In vitro shoot regeneration of tembesu (*Fagraea fragrans* Roxb.) from seed explants on different concentrations of sucrose and honey

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Tembesu (*Fagraea fragrans* Roxb.) is one of endangered tree species and has high economic value. Therefore it is necessary to have tembesu conservation efforts by planting them on a large scale, which requires a lot of seedlings. Another alternative to get the seedlings in large quantities is through in vitro culture. The effect of sucrose and honey on in vitro shoots regeneration of tembesu (*Fagraea fragrans* Roxb.) from seed explants was studied. The basal MS medium supplemented with 1 mg/l BAP (6-Benzylaminopurine) on different concentrations of sucrose (30, 40, 50, 60 g/l) and honey (6 and 12 ml/l). The result showed that treatment of 1 mg/l BAP with sucrose and honey in various concentrations can increase the number of shoots. The highest number of shoots was obtained on MS medium containing 1 mg/l BAP + 12 ml/l honey (40 shoots). The maximum shoot length was obtained on a media supplemented with 1 mg/l BAP + 30g/l sucrose. The maximum leaf number was obtained on MS medium without BAP + 30 g/l sucrose (control) and MS medium containing 1 mg/l BAP + 50 g/l sucrose.

Keywords: honey, in vitro, shoot regeneration, sucrose, *Fagraea fragrans*

INTRODUCTION

Tembesu (*Fagraea fragrans* Roxb.) plant is a potential timber crop and the fruit is beneficial in medicine. The specialty of tembesu wood include fine-textured, shiny, resistant to flooding for 10 to 15 years, as well as resistant to attack by termites and beetles. Based on the properties of wood, tembesu is included in strong class I-II and class I durable so that it can be used widely, both indoors and outdoors. Usefulness of tembesu is among others, the timber is used for building materials, furniture, bridges, ships, sculpture, and Chisel. Demand for industrial tembesu wood carving and furniture is increasing at 3,120 m³ per year, but the native distribution decreases (Wulandini et al., 2004; Wardani et al., 2006; Mindawati et al., 2014). Increased demand for woods that is not followed by the cultivation of the plants causing the tembesu plants become increasingly rare.

Therefore it is necessary to do the conservation of tembesu through planting tembesu on various fields. For that required seedlings in large quantities through plant propagation. Another alternative propagation of tembesu is through tissue culture techniques or in vitro techniques. Through tissue culture techniques large quantities of plant seedlings from small amounts of plant material can be generated (Behera and Sahoo, 2009).

Previous research on shoot regeneration of tembesu from seed explants on MS medium containing plant growth regulator of cytokinin was BAP (6-Benzylaminopurine) alone or in combination with auxin was NAA (1-Naphthaleneacetic acid). The highest number of shoots (10,53 shoots) were obtained on MS medium containing 1 mg/l BAP after 21 weeks of culture, but the shoots were small (1.42 cm)
(Fatonah and Isda, 2014). To increase the number of shoots and increase the growth of shoots, shoot regeneration needs optimization through the addition of plant growth regulator 1 mg/l BAP on MS medium and treatment of organic compounds as a carbon source that promotes growth, such as honey and increased concentrations of sucrose. The addition of an external carbon source on the culture medium enhances the proliferation of cells and regeneration of shoots (Rasheed and Yaseen, 2013). The increase in sucrose levels above 30 g/l (40, 50 and 60 mg/l) increased percentage of shoots formed. The treatment of 50 g/l sucrose on MS medium without growth regulators increases the shoot number of Citrus nobilis Kampar from cotyledon explants (Fatonah et al., 2016). The treatment of 6 ml/l of honey in medium MS promotes regeneration of shoots with the highest number of shoots at the orchid Grammatophyllum speciosum (Sari et al., 2011).

The objective of this study was to the effect of sucrose and honey on shoots regeneration of tembesu (Fagraea fragrans Roxb.) from seed explants.

MATERIALS AND METHODS

Experimental design
Optimization of in vitro shoot regeneration from seed of tembesu on optimization medium was the MS medium + 1 mg/l BAP with treatment of sucrose and honey at various concentrations, consisted of 7 treatments, namely: control (MS medium without the treatment of growth regulators + 30 g/l sucrose); 1 mg/l BAP + 30 g/l sucrose; 1 mg/l BAP + 40 g/l sucrose; 1 mg/l BAP + 50 g/l sucrose; 1 mg/l BAP + 60 g/l sucrose; 1 mg/l BAP + 6 ml/l honey; 1 mg/l BAP + 12 ml/l honey. Each treatment was repeated 5 times. The experiment was designed using completely randomized design. The basal nutrient medium consisted of MS (Murashige and Skoog 1962) medium with sucrose and vitamins, adjusted to pH 5.8 with 1 N NaOH and solidified with 0.8% agar. The medium was sterilized by autoclaving for 20 min 121° C with 17.5 psi for 15 minutes.

Source of Tissue, Preparation and Inoculation of Explants
Tembesu seeds were used as explant. Tembesu seeds were taken from mature red fruits and the pulp was removed to extract the seeds. Fruit was soaked in water, knead and filtered. Seeds were washed with running tap water for 30 minutes, and then soaked with detergent for 15 minutes. Thereafter, the seeds were rinsed three times in distilled water. In a laminar air flow cabinet (LAFC) they were dipped for 10 minutes in a fungicide (125 mg/l Dithane M-45), followed by soaking with bactericidal (125 mg/l Agarept) 0.125 g/l for 10 minutes and rinsed 3 times with sterile distilled water. Further seeds were soaked in a solution of NaOCl (bayclin) 10% for 5 minutes. The seeds were then rinsed three times in sterile distilled. Once again seeds were soaked in a solution of 70% alcohol for 2 minutes and rinsed again with sterile distilled water three times.

Seeds were inoculated in culture bottle, each bottle contains three seeds. After the inoculation of explants, culture bottles were transferred to the culture room. Cultures were maintained at 25±2°C under 24 h photoperiod was provided by cool white fluorescent light.

Observation and Statistical Analaysis
The variables observed in this study were the percentage of shoot regeneration, percentage of callus induction, days to shoot, number of shoots, shoot length, and number of leaves of shoot. Data from in vitro culture was analyzed using ANOVA (analysis of variance) and a further DMRT (Duncan's Multiple Range Test) using the SPSS computer program (ver. 16).

RESULTS

Morphogenesis potential from seed explants of Fagraea fragrans Roxb.
Research result showed that there was an effect of sucrose and honey concentration treatment on the percentage of shoot generation, callus induction and days to shoot (Table 1). The highest shoot regeneration percentage was found on seeds grown on MS medium with supplemented with 1 mg/l BAP + 12 ml/l honey (93.33%), which did not show significant difference compared to 1 mg/l BAP + 6 ml/l honey (86.66%). Treatment of 1 mg/l BAP + 30 g/l sucrose showed a little increase of shoot regeneration compared to control treatment (without BAP with 30 g/l sucrose), however sucrose concentration increase above 30 g/l (40, 50 and 60 mg/l) increased percentage of shoots from tembesu seed explant. Treatment of 6 or 12 ml/l honey increased higher shoot regeneration percentage than all of the sucrose treatment on MS medium. Treatment of 30 g/l on MS medium with 1 mg/l BAP showed higher shoot regeneration percentage compared to treatment
of sucrose with higher concentration (40, 50 and 60 g/l).

Table 1. The percentage of shoot regeneration, percentage of callus induction, and days to shoot from seed explants of Fagraea fragrans Roxb. after 28 weeks of culture on MS medium.

<table>
<thead>
<tr>
<th>BAP (mg/l)</th>
<th>Sukros (g/l)</th>
<th>Honey (ml/l)</th>
<th>Shoot regeneration percentage (%)</th>
<th>Callus induction percentage (%)</th>
<th>Days to Shoot (day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>30</td>
<td>0</td>
<td>60.01±27.88&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0</td>
<td>19.00±0.00&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td>30</td>
<td>0</td>
<td>73.34±27.88&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>26.69±9.141</td>
<td>15.4±3.13&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td>40</td>
<td>0</td>
<td>46.69±18.24&lt;sup&gt;b&lt;/sup&gt;</td>
<td>40.03±14.89</td>
<td>28.6±5.94&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td>50</td>
<td>0</td>
<td>46.69±18.24&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.02±13.96</td>
<td>23.6±1.67&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td>60</td>
<td>0</td>
<td>46.69±18.24&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
<td>28.4±2.80&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>6</td>
<td>86.66±18.26&lt;sup&gt;b&lt;/sup&gt;</td>
<td>86.66±18.26</td>
<td>28.00±2.74&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>12</td>
<td>93.33±14.91&lt;sup&gt;b&lt;/sup&gt;</td>
<td>93.33±14.91</td>
<td>27.20±2.17&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

(Mean value in the same column with different letter are statistically significantly different at p≤ 0.05 based DMRT)

Table 2. Effects of sucrose and honey on shoot regeneration and growth of shoot from seed explants of Fagraea fragrans Roxb. after 28 weeks of culture on MS medium.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BAP (mg/l)</th>
<th>Sukros (g/l)</th>
<th>Honey (ml/l)</th>
<th>Number of shoots/explant</th>
<th>Shoot length (cm)</th>
<th>Leaf number</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>0.00±0.11</td>
<td>2.55±0.03</td>
<td>9.50±2.88</td>
</tr>
<tr>
<td>1</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>18.00±3.33</td>
<td>3.80±0.60</td>
<td>7.00±1.15</td>
</tr>
<tr>
<td>1</td>
<td>40</td>
<td>0</td>
<td>0</td>
<td>16.00±2.83</td>
<td>2.75±0.02</td>
<td>6.75±1.50</td>
</tr>
<tr>
<td>1</td>
<td>50</td>
<td>0</td>
<td>0</td>
<td>18.00±2.83</td>
<td>2.72±0.10</td>
<td>9.00±1.15</td>
</tr>
<tr>
<td>1</td>
<td>60</td>
<td>0</td>
<td>0</td>
<td>05.75±2.77</td>
<td>1.12±0.19</td>
<td>7.25±2.98</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>34.25±1.37</td>
<td>1.40±0.32</td>
<td>8.00±1.41</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>12</td>
<td>0</td>
<td>40.00±2.34</td>
<td>1.17±0.32</td>
<td>5.00±0.81</td>
</tr>
</tbody>
</table>

(Mean value in the same column with different letter are statistically significantly different at p≤ 0.05 based DMRT)

Supplementation of sucrose on MS medium is generally with concentration of 30 g/l. Supplementary of sucrose above 30 g/l caused water potential decrease which caused the constraints of absorption of water, mineral, vitamin and growth regulator contained culture medium therefore it inhibited the growth and development on in vitro culture. According to Javed and Ikram (2008), sucrose treatment more than 30 g/l to culture medium induced osmotic stress which caused relative growth rate and cations (K<sup>+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup>, Mn<sup>2+</sup>, Fe<sup>2+</sup>) significantly decreased.

Callus was not formed on MS medium without supplemented with growth regulator. The treatment of 1 mg/l BAP with various concentrations of sucrose and honey on MS medium stimulated callus formation, except treatment of 1 mg/l BAP with higher sucrose concentration (60 g/l). Treatments of 30, 40, and 50 g/l sucrose showed lower callus induction percentages (20.02, 26.69 and 40%, respectively) than the 6 and 12 ml/l honey treatments (86.66 and 93.33%).

Treatment of honey in MS medium without sucrose showed higher percentage of shoots and callus formation than the treatment of sucrose. The results of this study indicate that honey can be used as a substitute for sucrose which is a source of carbon in the culture medium. The addition of honey with a low concentration showed higher yield than sucrose to increase the growth of explant form shoots and callus. This shows the ability of honey to promote growth and development. Honey contains carbohydrates as a carbon source (fructose, glucose, sucrose, maltose), polyphenols, various vitamins and various elements (Ca, Na, K, Fe, Cl, P, S, Mn) that are important in cell growth (Buba et al., 2013).

Seeds grown on MS medium supplemented with 1 mg/l BAP + 30 g/l sucrose showed the fastest day to shoots. The treatment of sucrose with a concentration higher than 30 g/l (40, 50 and 60 g/l) and the treatment of 6 and 12 ml/l of honey slowed the day to shoots, slower than the control treatment.
Higher concentration of sucrose treatment, and honey treatment, lowered the potential water on culture medium that inhibits the absorption of water, vitamins, and minerals, thus delaying shoot regeneration.

**Shooting Response from Seed Explants of Fagraea fragrans Roxb**

Results of analysis of variance showed that sucrose and honey significantly affect the number of shoots, shoot length, and leaf number of shoot. The mean number of shoots, shoot length, and leaf number of shoot from seed explants of *Fagraea fragrans* Roxb. after 28 weeks of culture on MS medium containing sucrose and honey on different concentrations are presented in Table 2. Response shoot regeneration from seed explants on different concentrations of sucrose and honey can be seen in Figure 1.

Seed explants on MS medium without plant growth regulator produced only one shoot, no additional number of shoots for 28 weeks of culture. The treatment 1 mg/l BAP with different concentrations of sucrose and honey was able to increase the number of shoots, except treatment 1 mg/l BAP with 60 g/l sucrose. Treatment of sucrose higher than 30 g/l sucrose (40 and 50 g/l)
was not significantly different with 30 g/l sucrose. The higher number of shoots is obtained on MS medium containing 1 mg/L BAP with the addition of honey, and the highest number of shoots is obtained on the honey treatment 12 ml/L (40 shoots). Shoots were axillary shoots from the leaves axis and adventitious shoots from the basal side of explants, from swelling of the cotyledons.

Shoot growth is indicated by the length of shoots and number of leaves. Seed explants grown on MS medium without plant growth regulator substances undergo elongation of shoots (2.55 cm). Treatment of growth regulators 1 mg/l BAP + 30 g/l sucrose increases the length of shoots up to 3.8 cm, whereas the increase in sucrose level above 30 g/l (40 and 50 g/l) causes no increase in the elongation of shoots, even the treatment of 60 g/l sucrose inhibits the elongation of shoots (1.12 cm). Treatment of 6 and 12 ml/l of honey also inhibits the elongation of shoots, each with shoot length of 1.4 cm and 1.17 cm.

The least leaf number of shoot was observed at the MS medium supplemented with treatment of 1 mg/l BAP at different concentrations of sucrose and honey, except treatment of 50 g/l sucrose. The highest number of leaves is obtained on MS medium without the addition of plant growth regulator and on MS medium supplemented with 1 mg/l BAP with 50 g/l sucrose. The lowest number of leaves is obtained on MS medium with the addition of 1 mg/l BAP and 12 ml/l of honey.

**DISCUSSION**

These results indicate that treatment of sucrose higher than 30 g/l did not increase the number of shoots, and did not decrease the number shoots up to a concentration of 50 g/l, while the treatment of 60 g/l sucrose decreased the number of shoots compared to the concentration of normal (30 g/l). With the same treatment (1 mg/l BAP + 30 g/l sucrose), the results of this study slightly increased the number of shoots (18 shoots) after 28 weeks of culture, when compared to the best treatment of shoot regeneration from seed explants of tembesu in the previous research (13.87) after 21 weeks of culture (Fatonah and Isda, 2014).

Response of shoot regeneration and sensitivity to sucrose depends on the species and type of plant explants. The growth and multiplication of shoots in vitro are affected by many factors, one of which is the concentration of carbon source added to the culture medium. The carbon sources serve as energy and osmotic agents to support the growth of plant tissues. The optimum sucrose concentration as an efficient carbon source has been examined in tissue cultures of some plant species, such as *Asparagus densiflorus*, *Stevia rebaudiana* in which 30 g/l sucrose enhanced shoot growth and development. Increasing sucrose concentration resulted in reduced shoot number (Preethi et al. 2011; Rasheed and Yaseen, 2013; Rahman et al. 2009). Higher concentrations of sucrose also reduced number of shoots of *Senna sophera* at 40 g/l and 50 g/l of sucrose (Parveen and Shahzad, 2014). Treatment of 50 and 60 g/l sucrose on MS medium supplemented with 2.0 mg/l BAP showed an increase in the number of shoots higher than sucrose treatment with lower concentration (40 g/l sucrose) from axillary bud explants of *Solanum viarum* (Mahadev et al., 2014).

The detrimental effect of a high sucrose concentration on shoot formation implies that the osmotic level in the medium may be inhibitory to further shoot development. Thus, high concentrations of sucrose seem to inhibit shoot growth and development. Although sucrose treatment above 30 g/l to 50 g/l does not increase the number of shoots, and does not cause any tissue damage, so it could potentially be used to regenerate shoots of *Fragraea fragrans* for efficiency of the in vitro culture through the reduction of sub-cultures. This is because the shoot regeneration of *Fragraea fragrans* is able to produce a lot of shoots, but require a longer incubation period. While the increase in sucrose levels above 30 g/l does not increase the number of shoots, if a longer incubation period goes without its sub culture it is likely to prolong the life of the shoots and prevents deaths of shoots. Increased sucrose concentrations increase the carbon supply required during in vitro cultures, thereby reducing subcultures. Sucrose is a carbohydrate as an energy source and carbon compounds required for the biosynthesis of cell metabolites. Sucrose is essential for a low photosynthetic activity of tissue during in vitro culture. Sucrose affects the physiology, growth and differentiation of cells (Rahman et al. 2009).

Treatment of honey produces more shoots than the treatment of sucrose. Treatment of 12 ml/l honey resulted more shoots (40 shoots) than 6 ml/l of honey (34.25 shoots) treatment on MS medium without sucrose. This result showed that honey can replace sucrose as a carbon source.
and is more effective to stimulate regeneration of shoots. Honey contains a variety of carbohydrates (fructose, glucose, sucrose, maltose), polyphenols, various vitamins and various elements (Ca, Na, K, Fe, Cl, P, S, Mn) are important in cell growth (Buba et al. 2013).

The results of this study showed high concentration sucrose treatment (60 g/l) and honey treatment decreased shoot elongation. Some researches indicate that shoot length decreases at higher concentration of carbon sources may be due to the inhibition of organogenesis, among other things shoot of *Stevia rebaudiana* (Preethi et al., 2011), shoots of maize (Gauchan, 2012), shoot of *Asparagus* (Rasheed and Yaseen, 2013). Higher concentrations of sucrose (40 and 50 g/l of sucrose) also reduced shoot length of *Senna sophera* (Parveen and Shazhad, 2014). Shoots induced on MS medium supplemented with 40 g/l sucrose resulted in maximum shoot length of *Solanum viarum* when compared to other concentrations. The least shoot length was observed at MS medium supplemented with 50 and 60 g/l sucrose. Shoot length declines at higher concentration of sucrose as carbon sources (Mahadev et al., 2014).

Elongation of the shoot proceeds through a combination of cell division and enlargement of the cells by the meristem. The final height of a shoot is determined by the rate and extent of the internodes. Plants are able to grow rapidly through elongation because of the structure of the plant cell. Plant cells have a large central vacuole. This central vacuole can be filled with water and expand the cell to great lengths. Reduction in cell water potential can decrease cell expansion (Sharp and Davies, 1979). Increasing levels of sucrose to 60 g/L and treatment 6 and 12 ml/l can reduce water potential and decrease cell expansion, resulting in the inhibition of the elongation of shoot.

Visual observations indicate that shoots that grow on MS medium without plant growth regulator showed larger leaves than other treatments. The treatment of 1 mg/L BAP in MS medium with different concentrations of sucrose showed leaf size greater than the honey treatment. This is related to the number of shoots that grow on each treatment on MS medium. Tembesu seeds grown on MS medium without plant growth regulator produced only one shoot, while the seeds grown on MS medium containing 1 mg/l BAP with the addition of 6 or 12 ml/l of honey produce many shoots (respectively 34.25 and 40 shoots). Treatment of 1 mg/l BAP + 12 ml/l of honey that produces the lowest number of leaves (5 leaves) also showed the highest number of shoots (40 shoots). In contrast, the control treatment that produced the highest number of leaves showed the lowest number of shoots. It shows that the shoots with small number (one shoot) in a culture bottle resulted in higher leaf growth as there was no competition for nutrients in the MS medium needed for the growth of shoots. Many shoots in the culture medium result in competition among the shoots to compete for nutrients so, so leaves grow more slowly. Moreover, the addition of cytokinin (1 mg/l BAP) increased cell division and shoot regeneration, whereas without plant growth regulator does not result in differentiation to form new shoots, but more differentiation to form new leaves and leaf enlargement.

These results indicate that the best shoot regeneration is obtained on MS medium containing 1 mg/l BAP + 12 mg/l of honey with the highest number of shoots (40 shoots). Shoots formed from this treatment are smaller, with shorter shoot length and smaller leaf size, and different color of the younger shoots as compared to treatment 1 mg/L with the addition of sucrose at various concentrations.

Some important things obtained from this study include: 1) the treatment 1 mg/l BAP + 50 g/l sucrose and treatment 1 mg/l BAP + 12 ml/l honey can be used by mikroproplantation tembesu from seed explants to reduce the subculture because the supply of carbon source is higher and the number of shoots formed is higher ; 2) the treatment of 1 mg/l BAP + 50 g/l and treatment 1 mg/l BAP + 12 ml/l honey on MS medium can be used in plant conservation tembesu in vitro short term (Slow-growth system), for as long as 28 weeks of culture without any subculture, no damage and no death of shoots, and if the culture age is enhanced to a few weeks, death of the shoot is unlikely to occur; 3) the treatment of 1 mg/l BAP + 30 g/l can be used for micropopagation with a shorter time, that is, without going through the stages of elongation shoot or rooting ex vitro because the results of this treatment have been already obtained which is 18 shoots per explant with the highest length of shoots (3.8 cm); 4) the treatment of 1 mg/l BAP + 12 mg/l of honey can be used in mikropopagation of tembesu to produce a lot of shoots, because after stage shoot induction for 28 weeks of culture without any subculture can produce shoots in a very large number (40 shoots), and if the
multiplication is done for each of these shoots, shoot multiplication medium is then performed to obtain more shoots more; 5) for the purpose of micropropagation, in vitro shoots that are formed by treatment of 1 mg/l BAP + 12 ml/l of honey then disubkultur for elongation of shoots or rooting in vitro, because of the results of these treatments, produced very small shoots (1.17 cm).

CONCLUSION
The treatment of 1 mg/l BAP with 12 ml/l honey showed the highest shoot formation percentage (93.33%). The treatment of 1 mg/l BAP with sucrose and honey in various concentrations can increase the number of shoots. The treatment of 30 to 50 g/l sucrose produce more shoots than the treatment of 60 mg/l of sucrose. The highest number of shoots was obtained on MS medium containing 1 mg/L BAP + 12 ml/l honey.

CONFLICT OF INTEREST
The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS
SF determines research ideas, is responsible for the preparation of tools and materials, conducting research, and writing research papers. MNI helps the preparation, conduct of research, and correct the writing. SF and MNI read and approved the final version.

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