Article

Effect of Growth Regulators on In Vitro Micropropagation of Stahlianthus thorelii Gagnep

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Abstract: Stahlianthus thorelii Gagnep is a plant belonging to the family Zingiberaceae, widely distributed in Asian countries like China, Thailand, India and Vietnam. In traditional oriental medicine, this plant is usually used to treat hemorrhage, heavy menstruation, poor digestion, rheumatism and bone/joint pain (tuberous roots). This research article presents the results of in vitro growth experiments on S. thorelii Gagnep using tubers as explants. The samples are grown in MS media enriched with BAP growth stimulant concentrations of 5.0 mg L\(^{-1}\) and a kinetin concentration of 4.0 mg L\(^{-1}\), yielding 5.55 ± 0.59 and 5.48 ± 0.87 shoots/explants, respectively. Once the plants reached a height of 3.0–4.0 cm, we inoculated 2.0–3.0 leaves with a MS rapid proliferation medium treated with BAP or NAA growth agents alone or in combination. The most shoots (7.54 ± 0.79 shoots/explants) were produced by the medium enhanced with 3.0 mg L\(^{-1}\) BAP and 0.5 mg L\(^{-1}\) NAA after 8 weeks of cultivation. The greatest root/shoot induction of 26.17 ± 1.5 was achieved with the medium that had been treated with 0.5 mg L\(^{-1}\) NAA and 0.5 mg L\(^{-1}\) IBA, which was prepared using the MS media that was administered alone or in combination with NAA and IBA for in vitro shoot rooting. Highest percentage of survival (100%) was observed when tissue cultured plantlets were acclimatized in soil:sand:compost (1:1:1).

Keywords: micropropagation; plant growth hormones; S. thorelii Gagnep; tissue culture

1. Introduction

Stahlianthus thorelii and Stahlianthus innocratus are members of the Zingiberaceae family and both are known as “Khuong tam that” used in Vietnamese and Chinese folk medicine, respectively, to treat inflammation, pneumonia, diarrhea, and anticancer [1,2]. S. thorelii Gagnep (Family Zingiberaceae), formerly known as Ginger Panax pseudocarrot, is a plant species that is dispersed throughout Asia, particularly in China, India, Laos, Thailand, Myanmar, and Cambodia through Malaysia, Indonesia, and Vietnam [3].

S. thorelii Gagnep is found across Vietnam, particularly in the northern regions of Laocai, Yenbai, Hagiang, Caobang, Bagiang, and Langson and southern regions, such as Kontum, Daclak, and Lamdong [4,5]. The demand for riodhachadhb, or medicines made from medicinal herbs, is increasing throughout the world, especially in Vietnam. This has resulted in an overuse of the country’s natural resources. S. thorelii Gagnep grows haphazardly, with no master plan for the development of the products [6]. For instance, vegetative propagation is presently the primary method used to cultivate S. thorelii Gagnep. The quantity of seedlings produced by this procedure is constrained, and the multiplication factor is tiny, ranging from 0.99- to 2.0-fold [7]. It is thought that S. thorelii Gagnep, in particular, can be propagated successfully by in vitro cultivation. Studies on the in vitro propagation of plants from the Zingiberaceae family have been conducted by a number of researchers, including Sunitibala et al. [8], Loc et al. [9], Kambaska et al. [10], Azhar et al. [11], and Sathyagowri et al. [12]. However, the findings of this research suggest that breeding practices and environments vary according to the specific variety. There have been no studies that specifically focused on S. thorelii Gagnep. To provide a source of
medicinal plants for mass production, this study aimed to identify the optimal conditions for each stage of the S. thorelii Gagnep in the in vitro propagation process.

2. Materials and Methods

Explant: The S. thorelii Gagnep wild varieties that were employed in this research study were obtained from the area around the study location in Vietnam. Explant is referenced through the description of Le Van Quang et al. [13].

Study Location: The Northeastern College of Biotechnology, Agriculture, and Forestry’s Biotechnology Laboratory served as the site of this experiment.

Experimental Layout

Shoot propagation: Young S. thorelii Gagnep tubers were washed 4–5 times in diluted soapy water and then rinsed for 2 h under running water. The clean samples were pre-disinfected with 70% alcohol for 20 s, followed by 20 min of disinfection with 0.1% HgCl2. The samples were placed on absorbent paper, and the hard outer shell was removed using piercing forceps and a knife to create a small, convenient sample after the tubers had been sterilized and washed 6–7 times with sterile distilled water. Then, inoculations were made into flasks containing MS media, 30 g/L saccharose, and 5.4 g/L agar [14]. The samples’ ability to regenerate new shoots was next tested by adding BAP or kinetin. We analyzed and plotted the correlation between BAP, kinetin content, and the number of shoots/explants.

Shoot Multiplication: For the in vitro shoots’ subculture, MS, 5.4 g/L agar, and 30 g/L saccharose medium were utilized. When the shoots were 1.0–2.0 cm long, separate supplements of BAP (0 to 4 mg L\(^{-1}\)) and NAA (0.0 to 1.0 mg L\(^{-1}\)), or a combination of BAP (0.0 to 4.0 mg L\(^{-1}\)) and NAA (0.0 to 1.0 mg L\(^{-1}\)), were applied. Using solely random variables, the experiment was planned (CRD).

Rooting: The effective shoots achieved an average height of 3.0–4.0 cm before the shoots emerged from the culture container; 2.0–3.0 leaves were added to MS media that also contained 5.4 g/L agar, 30 g/L saccharose, NAA (0.0–1.0 mg L\(^{-1}\)), IBA (0.0–1.0 mg L\(^{-1}\)), or a combination of NAA. After 2 weeks of culture, it was possible to observe the rate of germination, the typical number of roots, and the length of the roots.

Acclimatization of tissue cultured plantlets: Rooted plantlets were carefully removed from the medium washed in luke water to remove all traces of agar. They were dipped in the fungicide solution (Bullet® 2.0 mg/L) then acclimatized in a burned rice husk, soil, coconut fiber, sand, and acclimatized in a potting mixture of soil:sand:compost (1:1:1) or a mixture of soil:paddy husk (1:1 and 1:2). They were maintained in a mist chamber for 2 weeks and transferred to soil. Percentage survival was assessed after 4 weeks while maintaining the plants in an open environment. The whole process of in vitro micropropagation was shown in Supplementary Figure S1.

Experimental performance and data analysis: Before the plants were autoclaved at 121 °C for 20 min under pressure, all cultures were adjusted to a pH of 5.8. The studies were conducted in a completely random manner. In vitro culture conditions: Light intensity, temperature, and humidity were 2200–2500 lux, 22 ± 2 °C, and 60–70%, respectively. The data were processed with Microsoft Excel and IRRISTAT 5.0. A comparison equation was developed according to the estimation method of the difference of mean values using the LSD technique (95% confidence level).

3. Results

3.1. Shoot Induction In Vitro and Explant Viability

Although in vitro growth studies have shown that BAP cytokinins and kinetin promote in vitro plant morphogenesis, the requirements of BAP and kinetin differ between plant species and even in different cultivars within the same species.

In this experiment, the addition of BAP and kinetin to the medium had a positive effect on the in vitro shoot growth efficiency of red turmeric (Table 1, Figure 1).
Table 1. Effect of BAP and kinetin concentrations on shoot growth efficiency of *S. thorelii* Gagnep (second week of culture).

<table>
<thead>
<tr>
<th>Growth Regulators</th>
<th>Explants Inoculated</th>
<th>Shoots Initiated</th>
<th>Shoots/Explants (mean ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal medium (control)</td>
<td>44</td>
<td>42</td>
<td>0.95 ± 0.46^a^</td>
</tr>
<tr>
<td>BAP (mg L(^{-1}))</td>
<td>1.0</td>
<td>44</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>44</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>44</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>44</td>
<td>153</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>44</td>
<td>244</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>44</td>
<td>190</td>
</tr>
<tr>
<td>Kinetin (mg L(^{-1}))</td>
<td>1.0</td>
<td>44</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>44</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>44</td>
<td>168</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>44</td>
<td>241</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>44</td>
<td>146</td>
</tr>
</tbody>
</table>

CV% 6.5

Note: Different letter designations (a–g) in the same column indicate a significant difference at the 95% confidence level. The values represent mean ± standard deviation.

**Figure 1.** Evaluating the effect of BAP and kinetin on shoot regeneration of *S. thorelii* Gagnep.

The shoot growth factor increased as BAP and kinetin concentrations were increased from 0.0 to 6.0 mg L\(^{-1}\) and 0.0 to 5.0 mg L\(^{-1}\), respectively. The rate of shoot proliferation tended to decline when BAP and kinetin concentrations were increased to 6.0 and 5.0 mg L\(^{-1}\), respectively. The maximum shoot growth coefficients, 5.55 ± 0.59 shoots/explants for BAP and 5.48 ± 0.87 shoots/explants for kinetin, were produced by the formulations of 5.0 mg L\(^{-1}\) BAP and 4.0 mg L\(^{-1}\) kinetin. Numerous studies on the cultivation of plants belonging to the Zingiberaceae family (turmeric, galangal) have shown that the use of BAP provides good shoot regeneration.

As shown in Figure 2, BAP and kinetin concentrations were strongly correlated with the shoot multiplication coefficient, with correlation coefficients of \(R^2 = 0.903\) (BAP) and \(R^2 = 0.833\) (kinetin), respectively. As a result, the shoot multiplier rose as BAP and kinetin concentrations increased. However, the shoot multiplication coefficient tended to decline as the concentration was increased to 6.0 mg L\(^{-1}\) (BAP) and 5.0 mg L\(^{-1}\) (kinetin).
3.2. Effect of Type and Concentration of Cytokinin and Auxin on In Vitro Shoot Multiplication

The combination of BAP and other growth regulators may benefit plant in vitro shoot multiplication, according to Loc et al. [9]. To find the best formula for multiplying *S. thorelii* Gagnep’s quick shoots, concentrations of 0–4.0 mg L\(^{-1}\) of BAP and 0–1.0 mg L\(^{-1}\) of NAA were utilized alone or in combination. Table 2 and Figure 3 present the outcomes obtained.

Table 2. BAP and NAA’s impact on shoot multiplication (after 8 weeks of culture).

<table>
<thead>
<tr>
<th>Growth Regulators</th>
<th>No. of Shoots/Explants (mean ± S.E.)</th>
<th>Height of Shoots (cm) (mean ± S.E.)</th>
<th>Shoot Quality</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAP (mg L(^{-1}))</td>
<td>NAA (mg L(^{-1}))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>0.0</td>
<td>0.67 ± 0.17 (^{i})</td>
<td>0.76 ± 0.37 (^{j})</td>
</tr>
<tr>
<td>1.0</td>
<td>Absent</td>
<td>2.45 ± 0.50 (^{bi})</td>
<td>4.79 ± 1.11 (^{e})</td>
</tr>
<tr>
<td>2.0</td>
<td>0.5</td>
<td>4.31 ± 0.45 (^{e})</td>
<td>4.68 ± 1.06 (^{e})</td>
</tr>
<tr>
<td>3.0</td>
<td>0.75</td>
<td>4.74 ± 0.92 (^{d})</td>
<td>5.04 ± 1.12 (^{de})</td>
</tr>
<tr>
<td>4.0</td>
<td>1.0</td>
<td>3.97 ± 1.18 (^{cd})</td>
<td>5.52 ± 1.05 (^{d})</td>
</tr>
<tr>
<td>0.25</td>
<td>Absent</td>
<td>2.34 ± 0.48 (^{j})</td>
<td>6.19 ± 1.48 (^{e})</td>
</tr>
<tr>
<td>0.5</td>
<td>0.75</td>
<td>2.78 ± 0.86 (^{b})</td>
<td>6.91 ± 1.19 (^{ab})</td>
</tr>
<tr>
<td>1.0</td>
<td>1.0</td>
<td>2.51 ± 0.75 (^{b})</td>
<td>7.03 ± 1.23 (^{a})</td>
</tr>
<tr>
<td>1.0</td>
<td>0.25</td>
<td>2.85 ± 1.07 (^{a})</td>
<td>6.98 ± 1.19 (^{a})</td>
</tr>
<tr>
<td>2.0</td>
<td>0.5</td>
<td>6.06 ± 0.87 (^{c})</td>
<td>6.42 ± 1.02 (^{bc})</td>
</tr>
<tr>
<td>3.0</td>
<td>0.75</td>
<td>7.54 ± 0.79 (^{a})</td>
<td>6.81 ± 1.11 (^{ab})</td>
</tr>
<tr>
<td>4.0</td>
<td>1.0</td>
<td>7.01 ± 0.71 (^{b})</td>
<td>6.84 ± 1.42 (^{ab})</td>
</tr>
<tr>
<td>CV%</td>
<td></td>
<td>5.20</td>
<td>5.60</td>
</tr>
</tbody>
</table>

Note: + Shoots develop normally; ++ Green shoots, fat, balanced growth, large dark green leaves. Different letter designations (a–k) in the same column indicate a significant difference at the 95% confidence level. The values represent mean ± standard deviation.

The rapid growth of *S. thorelii* Gagnep shoots was impacted by the addition of BAP at concentrations of 0.0–4.0 mg L\(^{-1}\) and NAA of 0–1 mg L\(^{-1}\), alone or in combination. Depending on the empirical formula, the shoot growth coefficient changed. The increase in BAP from 0 to 0.2 mg L\(^{-1}\) tended to increase the shoot growth coefficient and shoot height. Shoot multiplier and shoot height often tended to decrease as the shoot height and shoot multiplication factor increased. The shoot multiplication coefficient was not
significantly affected by higher concentrations of NAA 0–1.0 mg L\(^{-1}\), but the shoot height was significantly affected. BAP and NAA together have a considerable impact on the shoot multiplier and shoot height. The shoot multiplier increased as the BAP concentration increased from 0 to 2 mg L\(^{-1}\) and the NAA concentration from 0 to 0.5 mg L\(^{-1}\). However, the growth multiplier tended to decrease as the BAP concentration increased from 2 to 4 mg L\(^{-1}\) and NAA concentrations from 0.5 to 1 mg L\(^{-1}\). The biggest shoot growth factor (Figure 3) was significant when 2.0 mg L\(^{-1}\) of BAP and 0.5 mg L\(^{-1}\) of NAA were applied, resulting in a shoot multiplier (no. of shoots/explants) of 7.54 ± 0.79. Shoot height was highest when NAA or NAA was administered in addition to BAP.

![Figure 3. Stahlianthus thorelii Gagnep’s rapid in vitro proliferation and the effect of growth regulators.](image)

3.3. The Impact of NAA and IBA on In Vitro Root Development

From the aforementioned tests, in vitro shoots (3.0–4 cm; 2.0–3.0 leaves) were extracted and placed on MS root induction medium with the addition of NAA, IBA plus NAA and IBA individually, or IBA with a NAA concentration of 0–1 mg L\(^{-1}\). The findings after 2 weeks of culture are displayed in Table 3 and Figure 4, respectively. The results showed that the MS control medium without added growth stimulants produced a significantly higher rooting rate of about 80% rooted shoots. This indicated that in vitro shoots could root well when placed in a MS basal medium with no growth promoters. However, the roots induced on the control medium developed weekly. In the mixed medium of 0.5 mg L\(^{-1}\) of NAA and 0.5 mg L\(^{-1}\) of IBA, the best rooting result was 26.17 ± 1.5 roots/shoots and the roots were hairy. The root length was significantly impacted by the mixture that included growth stimulants. The longest root lengths were seen in the treatments that included 0.25 mg L\(^{-1}\) NAA, 0.75 mg L\(^{-1}\) IBA, 0.25 mg L\(^{-1}\) NAA combined with 0.25 mg L\(^{-1}\) IBA, and 0.5 mg L\(^{-1}\) NAA combined with 0.5 mg L\(^{-1}\) IBA length.
Table 3. Evaluation of IBA and NAA’s effect on rooting growth (after the second week of culture).

<table>
<thead>
<tr>
<th>Growth Regulators</th>
<th>Rooting Rate (%)</th>
<th>No. of Roots/Shoots (mean ± S.E.)</th>
<th>Root Length (cm) (mean ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAA (mg L\textsuperscript{-1})</td>
<td>IBA (mg L\textsuperscript{-1})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>0.0</td>
<td>80</td>
<td>1.61 ± 0.4 \textsuperscript{k}</td>
</tr>
<tr>
<td>0.25</td>
<td>0.25</td>
<td>100</td>
<td>15.26 ± 1.9 \textsuperscript{f}</td>
</tr>
<tr>
<td>0.5</td>
<td>0.5</td>
<td>100</td>
<td>18.60 ± 1.1 \textsuperscript{e}</td>
</tr>
<tr>
<td>0.75</td>
<td>Absent</td>
<td>100</td>
<td>14.25 ± 1.5 \textsuperscript{h}</td>
</tr>
<tr>
<td>1.0</td>
<td>0.25</td>
<td>100</td>
<td>10.75 ± 1.2 \textsuperscript{i}</td>
</tr>
<tr>
<td>Absent</td>
<td>0.5</td>
<td>100</td>
<td>19.76 ± 1.9 \textsuperscript{d}</td>
</tr>
<tr>
<td>0.25</td>
<td>0.75</td>
<td>100</td>
<td>17.22 ± 1.8 \textsuperscript{f}</td>
</tr>
<tr>
<td>0.5</td>
<td>1.0</td>
<td>100</td>
<td>14.62 ± 1.7 \textsuperscript{h}</td>
</tr>
<tr>
<td>0.75</td>
<td>0.25</td>
<td>100</td>
<td>19.72 ± 1.4 \textsuperscript{d}</td>
</tr>
<tr>
<td>1.0</td>
<td>0.5</td>
<td>100</td>
<td>26.17 ± 1.5 \textsuperscript{a}</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>100</td>
<td>22.57 ± 1.5 \textsuperscript{c}</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>100</td>
<td>23.64 ± 2.4 \textsuperscript{b}</td>
</tr>
</tbody>
</table>

CV% 6.5 5.6

Different letter designations (a–k) in the same column indicate a significant difference at the 95% confidence level. The values represent mean ± standard deviation.

Figure 4. Effect of growth regulators of *S. thorelii* Gagnep root development.

3.4. Acclimatization of Tissue Cultured Plantlets

Highest percentage of survival (100%) was observed when tissue cultured plantlets were acclimatized in soil:sand:compost (1:1:1), coconut fiber 91.64%. Moreover, 80.40% of survival was observed when in vitro plantlets were grown in the mixture of soil:paddy husk (1:1), sand 58.70%, soil 53.54%. Furthermore, the lowest survival rate (30.27%) was observed in the mixture of soil:paddy husk (1:2), burned rice husk 16.13%. Reduction in water holding capacity in the presence of the high amount of paddy husk in this treatment could be the reason for the low survival percentage of plantlets in this potting mixture. The use of porous potting mixture with good water holding capacity which allows adequate drainage and aeration would provide better conditions for fast acclimatization of in vitro regenerated plants.

Therefore, the result obtained the highest percentage of survival (100%) observed when tissue cultured plantlets were acclimatized in soil: sand: compost (1:1:1), shoot length 29.40 ± 3.59 cm (Table 4 and Figure 5).
Table 4. Effect of various potting mixtures on hardening of in vitro rooted plantlets of *S. thorelii* Gagnep after 4 weeks.

<table>
<thead>
<tr>
<th>Potting Mixture</th>
<th>No. of Plantlets Transferred</th>
<th>Survival Rate (%)</th>
<th>New Leaf Formation (%)</th>
<th>Shoot Length (cm) (mean ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burned rice husk</td>
<td>36</td>
<td>16.13</td>
<td>43.70</td>
<td>10.20 ± 3.35 a</td>
</tr>
<tr>
<td>Soil</td>
<td>36</td>
<td>43.54</td>
<td>53.60</td>
<td>15.10 ± 3.81 d</td>
</tr>
<tr>
<td>Coconut fiber</td>
<td>36</td>
<td>91.64</td>
<td>72.40</td>
<td>25.00 ± 3.84 b</td>
</tr>
<tr>
<td>Sand</td>
<td>36</td>
<td>58.70</td>
<td>61.50</td>
<td>13.80 ± 4.24 d</td>
</tr>
<tr>
<td>Soil:sand:compost (1:1:1)</td>
<td>36</td>
<td>100.00</td>
<td>88.60</td>
<td>29.40 ± 3.59 a</td>
</tr>
<tr>
<td>Soil:paddy husk (1:1)</td>
<td>36</td>
<td>80.40</td>
<td>66.70</td>
<td>25.70 ± 3.69 b</td>
</tr>
<tr>
<td>Soil:paddy husk (1:2)</td>
<td>36</td>
<td>30.27</td>
<td>59.30</td>
<td>19.50 ± 3.46 c</td>
</tr>
</tbody>
</table>

CV% 7.00

Different letter designations (a–e) in the same column indicate a significant difference at the 95% confidence level. The values represent mean ± standard deviation.

![Plantlets](image)

**Figure 5.** Various potting mixtures on hardening of in vitro rooted plantlets of *S. thorelii* Gagnep after 4 weeks.

4. Discussion

4.1. Shoot Induction In Vitro and Explant Viability

*S. thorelii* Gagnep can reproduce asexually by means of tubers. Seeds of *S. thorelii* Gagnep tubers are mainly utilized for multiplication and production [6]. This methodology has a number of disadvantages, such as a low rate of multiplication and vulnerability to various diseases. The plant tissue culture technique has gained popularity as an alternative method of vegetative propagation of plants in recent years. As a modern technology, plant tissue culture has great potential to meet the increasing world demand. It has made significant contributions to the betterment of agricultural sciences in recent times, and today, it is an indispensable tool in modern agriculture. Due to tissue culture, we are able to micropropagate a large number of plants from a single seed or explants with select desirable traits; reduce the amount of space required for field trials; and generate disease-free plants through careful selection and sterile techniques.

Cytokinins have been implicated in many aspects of plant growth and development. The ratio of cytokinin to auxin determines the type of organs regenerated from undifferentiated callus tissue in vitro. Moreover, callus placed on media with a high cytokinin to auxin ratio usually produces many shoots and few roots, while callus placed on media with a low cytokinin to auxin ratio usually produces few shoots and many roots; equal concentrations result in the proliferation of undifferentiated callus [15]. Treating lateral buds with cytokinin causes them to end their dormancy and grow [16]. Application of cytokinins to whole plants or detached leaves tends to delay senescence [17].

Cytokinins, which play a major promoting role, were found to release these shoots from dormancy when exogenously applied to the axillary buds of intact peas (*Pisum sativum*) [18–21]. The main objective of this method was to regenerate directed tissue growth, a process controlled mainly by growth regulators (auxin/cytokinin ratios) introduced into the culture medium.
Cytokinins have been shown to influence sink/source relationships, germination, the formation of vascular tissue, and cotyledon expansion in many different plant species [22]. According to Behera et al. [11] and Nayak et al. [23], the MS basal medium, which uses the cytokinin BAP, gives good results in terms of shoot regeneration. Nayak et al. [23] showed that the MS basal medium with a BAP concentration of 3.0 mg L\(^{-1}\) produced the best results for galangal (*Alpinia galanga* L.). Behera et al. [24] also found that 2.0 mg L\(^{-1}\) of turmeric served as the optimal concentration of MS basal medium with BAP for shoot growth (*Curcumar longa* L.cv. Ranga). According to Bhattacharya’s [25] research, *Kaenpferia galanga* L., which is pathogen-free, grows best when the MS basal medium contains 4.0 mg L\(^{-1}\) kinetin and 3.0 mg L\(^{-1}\) BAP. According to Senarath et al. [26], a MS basal medium with 2.0 mg L\(^{-1}\) BAP had the maximum effect on *Kaempferia Galanga* rhizome shoot samples. According to Goyal et al. [27], shoot regeneration was very effective in *Curcuma longa* when the MS basal medium had a BAP concentration of 2.0 mg L\(^{-1}\). MS media supplemented with 5.0 mg L\(^{-1}\) BAP or 5.0 mg L\(^{-1}\) BAP and 5.0 mg L\(^{-1}\) kinetin or both produced the most shoots, with 5.55 and 5.48 shoots/explants, respectively. When three *Curcuma longa* L. cultivars—Faisarabad, Kasur, and Bannung—were grown on a medium enriched with 4.0 and 5.0 mg L\(^{-1}\) BAP, the highest shoot growth coefficients were attained. These outcomes are similar to those reported by Naz et al. [28].

4.2. Effect of Type and Concentration of Cytokinin and Auxin on In Vitro Shoot Multiplication

Shoot growth is a process controlled by a complex interplay of hormones, nutrients, and environmental cues [29–33]. The phytohormones auxin and cytokinin interact to regulate many plant growth and developmental processes [34].

Additionally, auxin and cytokinin act antagonistically to coordinate axillary bud development. Auxin is able to directly inhibit cytokinin biosynthesis through an *Auxin Resistant 1 (AXR1)*-dependent auxin signaling pathway, consequently suppressing axillary bud outgrowth [35]. On the other hand, exogenous cytokinin application is able to overcome the inhibitory effect of auxin on the axillary bud activity [36]. A recent study demonstrated that cytokinin determines the transcript accumulation of auxin efflux transporter *PIN3*, *PIN4*, and *PIN7* to promote shoot branching in *arr1* mutant [37]. These accumulating evidences in the model plant *Arabidopsis* provide an appealing model that high auxin level inhibits the activity of axillary bud, while cytokinin takes the opposite effect [37].

Cytokinins have been implicated in many aspects of plant growth and development. The ratio of cytokinin to auxin determines the type of organs regenerated from undifferentiated callus tissue in vitro. Callus placed on media with a high cytokinin to auxin ratio usually produces many shoots and few roots, while callus placed on media with a low cytokinin to auxin ratio usually produces few shoots and many roots; equal concentrations result in the proliferation of undifferentiated callus [15].

Numerous studies regarding ginger plants’ culture have demonstrated that using a MS base medium enriched with BAP in conjunction with NAA produces better results than using growth stimulants alone in the process of multiplying the shoots [38]. In a study by Senarath et al. [26], a MS basal medium supplemented with BAP (2.0 mg L\(^{-1}\)) and IBA (0.5 mg L\(^{-1}\)) improved *K. galanga* Linn (Zingiberaceae) in vitro. In a study by Goyal et al. (2010) [27] using a MS basal medium enriched with a mixture of 2.0 mg L\(^{-1}\) BAP and 3.0 mg L\(^{-1}\) kinetin, the most shoots of *Curcuma longa* Linn plants were generated. The best results for *Kaempferia parviflora* in terms of the shoot number, length of shoots, and root number were found when using a MS basal medium with 1.5 mg L\(^{-1}\) of BAP, according to research by Nur Adilla Khairudin et al. [39] (Zingiberaceae). The MS medium with BAP at 2.0 and 4.0 mg L\(^{-1}\) produced *Stahlianthus campanulatus* Kuntze the most shoots, according to research by W. Mongkolsawat et al. [40]. The MS medium with 2.2 mg L\(^{-1}\) BAP had the highest number of shoot multipliers at 3.67 shoots/explants, according to research on *Ellingera elatior* (Zingiberaceae) by Abdelmageed et al. [41]. The maximum average number of shoots (5.6 shoots/explants) was produced by the reference MS supplemented with 5.0 mg L\(^{-1}\) of BAP and 2.0 mg L\(^{-1}\) of IAA or 3.0 mg L\(^{-1}\) of BAP and 0.5 mg L\(^{-1}\)
IAA, according to a study by Faridah et al. [42]. The highest number of shoots for *Curcuma longa* Linn was produced in vitro by a MS basal medium supplemented with 2.0 mg L\(^{-1}\) of BAP, according to research by Rahman et al. [43]. The most shoots were produced in vitro in *Curcuma xanthorrhiza* Roxb plants when a basal medium with a BAP concentration of 2 mg L\(^{-1}\) was used, according to Suci Rahayu et al. [44]. The most shoots were produced in vitro by ginger (*Zingiber officinale* Rosco) plants using the MS basal medium with BAP (4.5 mg L\(^{-1}\)), according to research by Mohamed et al. [45]. In vitro *Zingiber officinal* Rosco treatment using a MS medium with BAP (9 mg L\(^{-1}\)) was highly effective, according to research by Abbas et al. [46].

### 4.3. The Impact of NAA and IBA on In Vitro Root Development

Auxins are involved in the formation of lateral roots, the regulation of the xylem development, and cambium growth in plants [47]. Adventitious root formation is stimulated by auxins and the response to auxin and its role in controlling the roots formation and their length and number are really important. Auxin role in root induction is significantly more highlighted than its initiation. In general, roots formation in plants occurs through two phases; the first phase is sensitive to auxin content but, the second phase is auxin insensitive. Roots primordia formation in cuttings is dependent upon internal auxin content and some synergistic components, such as diphenyls Yan et al. [48].

Auxin is considered a key regulator of root growth, gravitropism, and lateral root (LR) formation [49]. Exogenous auxin treatment has typically resulted in inhibited root growth rate [50–53]. Nevertheless, in Arabidopsis roots, it has been demonstrated that low concentrations of auxin can substantially stimulate primary root elongation [54]. Auxin action on root development is not only a question of concentration but also of its polar translocation [55].

According to a study by Loc et al. [9], ginger shoots successfully produced roots in a MS basal medium containing 2.0 mg L\(^{-1}\) of NAA. Abbas et al. [45] reported that the best rooting outcomes for ginger were obtained using a MS basal medium with 1.0 mg L\(^{-1}\) NAA (*Zingiber officinale* Rosco). In the in vitro research on *Curcuma longa*line conducted by Rahman et al. [43], the authors found that a MS basal medium with IBA (0.2 mg L\(^{-1}\)) was the best rooting medium. In a basal setting, ginger plants with NAA supplements stimulate rooting more effectively than those with IBA, according to the research by Kambaska and Santilata [10].

The results obtained were in conformity with Hartman et al. [56]. The application of auxins affected the regeneration of roots on cuttings. Auxin, in general, promotes the rooting of stem cuttings. However, the effectiveness varies with the nature and concentration of auxin. There was a reverse trend in the number of roots with an increasing auxin concentration.

### 4.4. Acclimatization of Tissue Cultured Plantlets

According to W. Mongkolsawat et al. [40], the plantlets with well-developed shoots and roots after acclimatization were successfully transplanted in soil and coconut fiber with 91.6% survivability potential. The survival of in vitro regenerated plants to ex vitro conditions depends on the conditions during transfer of in vitro plantlets and soil substrates; plant survival may be increased by the addition of moisture absorbing material to soil as stated in the study by Seran et al. [57]. Acclimatization of plantlets can be considered as one of the most important phases in tissue culture techniques. In this investigation, the mortality rate of the plantlets with well-developed roots that were acclimatized and hardened was low. On average, 80% of in vitro transferred plantlets survived in potted soil and did not show any morphological abnormalities. In conclusion, this protocol is a step forward toward the improvement of the propagation of *Z. zerumbet* species with a very important medicinal value [42].
5. Conclusions

Based on the results, the impacts of growing regulators on the in vitro plant *S. thorelii* Gagnep are described here. For the shoot regeneration stage, a basal MS growing medium with 5.0 mg L\(^{-1}\) of BAP or 4.0 mg L\(^{-1}\) of kinetin was observed to be suitable yielding 5.55 ± 0.59 and 5.48 ± 0.87 shoots/explants, respectively. In vitro shoots developed quickly in a base medium with the addition of 2 mg L\(^{-1}\) BAP and 0.5 mg L\(^{-1}\) NAA (7.54 ± 0.79 shoots/explants). The MS media with 0.5 mg L\(^{-1}\) of NAA and 0.5 mg L\(^{-1}\) of IBA (26.17 ± 1.5 Roots/Shoots) had the maximum rooting development. Highest percentage of survival (100%) was observed when tissue cultured plantlets were acclimatized in soil:sand:compost (1:1:1), with 29.40 ± 3.59 cm tree height.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture12111766/s1, Figure S1: In vitro micropropagation process of *S. thorelii* Gagnep.

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Abbreviations

- BAP: 6-Benzyl Amino Purine
- NAA: α-Naphthalene Acetic Acid
- IBA: Indol-3-Butyric Acid
- MS: Murashige and Skoog

References


