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Photoautotrophic Rooting Technology for Sugarcane In Vitro Plantlets: Postprint

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Abstract

The objective of investigating photoautotrophic rooting technology for sugarcane test-tube plantlets is to simplify tissue culture procedures and reduce production costs. In this experiment, rootless test-tube plantlets of sugarcane varieties GT44 and B9 served as experimental materials. The plantlets were first treated by foliar spraying with plant growth regulators, then acclimatized for 24 h, after which the treated plantlets were transplanted into a sand-soil mixed cultivation substrate to complete adventitious root formation and growth under greenhouse conditions; simultaneously, transplant survival rates and growth performance of rootless versus rooted test-tube plantlets were compared. The rooting rate investigation period ranged from day 3 to day 10 after transplantation, while survival rate was assessed on day 30. Results demonstrated that transplant survival rates of rootless plantlets treated with indole-3-butyric acid (IBA) and ABT2 rooting powder reached 96.3% and 97.7%, respectively, approaching those of conventionally rooted plantlets, with the rooting cost per plantlet being only 1/28 of the traditional method. For both GT44 and B9 varieties, the first visible roots emerged on day 4 after transplantation. Root regeneration of test-tube plantlets can be completed in non-sterile sand-soil substrate under greenhouse conditions without requiring sterile MS rooting medium and culture rooms; genotype and plantlet quality are key factors influencing photoautotrophic rooting of sugarcane test-tube plantlets; this technology offers greater advantages over conventional medium-based rooting, featuring simple operation, streamlined procedures, high rooting and survival rates, labor savings, energy conservation, reduced production costs, and high efficiency, enabling it to replace traditional rooting techniques for commercial application.

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Full Text

Photoautotrophic Rooting of Sugarcane Microshoots

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Abstract

This study aimed to simplify sugarcane tissue culture protocols and reduce production costs by developing a photoautotrophic rooting technique for sugarcane microshoots. Rootless microshoots of sugarcane varieties GT44 and B9 were treated with foliar sprays of plant growth regulators, acclimatized for 24 hours, then transplanted into a sand-soil substrate to complete adventitious root formation and growth under sunlight greenhouse conditions. We compared the transplant survival rates and growth performance between rootless and rooted microshoots. Rooting rate was monitored from day 3 to day 10 after transplantation, while survival rate was assessed on day 30. The results showed that rootless microshoots treated with indole-3-butyric acid (IBA) and ABT2 rooting powder achieved remarkably high survival rates of 96.3% and 97.7%, respectively, approaching the survival rate of conventionally rooted microshoots. Moreover, the cost per plantlet for this photoautotrophic rooting method was only 1/28 of that for conventional rooting methods. The first visible roots emerged on day 4 after transplantation for both GT44 and B9 varieties. Root regeneration could be completed in non-sterile sand-soil substrate under sunlight greenhouse conditions, eliminating the need for sterile MS rooting medium and culture room facilities. Genotype and microshoot quality were identified as key factors affecting photoautotrophic rooting. This technique offers significant advantages over conventional medium-based rooting, including simplified operations, streamlined protocols, high rooting and survival rates, reduced labor and energy consumption, lower production costs, and higher efficiency, making it suitable for commercial-scale application.

Keywords: sugarcane microshoots, plant growth regulators, foliar introduction, sand-soil substrate, photoautotrophic rooting

Introduction

Sugarcane tissue culture technology is widely used for rapid propagation of new varieties, production of virus-free seedlings, and transgenic sugarcane research,

generating substantial social and economic benefits in major sugarcane production regions worldwide. Conventional plant tissue culture involves four stages: primary culture (establishment), subculture proliferation, rooting culture, and nursery transplantation. The first three stages require sterile indoor conditions with controlled environments, complex procedures, high labor demands, and significant costs, resulting in high production costs for plantlets. For example, sugarcane healthy seedlings currently sell for 0.6-1.0 yuan per plantlet. With planting densities of 1,800-2,000 plants per acre, the seedling cost reaches 1,080-2,000 yuan per acre, 4.5-8.33 times higher than conventional stem planting costs. This high production cost limits farmer acceptance and constrains widespread adoption of sugarcane micropropagated seedlings.

Ex vitro rooting technology represents an advanced tissue culture technique developed in recent years and constitutes an important component of simplified micropropagation protocols. This technology involves treating proliferated microshoots with growth regulators before direct autotrophic rooting in greenhouse conditions, combining the rooting and acclimatization stages. It transforms the conventional heterotrophic rooting in culture rooms into autotrophic rooting under greenhouse conditions, eliminating the complex and costly in vitro rooting procedure. This approach reduces sterile operations, saves culture room space, simplifies production protocols, lowers costs, and improves efficiency. Studies have demonstrated that ex vitro rooting can reduce production costs by 35-75%.

The photoautotrophic rooting technique for sugarcane microshoots was developed based on ex vitro rooting principles. Rootless proliferated microshoots are sprayed with plant growth regulators and acclimatized for 24-48 hours in sunlight greenhouses before conventional planting. This innovation simplifies the conventional four-step protocol into three steps: establishment, proliferation, and nursery rooting/growth, eliminating the in vitro rooting stage. This simplification reduces production costs by over 50% and facilitates large-scale commercial production of healthy sugarcane seedlings.

Materials and Methods

Sugarcane varieties GT44 and B9 were obtained from the Sugarcane Research Institute of Guangxi Academy of Agricultural Sciences through multiple subcultures on MS medium supplemented with 6-BA and NAA. The experimental procedure involved: (1) foliar spraying of plant growth regulators (ABT2 rooting powder or IBA) onto proliferated rootless microshoots; (2) 24-hour acclimatization in sunlight greenhouse with uncontrolled environmental conditions (relative humidity 60-70%, temperature 20-40°C, light intensity 2,000-4,000 lx); (3) washing and disinfection by immersing microshoots in 500× dilution of Bright Shield (Syngenta) or 0.6% KMnO solution for 10 minutes with 1-2 agitations; (4) transplantation into a substrate mixture of fresh river sand and fully weathered yellow soil (1:1 v/v) in sunlight greenhouse with uncontrolled conditions (relative humidity 70-100%, temperature 20-40°C, light intensity 2,000-15,000

lx, primarily diffuse light).

1.1 Survival Rate Comparison Test

This experiment compared the transplant survival of conventionally rooted versus photoautotrophically rooted microshoots of variety B9. Two treatments were applied: (1) ABT2 solution at 200 $\text{mg} \cdot \text{L}^{1}$, and (2) IBA at 200 $\text{mg} \cdot \text{L}^{1}$ + 6-BA at 10 $\text{mg} \cdot \text{L}^{1}$, using patented methods reported by Liu et al. (2015) and He (2015). Treated rootless microshoots were acclimatized for 24 hours. The control consisted of rooted microshoots cultured for 15 days on MS medium supplemented with NAA at 10 $\text{mg} \cdot \text{L}^{1}$ and sucrose at 50 $\text{g} \cdot \text{L}^{1}$. Both rootless and rooted microshoots were transplanted simultaneously into sand-soil substrate in 6×9 cell trays. Survival rate was investigated 30 days after transplantation, with each replicate consisting of one 6×9 cell tray and three replications total. Survival was counted as cells containing at least one surviving plantlet. Rooting rate was surveyed from day 4 to day 10 after transplantation, sampling 10 cells per variety per time point to count total individuals and rooted individuals. Data analysis was performed using Microsoft Excel 2007. Survival percentage and rooting rate were calculated using Formula 1 and Formula 2, respectively.

1.2 Single Plantlet Rooting Cost Comparison Test

Uniformly grown GT44 proliferated microshoots were selected, with 99 bottles subjected to conventional rooting (15 days culture followed by acclimatization and greenhouse planting) and 120 bottles to photoautotrophic rooting (open cap, spray with ABT2 at 200 $\text{mg} \cdot \text{L}^{1}$ + D-proline at 60 $\text{mg} \cdot \text{L}^{1}$ + Tween at 0.2%, 24-hour acclimatization, then washing and transplantation). Both groups used sand-soil substrate (1:1 v/v). After 30 days, surviving plantlets (with at least one fully expanded leaf) were counted.

Results

2.1 Comparison of Transplant Survival Rates Between Rooted and Rootless Microshoots

Table 1 Comparison between the survival of sugarcane cultivar B9 microshoots achieved with photoautotrophic rooting and a conventional rooting technique.

Treatment	Average number of investigation holes	Average number of survival (holes)	Survival percentage
Rootless microshoots sprayed with ABT No.2 solution			96.3b
Rootless microshoots sprayed with mixed solution of IBA and 6-BA			97.7ab
Rooted microshoots in culture			99.6a

Note: The same lowercase letters represent no significant difference (p>0.05) and different lowercase letters represent significant difference (p<0.05).

Both treatments achieved high survival rates exceeding 96%. The IBA+6-BA treatment reached 97.7% survival, not significantly different from conventionally rooted microshoots ($P > 0.05$). In conventional sugarcane micropropagation, plantlets are first induced to root in culture rooms before nursery acclimatization. However, photoautotrophic rooting enables root induction in non-sterile greenhouse conditions, achieving survival rates comparable to conventional methods without requiring sterile rooting medium.

2.2 Changes in Rooting Rate During Photoautotrophic Rooting Process

No visible roots were observed on day 3 after transplantation for GT44 and B9 microshoots. Visible roots emerged on day 4, with rooting rates reaching 30–40%. By day 8, rooting rates approached 100% for both varieties. Varietal

differences were observed in root emergence timing; for example, GT49 first showed visible roots on day 9 with only 20% rooting rate, reaching 100% on day 20. Despite differences in rooting speed among GT44, B9, and GT49, above-ground leaf greening and new leaf growth were synchronized, with no observable differences.

Figure 1 [Figure 1: see original paper] The changes of rooting percentage from day 3 to day 10 after planting in soil-sand mixture in the greenhouse.

The conventional sugarcane micropropagation protocol consists of four steps: culture establishment, proliferation, rooting culture, and nursery transplantation, with the first three steps requiring sterile indoor conditions and sterile operations throughout. Adoption of photoautotrophic rooting simplifies this to a three-step protocol (see **Figure 2** [Figure 2: see original paper]). In the conventional protocol, proliferation and rooting culture compete for culture room facilities and equipment, creating workflow conflicts and limiting proliferation cycles. The simplified protocol separates proliferation (in sterile, closed conditions) from rooting (in open, non-sterile greenhouse conditions), eliminating conflicts and allowing all tissue culture facilities to be dedicated to proliferation. This change can dramatically increase subculture frequency and yield, theoretically enabling geometric progression of production capacity based on multiplication rates without additional infrastructure investment.

Figure 2 [Figure 2: see original paper] The change of conventional sugarcane tissue culture protocol from 4-step protocol to 3-step protocol with adoption of photoautotrophic rooting technique.

2.3.2 Simplified Protocol for Photoautotrophic Rooting

Conventional rooting culture is performed entirely in laboratories or sterile culture rooms, with microshoots placed in containers with rooting medium. This environment is characterized by closed conditions, poor ventilation, weak light intensity (<2,000 lx), constant temperature (30°C), and high humidity (100% RH). In contrast, photoautotrophic rooting involves completely different operational steps and environmental requirements (see **Figure 3** [Figure 3: see original paper]). The procedure is simple, conducted in ordinary sunlight greenhouses with non-sterile, semi-open conditions, good ventilation, high light intensity (>2,000 lx), fluctuating temperature and humidity (70–100%), and growth regulators applied via foliar spraying. The rooting substrate consists of fresh river sand mixed with yellow soil or fully oxidized farm soil (1:1 v/v), and microshoots are not continuously immersed in medium or auxin solution.

Figure 3 [Figure 3: see original paper] Comparison between the conventional rooting culture protocol and the photoautotrophic rooting protocol.

2.3.3 Cost Comparison Between Techniques

Based on the two protocols shown in Figure 3, 99 bottles of GT44 microshoots were subjected to conventional heterotrophic rooting and 120 bottles to photoautotrophic rooting. Transplant results are shown in **Table 2** . Using the average number of surviving plantlets per bottle and per hole from Table 2, the cost per plantlet was calculated as 0.00285 yuan for photoautotrophic rooting versus 0.0811 yuan for conventional rooting—only 1/28 of the conventional method cost (see **Table 3**). This represents a reduction of 0.08395 yuan per plantlet. Although statistical challenges exist due to the small size and clustered growth of microshoots, and uneven planting may introduce errors (99 bottles conventionally rooted produced 511 holes while 120 bottles photoautotrophically rooted produced 459 holes), the results clearly demonstrate that photoautotrophic rooting substantially reduces production costs.

Table 2 Collected data for cost comparison test between photoautotrophic rooting and conventional rooting culture protocol of sugarcane microshoots.

Table 3 Unit cost comparison between the conventional rooting culture protocol and the photoautotrophic rooting of sugarcane microshoots.

2.3.4 Comparison with Conventional Heterotrophic Rooting

Photoautotrophic rooting induces adventitious root development through foliar application of exogenous auxins followed by 24-hour acclimatization, enabling continuous rooting induction. Root primordia development and elongation occur under non-sterile sand-soil conditions with appropriate light, temperature, and humidity. Both microshoot and root quality exceed those from conventional sterile culture methods, achieving nursery survival rates above 96% with no significant difference from rooted microshoots. This novel technique offers substantial advantages over conventional medium-based heterotrophic rooting (see **Table 4**), including simplified operations, streamlined protocols, reduced inputs, and high efficiency, making it a viable replacement for commercial production.

Table 4 Comparison between the properties of photoautotrophic rooting technique and conventional rooting culture technique.

Discussion

3.1 Exogenous Plant Growth Regulator Treatment and Adventitious Root Development

The unique aspect of this study involves foliar application of plant growth regulator solutions to sugarcane microshoots, followed by 24-hour acclimatization in sunlight greenhouses. The combined action of light and plant hormones induces adventitious root initiation, primordia formation, and elongation through the epidermis. Conventional methods incorporate exogenous regulators directly into

sterile medium before microshoot inoculation, or immerse microshoots in regulator solutions before transfer to regulator-free medium. Previous ex vitro rooting studies have primarily used basal immersion or quick-dipping in auxin solutions, reporting optimal rooting rates of 72% for cut rose microshoots, 96.11% for *Momordica grosvenorii*, and 75% for blueberry. In contrast, our photoautotrophic technique achieved 100% rooting in sugarcane.

Exogenous auxins like IBA promote endogenous IAA accumulation, and high IAA concentration is critical for initiating adventitious root development. During photoautotrophic rooting, foliar spraying with ABT2 accelerated root emergence by 5 days and increased root number compared to controls, suggesting that exogenous auxin treatment enhances transport of endogenous auxins and carbohydrates to the leaf sheath base, thereby accelerating root initiation. Unsprayed controls must wait for leaf photomorphogenesis and photosynthetic recovery before accumulating sufficient endogenous auxins, resulting in slower rooting.

De Klerk (2002) divided apple microcutting adventitious root development into three phases: dedifferentiation (0–24 h), induction (24–96 h), and differentiation (after 96 h), with root primordia formation and epidermal breakthrough occurring around 120 h. Different genotypes show varying root development timelines; for example, *Eucalyptus globulus* × *maidennii* mini-cuttings required 0–5 days for induction, 5–15 days for primordia formation, and 15–45 days for elongation. In our study, GT44 and B9 showed first root emergence on day 4 (5 days post-treatment) and reached 100% rooting after day 9, while GT49 required 10 days for initial root emergence and 21 days for 100% rooting. De Klerk (2002) reported that auxin promotes root induction and primordia formation but inhibits elongation, a pattern consistent with our findings where exogenous auxin effects gradually diminished as root development progressed, unlike conventional heterotrophic rooting where continuous exposure to auxin-containing medium may inhibit primordia elongation.

3.2 Effect of Microshoot Quality on Photoautotrophic Rooting

Photoautotrophic rooting represents a transition from heterotrophic to autotrophic growth and from sterile to non-sterile conditions, involving shifts from constant temperature/humidity/weak light to fluctuating environmental conditions with high light intensity. This process subjects microshoots to multiple stresses including environmental, biological, high hormone treatment, and nutrient deficiency. Only high-quality microshoots can survive these stresses to complete adventitious root development. Quality requirements include plant height of 4–6 cm, robust stature, large leaf area, and green leaves—particularly important for foliar hormone application and carbohydrate supply. Microshoot morphology and physiology vary by genotype, affecting height, leaf characteristics, and stress tolerance, resulting in different rooting responses. GT44 and B9 showed very easy rooting, GT32 and GT49 were easy-rooting, while GT48 was difficult. Thomas (2000) demonstrated that leaf area, quality,

and position affect root development in grape cuttings, with large-leaf cuttings showing earlier and more vigorous rooting. Similarly, sugarcane microshoots with more, larger, and greener leaves exhibited higher rooting and survival rates.

3.3 Environmental Factors Affecting Photoautotrophic Rooting

3.3.1 Light Light plays a critical role in photoautotrophic rooting, with optimal intensity of 5,000–8,000 lx (range 2,000–15,000 lx). Diffuse light is preferred, requiring greenhouse plastic film to avoid direct sunlight while maintaining strong diffuse light. Weak or excessive light adversely affects growth and increases mortality. Photoautotrophic rooting involves transition from abnormal leaves developed under constant temperature, humidity, and heterotrophic conditions (lacking functional stomata, abnormal epidermal development, low photosynthetic efficiency) to normal leaf structure. Rodriguez et al. (2003) reported that sugarcane plantlet photosynthetic capacity normalized during acclimatization, with maximum photosynthesis increasing from 0.51 μmol CO m ^{2} s ^{1} in vitro to 3.54 μmol CO m ^{2} s ^{1} after 14 days, while transpiration rates decreased from 0.97 mol H O m ^{2} s ^{1} to 0.66 mol H O m ^{2} s ^{1} as stomatal function normalized. Conventional heterotrophic rooting uses weak light (2,000 lx) and closed conditions, resulting in poor photosynthesis and abnormal leaf development. If microshoots can achieve high photosynthetic efficiency and normal leaf structure after greenhouse transplantation, photoautotrophic rooting could replace conventional heterotrophic methods.

3.3.2 Temperature Temperature is a crucial environmental factor, with optimal rooting at 28–30°C. When average temperature dropped below 20°C, root emergence delayed to 15 days post-transplantation and survival rates decreased. Temperature regulation methods included plastic film covering in winter to increase temperature and light, and shade nets in summer to reduce temperature and light intensity. Fruit tree microshoots generally root best at 21–30°C, with apple showing optimal rooting at 28°C and reduced rates at 23°C and 21°C.

3.3.3 Humidity Humidity significantly affects photoautotrophic rooting but can be controlled. Low humidity causes dehydration and death, while excessive humidity promotes pathogen growth and disease. Greenhouse humidity was maintained at 70–100% with small ventilation holes in the arch, and soil fungicide applications every 5 days to control soil-borne diseases. Good drainage was essential to provide adequate water while avoiding waterlogging that could cause plantlet death and reduce survival rates.

Conclusion

Photoautotrophic rooting of sugarcane microshoots is achieved through foliar application of high-concentration exogenous hormones that induce endogenous auxin synthesis and accumulation at the leaf sheath base, initiating adventitious

root development. This process completes cell division, primordia formation, and elongation under non-sterile conditions. Genotype and microshoot quality are critical factors. This technique offers substantial advantages over conventional medium-based rooting, including simplified operations, streamlined protocols, high rooting and survival rates, reduced labor and energy consumption, lower costs, and higher efficiency, making it suitable for commercial production.

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