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The Effect of Moderate Pulsed Electric Field (PEF) on Microscopic Visualization of Glandular Trichome of Patchouli Leaves

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Abstract: Patchouli (*Pogostemon cablin*, Benth) is an herbal plant and become a leading export commodity. Almost 80% of the world's patchouli oil needs are supplied from the Indonesia. Patchouli oil is stored in the Glandular Trichomes (GT) which exist in all parts of the plant. The study aimed to determine the effects of pulsed electric field on the GT cells damage. The design of research was a completely randomized design and factorial treatments. The first study was conducted by voltages on 500-2000 V, exposure times of PEF 2 and 3 sec at a frequency of 500 Hz. The second study, treatment of 500-2000 Hz frequency, exposure times PEF 2 and 3 sec on the voltage of 500 V. The third study combined treatment mains voltages of 500, 750, 1000 and 2000 V, frequencies 500, 1000 and 2000 Hz and exposure times PEF 2 and 3 sec. The raw material in the form of fresh patchouli leaves the age of 7 months after planting and taken leaves of old. Data analysis is using software of SPSS 19 Version and to investigate the changes of GT cells used Scanning Electron Micrograph (SEM). The results showed that the number of GT cells on the lower surface of leaves (abaxial) and the upper surface of the leaf (adaxial) are 16-19 and 6-9 cells, respectively with a diameter of ±60-63 μm. In the area of 1 cm^2, the number of GT cell on abaxial and adaxial leaves are ±800 and ±355 cells, respectively or mean number of GT cells on whole leaves is ±1.155/cm^2. The electric field strength of PEF treatment has greater influence on GT cell damage than the frequency. PEF treatment by electric field strength of 125 V cm^{-1} makes GT cells patchouli damage. PEF frequency treatment makes GT cells wrinkled and PEF strength makes GT cell wall are erupted.

Key words: Patchouli, PEF, glandular trichome cells, SEM, frequency

INTRODUCTION

The patchouli plant Indonesia spread in the island of Sumatra, Java, Borneo and Celebes. The volume of production both the patchouli it is estimated that 800-1,000 MT per year. Patchouli oil is the result of secondary metabolites and stored in the glandular cells called trichomes (Schilmiller *et al*., 2008; Rusydi *et al*., 2013; Lange and Turner, 2013). It is applied in the pharmaceutical industry, food, fragrances, soaps and cosmetics (Donelian *et al*., 2009) as a fixative of other essential oils (Paul *et al*., 2010) and an aromatheraphy materials in two last decade.

The electroporation technology using a Pulsed Electric Field (PEF) has been applied in extraction process of agricultural products and the process of microbe sterilization of food and beverages. Electroporation has a promising future because almost no damage to the composition of the nutritional value of the product. The applications of PEF to the cell structure of plant tissues have been conducted on vegetables (Kusnadi and Sastry, 2012) disintegration of cells of plants and microorganisms (Toepfl, 2011), tobacco seeds (Hohenberger *et al*., 2011; Janositz and Knorr, 2010), the network red beet-root (Fincan *et al*., 2004), Alfalfa plants (Gachovska *et al*., 2009), apple slices (Chalermchat *et al*., 2010), wine volatile compounds (Garde-Cerdan *et al*., 2013) and grape seed polyphenols (Boussetta *et al*., 2013). However, the application of PEF on prior to the patchouli oil extraction has not been found.

In PEF process, the cell shape parameters and electrode distance has the effect on the extraction (Boussetta *et al*., 2013). PEF strength (E) and time (t) were identified as the main parameters in the processing shows the effect of the threshold electric field strength while the specific energy input (W) can be applied as a dose parameters. This condition depends on the application technique, the energy input required to permeabilization

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plant tissues and value in the range of 5-10 kJ kg^{-1} in solid materials and ±200 kJ kg^{-1} in liquid was reported by Toepfl (2011). The damage of the cell membrane as a result of PEF treatment was called 'electroplasmolysis' (McLellan *et al*., 1991; Angersbach *et al*., 2000). This treatment is based on the effect of the formation of the electric induction and growth of pores in the cell membrane as a result of the application of an electric field (Weaver and Chizmadzhev, 1996). The electroplasmolysis cellular network has grown rapidly with the development of a PEF application before processing (Barbosa-Canovas *et al*., 1999). PEF treatment of the biological tissue was performed to facilitate the pressing (McLellan *et al*., 1991; Knorr *et al*., 1994; Bazhal and Vorobiev, 2000; Bazhal *et al*., 2001; Bouzrara and Vorobiev, 2001) to increase difusities (Gulyi *et al*., 1994; Jemai and Vorobiev, 2002) drying (Bordiyan, 1991; Ade-Omowaye *et al*., 2001) and osmotic dehydration (Rastogi *et al*., 1999). It is important to note the application of the PEF, the electric field strength, pulse shape and the number of pulses is crucial to improve the technological process.

Chalermchart *et al*. (2010) say that to destroy tissue slices of apple necessary electric field strength ±900 V cm^{-1}. The optimal value of intensity PEF and the number of pulses in an attempt to break the cell walls of Glandular Trichoma (GT) patchouli leaves as the oil reservoir is very useful for determining the minimum energy required f or Patchouli oil extraction (Turk *et al*., 2012; Buckow *et al*., 2011). The study aimed to determine the effects of pulsed electric field (electric field strength and number of pulses) on the GT cells damage. Pretreatment with pulsed electric field strength and the number of pulses is expected to reduce energy consumption while extracting and obtained the high distillation efficiency.

MATERIALS AND METHODS

Material: Patchouli plant varieties Sidikalang (Aceh) obtained from the experimental garden in Kesamben, Blitar, East Java, age 7 months after planting. The samples were old leaves which taken as much as 2-4 leaves from the bottom.

Design of research: The design of research was a factorial completely randomized design with three replications to study the effect of voltage and exposure time PEF and interaction of each other to GT cells damage of patchouli leaves.

Pulsed electric field treatment: Pulsed electric field treatment on patchouli leaves with equipment as illustrated in Fig. 1. Patchouli leaves with conditions such as the stems of plants are placed into the chamber. The position of the leaves in the middle between the cathode-anode and the generator is turned on pulsed electric field according to treatment were tested. The first study to investigate at the influence of a voltage and exposure time PEF to change GT cells, performed at 500-2000 V power supply voltages, exposure times PEF 2 and 3 sec with a fixed frequency of 500 Hz. A second study to determination the effect on the frequency of GT cell changes of patchouli leaves, performed at a frequencies of 500-2000 Hz with exposure times PEF 2 and 3 sec on the fixed voltage equipment (500 V) and the cathode-anode distance of 10 cm. The third study was a combination of voltages, frequencies and PEF time exposure, respectively 500, 750, 1000 and 2000 V and 500, 1000 and 2000 Hz and 2 and 3 sec on the GT cells damage.

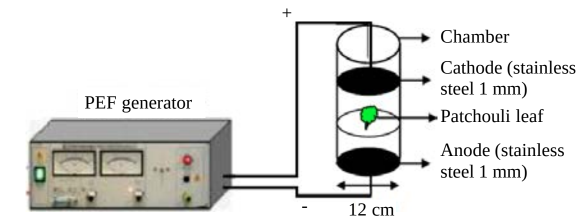

Fig. 1: PEF generator treatment

The microscopic analysis: Microscopic observations made with fresh leaves before and after PEF treatment using a Scanning Electron Micrograph (SEM) FEI-Inspect S25-EDAX. Fresh patchouli leaves picked from the stalk and taken to the laboratory, cut 1×1 cm then performed using a sputter coating with gold coated SC-7620 (Emitech). Other leaves after PEF cut 1×1 cm and made of gold using a sputter coater coating SC-7620 (Emitech). Samples were subsequently observed above.

The calculation of the number of GT cells: The calculation of the number of GT cells performed on patchouli leaves before and after PEF manually based on the results of SEM. The GT cell number is calculated from the average of 3 replications.

The data analysis: The data were analyzed using both ANOVA and Duncan test by a level of confidence of 95% ($p<0.05$) through a statistical software of SPSS 19 Version.

RESULTS AND DISCUSSION

The number of Glandular Trichomes (GT) cells: The GT cells are observed by SEM. The result shows that the GT

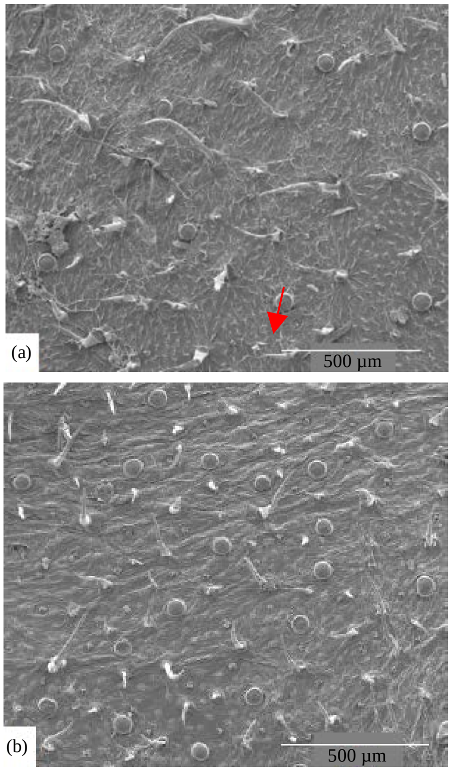

Fig. 2: Number of GT cells on: a) adaxial and b) abaxial patchouli leaves 200x

cells on the lower surface of leaves (abaxial) and the upper surface of the leaf (adaxial) is 16-19 and 6-9 cells, respectively. It has a diameter of 60-63 µm in width ±1500×1500 µm. This means that in an area of 1 cm^2, the number of GT cells is approximately ±800 for abaxial and ±355 for adaxial or mean number of GT cells is ±1155/cm^2 (Fig. 2).

The abaxial GT cell number is almost 2 times than adaxial and shows that the amount of oil stored in the abaxial also greater. Sandes states that the patchouli oil produced in peltate glandular trichomes located on both sides of the leaves and are round or oval. Patchouli glands are peltate glandular trichome types, located in the abaxial and adaxial and the number is very large and contains essential oils. The existence of the GT cells includes the number and density is very important for plants because it is a place to store patchouli essential oil. Glandular trichomes contain enzymes that synthesize compounds typical of mono-terpenes and sesqui-terpene and serves as a component of essential oil (Zaks *et al.*, 2008). The amount of oil in the leaves is higher than in stems and twigs of patchouli (Rusycli *et al.*, 2013; Paul *et al.*, 2010).

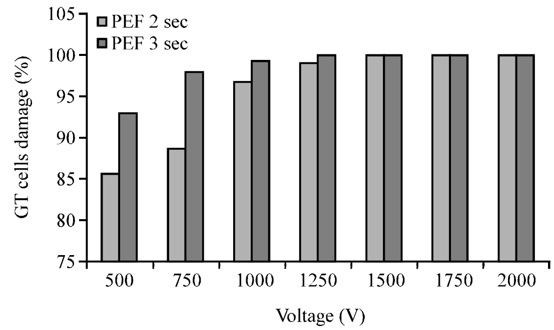

Fig. 3: GT cells damaged after voltages and exposure PEF times treatment

Table 1: Duncan analysis of the GT cells broken by voltages, frequency and exposure times

Duncan (GT cells broken)	
Voltage	Frequency
500^{a}	500^{a}
750^{b}	750^{b}
1000^{c}	1000^{c}
1250^{c}	1250^{d}
1500^{c}	1500^{e}
1750^{c}	1750^{f}
2000^{c}	2000^{f}

Percentage of GT cell damage due to PEF treatment: By the treatment of PEF with the voltage above 1,250 V for 2 sec, the GT cells of patchouli leave was 99% damage. When the exposure time was increased to 3 sec with a voltage of 1,250 V, all of the GT cells were become damaged (Fig. 3). To break/damage GT cells patchouli leaf, the conditions required voltage ±1,250 V or electric field (E) ±125 V cm^{-1} with a frequency of 500 Hz or number of pulses (n) ±1,500.

Statistical analysis shows the value of R^2 was 0.843 (84.3%) on significant level (α) of 0.5, this result indicates that voltage (electric field strength = E) plays a role in GT cell damage. Electric field strength is applied to the already capable to damage the cells of the patchouli leaf tissue and the electric field strength values above 125 V cm^{-1} visible all GT cells are damaged/broken. The Increasing of voltage >1,250 V with a frequency of 500 Hz and 3 sec PEF time exposure has a major influence on the amount of GT cell damage. The GT cells damage are presented in Fig. 3 and the results of the Duncan statistical analysis are presented in Table 1.

The significant interaction of treatment between voltage and exposure PEF times, this indicates that the patchouli leaf GT cell damage due to voltage treatment is very large. Patchouli GT cell damage caused by the difference the dielectric constant between in the GT cell and outside the cell (Jiahui *et al.*, 2009). GT cell patchouli with an electric field strength of 125 V cm^{-1} lower than for onions damage of 333 V cm^{-1} (Ersus and Barrett, 2010),

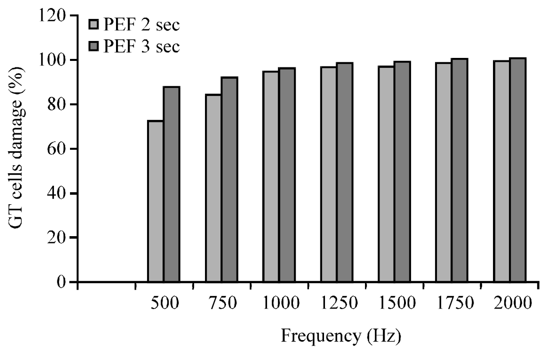

Fig. 4: GT cells damaged after frequencies and PEF times exposure treatment

whereas for the apple slices is 900 V cm^{-1} Chalermchat *et al*., 2010). Duncan test showed that the voltages of 500, 750 and 1000 V indicate difference GT cell damage whereas treatment voltage of 1000-2000 V there was no difference against GT cell patchouli damage. This shows that the GT cell damage of patchouli starts at voltages >1000 V (Table 1).

The effect of frequency and PEF times exposure (number of pulses = n) to the GT cell damage was investigated. After PEF treatment with some frequency variations and exposure times, the GT cells undergo changes in shape. Based on the results of Scanning Electron Microscope (SEM), it is known that by the frequency of 1,500 Hz treatment for 3 sec (n = ±4,500), most GT cells (98.7%) has been damaged/creased (but not broken) and 1.3% still intact while by the frequency of 2,000 Hz for 2 sec (n = 4,000), some 99% of GT cells damaged/wrinkled and 1% are still intact (Fig. 4). Duncan test showed the treatment frequency from 500-1,750 Hz, there are differences in the effect on GT cell damage of patchouli while between 1,750 and 2,000 Hz there is no difference (Table 1).

The GT cells damage due to the number of pulses (frequency) to the patchouli does not like the effect of a voltage because the influence of the number of pulses only occurs shrinkage in GT cell wall while the influence of a voltage to the GT cell as the ball broke (Fig. 5c). The effect of frequency has also been reported by Kulshrestha and Sastry (2010) on the potato slices and stated that no samples showed a marked change in dielectric constant with frequency of 5-20 kHz. The influence of the electric field strength (E) and the number of pulses (n) to the outbreak of onion tissue cells reported by Ersus and Barrett (2010) and stated that the number of cell rupture increases with increasing number of pulses. A total of 92.2% of the cells of the onion broke out after the application of an electric field of 333 V cm^{-1} and 100 pulses. Rupture of cells is essential for the optimal design process before extraction of the desired compound and/or drying plant tissue. The SEM results of the GT cells damaged by exposure to the PEF as shown in Fig. 5.

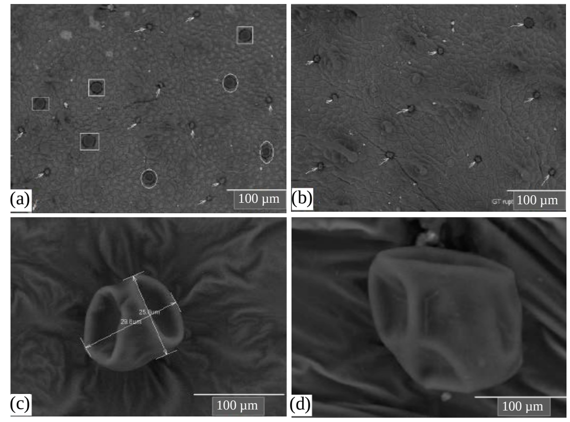

Fig. 5: a) The GT cells condition by PEF 1.500 V, 500 Hz, 2 sec (100x); b) the GT cells condition PEF 1.500 V, 500 Hz, 3 sec (100x); c, d) the GT cells broken/rupture (1.500x)

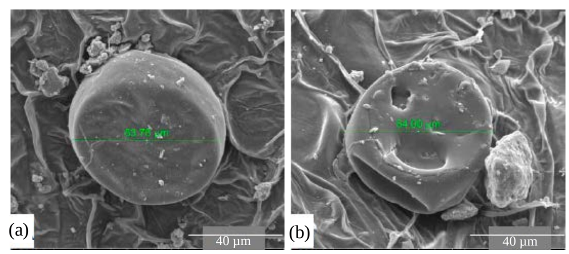

Fig. 6: a) GT cell structure before and b) after PEF treatment (2.500x)

The GT cell damage due to PEF treatment: After exposure to PEF, the GT cells are changes (Fig. 6). GT cells shape of patchouli become wrinkled or broken. It used as the basis for calculating the value of the electric field strength (E) which causes GT cell patchouli damage. The GT cells as patchouli oil storage started to crimp on a pulsed electric field of ±50 V cm^{-1}, the number of pulses (n) = 1,150 and erupted at E = 100 V cm^{-1} with n = 1,000 (Fig. 7). The changes in short time against GT cell patchouli damage assessment are very interesting because in 1 sec increments, it led to changes in the real GT cells. Bazhal suggest the optimal time PEF application for apple slice tissue damage estimated at <1 sec. Pulsed electrical pulses applied to the patchouli leaf intended to create a network of increased GT cell wall porosity, so the essential oils contained in the oil storage cells are taken and this is very important in the industry.

The application of electric field strength (E) is a non-thermal process and increase the value of E and a

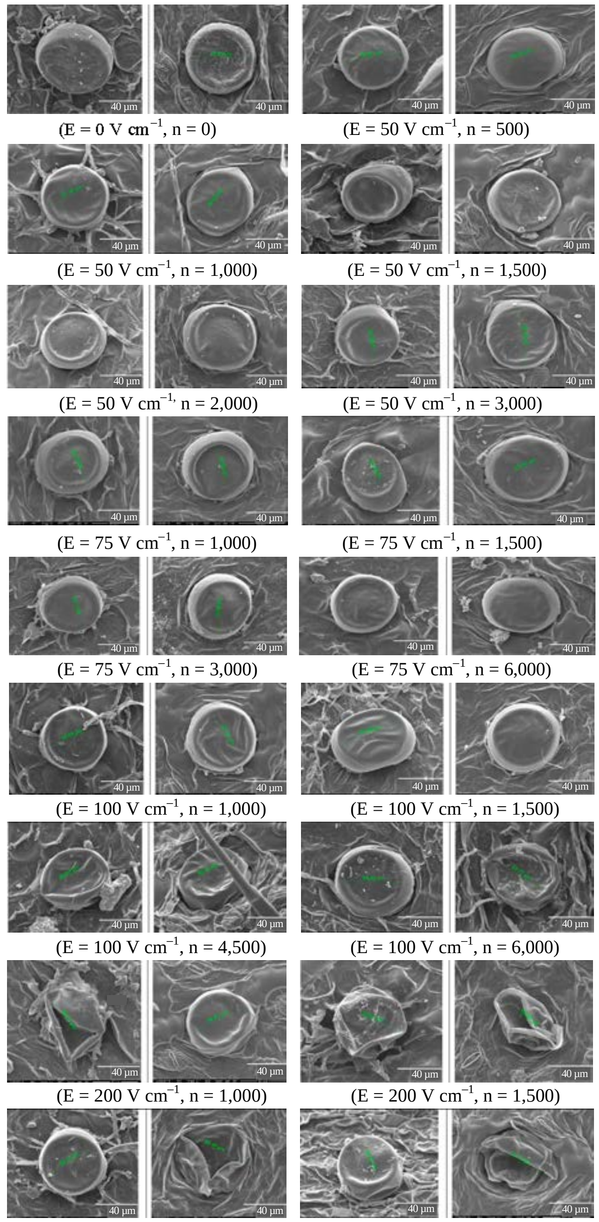

Fig. 7: Adaxial and Abaxial GT cell changes due to pulsed electric field (2.500x)

resulting increase in energy received materials, so that the larger GT cell damage (Walkling-Ribeiro *et al*., 2008). Bazhal *et al*. (2003a, b) examine the damage to some plant tissues obtained results E optimum value of each plant is different, is influenced by the type of network and the required value of E is higher when there is a secondary network. Optimal value E (E_{opt}) for fruit 500-600 V cm^{-1} except pears and bananas E_{opt} = 900-1,100 V cm^{-1} whereas for apples, cucumbers, carrots and potatoes value E_{opt} = 200-400 V cm^{-1}.

Electric field strength (E) is an important parameter which also controls the electroplasmolysis cellular network efficiency. Bazhal and Vorobiev (2000) classified PEF into 3 categories: low (E = 100-200 V cm^{-1}), medium (E = 300-1,500 V cm^{-1}) and high (E>1500 V cm^{-1}). The PEF application of the highest electric field and long time was cause of damage and loss quality of products. The optimization methods of electric field strength have not been many studies that reported by Lebovka *et al*. (2000). Patchouli GT cell damage due to pulsed electric field is low because the patchouli leaf tissue is soft tissue composed of cellulose. This finding is new data for GT cell patchouli damage and PEF treatment value of electric field strength E of ±125 V cm^{-1}.

The effect of pulsed electric field strength of the GT cell is determined by the of patchouli oil components. Patchouli Alcohol (PA) is the most important component of patchouli oil and the results of the analysis of the level of acidity (pH) of 6.5-6.8. The PEF effect on the GT cells look like a deflated ball (erupt) and tends to lead to the negative pole. At pH conditions mentioned above, patchouli oil has a positive pole (slightly acidic) and when subjected to a pulsed electric field, the oil tends toward the negative pole electrode (anode) to GT cell damage wrinkled form. The movement of patchouli oil molecules occurs due to the dielectric constant difference between inside and outside the GT cell as stated by Jiahui *et al.* (2009). Changes in the GT cell structure of patchouli due to the PEF treatment in a very short period of time, making it difficult for researchers determine exactly when the GT cell damage as shown in Fig. 7. It is appropriate opinion by Hohenberger *et al.* (2011) the cell damage caused by PEF is only within nanoseconds.

The application of pulsed electric field on optimal plant tissues broken was different for each type of plant and the affected by cell wall structure. The tissue damage is affected by onion epidermal cells, the number and size of the cell (Fincan and Dejmek, 2002). The amount of PEF strength, the force per unit charge between the electrodes and the voltage is proportional to the energy that passes through the electrodes. PEF strength value depends on the applied high-voltage pulse and the size of the chamber. The higher the voltage was caused the greater the electric field strength (Gongora-Nieto *et al.*, 2004). The influence of the electric field strength and the number of pulses on the cells treated separately in the GT cells results are very different. Application of pulsed electric strength on GT cell has bigger impact than the number of pulses, so that if GT cells desired do not damaged but only the cell porosity increased, it requires further research.

Optimal strength of the electric field (E_{opt}) plant depends on the type of network and higher for cells that have the secondary cell wall (Bazhal *et al.*, 2003a). Electroplasmolysis with pulsed electric field affects not only the plasmalemma membrane but also the integrity of the cell wall of apple tissue (Bazhal *et al.*, 2003b). Rupture and cell damage is determined by the magnitude of the voltage, number of pulses and the type of material as provided for by Praporscic *et al.* (2004) on the sugar beet crop. Cell damage caused by the voltage difference inside and outside the cell called trans-membrane potential (Jiahui *et al.*, 2009). The GT cells patchouli is a type of soft tissue is composed of cellulose. If passed pulsed electric field cell wall acts like a resistor.

Treatment of pulse number (n) of 6,000 with pulsed electric field (E) 50 V cm^{-1} results in GT cells shrinkage but not erupted. The GT cells damaged in electric field >100 V cm^{-1} with n = 1,000 (Fig. 7). If the electric field is continuously increased up to 200 V cm^{-1}, the cells GT looks erupted. Liu *et al.* (2014) state that the permeability of the membrane due to the electric field depends on the size of the cell. In addition, the percentage of porosity increases with increasing electric field due to the electroplasmolysis. In the electroplasmolysis, electric field strength, processing time, the electrical properties of the product, operating temperature and particle size of the product are an important factor.

CONCLUSION

Based on the results of the research, it can be concluded that the number of GT cells on the lower surface of leaves (abaxial) and the upper surface of the leaf (adaxial) are 16-19 and 6-9 cells, respectively with a diameter of ±60 to 63 μm. In the area of 1 cm^2 the number of GT cell on abaxial and adaxial leaves are ±800 and ±355 cells, respectively or mean number of GT cells on whole leaves is ±1.155/cm^2. The electric field strength of PEF treatment has greater influence on GT cell damage than the frequency. PEF treatment by electric field strength of 125 V cm^{-1} makes GT cells patchouli damage. PEF frequency treatment makes GT cells wrinkled and PEF strength makes GT cell wall are erupted.

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