In Vitro Propagation of Tribulus terrestris with IAA and BAP Concentrations

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Abstract
Tribulus terrestris is conventionally propagated using seeds but has limited germination capacity. One
approach that can be done for this plant multiplication using in vitro tissue culture. This study was
conducted to determine the growth potential of T. terrestris in vitro by the addition of auxin (IAA) and
cytokin (BAP) as growth regulators at several concentrations. The study was conducted at the
Laboratory of Plant Physiology and Biotechnology, Faculty of Agriculture, Universitas Sebelas Maret
Surakarta from February to December 2017. The experiment employed factorial Completely
Randomized Design (CRD) method with 2 factors, namely IAA concentration (0 ppm, 0.01 ppm, 0.2
ppm, 0.3 ppm), and BAP concentration (0 ppm, 0.3 ppm, 0.5 ppm, 0.7 ppm). The observed growth
parameters were shoot emergence time, leaf emergence time, root emergence time, number of leaves,
and number of roots. Data were analyzed using analysis of variance followed by DMRT with a 95%
confidence level. The results showed that the addition of IAA only affects leaf emergence time, while
the addition of BAP had a significant effect on the leaf emergence time and number of leaves. The
interactions between IAA and BAP had a significant effect on leaf emergence time.

Keywords: explant; plant growth; tissue culture

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Introduction
Tribulus terrestris L. (Zygophyllaceae) is a plant that originates from the Mediterranean
region and has been spread widely in warm climate regions such as Africa, Australia, and
Asia (1). This plant is used in the practice of Chinese medicine for several diseases (2).
Tribulus plant contains several active chemicals that are used as antibiotics and contain steroidal
substances that function as an aphrodisiac. One of the main characteristics of this plant is the
high amount of steroid chemicals contents. Steroid saponins, diosgenin, furostanol,
spirostanol, tigogenin, ruscogenin, chlorogenin, and gitogenin are important chemical
constituents which contents are very high in T. terrestris (3). The plant is rich in protein and
calcium and also contains oil, peroxidase, diastase, resins, and even various glucosides in
dried fruit from T. terrestris. These characteristics make T. terrestris a valuable
plant for medicines and other uses such as stimulants and stamina enhancers (4).
Tribulus terrestris is conventionally propagated using seeds. Based on a study
conducted on the multiplication of seeds, the germination rate capability by the seeds of this
plant is very small (5). In Indonesia, the cultivation of this plant is quite difficult
because it can only grow in certain conditions and times. In one year, cultivation can only be
done one season in early May to August. The characteristics of seed dormancy and low
germination from Tribulus terrestris makes this plant a suitable specimen for the study and
development of plant propagation methods by in vitro tissue culture methods. The plant’s
tissue culture aims to provide information about the correct method in propagation by
micropropagation for the purpose of seed
formation from cultured plantlets and other utilities. This also opens the pathway to medicinal substance production of *T. terrestris* secondary metabolite within the method of a bioreactor in vitro as the substitution for cultivation by means of a more sustainable method of agricultural production.

Modification of tissue culture media by regulating growth regulators needs to be done to increase the percentage of plant growth rate in tissue culture by means of propagation. There are two types of plant hormones (auxin and cytokinin) that are widely used in propagation in vitro. This experiment aims to observe whether the addition of IAA (*Indoleacetic Acid*) and BAP (*Benzyl Amino Purine*) at certain concentrations can promote *Tribulus terrestris* plant’s grown in tissue culture.

### Material and Methods

The research was conducted from February to December 2017 at the Laboratory of Plant Physiology and Biotechnology, Faculty of Agriculture, Universitas Sebelas Maret. The material used in this study were the seed sprouts of *Tribulus terrestris* plants as explants. The medium used was MS type, with aqua dest composition, sugar (80 g/L), macronutrient solution (50 ppm), micronutrient solution (5 ppm), vitamins (50 ppm), IAA (*Indoleacetic Acid*) and BAP (*Benzyl Amino Purine*), Kinetin (0.3 ppm), white agar (8 g/L), spiritus fuel, methanol, 70% alcohol, formalin, 5% chlorox, detergent, fungicide (Mankozeb) 0.1 g/100 mL, bactericidal (Agrept/Streptomycin) 0.1 g/100 mL, antibiotic solution (amoxycillin) 0.1 g/100 mL, ascorbic acid, and betadine 2 mL/100 mL. The tools used included autoclave, laminar airflow cabinet (LAFC), oven, bunsen lamp, hand sprayer, culture bottle, measuring cup, analytic scale, hot plate stirrer, tweezers, scalpel knife, pH meter, pipette, cutter, and petri dish. This research was carried out using a completely randomized design (CRD) with two factors. The first factor was the addition of auxin growth regulator of Indole-acetic Acid (IAA) (0; 0.1; 0.2; 0.3 ppm) and the second factor was the addition of cytokinin growth regulator of Benzyl Amino Purine (BAP) (0; 0.3; 0.5; 0.7 ppm). Research activities initialized by tools and materials preparation, explant sterilization, explant inoculation, and data observation. Data observation was done within 60 days after planting. The variables observed in this study were shoot emergence time (the day after planting/DAP), leaves emergence time (DAP), number of leaves, root emergence time (DAP), and number of roots. The obtained data were analyzed using analysis of variance followed by further DMRT (Duncan Multiple Range Test) with a confidence level of 95%.

### Results and Discussion

#### Shoot emergence time

The appearance of shoots is an indicator in tissue culture studies that plants can grow and develop with the given treatment. The growth and regeneration process is determined by three factors, which are cultivar selection of explant used with certain regeneration capability, explant source selection, and media adaptation to the given genotype (6). Embryo culture has shoot growth characteristics that emerged between cotyledons, characterized by the appearance of plumula. Adaptation of explants in in-vitro media is determined by nutrient availability factors, microclimate conditions that are following ex-vitro conditions, and additional concentrations of growth promoter (7). The mean value of shoots emergence time with various IAA and BAP concentrations were shown in Figure 1.

The fastest shoot emergence time was shown by the treatment of 0 ppm IAA and 0.7 ppm BAP which was 8.67 DAP, while the latest shoot emergence time shown by 0.3 ppm IAA and 0.3 ppm BAP which was 22.67 DAP. Based on the average value of shoot emergence time from IAA treatment independently, the response of the fastest shoot time was shown in the treatment of 0 ppm IAA, which was about 13 DAP on average, while the longest shoot emergence time was shown at 0.3 ppm IAA which is 19 DAP in average. This showed that the use of BAP independently without the addition of IAA has been able to give better shoot emergence times than the addition of IAA. Despite this result, another study on *Cucumis melo* L. (8) showed that IAA and BAP independently did not significantly affect the regeneration and shoot induction, but the combination of both proved highly affecting differentiation, development, and elongation of new buds.
Leaf primordia have accumulated IAA in apical tissue (9). This endogenous IAA content provides the needs of explants to carry out the growth process. The addition of exogenous IAA made the IAA concentration used by explant tissue to be excessive and not optimal, which resulted in growth inhibition. This was indicated by the lower average value of shoot emergence time from the use of IAA concentrations when the concentration was increased. An increase in IAA concentration did not affect the growth of shoot biomass on tomato seeds (10). Auxin at lower concentrations compared to cytokinins is needed in the shoot induction process because the combination of the two with the appropriate concentration will accelerate the process of cell division (11).

Table 1. The effect of BAP treatment on average shoot emergence time (DAP)

| BAP Concentration (ppm) | 0     | 0.3   | 0.5   | 0.7   |
|-------------------------|-------|-------|-------|-------|
| Shoot emergence time    | 17.91 | 20.5  | 15.58 | 14.41 |

Description: Number followed by the same letters showed no significant difference (P>0.05).

Based on the results in Table 1, the fastest shoot emergence time was indicated by the treatment of 0.7 ppm BAP independently which was 14.41 DAP; while the slowest shoot emergence time was shown in the use of 0.3 ppm BAP which was 20.5 DAP. Table 1 showed that a higher concentration of BAP in the treatments brought a faster explant shoot emergence time. The addition of BAP to tissue culture can increase the growth rate of shoots, so BAP is often used in tissue culture due to this several superior properties compared to other types of cytokines (12, 13).

Table 2. The result of the analysis of the various uses of IAA and BAP concentrations on the leaves emergence time.

| Treatments | Leaf Emergence Time |
|------------|---------------------|
| IAA        | 0.011*              |
| BAP        | 0.000**             |
| IAA * BAP  | 0.000**             |

Description: * has a significant effect (P<0.05), ** has a very significant effect (P<0.01)

Leaves emergence time

Leaf formation in explants has a function to carry out essential physiological processes such as photosynthesis which is used as a place energy generator for other physiological processes such as cell proliferation and organogenesis. Time of leaf emergence was positively correlated with leaf growth and biomass (14). The results of variance analysis
of leaves emergence time of *Tribulus terrestris* explants at several IAA and BAP concentrations are shown in Table 2.

Based on the results in Table 2, all treatments of IAA, BAP, and combination concentrations have a significant effect on the growth response of leaves emergence time. Changes in leaf growth, leaf area, and biomass were followed by changes in the content of cytokinin concentrations in the media and tissues (15). The effect of IAA and BAP concentrations combination on the response of leaves emergence time is shown in Table 3.

Table 3. Effect of IAA and BAP concentrations on leaves emergence time (DAP)

| IAA Concentration (ppm) | BAP Concentration (ppm) |
|-------------------------|-------------------------|
| 0                      | 18.33<sup>abcd</sup>  | 26.00<sup>def</sup> | 28.00<sup>ef</sup> | 16.67<sup>abc</sup> |
| 0.1                    | 26.00<sup>def</sup>    | 25.33<sup>def</sup> | 15.00<sup>ab</sup> | 16.00<sup>bc</sup> |
| 0.2                    | 23.33<sup>cde</sup>    | 24.00<sup>cde</sup> | 12.50<sup>a</sup>  | 25.00<sup>def</sup> |
| 0.3                    | 32.75<sup>f</sup>      | 26.33<sup>def</sup> | 19.33<sup>abcd</sup>| 22.33<sup>cde</sup> |

Description: Number followed by the same letters shows no significant difference in DMRT test of 5% level.

Table 3 showed the fastest response time for leaf emergence was indicated by the use of 0.2 ppm IAA and 0.5 ppm BAP treatments, which were 12.5 DAP; while the latest leaves emergence time was shown by 0.3 ppm IAA and 0 ppm BAP which was 32.75 DAP. The use of 0.2 ppm IAA concentration and 0.5 ppm BAP was able to increase the response rate of leaf emergence by 160% faster than the use of 0.3 ppm IAA and 0 ppm BAP with a very significant difference.

Based on the average value of the IAA treatment, the fastest response time of leaves appearance time was shown in 0.1 ppm IAA, which was 20.58 DAP, while the highest average value of leaves emergence time was shown by 0.3 ppm IAA which was 25.5 DAP. This showed that the use of IAA at low concentrations in this study gave better results in the growth response of leaf organogenesis compared to high IAA concentrations. The growth rate of shoots and the average time of shoot emergence decreased with increasing concentration of auxin in the media, which indicates that excess exogenous auxin actually reduces the rate of organogenesis (16). Depending on concentration and tissue, auxin stimulates or inhibits cell elongation. Thus, differential auxin is stimulated and distributed to all organs, such as roots or stems, leading to differential growth which results in organ formation (17).

The average time of leaf emergence on the use of BAP concentration independently showed the fastest value in the treatment of BAP 0.5 ppm which was 18.83 DAP. The average time of leaf emergence was indicated by the use of BAP with a concentration of 0 and 0.1 ppm with a value of 25.54 DAP on average. This data showed the different effects of BAP concentration on the growth response of shoots emergence time, with the fastest shoot emergence time indicated by BAP 0.7 ppm. Cytokinins have an important role in bud and root formation, regulate seed development, abiotic stress, and plant senescence (18, 19, 20, 21, 22). The role of BAP as the most stable type of cytokinin is to encourage cell proliferation, tissue lengthening, and tissue enlargement. Hamad and Taha (23) showed that a positive correlation between the number of shoots, total weight, total length, biomass, and propagule from the results of the tissue cell proliferation process was obtained from media added with the concentration of BAP.

**Root emergence time**

Roots emergence indicates good explant growth. Root induction is influenced predominantly by auxin activity in explants. Research about in vitro culture of Pecan plants by Zhang et al. (24) showed that exogenous auxin had an effective influence on root growth. IAA content in meristematic tissues can trigger root growth as apical meristem tissue. IAA as phytohormone can regulate many aspects of plant growth and development, such as branching in shoots and roots, as well as differentiation of the vascular vessel system (25, 26). Apart from playing a role in organogenesis in the apical meristem, IAA also determines the regulation of phyllotaxis (27). The effect of the use of IAA and BAP growth regulators on the root emergence time in *Tribulus terrestris* explants is presented in Figure 2.
Number of leaves

Leaves are one of the organs of plants that are very important especially for photosynthesis which is used as a food production factory and supports optimum growth (29). The number of leaves is one of the important indicators in determining tissue potential which is regulated by genetic factors and growth hormone. The analysis result of the effects of IAA and BAP growth regulators on the response of leaf growth in *Tribulus terrestris* are shown in Table 4.

Table 4. Analysis result of various IAA and BAP concentrations effect on the number of leaves.

| Treatment | Number of Leaves |
|-----------|------------------|
| IAA       | 0.518*           |
| BAP       | 0.050*           |
| IAA * BAP | 0.238*           |

Description: * has a significant effect, ns has no significant effect.

Table 4 showed that the addition of BAP independently has a significant influence on the number of leaves (P<0.05). The effect of IAA addition and the interaction between IAA and BAP on the number of leaves did not occur. Akhiriana et al. (30), suggested that the addition of IAA was independently able to affect the process of leaves formation, especially in a low concentration of 0.15 ppm. The previous research showed that significant changes in leaf growth, leaf area, and biomass were followed by changes in the content of cytokinin concentrations in the media and tissues (15). The average number of leaves from the treatment given by various IAA and BAP concentrations were shown in Figure 3.

Figure 3 showed the highest average response value of the number of leaves which were indicated by the treatment of 0.1 ppm IAA and 0.7 ppm BAP with 2.33 strands; while the least average number of leaves was 1 piece which obtained by 4 different treatments: IAA 0.1 ppm and BAP 0 ppm; 0.2 ppm IAA and 0 ppm BAP; 0.2 ppm IAA and 0.7 ppm BAP; and IAA 0.3 ppm and BAP 0 ppm. One of the roles of auxin in leaf growth is to help the development of meristem tissue of leaf induction (29). Cytokinin such as BAP in leaf formation is used for morphogenesis and leaves enlargement and the result will be used for growth and increasing the number of leaves (31).
Description: I0B0: IAA 0 ppm + BAP 0 ppm; I0B1: IAA 0 ppm + BAP 0.3 ppm; I0B2: IAA 0 ppm + BAP 0.5 ppm; I0B3: IAA 0 ppm + BAP 0.7 ppm; I1B0: IAA 0.1 ppm + BAP 0 ppm; I1B1: IAA 0.1 ppm + BAP 0.3 ppm; I1B2: IAA 0.1 ppm + BAP 0.5 ppm; I1B3: IAA 0.1 ppm + BAP 0.7 ppm; I2B0: IAA 0.2 ppm + BAP 0 ppm; I2B1: IAA 0.2 ppm + BAP 0.3 ppm; I2B2: IAA 0.2 ppm + BAP 0.5 ppm; I2B3: IAA 0.2 ppm + BAP 0.7 ppm; I3B0: IAA 0.3 ppm + BAP 0 ppm; I3B1: IAA 0.3 ppm + BAP 0.3 ppm; I3B2: IAA 0.3 ppm + BAP 0.5 ppm; I3B3: IAA 0.3 ppm + BAP 0.7 ppm.

Figure 3. The average number of leaves of T. terrestris explant with various concentrations of IAA and BAP

| Table 5. The effect of BAP concentrations on the number of leaves |
|---------------------------------|
| BAP Concentration Treatment (ppm) | 0   | 0.3  | 0.5  | 0.7  |
| Number of Leaves                | 1.25| 1.33a| 1.97b| 1.67ab|

Description: Number followed by the same letters shows no significant difference in DMRT test of 5% level.

The use of 0.5 ppm BAP concentration independently showed a significantly different and greater effect compared to the concentration of BAP 0 ppm and 0.3 ppm. The concentration of BAP of 0.7 ppm gave a relatively lower average number of leaves compared to the number of leaves from the treatment of a 0.5 ppm BAP concentration. Thus, the concentration of BAP of 0.5 ppm was assumed as the best concentration in this study with regard to the number of leaves. Table 5 shows that the number of leaves tends to increase in line with the increase in BAP concentration. This followed the previous research by Kristina (31) which stated that cytokines such as BAP in leaf formation have a role in morphogenesis and leaves enlargement which is used for supporting the growth and increase the number of additional leaves. The role of BAP in leaf formation is more dominant than the effect of IAA.

Higher number of leaves formed will give more energy for explant tissue to grow. The increasing number of leaves and leaf area width will support the growth and yield of plants, due to the increasing number of leaves that allow more sunlight to be received which improve photosynthesis rate, produce many photosynthates which then stored as carbohydrates (32).

Number of roots

High quantity, large, and long-sized roots are very useful for the absorption of nutrients from the media because the field of absorption of nutrients from the media will be wide. Roots can be formed optimally on the media if supported by the appropriate concentration of growth regulator, especially from the auxin group. The number of roots is an indicator of the ability of total tissue potential. Although in embryo culture the explants have formed roots, the supplementation of auxin and cytokinin will trigger explants to form additional roots. The effect of the use of IAA and BAP growth regulators on the number of Tribulus terrestris roots is shown in Figure 4.

Figure 5 showed that not all explant samples observed can grow roots. Root only appears in 4 treatments, which were 0 ppm IAA and 0.7 ppm BAP; 0.1 ppm IAA and 0.3 ppm BAP; 0.1 ppm IAA and 0.7 ppm BAP; 0.2 ppm IAA and 0.5 ppm BAP. The highest number of roots was produced by explants with the treatment of 0.1 ppm IAA and 0.7 ppm BAP which was 4 pieces, while the smallest number
of roots was shown by explants from 0 ppm IAA treatment and 0.7 ppm BAP.

Figure 4. *T. terrestris* explant with 0.1 ppm IAA and 0.3 ppm BAP with 3 strands of leaves

![Figure 4](image)

Figure 5. The number of root from various IAA and BAP concentrations

| Treatment combinations | Number of roots |
|------------------------|----------------|
| I0B0: IAA 0 ppm + BAP 0 ppm; I0B1: IAA 0 ppm + BAP 0.3 ppm; I0B2: IAA 0 ppm + BAP 0.5 ppm; I0B3: IAA 0 ppm + BAP 0.7 ppm; I1B0: IAA 0.1 ppm + BAP 0 ppm; I1B1: IAA 0.1 ppm + BAP 0.3 ppm; I1B2: IAA 0.1 ppm + BAP 0.5 ppm; I1B3: IAA 0.1 ppm + BAP 0.7 ppm; I2B0: IAA 0.2 ppm + BAP 0 ppm; I2B1: IAA 0.2 ppm + BAP 0.3 ppm; I2B2: IAA 0.2 ppm + BAP 0.5 ppm; I2B3: IAA 0.2 ppm + BAP 0.7 ppm; I3B0: IAA 0.3 ppm + BAP 0 ppm; I3B1: IAA 0.3 ppm + BAP 0.3 ppm; I3B2: IAA 0.3 ppm + BAP 0.5 ppm; I3B3: IAA 0.3 ppm + BAP 0.7 ppm. |

From the data, we cannot yet conclude the influence of each type of growth regulator treatment on the growth response of root growth. The addition of IAA can increase the formation of root numbers but this does not mean that without the external IAA, explants can’t grow roots. Root induction as meristem tissue is dominantly affected by the function of endogenous auxin such as IAA (33). It was because explants used in this research already have an endogenous growth regulator, so that the content of endogenous IAA might have been sufficient to grow roots. Root cells generally contain enough or almost enough auxin to elongate normally. Therefore, the addition of hormones is expected to stimulate faster and denser root growth.

The number of roots has a positive effect on explant growth. From all four explants that produce roots, their growth response by the number of leaves and the life span of the media which were relatively larger than the treatments that does not give rise to roots. This was due to the high number of roots increased the amount of nutrient uptake by explants. The inability of other explants in inducing roots was thought to be because the content of endogenous auxin was not able to stimulate the organogenesis process, especially the formation of lateral roots so that the addition of auxin concentration is needed.

**Conclusion**

The addition of IAA independently did not affect all explant growth variables observed, whereas the addition of BAP affects the growth response on leaves emergence time and the number of leaves. The combination treatment of 0.2 ppm IAA and 0.5 ppm BAP was a growth regulator concentration with the greatest effect on the leaves emergence time response, which was 12.5 DAP.

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**Conflict of Interest**

All authors declare no conflicts of interest in this paper.

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