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Effects of Different Electric Field on Mycelial Growth of Isolated Strain of Wild *Ophiocordyceps sinensis*

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The fundamental interaction of electric field with materials is that electric field exerts force on the charges in the materials. All plants, animals and other creature live under an electric field because of there is an electric field between the ionosphere and the Earth. The external electric influences cause the activation of ions, forcing on dielectric compounds and the polarization of dipoles in living cells. The experiment set up has represented natural electric field and interactions between ground and clouds. High voltage generator and Aluminium metal plates were used to produce +0.2 kV/cm and +0.1 kV/cm upward electric fields and –0.1 kV/cm downward electric field. Petri dishes which included *Ophiocordyceps sinensis* strain inoculated rice and adzuki bean were exposed to particular electric fields. The highest mycelial growth was obtained rice media in –0.1 kV/cm of electric field (63.03 mm, 25 days after inoculation) followed by adzuki bean media in –0.1 kV/cm of electric field (56.25 mm, 25 days after inoculation). However, on day 25 colony diameter value for rice media in –0.1 kV/cm of electric field was significantly higher than control ($P = 0.049 < 0.05$) followed by, adzuki bean substrates in –0.1 kV/cm of electric field had significantly higher colony diameter values than control ($P = 0.029 < 0.05$). Other electric fields (+0.1kV/cm and +0.2kV/cm) on both type of media were not significantly different which measurements were taken on day 25 ($P > 0.05$) compared to control. The moisture contents of the aggregated grown mycelia were comparatively exceeded in –0.1 kV/cm of electric field treated sample in both types of media. Positive electric field treated samples have been shown less water content compared to control. This study shows that inverse electric field has significant effect on enhancement of the mycelial growth of *O. sinensis*.

Key words: Electric field; *Ophiocordyceps sinensis*; Electroporation

INTRODUCTION

The entire lives on the Earth live under an electric field because there is an electric field between the Earth and clouds or ionosphere. The naked Earth bears a large negative electric charge, generating a vertical electric field at its surface. In the fair weather area the magnitude of the electric field is –1V/cm (Biemond, 2010). Biological effects of extremely high voltage pulse electric fields and current on living organisms have been explored in many studies. Cultivation systems have been improved with electricity in the field of the green house crops such as tomato, lettuce, strawberry and various types of flowers. The electric field effects on membrane bilayers, cells, tissues and animals have been found by inductive or capacitative coupling applications of direct current (DC) or alternating current (AC) field pulses (Astumian and Hermann, 1991). Those effects and methods have some sort of synonyms and definitions which are used in current literature as follow, Electroporation (pore formation in lipid bilayers), Electropercolation (permeabilization), Electroincorporation (uptake of substances from outside in to cell), Electrotransconformation (change of conformation of membrane protein), Electrorelease (release of cell ingredients).

Electric field or pulse treatments on plants

The electro culture is a practice of exposing plants to strong electric fields and electric currents in order to stimulate growth. The beneficial effects of electric fields on plants have generally accepted (Movahed and Li, 2012). Bai *et al.* (2003) demonstrated that the electrostatic fields with certain intensity can increase the content of free radicals in seeds. Significantly higher frequencies of regeneration and morphogenesis have been noted in the electroporated protoplasts. Electric field exposed broad beans shows a considerable increase in each of the chlorophyll content and the carbohydrate amount by increasing the exposure periods, and also considerable changes in the elements level has been recorded for the exposed seeds (Hanafy *et al.*, 2001).

Application of a constant electric field, an enhancement of the mitotic activity in tomato and onion roots has been observed (Nechitailo and Gordeev, 2004). Electrical field treated hypocotyls of moth bean showed marked increase in stimulation of shoot bud formation. The electrostimulation persist over many cell generations and, enhancement of growth and organogenesis has applied in the multiplication of elite individuals and in maximizing the recovery of genetically engineered plants (Davey *et al.*, 1996). The height of stems and the length of roots are higher and longer than of those without electric field (Kiatgamjorn *et al.*, 2004). Field tests have reported greater than 10% incensement in the field

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of maize and wheat, after submitting the seeds to carefully controlled electric fields. Predominantly weak periodic electric fields are more bioactive and suitable for influencing metabolic processes, gene expression, enzyme activity and membrane transport (Shabrangi and Majd, 2009).

Electric field or pulse treatment on mushrooms

Several occasions have been reported that mycelia of fungi were treated with pulse electric field. The electrical sources such as high voltages in sinusoidal waves or pulses in mushrooms cultivation have been reported and the yields of mushroom had been increased (Farooq *et al.*, 2014; Ohga and Iida, 2001; Takai *et al.*, 2009). The pulsed power resulted to more production of fruit body in all tested mushrooms and significantly higher harvest has been achieved (Ohga *et al.*, 2004). It has been reported that the fruit bodies of treatment plots were 67% heavier than those from the control plots in the first harvest (Islam and Ohga, 2012).

Why electric field treatment use for *Ophiocordyceps sinensis* ?

The *Ophiocordyceps sinensis* fungus is grown on high elevation, around 3500 m–5000 m mount peeks (Holliday and Cleaver, 2004). In order to rising of elevation, various types of growth factors have been changed specially, temperature and relative humidity. At a place like Himalayas, the concentrations of aerosol particles are low, as a result of that the concentrations of air ions go high and negative potential gradients as well as negatively charged raindrops can be observed (Herve *et al.*, 2008). The enhancement factor of electric field is 2.75 due to the mountain itself with reference to the mountains surrounding terrain (Zhou *et al.*, 2011). The natural habitats of *O. sinensis* are exposed to natural electric field which is generated in between the Earth and clouds or ionosphere. The strength of the electric field is governed by the amount of charge of the cloud and the distance between ground and charged cloud. According to clouds separation, those clouds could be positive or negative which directly control the direction of electric field.

MATERIALS AND METHODS

Cereal grains media preparation

The rice and adzuki grain bean media were used for cultivation of *O. sinensis*. Those grains were cooked by simmering until they were soaked and began to soft and explode. After cooking, the grains were washed with cold water to remove excess starch and debris. The initial pH values of both media were measured by dissolving ruptured grains in distilled water. It was in favorable pH range in between 6–8 according to pH meter (Chioza and Ohga, 2013). Hundred grams of each media were then put into 90 mm (diameter) × 40 mm (height) of glass petri dishes. Twelve Petri dishes, each corresponding to one grain type, were prepared for both rice and adzuki bean media. All of the petri dishes with grains were sterilized at 121°C for one hour using an autoclave. Inoculation was done following an overnight cooling of the substrates. A 5 mm of diameter piece of potato dextrose agar with actively growing *O. sinensis* mycelia was placed at the centre of the surface of the substrate in each Petri dish.

Fungal materials

The fungal strain used in this study was obtained from the mushroom culture bank at the Laboratory of Forest Resources Management, Kyushu University. It is assigned to accession number KUMB1081 in the culture bank. The inoculated substrates were then incubated at 20°C (Amin *et al.*, 2008) with special electric field treatments which has been explained as followed. Colony diameter measurements were made on day 25.

Application of electric field treatment

The high voltage generator was used to create high electric field. Positive polarity output of the generator was divided to several out puts. Those output terminals were connected with Aluminum metal plates and produced different vertical electric field. Three different uniform electric fields were created. Electric field intensity of 0.2 kV/cm, 0.1 kV/cm, –0.1 kV/cm and 0 kV/cm (control) were used. The electric field intensity is,

$$E = \frac{V}{d^{\eta}}$$

$$E = {}^{\eta} * E^{max}$$

V = Voltage between electrodes (V)
d = Distance between electrodes (m)
E = Average electric field
E^{max} = Maximum electric field
η = Field utilization factor

The field utilization factor of this research was 0.8 (Kiatgamjorn *et al.*, 2004) and according to that high voltage generator was controlled to 1.25 kV output voltage to obtaining required electric fields. Low frequency (50 Hz) and prolong treatments were prepared. Electromagnetic fields can be more bioactive than continuous fields of the same characteristics and previous experiment has been conducted by negative pulses being applied from high voltage generator to soil near the plant via a high voltage lead terminated with a needle (Tikhonov *et al.*, 2004). Present experiment, two positive electric fields, one negative electric field and control were used as experiment plots. It has been reported, the number of stained cells then increased with the increase in field strength. Nearly all of the cells were stained when 1.4kV/cm of electric field was used (Teissie and Ramos, 1998). The dielectric breakdown mainly, depending on the maximal field strength as well as on the field pulse duration. The result with vertical electric field direction is higher for the stem and longer for the root than horizontal electric field direction on the bean sprout growing (Kiatgamjorn *et al.*, 2004).

The previous assumptions are being considered, low

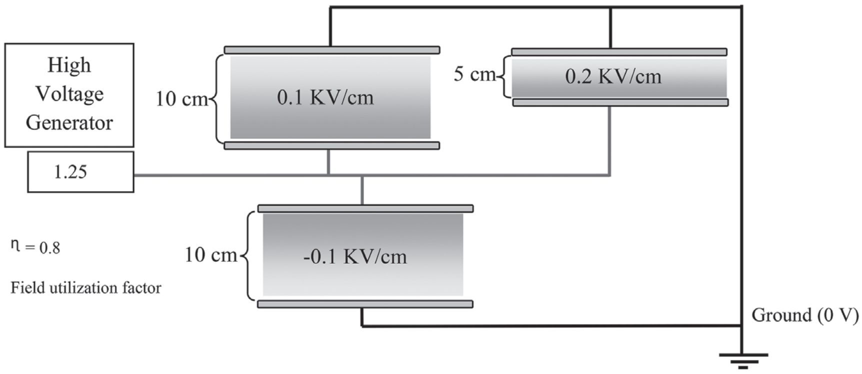

Fig. 1. The block diagram of the experimental apparatus: According to field utilization factor, component arrangements and distances between metal plates have been decided.

strength pulse electric fields were generated in this research. The direction of the field was vertical and 0.2 kV/cm, 0.1 kV/cm and –0.1 kV/cm of electric fields were produced by using Aluminium metal plates. Generation of high electric field was described by Tirtha *et al.* (2006). The high electric field generation set up which was used for studying the effect of high electric field on *O. sinensis* mycelial growth is shown in Figure 1.

Measurements and observation

Colony diameter and moisture content: The diameters of mycelial colonies were measured by using Vanier caliper which has two decimals accuracy. Perpendicular two measurements from each dish were taken. Same action was taken for all dishes. Moisture measurements were automatically calculated by Sartorius– MA 35 moisture analyzer. Identical size cylindrical piece from most upper layer of the colony was strategically obtained. A hole borer (5mm diameter), a push rod and a sharp blade were used for isolate a piece of sample. The size, shape and place where the sample has obtained were identical to each sample. The moisture contents of the top layer of samples were measured on day 25.

Data analysis

Identification of statistical differences within treatments was done by Analysis of Variance (ANOVA) followed by Tukey's post hoc test in one–way ANOVA. Two–way ANOVA was used to analyze combine effect of grains media and electric fields. The Pearson correlation analysis was done and recognized correlation between mycelial growth and moisture content of surface layer of the media. All the analyses were done with 0.05 significance levels. The analyses were done using Minitab 17 statistical software (Minitab Inc.) and Microsoft Excel. All graphs are presented with standard error bars.

RESULTS AND DISCUSSION

The measurements of the colony diameter of individual dishes and measurements of the moisture percentage on day 25 were presented in the Table 1.

Two different parameters and their results have been discussed as followed. At first, mycelial growths on rice and adzuki bean media in the presence of three types of electric fields have been discussed by utilizing results of previous research. Water content observations and possible effects by electric fields have been discussed later part.

Table 1. The measurements of mycelial growth and moisture percentage of *O. sinensis* in rice and adzuki bean media under different electric fields have been deputed. Each row represents the mean value of three replicates measured on the 25th day after inoculation. Each value represents mean ± SD of three replicates. Values in same media not sharing a letter are significantly different ($P < 0.05$).

Media type	Treatment	Colony diameter (mm)	Moisture percentage (%)
Rice	0.1 KV/cm	49.93 ± 5.95 [b]	52.43 ± 1.92 [d]
Rice	0.2 KV/cm	55.06 ± 2.46 [ab]	58.59 ± 1.29 [c]
Rice	– 0.1 KV/cm	63.03 ± 5.45 [a]	74.05 ± 4.64 [a]
Rice	0 KV/cm (Control)	50.12 ± 4.30 [b]	66.25 ± 1.86 [b]
Adzuki beans	0.1 KV/cm	50.41 ± 3.90 [ab]	48.75 ± 4.24 [b]
Adzuki beans	0.2 KV/cm	48.18 ± 5.68 [ab]	53.78 ± 2.07 [b]
Adzuki beans	– 0.1 KV/cm	56.25 ± 2.55 [a]	66.44 ± 3.79 [a]
Adzuki beans	0 KV/cm (Control)	44.25 ±4 .60 [b]	56.57 ± 2.46 [b]

Fig. 2. The actual arrangement of the experimental setup: Safety precaution has been done.

The mycelial growth of rice grain media and adzuki beans media

The rice grain media and adzuki beans media have obtained similar pattern in terms of growth. Meanwhile, rice media has acquired highest growth compared to adzuki bean media in terms of colony diameter. The *O. Sinensis* mycelial growth of the two different media in the presence of dissimilar electric fields, there is no any significant different (P > 0.05) between two types of media. Both media were shown that higher growth rate in the presence of –0.1 kV/cm of electric field.

Rice grain media

As shown in Figure 3, the highest growth in terms of colony diameter (as measured on the top surface of the substrate) was obtained –0.1 kV/cm electric field in rice media (63.03 mm, 25 days after inoculation) followed by 0.2 kV/cm (55.06 mm, 25 days after inoculation). The colony diameter measurements which were taken on day 25 showed no significant differences (P > 0.05) between control (0 KV/cm) and positive electric fields (0.1 KV/cm, 0.2 kV/cm). However, on day 25 colony diameter value for –0.1 kV/cm in rice media was significantly (P = 0.049 < 0.05) higher than their own control (0 KV/cm).

Aduzki bean grain media

As shown in Figure 3, the highest growth in terms of colony diameter was given by –0.1 kV/cm of electric field in adzuki bean media (56.25 mm, 25 days after inoculation) followed by 0.1 kV/cm of electric field (50.41 mm, 25 days after inoculation). Colony diameter measurements were taken on day 25 showed no significant differences (P > 0.05) between control (0 kV/cm) and positive electric fields (0.1 kV/cm, 0.2 kV/cm). However, on day 25 colony diameter value for –0.1 kV/cm in adzuki bean media was significantly (P = 0.029 < 0.05) higher than their own control (0 kV/cm).

Moisture content in rice media

As shown in Figure 4, the highest moisture content (as measured moisture percentage of the cylindrical piece taken from surface layer of substrate) was provided –0.1 kV/cm of electric field in rice media (74.05%, 25 days after inoculation) followed by control (0 kV/cm) of electric field (66.25%, 25 days after inoculation). Moisture percentage of each and every electric field on day 25 showed significant differences from each other (P < 0.05). However, on day 25 the lowest moisture content was represented by 0.1 kV/cm of electric field in rice media.

Moisture content in adzuki bean media

The adzuki bean media in figure 4, the highest moisture content was granted –1 kV/cm of electric field (66.44%, 25 days after inoculation) which was significantly different (P < 0.05) with control (0 kV/cm) of electric field (56.57%, 25 days after inoculation) and other two electric fields (0.1 kV/cm, 0.2 kV/cm) in adzuki

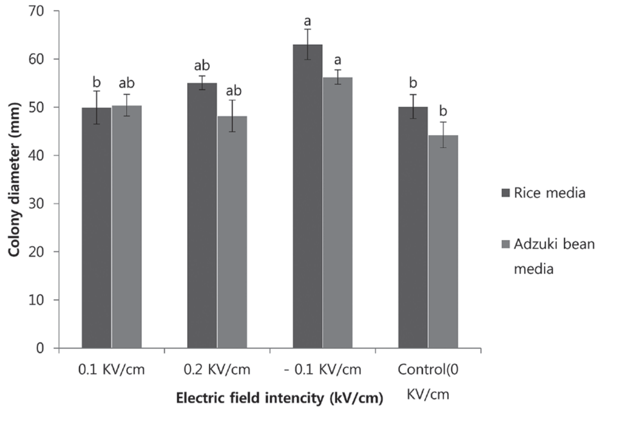

Fig. 3. Mycelial growths of *O. sinensis* in rice and adzuki bean media under different electric fields have been deputed. Each bar represents the mean value of three replicates measured on the 25th day after inoculation. Values in same media not sharing a letter are significantly different (P < 0.05). The error bars represent standard error.

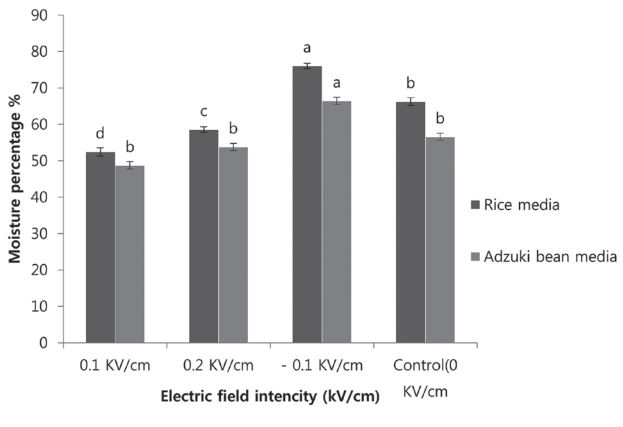

Fig. 4. Moisture contents of *O. sinensis* mycelial harvest in rice and adzuki bean media under different electric fields have been shown. Each bar represents the mean value of three replicates measured on the 25th day after inoculation. Values for each media not sharing a letter are significantly different (P < 0.05). The error bars represent standard error.

bean media on day 25. Moisture percentage of positive electric fields (0.1 kV/cm, 0.2 kV/cm) on day 25 showed no significant differences ($P > 0.05$) with control (0 kV/cm).

It was observed that significant effect which has been done by down ward electric field. It is still not clear whether the observed effects of the electrical treatments were simply the result of a metabolic change induced by the electric field applied at the growth stage. Previous researches results have to be taken in to consider clarifying suggestions that can be made.

Electric field effect on cell membrane

The cell membrane is most important outer cover and contains functional protein modules imbedded in a bimolecular lipid sheet or bilayer. The bilayer serves both as an electrical insulating and diffusion barrier as well as a supporting matrix for the protein modules (Hans and Chilcott, 2002). Almost all chemical and enzymatic metabolic reactions are taken place in cellular sap. If cellular sap is facilitated with required molecular, ions and energy, the metabolic reactions would have to be forwarded for desirable results. The site of the electric field interaction is the lipid portion of the cell membrane (Weaver and Chizmadzhev, 1996). The literature is currently lacking a computationally efficient method for predicting the molecular motion of large molecules in diverse matrices influenced by electric fields.

The thickness of a plasma membrane is estimated to be about 7–8 nanometers. Typical value for trance membrane potential ($\Delta\Psi$) across the membrane of a resting cell is around 70 mV, corresponding to the electric field strength of ~10^7 V/m inside the membrane. In contrast, the dipole potential ΨD changes sharply across the head group area resulting in much stronger electric fields, on the order of 10^9 V/m. Therefore, it is possible that the mechanically induced changes in the much stronger electric field of the dipolar potential ΨD could be capable of exerting strong influence on the conformational dynamics of membrane proteins (Warshaviak *et al.*, 2011). It has been calculated that a voltage difference much larger than 200 mV could cause dielectric breakdown.

The extremely high electric field strengths in a cell membrane may give rise to electrostriction effects in proteins imbedded in the bilayer and this can have profound effects on the molecular organization of the proteins in these membranes (Hans and Chilcott, 2002). Electroporation was attributed to electrically mediated pore formation, which conferred an increased capacity for the treated mycelia to take up specific components from the surrounding culture medium. A decreasing in membrane thickness influences its dielectric breakdown (Moon and Chung, 2000). It results in a dramatic increase both in membrane conductance, permeability and structural changes of membrane. That could also be involved in molecular uptake process. The molecular transformations underlying the dielectric breakdown may be reversible or irreversible. The Pulsed electric field exposures create irreversible membrane permeabilization that allows for bulk flow of macromolecules (Chen *et al.*, 2006).

The dipolar potential and bulk flow of micro molecules across the cell membrane may have increased the concentration of the required inputs those responsible for forwarding desired reactions. In this context, previous findings and the present results suggest that the up taking of media components was indeed enhanced after electrically stimulating the protoplasts and was probably due to a change in membrane permeability.

Electric field effect on cell metabolism

High voltage, short duration electrical pulses stimulate DNA synthesis in isolated higher plant protoplasts (Davey *et al.*, 1996). Identical effects on fungus nucleus can be expected. Though *O. sinensis* represents multi nucleus structure, increasing of nuclear component and DNA may have direct effect on size of the protoplast and division of them. In order to that the rate of cell division and growth may also be higher in the electro treated protoplasts (Kiatgamjorn *et al.*, 2004).

Electric treatments are assumed to enhance seed vigor by inducing the biochemical processes that involve free radicals and by stimulating the activity of proteins defined as hetero polymers of amino acids, they contain a mixture of neutral, polar, and charged side chains (Shabrangi and Majd, 2009). As the results, mushroom fruit bodies were actively developed (Ohga and Iida, 2001). The enzymes are also proteins that are folded into complex shapes that allow smaller molecules to fit into them. Structures of protein have positive and negative charge points according to types of amino acid that particular proteins have been constructed. Those charge points and active site may have been affected by electric field hence enzymatic activities might be increased.

Electric field effect on ions of the cell

The basic mechanism is the forced vibration of all the free ions on the surface of a cell plasma membrane, caused by an external oscillating field (Shabrangi and Majd, 2009). It has been reported that external electric influence both the activation of ions (Moon and Chung, 2000). Tikhonov *et al.*, 2004 proposed that generation of NAI by plants could be enhanced by electrization of soil by high voltage pulses, thus providing for the high level of NAI. The concentration of NAI generated by plants increased upon electrical stimulation by a factor of hundreds and thousands over the background level. Different plant species had different capacities to generate NAI in response to electrostimulation. Surface charge is the electrical potential difference between the inner and the outer surface. There are many different processes which can lead to a surface being charged, including adsorption of ions and the application of an external electric field. Electric field, which causes particle repulsions and attractions, is responsible for many colloidal properties (Butt *et al.*, 2006).

The effect of electric field direction on the growth of mycelia is presented. Negative electric field stimulated highest mycelial growth. Similarly, the result with vertical electric field direction is higher for the stem and longer for the root than horizontal electric field direction on the bean sprout growing (Kiatgamjorn *et al*., 2004). Both sides of every cell membrane, there are large numbers of free ions (K^+, Na^+, Cl^-, Ca^{2+}, etc…) which control the cell volume, play an important role in signal transduction processes. However, dielectric properties or permittivities are intrinsic properties that determine the interaction of electromagnetic energy with materials (Guo *et al*., 2010). The weakening or strengthening of the hydrogen bond can be explained in terms of a high polarizability of the molecular orbital with respect to the direction of the electric field applied (Mata *et al*., 2009). The hydrogen bonds of building materials of the *O. sinensis* may be affected by electric field and induced their propagation in order to uplift mycelia growth.

Electric field effect on water potential

The Identical water content in every sample of individual grain media has to be presented. The reason for diverse moisture content measurements which were taken on day 25 would be the characteristic effect of exposed electric field. The electric field has much higher effect on water molecules which is considered as base of the life. The electric field, temperature, pressure or density can cause fluctuations in the strength or distance of the hydrogen bond of water (Mallik and Chandra, 2008). It has been identified some interaction between water and electric fields. Water related studies have been used electric fields effect on water because it would not be negligible. Electrical potential (Ψe) results from the electrical field which may be subjected during laboratory and field experiments. The mycelial growth and physiological condition were judged from water potential at the time of electric impulse treatment. The water potential (Ψ) of the substrate has clear relationship to capacity of fruit body formation (Ohga *et al*., 2004).

Interfacial water, which is close to macromolecules and membranes, being part of a network of biological interfaces, are dynamically oriented and exhibit restricted motion (bound). In consequence, the mobility and the ordering of water molecules are very different from those of pure bulk or “free” water (Sun, 2000). The electrical fields interact with ions in the xylem sap and can cause indirect effects on water. For example, such effects exist in electro osmosis. Electrical forces play a very important role in heterogeneous systems near cell walls and membrane surface. Same effect can be occurred on *O. sinensis* mycelia. High concentration of ions might have been regulated around surface area where high interaction has been taken place by electric field. In order to high ion concentration of mycelia nearby surface area, water potential of particular area has to be increased. That may be the reason for having significant higher water content nearby surface area of sample media which were treated by negative electric field.

The electric field can be used to phase transition and deform water droplets (Takeda *et al*., 2002) to change the adsorption velocity (Yoshida *et al*., 2009). Same type of effect can be expected in top layer of grains media. The phase transited or deformed water droplets or molecules which were influenced by electric field, might have high opportunity to being inserted in to mycelial body with increased absorption velocity. In order to that growth and developments of the mycelia have to be accelerated.

CONCLUSION

Detailed knowledge of the precise mechanisms of action of electrical pulses and fields on the stimulation of growth and morphogenesis in fungi are still lacking. Despite some ideas about the underlying physical processes and biochemical explanation, the complete understanding of the mechanism underlying the accelerated mycelia growth and development is lacking. One of the most important applications of cell electroporation is cell transfection which is nanopores created in the cell membrane are used as a pathway to insert biological molecules into the cell. The downward electric field makes force on negative ions and desirable molecule, and holds them nearby surface area by creating nutrient and reactant rich micro environment. Whatever treatments are done to enhance the growth, considerable output will not be given otherwise it is not affected on range of necessary nutrients intake specially, major and trace elements, organic compounds and water. So, treatments should be simultaneously induced feed intake of the cell or organism. The possible synergistic effects of electrical and chemical parameters require further investigation.

AUTHOR CONTRIBUTIONS

The whole manuscript was written by Sanath Gamage who designed and developed the research and experiment setup. Shoji Ohga advised and supervised whole research and manuscript. Both authors assisted in editing the manuscript and approved the final version.

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