Increase in vitamin levels to improve shoot growth of diploid, triploid and tetraploid taro cultured in liquid medium

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Abstract. Taro (Colocasia esculenta (L.) Schott) is one of important food crop in many countries including Indonesia, which has big diversity of taro germplasm. Tissue culture is an in vitro technique to mass-propagated propagules. Modification of the medium compositions is required to obtain the best growth of plantlets. This research was aimed to accelerate growth of taro shoot cultures by increasing of the vitamin levels added on MS liquid medium using three different ploidy levels of taro. Experiments were designed by increasing three kind of vitamin B i.e. thiamine, pyridoxine and nicotinic acid to 100-fold of the normal concentrations. Shoots were cultured for 4 weeks. Growth was determined by recording shoot numbers, petiole length, leaf and root numbers of culture. The results showed that increase in vitamin B levels affected growth of taro culture. Increase in nicotinic acid gave the best petiole length for Bentul diploid (5.39 cm), increase in pyridoxine concentration enhanced root numbers (6.7 roots). For Satoimo triploid, increase in thiamine combined with nicotinic acid gave the best for root numbers (8.7 roots). For Bentul tetraploid, increase in pyridoxine accelerated shoot numbers (1.3 shoots). Growth of Satoimo triploid was the best compared to both Bentul diploid and tetraploid.

1. Introduction

Taro (Colocasia esculenta (L.) Schott) is place as second rank of food crop in many countries in the world after cereals food crops. In tropical Asia countries, taro has been cultivated since more than 10,000 years ago [1,2]. In Indonesia, taro is a minor food crop, it consumed as snacks, but as staple food in Papua and Mentawai islands [3]. Nowadays, taro has been cultivated in many parts of Indonesia as an alternative source of carbohydrate to support the national program in Indonesia for food security.

Genetic improvement of taro to increase its productivity as well as to obtain varieties tolerant to biotic and abiotic stresses can be done by biotechnology such as by in vitro polyploidization. This technique is very useful to accelerate new varieties development as well as to support the understanding of evolution processes as many scientists have been reported on Hibiscus moscheutos [4], Lilium davidii var. unicolor [5], Raphanus sativus L. [6], Persian poppy or Papaver bracteatum Lindl. [7], Lycium ruthenicum [8], Colocasia esculenta var Bentul [9] and var Kaliurang [10].

Most polyploid plants have better characteristics as compared to their wild accession of diploids. They have thicker leaves, darker leaf color, bigger in plant size, last longer flowering, more vigorous,
higher in biomass, as well as more tolerant to stress, diseases, pests and having higher of secondary metabolite products [11]. However, growth polyploids plants commonly, lower than that of diploids, longer to form flowers, and having lower fertility. These all are due to abnormality during meiosis and mitosis processes [6,12,13]. This also the case of Bentul tetraploid plants which had lower growth than that of the diploids [9].

Many efforts can be done to enhance growth of in vitro shoot culture. One of them is by addition of plant growth regulators such as cytokinins and auxins. Application of plant growth regulators for a long period may cause somaclonal variation in tissue culture, which is a disadvantage for micropropagation. Increase in vitamin levels may offer to overcome the problem. In many plants, increase in vitamin concentrations enhance growth of callus, somatic cells, root formation and embryo development [14]. Common basal culture medium such as MS [15] medium consists of vitamin B namely thiamine (vitamin B1), nicotinic acid (vitamin B3) and pyridoxine (vitamin B6). Vitamins are catalyst in the plant [16]. Vitamins are also cofactors for carboxylase reaction and biosynthesis of amino acids [17]. Pyridoxine (vitamin B6) is not only active as cofactors, but also have important role in enzymatic reaction in protein biosynthesis [18]. Vitamins also affect growth and development of roots, besides to involve as antioxidant in the osmotic stress mechanism [19]. In addition, pyridoxine, thiamine and nicotinic acid may stimulate embryogenic callus development in Phoenix dactylifera L. (date palm) and Agave angustifolia Haw [18,20].

Besides increase in the vitamin concentrations, use of liquid media has been applied in several plant tissue culture to accelerate growth rate dan shoots proliferation as reported in potato [21], taro [22] and redberry (Vaccinium vitis-idaea subspecies Minus) [23]. A combination between increases in vitamin levels with the use of liquid medium may also increase shoot development of taro. The aim of the research was to accelerate growth of taro shoot cultures by increasing the vitamin levels added on MS liquid medium using three different ploidy levels of taro.

2. Materials and Methods

2.1. Materials

Experiments used one month old of in vitro shoots of taro Bentul diploid, Satoimo triploid and Bentul tetraploid from the Laboratory of Plant Cell and Tissue Culture, Research Centre for Biotechnology, Indonesian Institute of Sciences (LIPI). Tetraploid Bentul was obtained by induction of oryzalin [9].

2.2. Methods

Culture medium used in this research was MS medium [15] containing 2 mg l⁻¹ BAP and 2 mg l⁻¹ glycine. Vitamin B supplemented at normal and 100-fold of the normal concentration was thiamine (vitamin B1), nicotinic acid (Vitamin B3) and pyridoxine (vitamin B6). Their concentrations and code of the media compositions are shown on table 1, respectively. The experiment was carried out with a completely randomized design with three replicates. Each replicated consisted of three shoots of one month old, which was cultured on MS solid medium without addition of plant growth regulators. About 1 cm long of shoots was isolated from their leaves and roots, and then they were cultured on treatment media. All cultures were incubated in a culture room at 25-26°C with 24h in light using continuous white TL lamp. Liquid medium was shaken at 100 rpm. Growth was observed by recording number of multiple shoots, length of petioles, number of leaves and number of roots, recorded every week until four weeks of culture. Data of each treatment after 4 weeks of culture was subjected to statistical analysis by determining ANOVA and significant differences among means, followed by Duncan Multiple Range Test. Data were analyzed by software SPSS version 25.
Table 1. Combination of vitamin concentration used (thiamine, pyridoxine and nicotinic acid) at normal and 100-fold of the normal concentrations.

| Medium code         | Thiamine (mg/l) | Pyridoxine (mg/l) | Nicotinic acid (mg/l) |
|---------------------|-----------------|-------------------|----------------------|
| T1-P1-A1 (control)  | 0.1             | 0.5               | 0.5                  |
| T1-P100-A1          | 0.1             | 50                | 0.5                  |
| T1-P1-A100          | 0.1             | 0.5               | 50                   |
| T1-P100-A100        | 0.1             | 50                | 50                   |
| T100-P1-A1          | 10              | 0.5               | 0.5                  |
| T100-P100-A1        | 10              | 50                | 0.5                  |
| T100-P1-A100        | 10              | 0.5               | 50                   |
| T100-P100-A100      | 10              | 50                | 50                   |

3. Results and Discussion

3.1. Number of shoots
Taro shoot cultures increased in shoot numbers in all treated media. The ploidy level gave varied responses in number of shoots formed. Bentul diploid grown on medium containing normal concentration of thiamine showed similar pattern in growth from 2 to 4 weeks of cultures both in combination with normal or increased in pyridoxine and nicotinic acid concentrations (Figure 1A), however it had better growth when all thiamine, pyridoxine and nicotinic acid were increased to 100-fold. Increase in pyridoxine or nicotinic acid alone did not enhance number of shoots in Bentul diploid (Figure 1B).

Number of shoots of Satoimo (triploid) was better compared to Bentul diploid from 2 to 4 weeks in culture. Both normal and increased in thiamine levels gave high shoot number development (Figure 1C and D). Increased in all three of vitamin B inhibited growth of Satoimo (Figure 1D). In Bentul tetraploid, normal thiamine concentration in combination with 100-fold of pyridoxine and normal nicotinic acid enhanced their growth (Figure 1E). This result was similar to that of Satoimo, however, increase in thiamine concentration inhibited growth of shoots (Figure 1F).

Increase in shoot numbers of Bentul diploid on MS medium containing thiamine at 100-fold of the normal concentration was similar to Bentul diploid when it was treated with increase in thiamine level at 10-fold in combination with 2 mg/l adenine sulphate dan 2 mg/l BAP [24]. This finding was also found in other plant species such as in Rosa hybrid L. cv “Sarra” that increase in thiamine to 8-fold enhanced axillary bud proliferation [25]. In potato, increase in thiamine up to 10-fold also increased in shoot proliferation [26]. In Agave angustifolia Haw., increase in thiamine level not only accelerate shoot proliferation, but also that thiamine acts as essential substance to induce somatic cell develop to embryogenic cells [18].

As vitamin B1, thiamine is a micronutrient transported actively into plant cells against concentration gradient [27]. In plants, thiamine is recognized as a co-factor for various metabolisms such as for acetyl-CoA biosynthesis, amino acid biosynthesis, the Krebs cycle and the Calvin cycle [28]. In addition, thiamine also acts as a protection when biotic or abiotic stress occurs. When abiotic stress occurs, such as in drought, high salinity, heavy metals, changes in temperature or light intensity, thiamine acts as an antioxidant. In biotic stress, thiamine has the role in activating SAR (Systemic Acquired Resistance) against pathogen attack [29,30].

In Satoimo triploid taro and Bentul tetraploid, the highest increase in number of shoots was obtained in the treatment of pyridoxine at 100-fold concentrations. Pyridoxine is vitamin B6, it plays a role in transaminase reactions and amino acid decarboxylation [18]. In addition, pyridoxine not only
involves in plant development, but also it involves in environmental stress tolerant, and to function as an antioxidant [19].

3.2. Length of petiole
In the medium containing normal level of thiamine, length of Bentul diploid petiole increased from one to two weeks of culture, then remained stable afterward, except shoots grown on MS containing higher pyridoxine concentrations with normal nicotinic acid and 100-fold of nicotinic acid (figure 2A). Increase in vitamin level gave same growth of petiole from week-1 to week-4 of culture (figure 2B). High concentrations of vitamin B did not enhance growth of petiole in Satoimo triploid. Growth pattern of both petiole length in the medium containing normal and high thiamine level in combination with normal and higher concentration of both pyridoxine and nicotinic acid was similar (figures 2C and D). The growth pattern of petiole length on Bentul tetraploid cultured in MS medium containing normal and high concentration was similar. Medium containing thiamine and nicotinic acid 100-fold with pyridoxine at normal concentration increased in growth of the petiole (figures 2E and F). Thus, increase in concentration of nicotinic acid to 100 fold enhanced growth of petiole length.

A similar response was also shown by Allium cepa plants which indicated the height increased after spraying with nicotinic acid on their leaves [31]. In addition, seeds of Ricinus communis had high growth after soaking in nicotinic acid for 48h. Its oil content and alkaloids also increased [32]. Nicotinic acid (vitamin B3) is required by plants for the synthesis of amino acids and plays an important role in carbohydrate metabolism [14]. Nicotinic acid also involves in the tolerance mechanism of wheat plants to salinity stress. Application of nicotinic acid and tryptophan in wheat
under salinity stress conditions reduces the content of Na⁺ and Cl⁻ ions and increase the K⁺, Ca²⁺ and Mg²⁺ ions in wheat shoots [33].

![Figure 2](image_url)

**Figure 2.** Petiole length of taro cultured for 0-4 weeks on MS liquid medium containing normal concentrations of thiamine and 100-fold for Bentul diploid (A and B); Satoimo triploid (C and D); and Bentul tetraploid (E and F).

### 3.3. Number of leaves

The growth pattern of the number of leaves from the beginning of culture until four weeks of culture on taro Bentul diploid in all treatment media was not different. Increasing the concentration of three types of vitamin B did not increase the growth of leaf numbers (figures 3A and B). Growth of Satoimo triploid taro leaf numbers was faster than that of Bentul diploid. The highest increase in leaf numbers was achieved in the T₁-P₁₀₀⁻A₁ medium, namely MS media containing pyridoxine concentration 100-fold in combination with thiamine and nicotinic acid at normal concentrations (figure 3C). Increase in concentration of thiamine slightly decreased the growth of number of leaves (figure 3D). Growth pattern of leaf numbers in Bentul tetraploid was similar to Bentul diploid. Increase in B vitamin levels did not change the growth pattern it gave the same pattern as normal vitamin B concentrations (figures 3E and F).

Increase in concentration of pyridoxine to 100-fold on taro Satoimo increased the number of leaves (figure 3). The same response was also shown in the administration of pyridoxine through foliar spray of *Sesamum indicum* plants at 200 ppm. It could increase the number of leaves per plant, height of plants, total leaf area per plant, plant dry weight, as well as oil content in seeds [34]. Pyridoxine or vitamin B6 involves in nitrogen metabolism and the defense mechanism of plants against pathogens through increase in salicylic acid [35]. Vitamin B6 also functions as a photoprotector in chloroplasts, which limits the accumulation of free radicals and prevents oxidative damage [36].
Figure 3. Leaf numbers of taro cultured for 0-4 weeks on MS medium liquid containing normal concentrations of thiamine and 100-fold for Bentul diploid (A and B); Satoimo triploid (C and D); and Bentul tetraploid (E and F).

3.4. Number of roots

The results showed that in Bentul diploid taro the highest increase in the number of roots was found in MS media containing pyridoxine concentration 100-fold in combination with thiamine and nicotinic acid at normal concentrations (T1-P100-A) (figure 4A). Increase in three concentrations of vitamin B decreased the number of roots up to four weeks of culture (figure 4B). In the contrary, in Satoimo triploid, normal pyridoxine concentrations combined with thiamine and nicotinic acid at 100-fold (T100-P1-A100) gave the highest increase in the number of roots (figure 4C). The growth patterns in root numbers from one week to four weeks of culture were also similar (figures 4C and D). In Bentul tetraploid, the treatment of all vitamins at normal concentrations (T1-P1-A) gave the highest increase in the number of roots (figure 4E). The increase in the number of roots still occurred until four weeks of culture (figures 4E and F).

Our finding showed that the growth of the root numbers in taro showed a variety of responses at different levels of ploidy. In Bentul diploid taro, the addition of pyridoxine concentration to 100-fold increased the number of roots, however, increase in thiamine and nicotinic acid to 100-fold did not increase the number of roots. In Bentul tetraploid, pyridoxine, thiamine and nicotinic acid at normal concentrations increased the growth of the root numbers. These results could be related to the effect of pyridoxine on root development. Research conducted on Arabidopsis mutant that have disturbed pyridoxine biosynthesis showed that interference of root development occurred [37]. Correlation between increased in ploidy level and vitamin B endogenous in tetraploid plants still needs to be investigated further.
Figure 4. Root numbers of taro cultured for 0-4 weeks on MS liquid medium containing normal concentrations of thiamine and 100-fold for Bentul diploid (A and B); Satoimo triploid (C and D); and Bentul tetraploid (E and F).

Statistical analysis indicated that the average value of growth parameters at 4 weeks of culture was affected by different levels of ploidy (table 2). In Bentul diploid taro, the length of the petiole and the number of roots was significantly different with or without increase in the vitamin levels, while the number of shoots and number of leaves were not. The average range of petiole lengths was from 3.22 to 5.39 cm, while the average range of roots was from 2.2 to 6.7 cm. The highest length of petiole was found in MS medium containing nicotinic acid at 100-fold concentrations in combination with thiamine and pyridoxine at normal concentrations. The highest number of roots was found on the medium containing high pyridoxine level in combination with 100 high thiamine level and nicotinic acid at normal concentrations (table 2). The highest length of the petiole and the highest number of roots were found on the medium containing thiamine at normal concentrations. Combination with nicotinic acid at concentration 100-fold produced the highest petiole length, while combination with pyridoxine concentration at 100-fold produced the highest number of roots.
Table 2. Effect of vitamins at different concentrations on growth of taro Bentul diploid shoots grown in MS liquid medium after 4 weeks in culture.

| Concentration of vitamin (mg/l⁻¹) | Shoot numbers | Petiole length (cm) | Leaf numbers | Root numbers |
|-----------------------------------|---------------|---------------------|--------------|--------------|
| Thiamine 0.1 Pyridoxine 0.5 Nicotinic acid 0.5 | 0.3 | 4.17abc | 2.0 | 4.4ab |
| Thiamine 0.1 Pyridoxine 50 Nicotinic acid 0.5 | 0.4 | 4.72bc | 1.9 | 6.7b |
| Thiamine 0.1 Pyridoxine 0.5 Nicotinic acid 50 | 0.6 | 5.39c | 2.0 | 4.0a |
| Thiamine 0.1 Pyridoxine 50 Nicotinic acid 50 | 0.4 | 4.00ab | 1.8 | 4.4ab |
| Thiamine 10 Pyridoxine 0.5 Nicotinic acid 0.5 | 0.6 | 3.22a | 1.9 | 3.6a |
| Thiamine 10 Pyridoxine 50 Nicotinic acid 0.5 | 0.6 | 3.44ab | 1.8 | 3.0a |
| Thiamine 10 Pyridoxine 0.5 Nicotinic acid 50 | 0.4 | 3.61ab | 2.0 | 2.2a |
| Thiamine 10 Pyridoxine 50 Nicotinic acid 50 | 1.0 | 3.67ab | 2.0 | 4.1a |

Notes: Number followed with the same letters on the same column is not significantly different according to Duncan Multiple Range Test at α = 5%

Statistical analysis in Satoimo triploid taro showed that increase in vitamin B concentration only gave significantly different on root number parameters. The average range of roots was from 4.6 to 8.7. The highest root numbers were found in MS medium containing thiamine and nicotinic acid at 100-fold concentrations in combination with pyridoxine at normal concentrations. The lowest root numbers were found in MS medium containing high concentrations of thiamine combined with pyridoxine and normal nicotinic acid. Increasing the concentration of B vitamins did not affect the number of shoots, length of petiole and number of leaves (table 3).

Table 3. Effect of vitamins at different concentrations on growth of taro Satoimo triploid shoots grown in MS liquid medium after 4 weeks in culture.

| Concentration of vitamin (mg/l⁻¹) | Shoot numbers | Petiole length (cm) | Leaf numbers | Root numbers |
|-----------------------------------|---------------|---------------------|--------------|--------------|
| Thiamine 0.1 Pyridoxine 0.5 Nicotinic acid 0.5 | 2.8 | 2.46 | 5.4 | 6.9ab |
| Thiamine 0.1 Pyridoxine 50 Nicotinic acid 0.5 | 3.3 | 2.10 | 7.6 | 6.2ab |
| Thiamine 0.1 Pyridoxine 0.5 Nicotinic acid 50 | 2.9 | 2.13 | 6.6 | 6.4ab |
| Thiamine 0.1 Pyridoxine 50 Nicotinic acid 50 | 2.7 | 2.81 | 4.9 | 7.2b |
| Thiamine 10 Pyridoxine 0.5 Nicotinic acid 0.5 | 3.2 | 2.59 | 5.8 | 4.6a |
| Thiamine 10 Pyridoxine 50 Nicotinic acid 0.5 | 2.6 | 2.68 | 4.6 | 6.1ab |
| Thiamine 10 Pyridoxine 0.5 Nicotinic acid 50 | 2.8 | 2.71 | 6.1 | 8.7ab |
| Thiamine 10 Pyridoxine 50 Nicotinic acid 50 | 2.2 | 2.46 | 6.0 | 7.2ab |

Notes: Number followed with the same letters on the same column is not significantly different according to Duncan Multiple Range Test at α = 5%

Table 4 shows that in Bentul tetraploid taro, an increase in B vitamins only significantly affected the number of shoots. The average range of shoots was from 0.2 to 1.3. The highest number of shoots was found in MS medium containing high pyridoxine (100-fold of the normal concentration) combined with thiamine and nicotinic acid at normal concentration. Increase in concentration of vitamin B did not affect the length of the petiole, the number of leaves and roots were not significantly different (table 4).
Table 4. Effect of vitamins at different concentrations on growth of taro Bentul tetraploid shoots grown in MS liquid medium after 4 weeks in culture.

| Concentration of vitamin (mg l⁻¹) | Shoot numbers | Petiole length (cm) | Leaf numbers | Root numbers |
|-----------------------------------|---------------|---------------------|--------------|--------------|
| Thiamine 0.1                      | 3.77          | 2.3                 | 8.3          |              |
| Pyridoxine 0.5                     | 3.57          | 2.2                 | 7.8          |              |
| Nicotinic acid 0.5                 | 3.00          | 2.4                 | 5.9          |              |
| Thiamine 0.1 50                    | 4.11          | 2.7                 | 6.7          |              |
| Pyridoxine 0.5 50                  | 3.99          | 2.4                 | 6.8          |              |
| Nicotinic acid 0.5 50              | 4.08          | 2.8                 | 7.9          |              |
| Thiamine 10 0.5                    | 3.99          | 2.4                 | 6.8          |              |
| Pyridoxine 10 0.5                   | 4.23          | 2.2                 | 6.0          |              |
| Nicotinic acid 10 0.5               | 3.66          | 2.6                 | 8.1          |              |

Notes: Number followed with the same letters on the same column is not significantly different according to Duncan Multiple Range Test at α = 5%

Performance of Bentul diploid, Satoimo triploid and Bentul tetraploid after 4 weeks in culture are shown in figures 5, 6 and 7, respectively. Bentul tetraploid plantlets had shoots and roots that are more vigorous than that of Bentul diploid in all treatment media.

Figure 5. Performance of in vitro shoots of taro Bentul diploid after 4 weeks culture on MS liquid medium containing normal and 100-fold of vitamin concentrations. (A) normal vitamins; (B) increase in pyridoxine level; (C) increase in nicotinic acid level; (D) increase in pyridoxine and nicotinic acid levels; (E) increase in thiamine level; (F) increase in thiamine and pyridoxine levels; (G) increase in thiamine and nicotinic acid levels; and (H) increase in thiamine, pyridoxine and nicotinic acid levels.
**Figure 6.** Performance of *in vitro* shoots of *taro* Satoimo triploid after 4 weeks culture on MS liquid medium containing normal and 100-fold of vitamin concentrations. (A) normal vitamins; (B) increase in pyridoxine level; (C) increase in nicotinic acid level; (D) increase in pyridoxine and nicotinic acid levels; (E) increase in thiamine level; (F) increase in thiamine and pyridoxine levels; (G) increase in thiamine and nicotinic acid levels; and (H) increase in thiamine, pyridoxine and nicotinic acid levels.

**Figure 7.** Performance of *in vitro* shoots of *taro* Bentul tetraploid after 4 weeks culture on MS liquid medium containing normal and 100-fold of vitamin concentrations. (A) normal vitamins; (B) increase in pyridoxine level; (C) increase in nicotinic acid level; (D) increase in pyridoxine and nicotinic acid levels; (E) increase in thiamine level; (F) increase in thiamine and pyridoxine levels; (G) increase in thiamine and nicotinic acid levels; and (H) increase in thiamine, pyridoxine and nicotinic acid levels.
4. Conclusion
Increase in concentrations of three types of B vitamins (thiamine, pyridoxine and nicotinic acid) affected the growth of taro which has a different ploidy level. Growth responses of in vitro growth of taro Bentul diploid, Satoimo triploid and Bentul tetraploid varied. In Bentul diploid, medium containing 100-fold concentrations of pyridoxine and nicotinic acid enhanced the length of petiole and number of roots. In Satoimo triploid, medium 100-fold concentrations of thiamine and nicotinic acid stimulated formation of roots. In Bentul tetraploid taro, medium with high pyridoxine concentration increased number of shoots.

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