In vitro growth response of Patchouli (Pogostemon cablin) cultured in medium containing Methylobacterium spp. filtrate

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Abstract. Mass seeds production through micropropagation can support modern agriculture, but this technique is expensive and largely determined by a medium formulation. The addition of synthetic Plant Growth Regulator (PGR) has a residual effect, meanwhile natural PGRs is more secure but expensive. In vitro testing of PGR activity obtained from bacteria was the first step to substituted synthetic PGRs to become nature PGR. Research to observe PGR activity obtained from four strains of Methylobacterium spp that had been analyzed of the capability of PGRs synthesis was done on in vitro patchouli cultured. There are three activities: 1) The sterilized technique of filtrate using filtration techniques, humid heat, and a combination of both; 2) Testing of Methylobacterium spp. from that production of cytokinins and compared its activity with commercial PGRs (BA and zeatin); and 3) auxin (NAA and IAA). The research showed that strain TD-J2 filtrate added to medium was similar to BA activities, and TD-J7 was similar to zeatin. Auxin activity derived from strain TD-J10 showed the capacity to induces root formation. The research result indicates that synthesis PGR derived from Methylobacterium spp. has similar activity with commercial PGRs.

1. Introduction

The development of modern farming is currently very fast because agriculture can be done in sub-optimal land. The progressive expansion of planting land causes the demand for superior seeds to increase rapidly as well. The demand for superior seeds in large quantities, simultaneously and uniformly, is difficult to fulfill with conventional propagation, so innovation for this is needed. Micropropagation is an alternative to meet the demand for these seeds, although the technique is largely determined by the formulation of the medium.

The medium formulation for micropropagation is a factor that determines the success of mass and uniform seed production as well as determining the price of the seed [1]. Until now, most chemicals-synthetic that are used to make medium in the in vitro technique, are imported materials and the price depends on fluctuations of US dollar values. Besides, procurement often faces obstacles in the import
process so that sometimes some materials are difficult to obtain in the local market or require a long time to purchase. At present the use of natural substitutes to replace expensive synthetic materials in tissue culture mediums has begun to be widely used such as the use of seaweed products [2], microalgae *Chlorella sorokiniana* [3], and *Ascophyllum* [4].

Furthermore, the use of synthetic chemicals is generally difficult to degrade in plant tissues derived from *in vitro* propagation. This often has a residual effect on growth regulators when plants are planted in the field. The residual effect of these growth regulators often causes the vegetative phase of plants to be longer, this does not cause big problems when plants are harvested in the vegetative phase but harvesting reproductive products such as flowers and fruit is often an obstacle. The use of natural growth regulators is safer for plants because it can be quickly absorbed and utilized then the residue is quickly degraded so there is no residual effect on the plants it produces. Although it is safer, the price of natural growth regulators is very expensive and often difficult to obtain so that it will increase the selling price of seeds obtained from *in vitro* techniques.

The substitute of commercial natural growth regulators with other substitutes that have the same activity but the price is cheaper, easier to obtain, and faster absorbed, utilized, and degraded by plant tissue is one of the important things when using *in vitro* techniques to provide mass and uniform seedlings. Many research results have reported the activity of growth regulators secreted by several types of microbes in their life cycle [5].

The use of microbes on a plant *in vitro* culture has been widely used but it is more as a selection component in increasing resistance to biotic stress [6,7]. Until now many toxin components from pathogens have been isolated, whereas other products from microorganisms have not been widely used in *in vitro* culture of plants [5].

*Methylobacterium* spp. or known as PPFM - Pink Pigmented Facultative Methylotroph is a normal microbiota in phyllosphere in almost all types of plants, mosses, and ferns [8]. The presence of these bacteria in the phyllosphere of several tropical plants in Indonesia is quite high, ranging from 105 CFU / gram of leaves [9]. PPFM bacteria have characteristics as facultative methylotrophs in nature; its presence spreads significantly in several types of plants in quite large quantities; stimulates growth; plays a role in nitrogen fixation; triggering seed germination; stimulates root growth and can synthesize cytokinins [5]. This bacterium also has a redox cofactor that is characterized by antioxidants and vitamins (methoxantin) [10]. *Methylobacterium* spp. bacteria are reported to be able to produce growth regulators from the cytokinin group [11,12], auxin, GA [13] also produce vitamin B12 [14]. Even a little, these bacteria have begun to be used in *in vitro* culture such as in rice culture [15], *Nicotiana. tabacum, Lycopersicon esculentum, Sinapis alba* and *Fragaria vesca* [14], and wheat.

Seventeen isolates from the phyllosphere of rice, maize, soybean, cucumber, sweet potato, red chili peppers, beans, avocados, pumpkins, white eggplants, and tomatoes that grow in East Kalimantan isolated successfully [16]. Some isolates were then tested for their ability to synthesize growth regulators and the interim results showed that some isolates were able to synthesize cytokinins, auxins and finally were known to be able to synthesize Gibberelllic Acid (GA3).

Patchouli (*Pogostemon cablin*) is a very responsive plant for *in vitro* culture. Media formulations for propagation, root induction, and callus formation were obtained at ICABIOGRAD. These plant cultures give very different responses when certain PGR are added. So that the testing of PGRs activity produced by bacteria can be done on patchouli patches *in vitro*. Testing is done by comparing the response of patchouli buds to media with the addition of PGR synthetic and commercial natural with the media added with the filtrate of several bacterial strains that have been analyzed for PGR synthesizing.

This study aims to observe the response of single node patchouli (*P. cablin*) on media containing commercial PGRs compared with the response to the media with the addition of *Methylobacterium* spp. filtrate.
2. Methods

The research was conducted in the *in vitro* culture laboratory of the Research Group of Cell and Tissue Biology, Indonesia Center of Agricultural of Biotechnology and Genetic Resources Research and Development, ICABIOGRAD.

Plant material used is patchouli *in vitro* shoots (BB BIOGEN collection). The PGRs producing bacteria tested were *Methylobacterium* spp-PPFM (BB BIOGEN CC), with the tested strain originating from East Kalimantan and isolated from the surface of “Siam” squash and corn leaves.

The study was conducted in 3 experiments to find out the growth response of patchouli single node culture by different sterilization technique of *Methylobacterium* sp filtrate strain TD-L2; the use of *Methylobacterium* spp strains TD-J2 and TD-J7 from East Kalimantan producing cytokinins on the growth of patchouli single node culture; the use of *Methylobacterium* sp strains TD-J10 from East Kalimantan producing auxin on the growth of patchouli three-node culture.

2.1. The growth of patchouli single node culture by a different technique of sterilization of *Methylobacterium* sp filtrate strain TD-L2

Experiment Plant material was *in vitro* shoots of patchouli (*P. cablin*). The explant was a single node of the patchouli culture, 8 weeks after culture. The filtrate used was derived from a strain of the bacterium *Methylobacterium* sp isolated from the phyllosphere of “siam” squash leaves.

The basic medium were MS medium containing PGRs, 6-Benzyladenine-BA; 1-Naphthaleneacetic-NAA; Indole-3-acetic acid-IAA at a concentration of 0.1 mg/l as a comparison. To observe activities of bacterial filtrate after sterilization *in vitro* culture, TD-L2 strains were used with a concentration of 10; 20; 30; 40;50% (v/v culture media). Three sterilization techniques of bacterial filtrate were tried: sterilization with filter sized 0.22 µM; humid heat by autoclaved; filtered and autoclaved at a 1.5 psi for 20 minutes. Three single nodes were cultured in the medium treatment; each treatment was repeated 3 times. The culture was incubated in the incubation room in light conditions with a fluorescent light, 16 hours/day, at temperature of 21-25°C. Observation of number and height, number of shoots, number of leaves, the formation of roots and callus, carried out every week for four weeks after culture.

2.2. The use of *Methylobacterium* spp strains TD-J2 and TD-J7 producing cytokinins on the growth of patchouli single node culture

The plant material used in this study was a single node of patchouli cultured on the medium without the addition of plant growth regulators. The basic medium was MS with the addition of 30 g/l sugar and solidified with 2.5 g/l phytagels (Sigma).

The bacterial strain was *Methylobacterium* spp from East Kalimantan TD-J2 and TD-J7 strains that isolated from the corn leafphyllosphere. TD-J2 and TD-J7 filtrate sterilized by humid heat by autoclaved at 121°C for 20 minutes. Concentration of bacterial filtrate addition in culture medium was 0, 10, 20, 30%, as control were MS0, MS + BA 0.1 mg /l, MS + Zeatin 0.1 mg /l and bacterial growth medium. Three single nodes were cultured in the medium treatment; each treatment was repeated 3 times.

The culture was incubated in the incubation room in light conditions with a fluorescent light, 16 hours/day, at temperature of 21-25°C. Observation of number and height of shoots, number of leaves, the formation of roots and callus, carried out every week for eight weeks.

2.3. The use of *Methylobacterium* sp strains TD-J10 producing auxin on the growth of patchouli three-node culture

Plant materials used in the study were three nodes patchouli that was cultured on the medium without the addition of plant growth regulators. The basic medium was MS containing 30 g/l sugar and solidified with 2.5 g/l phytagels.
The bacterial strain was *Methylobacterium* sp from East Kalimantan TDJ10 strain isolated from the corn filosphere. The filtrate sterilized by humid heat by autoclaved at 121°C for 20 minutes. Concentration of bacterial filtrate addition to culture medium was 0, 10, 20, 30%, as control were MS0, MS + 0.1 mg/l NAA, MS + 0.1 mg/l IAA and bacterial growth media. Three single nodes were cultured in the medium treatment; each treatment was repeated 3 times.

The culture was incubated in the incubation room in light conditions with a fluorescent light, 16 hours/day, a temperature of 21-25°C. Observation of shoot time initiating, number and height of shoot, number of leaves, number and length of roots, callus formation, carried out every week for eight weeks.

3. Results and Discussion

3.1 The growth of patchouli single node culture by a different technique of sterilization of *Methylobacterium* sp filtrate strain TD-L2

Preliminary research (data not presented) showed constraints of the level of sterility of bacterial filtrate for use in tissue culture which requires a high level of medium sterility. The sterilization technique must also consider whether the materials contained in the filtrate are thermolabile or not so that the materials are not damaged or decreased inactivity after media sterilization.

Table 1. Growth Response of patchouli single node on activity testing bacterial filtrate TD-L2 strain of *Methylobacterium* sp., 4 Weeks after culture

| Sterilize technique       | Average of shoots numbers | Average of shoots height | Average of nodes numbers | Averages of leafs number | Root formation | Callus formation |
|--------------------------|---------------------------|--------------------------|--------------------------|-------------------------|----------------|------------------|
| MS+BA0.1mg/l             | 14.3                      | 0.4                      | 12.1                     | 3.4                     | -              | -                |
| MS+IAA0.1mg/l            | 3.7                       | 0.3                      | 6.7                      | 3.6                     | +              | ++               |
| MS+NAA0.1mg/l            | 7.3                       | 0.3                      | 8.3                      | 12                      |                | ++               |
| Filter 10%               | 1.3                       | 0.3                      | 2.2                      | 4.0                     | ++            | +                |
| Filter 20%               | 0.2                       | 0.1                      | 1.0                      | 2.8                     |               | -                |
| Filter 30%               | contaminated              |                          |                          |                         |               |                  |
| Filter 40%               | -                         | -                        | -                        | -                       |               |                  |
| Filter 50%               | contaminated              |                          |                          |                         |               |                  |
| Filter+Otklf 10%         | 1.1                       | 0.4                      | 2.3                      | 5.3                     | ++            | ++               |
| Filter+Otklf 20%         | 2.5                       | 0.6                      | 5.3                      | 0.5                     |               | +                |
| Filter +Otklf 30%        | 0.6                       | < 0.1                    | 0.6                      | 2.0                     |               | +                |
| Filter +Otklf 40%        | -                        | -                        | -                        | -                       |               | ++               |
| Filter+Otklf 50%         | contaminated              |                          |                          |                         |               |                  |
| Otklf 10%                | 3.3                       | 0.2                      | 4.7                      | 10.3                    | +             |                  |
| Otklf 20%                | -                        | -                        | -                        | -                       | +             |                  |
| Otklf 30%                | 1.0                       | < 0.1                    | 1.0                      | 1.3                     |               | ++               |
| Otklf 40%                | 0.75                      | 0.2                      | 1.0                      | 2.0                     | ++            | +                |
| Otklf 50%                | 1.0                       | 0.2                      | 1.0                      | 2.0                     |               | +                |

Note: MS= Murashige and Skoog, 1962; BA = Benzyladenine; IAA= Indole-3- acetic acid; NAA=1-Naphthaleneacetic; Filter = filtration by 0.22 µM; Filter+Otklf = Sterilize by filtration and humid heat; Otklf = Sterilize by humid heat; - = not formed; + = formed
The activity of bacterial filtrate on \textit{in vitro} patchouli culture was observed by comparing it with synthetic plant regulators from the cytokinin (BA) and auxin (NAA and IAA) groups. After four weeks, the single node culture showed various responses at the trial medium formulations as presented in table 1.

From the results of the analysis conducted by [17], TD-L2 is a \textit{Methylobacterium} sp bacterium isolated from the phyllosphere of “Siam” squash leaves with high levels of auxin, 12, 68 ppm by spectrophotometer and HPLC. The TD-L2 strain is also able to synthesize cytokinins quite high at around 49.74 ppm [17].

Sterilization by 0.22 µm filter shows a higher risk of contamination compared to sterilization by autoclaving which utilizes humid heat at a pressure of 1.5 psi or equivalent to 121°C for 20 minutes. This happens because the higher of filtrate concentration is added to the MS medium, which will prolong the filtration process, thereby increasing the potential for contamination.

Filter sterilization shows good results on the addition of filtrate to a concentration of 20%, but the addition of a higher concentration will increase contaminated trigger. Contamination occurs in cultures with high concentrations of addition 30 and 50% v/v media.

Table 1 shows that the response of patchouli explant growth was still visible even though the filtrate has been heating. The activity of bacteria produced in the filtrate is still visible, and can be seen in the formation of callus and growth of shoots, although not as good as in the medium with the addition of growth regulators BA (cytokinins) (figure 1). In rice culture, the addition of \textit{Methylobacterium} sp. to \textit{in vitro} culture can increase callus growth [15]. These results indicate that the bacterial strain used in this test can synthesize auxin with sufficient concentration to be able to influence the growth of patchouli culture. In several reports it is known that many strains of \textit{Methylobacterium} sp. can synthesize auxin [15].

![Figure 1](image1.png)

**Figure 1.** The growth response of patchouli culture to the plant growth regulators and Filtrate of TD-L2 strain of \textit{Methylobacterium} sp. from East Kalimantan with different filtrate sterilization techniques. A. Medium with 0.1 mg/l BA; B. Medium with 0.1 mg/l Zeatin; C. Medium with 0.1 mg/l NAA; D. Medium with 10% v/v TD-L2 strain filtrate; E. Medium with 20% v/v TD-L2 strain filtrate. F. Medium with 50% v/v TD-L2 strain filtrate (contaminated)

In the preliminary results it is known that the addition of filtrate with a low concentration of 10 to 30%, has shown the activity of the compounds produced by \textit{Methylobacterium} sp. in patchouli culture even though the strain used, TD-L2, does not include producing plant growth regulators with the highest concentration.
Sterilization with humid heat (autoclaves) at 121°C for 20 minutes does not inactivate the filtrate activity in patchouli in vitro culture. Additions in concentrations of more than 30% tend to trigger the growth of other microorganisms, contamination of both bacteria and fungi.

3.2 The use of Methylobacterium spp. strains TD-J2 and TDJ 7 producing cytokinins on the growth of patchouli single node culture

After analyzing the levels and types of plant growth regulators using a spectrophotometer and HPLC Waters, it is known that almost all Methylobacterium spp. collected by BB BIOGEN/ICABIOGRAD can synthesize plant growth regulators from the auxin and cytokinin groups either one or both with different concentrations [16]. The strain that can synthesize the highest cytokinins is used to test whether the cytokinin has the same activity as commercial synthetic and natural cytokinins, the strain is TD-J2 [17] and was isolated from the corn leaf phyllosphere.

At the beginning of culture, almost all cultures did not show growth response, both in the control medium and medium containing the filtrate. Even on medium with the addition of filtrate, some cultures withered. This happens because of the influence of methanol which is part of the bacterial growth medium and it is used as an ingredient in making the filtrate in this study, has caused symptoms of withering in culture. These symptoms of withering stopped after few weeks cultured.

After four weeks planting, a different response was observed from patchouli explants (table 2). No growth was observed in the explants cultured on growth medium or bacteria. This shows that there is no material dissolved in bacterial culture medium that can affect the growth of patchouli explants.

The growth response of explants cultured on the medium with the addition of filtrate was better than the growth of explants cultured on the control medium (without the addition of synthetic PGR or filtrate), except for callus formation (table 2).

Shoot initiation of explants cultured on medium with filtrate were faster compared to the medium with the addition of BA 0.1 mg/l. The average number of initiated shoots and several nodes was also higher than that of the medium with the addition of BA 0.1 mg/l. This will be very beneficial for mass seed propagation. Direct root formation in the media and low callus formation make this medium formulation even better for mass seed propagation.

The identification and measurement by chromatography, revealed that Methylobacterium sp. strain TD-J2 can synthesize growth regulator cytokinins, trans zeatin, which is high, 89.21 ppm [16]. After being sterilized at 121 °C, the activity in patchouli in vitro culture showed that it can substitute the activity of BA even for single node culture. Growth response up to 4 weeks after culturing was better than BA (Table 2; Figure 2). These results are similar to the reports of Koenig et al. [11] and Ivanova et al. [12] who reported that many strains of Methylobacterium sp. were able to synthesize cytokinins. This good growth response is suitable for the need for mass patchouli seedlings.

Patchouli culturing is carried out until the 8th week, to see whether the filtrate activity of patchouli culture disappears or remains compared to the addition of synthetic plant growth regulators. After 6 weeks of culture, observations can no longer be counted quantitatively because the growth of shoots is very large and rosette, so it is difficult to count each shoot. Growth measurement is done by converting the percentage of shoot growth area to the area of the medium jar base (table 3.).
Table 2. Growth response of single node patchouli on medium containing *Methylobacterium* sp.strain TD-J2 strain, 4 Weeks After Cultured

| Medium                          | Average of shoot initiation (WAC) | Average of shoot numbers | Average of height shoot (cm) | Average of nodes numbers | Average of Leafs numbers | Root formation | Callus formation |
|---------------------------------|-----------------------------------|--------------------------|-----------------------------|--------------------------|--------------------------|-----------------|-----------------|
| MS+BA 0.1mg/l                   | 2.5±0.2                           | 1.6±0.1                  | 0.1±0.05                    | 5.2±1.9                  | 10.4±3.9                  | -               | ++              |
| MKB                             | 0                                 | 0                        | 0                           | 0                        | 0                        | -               | -               |
| MS without filtrate             | 3.0±0.2                           | 2.4±0.3                  | 0.3±0.1                     | 9.6±3.6                  | 18.8±7.1                  | ++              | ++              |
| MS+10% filtrate                 | 2.3±0.3                           | 4.0±0.3                  | 0.2±0.1                     | 10.8±4.1                 | 51.6±19.5                 | ++              | +               |
| MS+20% filtrate                 | 2.3±0.3                           | 6.0±0.1                  | 0.2±0.1                     | 15.6±5.9                 | 31.6±11.9                 | ++              | +               |
| MS+30% filtrate                 | 2.2±0.3                           | 5.2±0.2                  | 0.2±0.1                     | 12.4±4.7                 | 24.4±9.2                  | ++              | +               |

Note: MKB = Bacteria culture médium; WAC = Weeks after culture; - = not formed; + = formed a little; ++ = formed a lot

Figure 2. Growth Response of single node patchouli in medium formulations with the addition of TD-J2 strain *Methylobacterium* sp. strain from East Kalimantan, 4 weeks after cultured. A. Medium with 0.1 mg/l BA; B. Bacterial medium; C. MS Medium without PGRs; D. Medium with 10% v/v TD-J2 strain filtrate; E. Medium with 20% v/v TD-J2 strain filtrate; F. Medium with 30% v/v TD-J2 strain filtrate
Table 3. Growth Response of single node patchouli in medium containing TD-J2 strain *Methylobacterium* sp., 8 weeks after cultured

| Medium                | Average Percentage of media area covered by shoots | Average Percentage of media area covered by roots | Average Percentage of media area covered by callus |
|-----------------------|----------------------------------------------------|--------------------------------------------------|---------------------------------------------------|
| MS+BA 0.1mg/l         | 30.0                                               | 2.4                                              | 62.4                                              |
| MKB                   | 0                                                  | 0                                                | 0                                                 |
| MS without filtrate   | 40.8                                               | 18.4                                             | 41.6                                              |
| MS+10% filtrate       | 40.0                                               | 20.8                                             | 23.2                                              |
| MS+20% filtrate       | 67.6                                               | 19.2                                             | 27.6                                              |
| MS+30% filtrate       | 55.2                                               | 16.0                                             | 18.4                                              |

Note: MKB = Bacteria culture médium; MS with 30% TD-J2 filtrate; WAC = Weeks after culture;

After 8 weeks of culture, it turns out that the activity of bacterial filtrate in culture persists and tends to remain better than the medium with the addition of synthetic cytokinins, BA. MS medium with the addition of 20% filtrate gave the best shoot formation response (67.6%) compared to medium with other filtrate and control medium, MS0 and MS + BA 0.1 mg / l.

Callus growth in the medium with the addition of filtrate turned out to be faster, so that in the 8th week of culture, callus growth was faster than root growth, although the formation of this callus was still less than the control medium (without PGR).

The very fast growth of shoots causes a large number of shoots and these shoots are difficult to elongate. This phenomenon is very well related to the mass production of seedlings because each shoot can be separated for the elongation of shoots and the formation of roots.

To observe the trans zeatin activity synthesized by *Methylobacterium* sp. on *in vitro* tissue culture, the filtrate of the TD-J7 bacterial strain based on measurements was able to synthesize the trans-zeatin quite high. Zeatin is a thermolabile compound so that sterilization is not done by autoclaving but using a filter with a size of 0.22 µm. As a control to observe the filtrate activity used by MS media with the addition of 0.1 mg/l Zeatin. From observations, it was seen that the addition of zeatin to the single node patchouli culture induced a very rapid callus formation, so that the growth of shoots was inhibited, both at the time of initiation, number and height of the shoots and nodes (table 4).

Single nodes cultured on a medium enriched with bacterial filtrate showed the same callus formation response as explants cultured on the zeatin-enriched medium. However, the single node cultured on the medium with filtrate less than 30% still showed shoot growth with an average shoot growth similar to the response of explants on the medium without the addition of PGRs. Shoots that grow on medium with filtrate show inhibition in their growth, because at the base, a callus is formed which grows rapidly and inhibits shoot growth. In the medium with the addition of 30% filtrate, explants showed the same callus growth and inhibition of shoot formation as the control medium. These results indicate that the zeatin produced by strain TDJ7 has just shown the same results as zeatin 0.1 mg / l at 30% concentration. Callus formed from explants cultured on the medium with the addition of filtrate showed a better callus performance than the callus produced on the medium with the addition of zeatin, which was indicated by a more friable callus performance with yellowish-white color. In rice callus culture, the addition of *Methylobacterium spp* can increase callus growth [15]. Generally, the yellowish-white and crumb callus will be more easily regenerated than the compact callus [18].
Table 4. Growth response of single node patchouli on medium containing *Methylobacterium* sp. strain TD-J7, 4 Weeks After Cultured

| Medium                          | Average of shoot initiation (WAC) | Average of shoot numbers | Average of shoot height (cm) | Average of nodes numbers | Average of leaf numbers | Root formation | Callus formation |
|---------------------------------|-----------------------------------|--------------------------|----------------------------|--------------------------|-------------------------|----------------|-----------------|
| MS+ 0.1 mg/l zeatin             | 4.0±1.5                           | 1.0±0.4                  | 0.1±0.06                   | 1.0±0.4                  | 2.2±0.9                 | -             | ++++            |
| MKB                             | 0                                 | 0                        | 0                          | 0                        | 0                       | -             | -               |
| MS without filtrate             | 1.33±0.5                          | 2.7±1.1                  | 0.8±0.4                    | 10.9±4.1                 | 21.7±2.2                | +             | +               |
| MS+10% filtrate                 | 2.0±0.8                           | 3.3±1.3                  | 0.1±0.06                   | 6.3±2.4                  | 12.3±4.7                | -             | ++++            |
| MS+20% filtrate                 | 3.0±1.2                           | 3.0±1.2                  | 0.4±0.2                    | 7.0±2.7                  | 12.0±4.5                | -             | ++++            |
| MS+30% filtrate                 | 5.0±1.9                           | 0.3±0.2                  | 0.2±0.1                    | 3.3±1.2                  | 6.7±2.6                 | -             | ++++            |

Note: MKB = Bacteria culture medium; WAC = Weeks after culture; - = not formed; + = formed; ++++= formed very much

Figure 3. Growth Response of single node patchouli in a medium formulations with the addition of TD-J7 strain *Methylobacterium* sp. strain from East Kalimantan. A. Medium with 0.1 mg/l zeatin; B. Bacterial medium; C. MS Medium without PGRs; D. Medium with 10% v/v TD-J7 strain filtrate; E. Medium with 20% v/v TD-J7 strain filtrate. F. Medium with 30% v/v TD-J7 strain filtrate.

The testing with high trans zeatin producing strains, resulting that the addition of the filtrate cannot be used for plant propagation purposes but is better for plant breeding. Improvement of plant characteristics *in vitro* is generally carried out at the cellular stage where it is necessary to have an embryonic callus induction technique that can regenerate one cell into a complete plant.
At the 8th week of culture, the explant growth response was still the same as the 4th week. At the 8th week, the growth of the shoots remained stunted without the formation of roots and the visual callus that formed was still constant. Callus in media with bacterial filtrate is more embryonic than callus from media with zeatin which is shown in yellowish-white with several nodular green and friable callus.

3.3 The use of Methylobacterium sp. strains TD-J10 from East Kalimantan producing auxin on the growth of patchouli three-node culture

The addition of low concentration auxin, 0.1 mg/l, into the medium for patchouli culture and several other plant species, is generally added for induction of root formation so that the plantlets formed can be acclimatized. Some strains of the Methylobacterium sp. can synthesize auxin [16]. Many strains of Methylobacterium are capable of synthesizing auxin (16), including TD-J10, a strain originating from East Kalimantan. This strain is capable of synthesizing auxins up to 15.14 ppm (16) which is high enough for the species of Methylobacterium. Up to 4 weeks after culture, explants cultured on the medium with the addition of synthetic auxin were not able to form shoots but only formed roots from week 2 (Table 5). Meanwhile in the medium with the addition of TD-J10 filtrate, roots also began to form from week 2, but the amount was less than the growth of roots in the medium added with synthetic auxins, even after 4 weeks of culture (Figure 4).

This occurs because the filtrate Methylobacterium sp. is not only auxin synthesized but also cytokinins and other organic materials so that cytokinin activity is still visible which is indicated by the formation of shoots and nodes [11,12,14].

Table 5. Growth response of three nodes patchouli on medium containing Methylobacterium sp. strain TD-J10 strain from East Kalimantan, 4 Weeks After Cultured

| Medium                  | Average of shoot initiation (WAC) | Average of shoot numbers | Average of shoot height (cm) | Average of nodes numbers | Average of Leafs numbers | Average of root initiation (WAC) | Average of roots numbers | Average of roots length |
|-------------------------|-----------------------------------|--------------------------|----------------------------|--------------------------|--------------------------|-----------------------------|--------------------------|------------------------|
| MS+0.1mg/INAA           | -                                 | -                        | -                          | 9.7±0.5                  | 19.2±0.5                 | 2.2±0.2                     | 12.3±0.5                 | 0.3±0.1                |
| MS+0.1mg/IAA            | -                                 | -                        | -                          | 9.6±0.4                  | 13.2±0.6                 | 3.9±0.3                     | 14.1±0.5                 | 0.3±0.09               |
| MKB                     | -                                 | -                        | -                          | 11.7±0.5                 | 23.4±0.4                 | -                           | -                        | -                      |
| MS without filtrate     | 2.3±0.2                           | 2.1±0.2                  | 0.3±0.07                   | 19.8±0.3                 | 36.0±0.6                 | 1.7±0.3                     | 4.3±0.6                  | 0.2±0.1                |
| MS+10% filtrate         | 4.0±0.5                           | 0.3±0.1                  | -                          | 15.6±0.4                 | 31.2±0.3                 | 3.0±0.3                     | 3.5±0.5                  | 0.2±0.1                |
| MS+20% filtrate         | 3.0±0.4                           | 3.6±0.4                  | 0.3±0.07                   | 19.8±0.5                 | 39.3±0.3                 | 3.0±0.2                     | 5.4±0.4                  | 0.1±0.05               |
| MS+30% filtrate         | 3.7±0.4                           | 2.7±0.2                  | 0.1±0.02                   | 18.9±0.5                 | 38.1±0.4                 | 3.5±0.4                     | 6.0±0.3                  | 0.1±0.04               |

Note: MKB = Bacteria culture medium; WAC = Weeks after culture.

Until the 8th week after culture, root formation (Table 6), as well as shoot growth, were still slightly increased. At low filtrate concentrations (20%), auxin activity was higher than cytokinin activity, this was indicated by higher root and callus growth than shoot growth. Meanwhile, at a higher filtrate concentration (30%), cytokinin activity increased, as indicated by the growth of shoots, roots, and callus which had almost the same growth rate (Table 6).
Figure 4. Growth Response of single node patchouli in medium formulations with the addition of TD-J10 strain *Methylobacterium* sp. strain from East Kalimantan, 4 weeks after cultured. A. Medium with 0.1 mg/l NAA; B. Medium with 0.1 mg/l IAA; C. MS Medium without PGRs; D. Medium with 10% v/v TD-J10 strain filtrate; E. Medium with 20% v/v TD-J10 strain filtrate. F. Medium with 30% v/v TD-J10 strain filtrate

Tabel 6. Growth response of three nodes patchouli on medium containing *Methylobacterium* sp. strain TD-J10, 8 Weeks After Cultur.

| Medium                  | Average Percentage of media area covered by shoots | Average Percentage of media area covered by roots | Average Percentage of media area covered by callus |
|-------------------------|---------------------------------------------------|--------------------------------------------------|--------------------------------------------------|
| MS+0.1 mg/l NAA         | 1.8                                               | 15.0                                             | 0.9                                               |
| MS+0.1 mg/l IAA         | 6.6                                               | 15.0                                             | 9.9                                               |
| MKB                     | 0                                                 | 0                                                | 0                                                 |
| MS without filtrate     | 11.7                                              | 16.5                                             | 16.8                                              |
| MS+10% filtrate         | contaminated                                      | contaminated                                     | contaminated                                      |
| MS+20% filtrate         | 9.9                                               | 11.7                                             | 20.1                                              |
| MS+30% filtrate         | 13.2                                              | 13.2                                             | 15.0                                              |

Note: MKB = Bacteria culture médium; WAC = Weeks after culture

4. Conclusions

The test in the activity of the filtrate *Methylobacterium spp* in several strains from East Kalimantan can be concluded as follow: Sterilization can be done with humid heat, autoclaving at a pressure of 1.5 psi at temperature of 121°C for 20 minutes without losing its activity. The addition of 20% filtrate from the strain...
which synthesizes cytokinin has better activity than synthetic plant growth regulator BA at 0.1 mg/l single
node patchouli culture. The addition of filtrate from strains that synthesize high auxin was still mixed with
cytokinin activity which can also be synthesized by the same strain.

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