Establishment of an efficient regeneration system of ‘ZiKui’ tea with hypocotyl as explants

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Abstract

Zikui (Camellia sinensis cv. Zikui) is a recently discovered cultivar of local purple tea in Guizhou, China and it is a perennial woody plant, mainly propagated by cuttings. It is also a model plant for the study of anthocyanins, but the limited germplasm resources and the limitation of traditional reproduction seriously hinder its application. Here, an efficient regeneration system is established by using hypocotyl as explants for the first time. Different plant growth regulators (PGRs) were evaluated during different regeneration processes including callus and root induction. According to our findings, using the optimal disinfection conditions, the embryo contamination rate was 17.58 %. Additionally, the mortality rate was 9.69 %, while the survival rate was measured as 72.73 %. Moreover, the highest germination rate of 93.64 % was observed under MS+2.40 mg/L GA3 medium conditions. The optimal callus induction rate was 95.19 %, while the optimal adventitious bud differentiation rate was 20.74%, Medium with 1.6mg L−1 IBA achieved 68.6% rooting of the adventitious shoots. The survival rate was more than 65% after 6 days growth in the cultivated matrix. In summary, our research aims to establish a regeneration system for Zikui tea plants and design a transformation system for tea plant tissue seedlings. This will enable the transfer of the target gene and ultimately facilitate the cultivation of new tea varieties with unique characteristics.

Introduction

Tea plants (*Camellia sinensis L.*) are extensively cultivated worldwide, and tea is also among the top three non-alcoholic beverages globally. Purple leaf tea is a type of tea germplasm resource characterized by purple buds and leaves, and it contains a high amount of anthocyanin. Anthocyanins exhibit significant antioxidant properties, thereby enhancing the health benefits of purple bud varieties in tea production. Consequently, it is a crucial area of concentration in the breeding and investigation of tea tree. It is a dire need to establish the protocols for successful tissue culture propagation of the Zikui tea plant.

The Zikui is abundant in China, and a unique purple tea tree known for its high content of anthocyanins. The green tea made from purple tea tree has a mellow taste, a long-lasting aroma and a little bitter. Anthocyanins are pigments soluble in water, possess numerous beneficial properties including free radical scavenging, anticancer effects, and prevention of cardiovascular and cerebrovascular disorders. Its health function has also become one of the important qualities of purple tea breeding. The anthocyanins in purple tea are rich in resources, safe and non-toxic, with diverse biological activities, and have become a hot research and development topic in the field of food and health products. Zikui trees have an exceptionally elevated concentration of anthocyanins, reaching 4.97mg/g. This amount surpasses that of other tea tree varieties by more than threefold. Additionally, the antioxidative capability of this compound is remarkably remarkable, being 50 times stronger than vitamin E and 20 times more potent than vitamin C. Anthocyanins can be extracted from the Zikui tea tree as well. Moreover, the anthocyanin content of purple tea tree reached 0.5-1.0% of dry matter weight. In addition,
anthocyanins have attracted increasing attention from researchers due to their various bioactive effects such as antibacterial, salt resistance, and lipid reduction, as well as their beneficial effects on cardiovascular diseases, eye diseases, neurodegenerative diseases, and cancer\textsuperscript{15-16}. To address the issue of limited tea seedlings, one possible solution is to integrate the Zikui tissue regeneration system with various biotechnologies, including genetic engineering\textsuperscript{17}. This combination can offer sterile resources and serve as a valuable technical resource for future investigations into the mechanisms underlying the transformation of tea into a purple variant. The important prerequisite for establishing a genetic transformation system for tea trees is to establish a regeneration system for tea tree differentiation through tissue culture technology\textsuperscript{18}. The genetic transformation of purple sunflower tea trees can be greatly influenced by the presence of the exceptional and uncommon compound known as anthocyanins. This particular substance holds remarkable scientific research value due to its rarity and unique characteristics.

For the purpose of investigating the propagation mechanism in tea trees, a novel regeneration system is successfully developed using hypocotyl as explants. Through tissue culture, we were able to achieve good treatment conditions and develop suitable culture medium formulations, thereby enhancing the research achievements of tea plant in vitro culture technology. As a next step, the combination of tea plant tissue culture and transgenic technology will be explored to establish an advanced tea plant tissue culture seedling transformation system. This system would facilitate the transfer of target genes using the developed regeneration system.

**Materials and methods**

**Plant materials**

Efficient regeneration system was developed by inducing selected Zikui seed embryos, collected from October to November, as explants, with various plant growth regulators (PGR). To facilitate the growth of fully adapted plants within their respective environments, this study focused on exploring the mechanisms involved in embryo germination, cotyl callus formation, differentiation, seedling vigor, and rooting processes.

**Seed disinfection**

After harvesting, the Zikui seeds were extracted by removing the outer peel and then removing the seed shells using a sheller. Subsequently, high-quality seeds free from pollution were meticulously chosen and soaked in a beaker containing a solution of dishwashing liquid for a duration of 8 minutes. The seeds were stirred gently and rinsed with tap water for over 10 minutes. Subsequently, they were soaked in a carbendazim solution with a concentration of 0.50% for 10 minutes, with continuous stirring. Following the soaking, the seeds were rinsed again with tap water for more than 10 minutes, while being continuously stirred. Finally, the seeds were placed on a super-clean table for further use. The seeds were placed on the clean bench for subsequent use. The experiment included two disinfectants: 75% ethanol
disinfection for 1, 2, and 3 minutes, and 20% sodium hypochlorite for 7, 10, and 13 minutes. This resulted in a total of 9 treatment groups. Following disinfection with 75% ethanol, the seeds are rinsed three times with sterile water and further disinfected using 20% sodium hypochlorite. To mitigate any potential harm caused by residual disinfection agents, the seeds undergo a thorough rinsing process, consisting of more than seven cycles of sterile water. Following the oscillation, the water from the seed was removed using a sterile blotting paper. The seed embryo was then extracted using a sterile blade and subsequently placed into the seed embryo germination medium.

Contamination rate (%) = \( \frac{\text{Number of contaminated explants}}{\text{total number of inoculated explants}} \times 100\% \)

Survival rate (%) = \( \frac{\text{Number of active explants}}{\text{total number of explants inoculated}} \times 100\% \)

Mortality rate (%) = \( \frac{\text{Number of non-viable explants}}{\text{total number of explants inoculated}} \times 100\% \)

**Determine the suitable medium for embryo germination**

The above sterilized embryos were inoculated into seed embryo germination medium with different GA\(_3\) concentrations, which was MS as the base medium, and the seed embryo germination medium with different GA\(_3\) concentrations was added into the MS as the base medium, and 4 treatment groups with different GA\(_3\) ratio (0.00 ~ 3.60mg/L) were added. Each treatment group was repeated 3 times and 110 doses per dose. The germination of seeds was observed and recorded, and the germination number was recorded after 60 days.

Seed embryo germination rate (%) = \( \frac{\text{number of seed embryo germination}}{\text{total number of seed embryo inoculation}} \times 100\% \)

**Dark culture days of hypocotyl differentiation were determined**

After the hypocotyl of the seedlings in the above germination medium grew out, the hypocotyl of the seedlings was cut into small segments of about 1cm, which were used as explants for callus induction and differentiation, and inoculated into MS + 2.00mg/L 6-BA + 0.80mg/L IBA callus and differentiation medium. After being cultured for different periods (0, 7, 14 days) in a dark environment, they were transferred to a light condition for further culture. The experiment consisted of three treatment groups, with each group being repeated three times, resulting in a total of 90 inoculations per group.

Callus induction rate (%) = \( \frac{\text{number of callus induced by explants}}{\text{total number of explants inoculated}} \times 100\% \)

Adventitious bud differentiation rate (%) = \( \frac{\text{number of callus differentiation buds}}{\text{total number of explants inoculated}} \times 100\% \)
Callus and adventitious bud differentiation were induced

The hypocotyl of the seedlings was cut into small segments measuring approximately 1cm. These segments were then used as explants for callus induction and differentiation. They were inoculated into the hypocotyl differentiation medium, which contained varying proportions of plant growth regulators. The base medium for axial differentiation was WPM. Six treatment groups were formed by using various concentrations of IBA (0.10, 0.30mg/L), NAA (0.10, 0.30mg/L), and 2,4-D (0.10, 0.30mg/L) in a 2.00mg/L 6-BA solution. Each treatment group was repeated three times, resulting in a total of 90 doses. After a period of 30 days, the growth state and number of callus were observed. Subsequently, at the 60-day mark, the growth of adventitious buds and the rate of explant differentiation were observed once more.

Callus induction rate (%) = (number of callus induced by explants/total number of explants inoculated) ×100%

Adventitious bud differentiation rate (%) = (number of callus differentiation buds/total number of explants inoculated) ×100%

Strengthen seedlings and take root

The Adventist bud was cut from the callus and the lower end was cut diagonally. The seedlings were inoculated and cultured in MS + 2.00mg/L 6-BA + 0.60mg/L IBA medium. The lower end of a robust, rootless seedling was measured approximately 5cm in height and was diagonally cut from the strong seedling medium. It was then soaked in a sterile solution of 60.00mg/LIBA for 8 minutes and inoculated into the rooting medium. The aim was to determine the optimal concentration of 1/2 MS + 1.60mg/L IBA for root development.

Transplanting seedlings

The aseptic vaccine in the tissue culture bottle was moved to the greenhouse, and the bottle cap was opened during the process of seedling cultivation. The seedling cultivation time was 6d under natural light. After the cultivation of seedlings, the plants were rinsed with the medium and transplanted into the prepared V-yellow loam: V-vermiculite = 2:1 transplanting medium.

Plant ethics statement

Zikui samples were collected from the Agricultural Bioengineering Research Institute of Huaxi District, Guiyang City, Guizhou Province(lat 26 ° 11 ′-26 ° 34 ′ N, long106 ° 27 ′-106 ° 52 ′E), and the Tea Research Institute of Meitan County(lat 27 °20 ′ N, long 107 ° 15 ′E) Guizhou Province, China, between October and November 2019. The samples were collected by Professor Li Yan and Researcher Zhou Guolan. It is in the custody of Professor Li Yan of Guizhou University. The research was carried out at the Institute of
Agricultural Bioengineering. The study complies with relevant institutional, national and international guidelines and legislation.

**Statistical analysis**

The sampling and inoculation of explants were randomly conducted. The experimental data were collected and summarized using Microsoft Excel. After converting the original data, ANOVA and LSD multiple comparison analysis were performed on the experimental data using SPSS22.0.

**Culture conditions**

In the process of regenerating Zikui tea tree culture, WPM was used as the base medium for cotyledon callus differentiation, while MS was used as the base medium for the remaining steps. For callus differentiation, the composition of the medium included 29.78 g/L of WPM powder, 10.00 g/L of sucrose, and 1.00 g/L of AGAR. During rooting induction, the medium consisted of 2.22 g/L of MS powder, 2.50 g/L of plant gel, and 15.00 g/L of sucrose. For other processes, the medium contained 4.43 g/L of MS powder, 30.00 g/L of sucrose, and 8.00 g/L of AGAR. The pH value of the medium was maintained between 5.80 and 6.00. The tissue culture room was set to a light intensity of 2000 lx, a temperature of \((23 \pm 2)\,^\circ \text{C}\), and a light time of 12 h/d.

**Result**

**Effect of disinfection time on survival rate of seed embryo**

The establishment of a germ aseptic system involves obtaining sterile cotyls and providing materials for the construction of a subsequent regeneration system. Research has demonstrated that varying the duration of disinfection significantly affects the rate of bacterial infection and mortality in seeds (Table 1). Through the comparison of 9 groups, it was observed that after 20 days, the pollution rate and mortality rate of 8 were lower compared to the other groups. The survival rate of this group reached 72.73%, which was the highest among all the groups and thus considered the most effective groups. Therefore, the disinfection time of Zikui tea seed embryos was 75% alcohol disinfection for 3min and 20% sodium hypochlorite immersion for 10min.

When the disinfection time for 75% alcohol remained constant, the pollution rate gradually decreased with a 20% sodium hypochlorite increase in disinfection time. The lowest pollution rate recorded was 15.45%, while the highest pollution rate reached 41.82%. The survival rate initially increased and then decreased, with the lowest survival rate observed at 53.94% and the highest survival rate at 72.73%. When the disinfection time of 20% sodium hypochlorite remained unchanged, the contamination rate gradually decreased and the survival rate gradually increased with an increase in the disinfection time of 75% alcohol. Moreover, as the soaking time of the two disinfection reagents increased, the mortality rate also increased correspondingly, reaching a peak of 17.88%. Disinfection reagents have the capability to not only eliminate fungi and bacteria, but also have the potential to harm the structures, including the cells, of...
the material itself. The longer the disinfection time, the more damaged the seed embryo will be, and even die.

### Table 1
Effect of disinfection time on survival rate of seed embryo

<table>
<thead>
<tr>
<th>75% alcohol (min)</th>
<th>20% sodium hypochlorite (min)</th>
<th>Number of explant (per unit)</th>
<th>Survival rate (%)</th>
<th>Pollution rate (%)</th>
<th>Mortality rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>100</td>
<td>53.94 ± 7.09c</td>
<td>41.82 ± 2.41a</td>
<td>4.24 ± 1.39a</td>
</tr>
<tr>
<td>1</td>
<td>15</td>
<td>100</td>
<td>60.91 ± 1.57bc</td>
<td>31.82 ± 1.57bc</td>
<td>7.27 ± 0.00a</td>
</tr>
<tr>
<td>1</td>
<td>20</td>
<td>100</td>
<td>64.85 ± 2.10bc</td>
<td>30.61 ± 1.89bc</td>
<td>4.54 ± 1.57d</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>100</td>
<td>55.45 ± 2.73c</td>
<td>40.00 ± 2.41a</td>
<td>4.55 ± 0.91d</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>100</td>
<td>64.24 ± 5.01bc</td>
<td>28.18 ± 2.41c</td>
<td>7.58 ± 2.78bc</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>100</td>
<td>65.76 ± 2.29b</td>
<td>25.15 ± 2.78cd</td>
<td>9.09 ± 0.91bc</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>100</td>
<td>67.88 ± 0.52ab</td>
<td>23.33 ± 1.05d</td>
<td>8.79 ± 1.39bc</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>100</td>
<td>72.73 ± 1.82a</td>
<td>17.58 ± 1.39e</td>
<td>9.69 ± 0.52b</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>100</td>
<td>66.67 ± 2.78b</td>
<td>15.45 ± 1.82e</td>
<td>17.88 ± 1.05a</td>
</tr>
</tbody>
</table>

The different letters in the table are significant at the 0.05 level.

**Note**

Mean ± S.D

### The Effect of GA₃ on Germination of Seed Embryos

Gibberellin, a widely used and effective plant growth regulator, stimulates cell elongation and promotes plant growth and development. It was found that adding GA₃ in the medium could effectively promote embryo germination and cotyl growth. (Fig. 1). In this study, the hypocotyl was chosen as the required explants. Group 3 exhibited the highest germination rate, likely due to the long hypocotyl and shorter root hair. The optimal treatment group in this experiment was treatment group 3, which utilized MS + 2.4mg/LGA3 as the seed embryo germination medium for Zikui Tea tree. GA₃ was used to induce the
breaking of seed dormancy and promote germination. It was observed that the germination rate was lower in MS medium without plant growth regulators after removing the cotyledon from the tea embryo. (Fig. 2A). The germination medium was supplemented with GA$_3$ of varying concentrations and ratios (Table 2). The results showed a significant decrease in the germination period of seeds following the addition of GA$_3$. The germination rate increased from 82.73% to over 90.91% when 1.20mg/L GA$_3$ was added (Fig. 2B). The germination rate for 2.4mg/L GA$_3$ was 93.64% (Fig. 2C), and for 3.6mg/L GA$_3$ it was 92.12% (Fig. 2D). There was no significant difference in the germination rate among the three treatment groups. Additionally, the germination period was shortened. However, the length of hypocotyl initially became shorter and then longer with increasing concentration. This suggests that the concentration of GA$_3$ may have a certain effect on the elongation of the cotyl, with both high and low concentrations potentially impacting it.

**Table 2**

<table>
<thead>
<tr>
<th>GA$_3$ (mg/L)</th>
<th>Inoculated number</th>
<th>Germination rate(%)</th>
<th>Germination at 60d</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.20</td>
<td>110</td>
<td>90.91 ± 1.57a</td>
<td>Germination time is short, hypocotyl is short, root hair is long</td>
</tr>
<tr>
<td>2.40</td>
<td>110</td>
<td>93.64 ± 1.82a</td>
<td>Germination time is short, hypocotyl is long, root hair is short</td>
</tr>
<tr>
<td>3.60</td>
<td>110</td>
<td>92.12 ± 1.89a</td>
<td>Germination time is short, hypocotyl is short, root hair is short</td>
</tr>
</tbody>
</table>

The different letters in the table are significant at the 0.05 level

**Note**

Mean ± S.D

**Effect of dark culture days on hypocotyl callus formation and differentiation**

During callus induction, it was observed that dark culture conditions were more conducive to the dedifferentiation process into callus. On the other hand, under the influence of light, the formation of vascular bundles and other tissues was more prominent, leading to a disorder in the dedifferentiation process, which was found to be less favorable for callus generation. The growth rate of callus in plant tissue culture was faster in the absence of light. Therefore, this study determined the optimal dark culture days for cotyledon differentiation in a medium with the same composition. And examined the relationship between the duration of dark embryo days and the rate of differentiation (Table 3). The
findings suggested a positive correlation between these two variables. However, it should be noted that the observed change range was relatively small. This could possibly be attributed to the fact that the medium used in this study might not the most optimal for cotyledon differentiation. During the dark embryo development period, which ranged from 0 to 7 days and eventually reaches 14 days, there was a noticeable increase in the number of cotyledon-induced calluses. In fact, the callus induction rate reached an impressive 93.64%. Hence, it can be concluded that the ideal duration for promoting cotyledon differentiation through dark culture is 14 days.

Table 3
Effect of dark culture days on hypocotyl callus formation and differentiation.

<table>
<thead>
<tr>
<th>Dark cultivation time (d)</th>
<th>Number of vaccinations</th>
<th>Callus induction (%)</th>
<th>Differentiation rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>90</td>
<td>82.22 ± 2.22b</td>
<td>3.70 ± 0.64b</td>
</tr>
<tr>
<td>7</td>
<td>90</td>
<td>84.44 ± 2.22b</td>
<td>4.81 ± 0.64ab</td>
</tr>
<tr>
<td>14</td>
<td>90</td>
<td>93.64 ± 1.82a</td>
<td>5.93 ± 0.64a</td>
</tr>
</tbody>
</table>

The different letters in the table are significant at the 0.05 level

Note

Mean ± S.D

Effect of plant growth regulators on hypocotyl callus induction and differentiation

The induction of callus and buds were performed using the same medium. Different types and concentrations of plant hormones had different effects on callus induction and organ differentiation. The study showed that in six treatment groups (Table 4). The Callus induction rate did not show a significant difference between group 1 and group 4. However, the differentiation rate of Group 1 was 20.74% (Fig. 3D), which was the highest among all the treatment groups. Based on factors such as callus growth status, it can be concluded that Group 1 performed the best among the 6 treatment groups (Fig. 4). Therefore, the callus induction and differentiation culture base of Zikui tea tree was WPM + 2.00mg/L 6-BA + 0.10mg/L NAA.

The results showed that the recovery rate of 6 groups ranged from 88.89–97.04%, and the callus induction rate of 6 groups was very high. Under the conditions of 6-BA and NAA, the callus appeared green and the callus structure was tight, and the adviced-bud clustered with subsequent differentiation were abundant (Fig. 3A). The callus appeared yellowish green and had a tight structure, with a slight amount of browning observed at a later stage (Fig. 3B). Under the conditions of 6-BA and 2,4-D, the callus displayed a mixture of green and white colors. The structure of the callus was loose, and there were only
a few indefinite bud clusters present (Fig. 3C). As the concentrations of IBA, NAA and 2,4-D regulators increased from 0.1mg/L to 0.3mg/L, the differentiation rates were found to be decreasing, and the concentration of 0.1mg/L was more suitable for differentiation.

### Table 4
Effect of plant growth regulators on hypocotyl callus induction and differentiation.

<table>
<thead>
<tr>
<th>Processing condition</th>
<th>Inoculated number</th>
<th>Callus induction rate (%)</th>
<th>Induction rate of bud differentiation (%)</th>
<th>Callus growth status</th>
</tr>
</thead>
<tbody>
<tr>
<td>WPM + 2.00mg/L 6-BA + 0.10mg/L NAA</td>
<td>90</td>
<td>95.19 ± 0.64a</td>
<td>20.74 ± 0.64a</td>
<td>The incision's edge is expanded, appearing green, and it covers the cotyl's surface. It has a tight structure and numerous adventitit bud clusters.</td>
</tr>
<tr>
<td>WPM + 2.00mg/L 6-BA + 0.30mg/L NAA</td>
<td>90</td>
<td>96.30 ± 1.70a</td>
<td>15.93 ± 1.70c</td>
<td>The edge of incision appears swollen and green, extending over the surface of the cotyl. The structure is tightly packed with only a few adventitious bud clusters.</td>
</tr>
<tr>
<td>WPM + 2.00mg/L 6-BA + 0.10mg/L IBA</td>
<td>90</td>
<td>91.85 ± 1.28b</td>
<td>19.03 ± 1.11ab</td>
<td>The edge of incision appeared expanded and had a yellow-green color, which covered the surface of the cotyl. The structure was tightly packed, with numerous adventitious bud clusters and a slight amount of browning.</td>
</tr>
<tr>
<td>WPM + 2.00mg/L 6-BA + 0.30mg/L IBA</td>
<td>90</td>
<td>97.04 ± 1.70a</td>
<td>18.15 ± 1.70b</td>
<td>The edge of the incision appeared to be expanded and had a yellow-green color, spreading over the surface of the cotyl. The structure was tightly packed with numerous adventitious bud clusters and a slight amount of browning.</td>
</tr>
<tr>
<td>WPM + 2.00mg/L 6-BA + 0.10mg/L 2,4-D</td>
<td>90</td>
<td>90.00 ± 1.11bc</td>
<td>15.56 ± 1.11c</td>
<td>The edge of the incision appears to be primarily swollen at one end, exhibiting a combination of white and light green colors.</td>
</tr>
<tr>
<td>WPM + 2.00mg/L 6-BA + 0.30mg/L 2,4-D</td>
<td>90</td>
<td>88.89 ± 2.22c</td>
<td>8.52 ± 0.64d</td>
<td>The edges of the incisions were predominantly swollen at one end, appearing white or light green. They covered the surface of the cotyl and exhibited a loose structure with only a few adventitious bud clusters.</td>
</tr>
</tbody>
</table>

**Effect of plant growth regulator on strong seedling**

Strong seedling culture could enhance the plant robustness, thereby establishing a solid foundation for the subsequent rooting stages. Previous research demonstrated that varying ratios of plant growth
regulators have a noteworthy impact on the strength of plant seedlings (Table 5). When 2.00 mg/L 6-BA + 0.60mg/L IBA were added to the MS medium, the plant height difference reached 2.12cm after 45 days, which was the highest among all treatments. Therefore, this combination was considered as the optimal treatment (Fig. 5).

The difference in plant height was 1.51 cm when no hormone was used. However, when 2.00 mg/L of 6-BA was applied, the difference in plant height between the 6th and 7th treatment groups increased to 1.81cm. Therefore, it can be concluded that 2.00mg/L of 6-BA is the optimal concentration.

<table>
<thead>
<tr>
<th>6-BA (mg/L)</th>
<th>IBA (mg/L)</th>
<th>Inoculated number</th>
<th>Plant height difference (cm)</th>
<th>45d plant growth status</th>
</tr>
</thead>
<tbody>
<tr>
<td>–</td>
<td>–</td>
<td>120</td>
<td>1.51 ± 0.17c</td>
<td>The stem is thin and tall, with poor growth</td>
</tr>
<tr>
<td>1.20</td>
<td>–</td>
<td>120</td>
<td>1.28 ± 0.22c</td>
<td>Stem thin and short, poor growth</td>
</tr>
<tr>
<td>2.00</td>
<td>–</td>
<td>120</td>
<td>1.40 ± 0.10c</td>
<td>The stem is thin and short, the base has tufted bud differentiation, growing well</td>
</tr>
<tr>
<td>1.20</td>
<td>0.60</td>
<td>120</td>
<td>1.31 ± 0.03c</td>
<td>Stem thick and short, poor growth</td>
</tr>
<tr>
<td>1.20</td>
<td>0.90</td>
<td>120</td>
<td>1.45 ± 0.07c</td>
<td>The stem is thick and tall and grows moderately</td>
</tr>
<tr>
<td>2.00</td>
<td>0.60</td>
<td>120</td>
<td>2.12 ± 0.09a</td>
<td>The stem is thick and tall and grows well</td>
</tr>
<tr>
<td>2.00</td>
<td>0.90</td>
<td>120</td>
<td>1.81 ± 0.18b</td>
<td>The stem is thick and tall, the base has tufted bud differentiation, the growth is good</td>
</tr>
</tbody>
</table>

**Effect of plant growth regulators on plant rooting**

NAA and IBA are plant growth regulators that could be alone or in combination. They play a certain role in promoting plant cell division and promoting plant development. The results showed that the ratio of different plant growth regulators had a significant effect on plant rooting in tissue culture seedlings (Table 6). Under the condition of 1/2MS + 1.60mg/LIBA, the rooting rate after 60 days was 68.57%. These values were the highest among all the treatment groups (Fig. 6). Therefore, the medium suitable for rooting of tissue culture seedlings of Zikui tea tree was 1/2MS + 1.60mg/LIBA.
Table 6
Effects of different ratios of plant growth regulators on plant rooting.

<table>
<thead>
<tr>
<th>Processing condition</th>
<th>Inoculated number</th>
<th>Rooting rate (%)</th>
<th>Mean number of roots</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/2MS</td>
<td>35</td>
<td>8.89 ± 1.65ef</td>
<td>2.00 ± 0.00f</td>
</tr>
<tr>
<td>1/2MS + 0.50mg/L NAA</td>
<td>35</td>
<td>26.67 ± 1.65d</td>
<td>3.67 ± 0.58e</td>
</tr>
<tr>
<td>1/2MS + 0.50mg/L IBA</td>
<td>35</td>
<td>30.48 ± 1.65c</td>
<td>6.33 ± 0.58c</td>
</tr>
<tr>
<td>1/2MS + 1.60mg/L NAA</td>
<td>35</td>
<td>56.19 ± 1.65b</td>
<td>7.33 ± 0.58b</td>
</tr>
<tr>
<td>1/2MS + 1.60mg/L IBA</td>
<td>35</td>
<td>68.57 ± 2.86a</td>
<td>8.67 ± 0.58a</td>
</tr>
<tr>
<td>1/2MS + 0.25mg/L IBA + 0.25mg/L IBA</td>
<td>35</td>
<td>10.48 ± 1.65e</td>
<td>5.33 ± 0.58d</td>
</tr>
<tr>
<td>1/2MS + 0.80mg/L NAA + 0.80mg/L IBA</td>
<td>35</td>
<td>6.67 ± 1.65f</td>
<td>4.33 ± 0.58e</td>
</tr>
</tbody>
</table>

Effect of seedling refining time and transplanting medium on survival rate of plants

The results of the six sets of data (Table 7) indicated that the survival rate progressively increased as the seedling cultivation time increased. When the seedling cultivation time reached 6 days, the survival rate exceeded 59.42%, suggesting that this duration was considered suitable for seedling cultivation. The survival rate of V yellow loam with V vermiculite = 2:1 ratio was higher than that of V nutrient soil with V vermiculite = 2:1 ratio. When the ratio of V yellow loam: V vermiculite = 2:1 and seedling cultivation time was 6d, the survival rate was the highest (65.22%) among the 6 treatment groups. Moreover, under these conditions, the plants grew vigorously after transplantation, the leaves extended, and the roots continued to extend downward (Fig. 7). Therefore, the seedling cultivation time of Zikui was 6d, and the transplanting medium was selected V yellow loam: V vermiculite.

Table 7
Effects of different ratios of plant growth regulators on plant rooting.

<table>
<thead>
<tr>
<th>Seedling refining time (d)</th>
<th>Nutrient soil(2:1)</th>
<th>Perlite</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Yellow loam: vermiculite</td>
<td>27.54 ± 2.51d</td>
</tr>
<tr>
<td>4</td>
<td>Nutrient soil: vermiculite</td>
<td>20.29 ± 2.51e</td>
</tr>
<tr>
<td>6</td>
<td>Yellow loam: vermiculite</td>
<td>65.22 ± 4.35e</td>
</tr>
<tr>
<td>6</td>
<td>Nutrient soil: vermiculite</td>
<td>59.42 ± 2.51b</td>
</tr>
<tr>
<td>8</td>
<td>Yellow loam: vermiculite</td>
<td>42.03 ± 2.51c</td>
</tr>
<tr>
<td>8</td>
<td>Nutrient soil: vermiculite</td>
<td>40.58 ± 2.51c</td>
</tr>
</tbody>
</table>
Discussion

The aseptic system plays a critical role in the success of the plant tissue culture process\textsuperscript{19}. Zikui tea tree, being a perennial woody plant, is susceptible to contamination by various microorganisms and bacteria as a result of prolonged exposure to the external environment. Therefore, selecting and controlling the disinfection time is a vital step in minimizing material pollution\textsuperscript{20}. Currently, commonly employed bactericidal methods include alcohol, mercury chloride, sodium hypochlorite, bleaching powder, hydrogen peroxide, and neogeramine\textsuperscript{21}. In a study conducted by Wu\textsuperscript{22}, the ‘Yucha No.1’ embryo was disinfected by cleaning it first and then treating it with 75% alcohol for 30 seconds. This was followed by sterilization using a 0.10% mercuric chloride solution for 6 minutes. These conditions led to a relatively low contamination rate and mortality rate of the embryo. In a similar study, Wang\textsuperscript{23} successfully established a sterile system by collecting tea tree stem segments and leaves. The explants were disinfected with 75% alcohol for 1 minute and then washed with 4% sodium hypochlorite for 20 minutes. The experiment determined that a combination of 75% alcohol disinfection for 3 minutes and 20% sodium hypochlorite disinfection for 10 minutes is the optimal method for disinfection. This method proved to be effective while also minimizing damage to external implants.

The concentration ratio and type of plant growth regulators play a significant role in inducing callus and differentiating organs in plants\textsuperscript{24}. In a study on the establishment of a regeneration system based on tea tree cotyledons, Xie\textsuperscript{25} observed a callus induction rate of 95.5% in ‘Zhonghuang No. 1’ cotyledons using MS + 2mg/L 6-BA + 0.3mg/L IBA medium. In their study, Chen\textsuperscript{26} treated the cotyl of ‘Yucha No. 1’ with a medium containing MS + 2mg/L 6-BA + 0.3mg/L IBA, resulting in a callus induction rate of 71.7%. In this study, we used a culture condition of WPM + 2.00mg/L 6-BA + 0.10mg/L NAA. The callus induction rate of Zikui tea tree was determined to be 95.19%, indicating a high rate of callus induction. However, the adventitious bud differentiation rate was found to be only 20.74%, suggesting that the callus of Zikui tea tree encountered difficulties in re-differentiating buds. Genotype plays a significant role in tissue and organ differentiation. The low differentiation rate could be attributed to the high content of their own genes or anthocyanins.

In this study, GA\textsubscript{3} was utilized to alleviate seed dormancy and enhance seed germination. Previous research conducted by Yang\textsuperscript{27} indicated that a high concentration of GA\textsubscript{3} may impede the germination process of Toon sinensis seeds to a certain extent. However, Li\textsuperscript{28} found that the germination rate decreased when the concentration of GA\textsubscript{3} reached 3.60mg/L. This decrease could be attributed to the excessive influence of plant hormones on the germination of tissue culture seedlings. This study revealed that varying concentrations of GA\textsubscript{3} had the ability to either enhance or hinder the germination and growth of tissue culture seedlings.

The adoption of the plant tissue culture method involves using Zikui tea tree embryos as explants. It is crucial to establish the optimal sterilization and debacteria conditions for the explants. Additionally, careful consideration should be given to the induction differentiation medium, media for advanced
budding of strong seedlings, and the steps for transplanting and seedling cultivation. If seedlings are not cultivated properly, their root system may wither easily, resulting in transplantation failure. Additionally, if seedlings are transplanted without proper disinfection and heat preservation, they can be easily contaminated by external factors and may even die. Substrate selection, disinfection, and heat preservation are all crucial steps in the transplantation process. When using a transplanting medium consisting of yellow loam mixed with vermiculite in a ratio of 2:1, the plant growth was observed to be optimal, with a high survival rate of 90%.

Anthocyanidin has received considerable attention in recent years. However, the lack of pure anthocyanidin in the market has prompted researchers to shift their focus towards rare purple tea trees, which are rich in anthocyanidin. Establishing a regeneration system for obtaining a substantial quantity of Zikui tea plants can serve as a significant method for further research and extraction of anthocyanidin. In the process of regenerating purple tea trees that are rich in anthocyanidin, it has been observed that exposure to strong light and high temperature generally leads to an increase in the anthocyanidin content. During the process of plant tissue culture, the presence of anthocyanins, a natural product with strong antioxidant capacity, can cause plants to turn brown and eventually die. This is because anthocyanins can lead to a darker and more pronounced purple color in the leaves. Shan discovered that callus-inducing methods could effectively induce a large number of callus containing anthocyanins. Wang further demonstrated that anthocyanin synthesis is dependent on light. Therefore, in this study, dark culture conditions were employed to investigate the impact on callus induction. The results showed that after 14 days, the callus induction rate reached an impressive 93.64%, effectively suppressing anthocyanin synthesis.

In this study, the tissue culture method was employed to investigate the Zikui tea tree embryo as explants. The focus was on determining the optimal sterilization conditions and induction differentiation medium for the explants. The study also examined the optimal conditions for transplanting and refining adventitious shoots. Specifically, the research focused on understanding the regeneration system of the Zikui tea plant, which can offer valuable insights for future genetic transformation studies on Zikui tea plant genes.

**Conclusion**

This study successfully developed an efficient regeneration system using the hypocotyls of Zikui tea as explants. Throughout the cultivation process, optimal treatment conditions and appropriate medium formulations were achieved. It is worth noting that although Zikui tea is a purple tea variety, its plant color remains indistinguishable from that of ordinary green tea varieties during tissue culture. This observation suggests that the regulation of related genes within the plant body may play a role in determining the plant color. The establishment of a tissue culture and regeneration system for Zikui tea can be complemented by other biotechnology methods, such as genetic engineering, to provide sterile materials and serve as a valuable reference for future research on the mechanism of tea tree purple transformation.
Declarations

Author Contribution

Yan Li, Jiong Yi Jin and Yu Lu Chen designed the experiment and analyze the data; Jiong Yi Jin, Yu Lu Chen, Ju Cai, Litang Lv, Xiaofang Zeng, Jian Rong Li, Sumeera Asghar and Yan Li performed the experiments; Jiong Yi Jin wrote the manuscript.

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Competing of Interest: The authors declare that they have no conflict of interest to report regarding the present study.

Data availability

All data are available in the manuscript.

References


Figures

Figure 1

Growth of embryo germination induction at different time. A: Growth after 10d after culture; B: Growth after 25 days of cultivation; C: Growth after 45 days of cultivation; The ruler in the figure is 1cm.

Figure 2

Growth of embryos germinated at different concentrations of GA$_3$ for 60 days. A: The growth of embryo inoculated on blank medium for 60 days; B: The growth of embryo inoculated on MS+1.20mg/L GA$_3$ medium for 60 days; C: The embryo was inoculated on MS+2.40mg/L GA$_3$ medium for 60 days; D: Embryo was inoculated to MS+3.60mg/L GA$_3$ medium for 60 days of growth. The ruler in the figure is 1cm.
Figure 3

Differentiation of the cotyl at 60d under different differentiation medium conditions. (A) The cotyl was inoculated on WPM+2.00mg/L 6-BA+0.10mg/L NAA medium for 60 days; (B) The cotyl was inoculated on WPM+2.00mg/L 6-BA+0.10mg/L IBA medium for 60 days; (C) Cotyl inoculation to WPM+2.00mg/L 6-BA+0.10mg/L 2,4-D culture for 60 days; (D) Zoomed-in image of cotyl inoculation on WPM+2.00mg/L 6-BA+0.10mg/L NAA substrate for 60 days. The ruler in the figure is 1cm.

Figure 4

Callus formation and differentiation of the cotyl in WPM+2.00mg/L 6-BA+0.10mg/L NAA. (A) The callus induced by hypocotyl explants and adventitious buds were cultured in WPM+2.00mg/L 6-BA+0.10mg/L NAA medium; (B) Callus induced by hypocotyl explants and adventitious buds were cultured in WPM+2.00mg/L 6-BA+0.10mg/L NAA medium for 15 days; (C) Callus induced by hypocotyl explants and adventitious buds were cultured in WPM+2.00mg/L 6-BA+0.10mg/L NAA medium for 30 days. The ruler in the figure is 1cm.
Figure 5

Growth of seedlings in strong seedling medium (A) seedling growth at 0d in MS+2.00mg/L+6-BA+0.60mg/L IBA seedling medium; (B) Seedling growth in MS+2.00mg/L 6-BA+0.60mg/L IBA strong seedling medium for 25 days; (C) Growth of axillary buds in MS+2.00mg/L 6-BA+0.60mg/L IBA seedling culture for 45 days. The ruler in the figure is 1cm.

Figure 6

Rooting of plants in rooting medium for different days. (A) Growth of plants in 1/2 ms +1.60mg/L IBA rooting medium for 0d; (B) Growth of plants in 1/2 ms +1.60mg/L IBA rooting culture for 45 days; (C-D) Growth of plants in 1/2 ms +1.60mg/L IBA rooting medium for 60 days. The ruler in the figure is 1cm.
Figure 7

Living plant of Zikui. (A) The growth of plants in V yellow loam: V vermiculite =2:1 in the transplanting medium for 0d; (B) Growth of plants in V yellow loam: V vermiculite =2:1 transplanting medium for 60 days. The ruler in the figure is 1cm.