systems may have an important function for seeds to germinate even under 200 mM NaCl salt concentration.

Due to an increase in seed-related contaminations and the reduction in crop yields, alternative decontamination methods are on the high demand. The U.S. Food and Drug Administration (52) recommended the application of calcium hypochlorite solution at 20.000 ppm providing 1-3 log cfu g^{-1} inactivation for seed disinfection. The treatment of mung bean seeds by moderate temperatures is one of the most popular decontamination method in Japan (9). The application of moderate temperatures (57 or 60 °C) for 5 min provided 1 log reduction

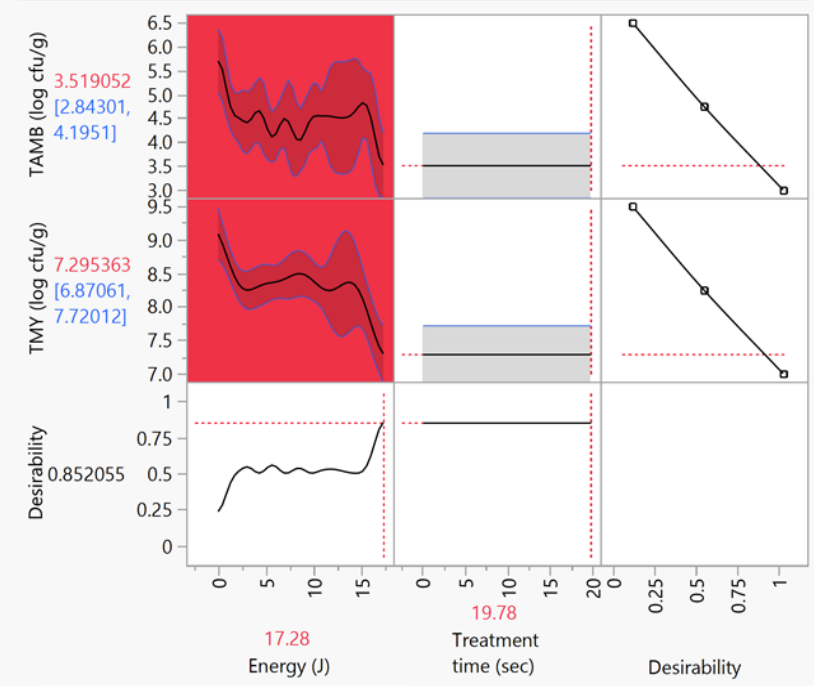

Figure 4. Optimization of Gaussian process model.

in *Salmonella* ssp. without adversely affecting germination abilities of seeds. Although the applied temperatures and treatment times were not effective to provide the seed disinfection (38), the increased temperature significantly decreased the seed germination. The combination of heat treatment with chlorine-based or organic sanitizers to achieve acceptable microbial inactivation with preserving germination abilities was not adequate for a complete inactivation of microbial load (25). Inhibitory effect of peroxyacetic acid, ethanol, fatty acids, and lactic acid for seed disinfection (14,17) was not satisfactory for seed disinfection. Among the physical treatments, cold atmospheric pressure plasma (CAPP) treatment of sprout provided an 8.8-log reduction after 10 min in *Staphylococcus aureus* and *Listeria monocytogenes*. A 5.2-log reduction in *Escherichia coli* and 1-2 log reductions in *Geobacillus stearothermophilus* endospores on lentils were accomplished with shorter CAPP treatment of 3 min (59). High pressure processing was also evaluated for seed disinfection. Application of pressure at room temperatures with 500-600 MPa for 2 min provided 3.50 log reductions on alfa alfa seeds (44). Irradiation treatment at 2.0 kGy provided 3.18 log reductions in endogenous microflora, but showed adverse effects on the germination properties and physical properties of seedling in addition to nutrient loss such as vitamin C (49). Inactivations of TMAB and total fungi (TF) ranged from 0.22 to 2.85 log in cabbage, lettuce, garden rocket, and wheat by PEF treatment (28). Both increased treatment time and frequency enhanced inactivation of *Fusarium graminearum*, *Xanthomonas campestris* pv. *campestris*, *Alternaria brassica*, and *Drechslera graminea* inoculated onto red cabbage seeds (29).

Overall, the PEF treatment enhanced germination rate and normal seedling rate with earlier germination, better body and root formations, and resistance to salt stress. EC was mostly affected by time rather than the PEF treatment. The changes in the conductivity under the different energy levels still remain poorly understood due to the existing knowledge gaps about the physiological and biochemical mechanisms of the PEF treatment for seeds. PEF appeared to influence the biochemical processes involving free radicals and antioxidant enzyme activity, thus resulting in seed invigoration (20). Adverse effects of active radicals on seed deterioration have been long known (46). Highly aggressive free radicals produced by autoxidation in dry seeds can react with the majority of biomolecules, causing cellular damages such as membrane dysfunction, and enzyme inactivation. Free radical production is elevated rapidly increasing respiratory activities resulting in oxidative stress to cellular components. The success of germination largely depends upon the activity of antioxidative systems to prevent cellular components from being damaged by the free radicals (8,11).

Modelling studies performed with PEF treatment of wheat grains revealed 93.9, 85.3, 65.0, and 58.2% variations in *A. parasiticus* % inhibition, peroxide number, b* value, and total color difference, respectively with the most optimal operational conditions of 19.58 s treatment time, 107.54 Hz frequency, and 3.84 J of, energy for the 12 responses (15). Lower PEF treatment values of frequency (161.8 Hz), energy (6.1 J), and treatment time (19.5 s) with 0.52 desirability were determined as optimal settings for PEF treated wheat grains (15).

Conclusion

Demands for a reduction in the chemical use in the agriculture and chemical-free crop production have increased recently due to their adverse effect on the environmental and public health. The PEF treatment is of a high potential for the chemical-free seed provision and organic farming as it provides healthy seeds and propagation materials. This is the first report involving effect of the PEF treatment at the different energies applied to cucumber seed with the improvement of seed vigor, germination, and salt tolerance. The PEF-treated seedling had more leaves, stronger root formation, and longer fine roots. The significant reduction in the endogenous microflora without adversely affecting the seed germination ability presented the superiority of PEF for seed vigor. The PEF treated cucumber seeds increased the germination rate by 9% and normal seedling rate by 25.73% with earlier germination. Increased salt tolerance, improved germination rate and normal seedling and shortened germination time are important indicators as they affect quality, yield, and profitability. The exact mechanism of PEF on the seed metabolism is not clear, but it is possible that membrane permeability and other metabolic activities for plant tissue might be influenced by the PEF treatment, and the impact of PEF was identical to the other stress conditions. PEF can be a feasible alternative to the chemical applications, but further studies are needed to better quantify the seed responses to PEF-related stresses and associated biochemical changes such as enzyme and free radical activities.

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