*J Bangladesh Agril Univ 22(2):**178-184**, 2024* https://doi.org/10.3329/jbau.v22i2.74576

ISSN 1810-3030 (Print) 2408-8684 (Online)

Journal of Bangladesh Agricultural University

Journal home page: http://baures.bau.edu.bd/jbau

Research Article

Effect of Plant Growth Regulators on Mass Propagation of Strawberry (*Fragaria* sp.)

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ARTICLE INFO

Article history
Received: 05 April 2024
Accepted: 25 June 2024
Published: 30 June 2024

Keywords
Plant growth regulators,
In vitro culture,
Disease-free plantlets,
Strawberry,
Runner tips

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ABSTRACT

Efficient *in vitro* regeneration protocols for strawberries are crucial for accelerating the propagation of superior cultivars and improving overall productivity through controlled manipulation of plant growth regulators. This study investigated the influence of different concentrations and combinations of plant growth regulators on *in vitro* regeneration process of strawberries. BARI Strawberry-1 runner tips were sterilized and placed on Murashige and Skoog medium (MS) supplemented with various combinations of cytokinins, including 6-benzylaminopurine (BAP) and kinetin (KIN), to initiate shoot growth. Notably, the combination of 1.5 mg/L BAP + 1.0 mg/L KIN led to the shortest duration for shoot initiation (9 days), resulting in the highest number of shoots per explants (6.33), longest shoot length (2.87 cm), and maximum number of leaves per shoot (4.00). Additionally, this combination also exhibited the highest rate of shoot regeneration (70.33%). Subsequently, the proliferated shoots were transferred to MS medium supplemented with various concentrations and combinations of auxins, specifically 3-indolebutyric acid (IBA) and 1-naphthaleneacetic acid (NAA), for root induction. The *in vitro* regenerated shoots cultured in MS medium supplemented with 1.5 mg/L IBA + 1.0 mg/L NAA showed the shortest duration for root formation (8 days), the highest number of roots per explants (4.00), and the longest root length (2.77 cm). Furthermore, this combination also resulted in the highest rate of root regeneration (40%). These findings contribute valuable insights for the mass production of healthy strawberry plantlets using *in vitro* culture techniques.

Introduction

Strawberry (*Fragaria X ananassa* Duch.) is a highly valued fruit known for its succulence, nutritional richness, and sensitivity to climate variations. A crossbreed of *Fragaria chiloensis* L.P. Mill., and *Fragaria virginiana* Dutch, it belongs to the Rosaceae family and Rosoideae subfamily, with approximately 20 recognized species. Renowned for its abundance in essential nutrients such as vitamins C, B_1, and B_2, protein, calcium, potassium, and copper, strawberries also boast low-calorie carbohydrates, ample fiber, and a wealth of natural antioxidants like carotenoids, phenols, and flavonoids. Moreover, they contain notable levels of ellagic acid, a compound with purported anticancer properties (Nehra *et al*., 1994; Lakhwinder *et al*., 2023). Moreover, strawberries hold significant economic and commercial importance, commonly enjoyed fresh or processed into products like jams and jellies. This popularity has led to extensive study of strawberries from agronomic, genomic, and nutritional perspectives (Danial *et al*., 2016). Cultivated as a high-value fruit in various regions, including Bangladesh, major strawberry-producing countries worldwide include the USA, Spain, Japan, Poland, Korea, and the Russian Federation. In Bangladesh, commercial cultivation of strawberries is emerging in regions such as Tangail, Rajshahi, Joypurhat, Bogra, Cumilla, Sathkhira, Khagrachari, and Coxbazar (Hossan *et al*., 2013).

The cultivation of strawberries holds immense significance in Bangladesh, particularly during periods when native fruits are scarce. Its cultivation provides a valuable opportunity for smallholder growers to augment their income, even with limited available land. Despite being regarded as a viable crop option for growers, the widespread adoption of strawberry cultivation in Bangladesh is hindered by the substantial expenses associated with seedling collection (Khatun *et al*., 2019). Traditionally, strawberries have been propagated through established runners (Biswas *et al*., 2008). However, this method has proven unsuitable due to susceptibility to various diseases and infections,

Cite This Article
Rimy, F.A., Karmakar, B., Haque, M.S., Yasmin, S. and Khatun, F. 2024. Effect of Plant Growth Regulators on Mass Propagation of Strawberry (*Fragaria* sp.). *Journal of Bangladesh Agricultural University,* 22(2): 178-184. https://doi.org/10.3329/jbau.v22i2.74576

leading to a scarcity of healthy stocks for propagation. Additionally, the conventional propagation system is unable to meet the market demand adequately. In contrast, micro-propagated strawberry plantlets have shown superiority in various characteristics compared to conventionally propagated runner plants (Zebrowska *et al*., 2019).

In vitro micropropagation of strawberries has been adopted due to its numerous advantages, including the rapid multiplication of disease-free stock and the enhanced ability of these crops to generate runners for field planting (Angrej *et al*., 2021). Meristem tips, typically sourced from disease-free crop runners, are commonly utilized to establish *in vitro* cultures, which serve as a means for mass propagation or as a source of plant material for rejuvenation and transformation experiments (Palei *et al*., 2015).

Furthermore, *in vitro,* plantlets exhibit greater uniformity, with a 24% increase in fruit yield compared to crops propagated using traditional methods (Naing *et al*., 2019). *In vitro* cultivation of disease-free plantlets involves the application of growth regulators (PGRs), such as auxins and cytokinins, which play crucial roles in the growth and development of strawberry plantlets. The growth-promoting effects of these PGRs are specific to each species and hybrid and therefore require empirical determination (Khan *et al*., 2019). Consequently, the present study aims to establish an effective and reproducible protocol for the mass production of disease-free strawberry plantlets *in vitro*.

Materials and Methods

Study site and plant material

The experimental period spanning from May 2019 to March 2020 took place at the Plant Tissue Culture Laboratory, Department of Biotechnology, Bangladesh Agricultural University (BAU), Mymensingh. BARI Strawberry-1 served as the primary source of explants. Shoot tips from mature strawberry plants were carefully selected for *in vitro* regeneration. These mature plants were procured from the Bangladesh Agricultural Research Institute, Gazipur. Only healthy and actively growing runner tips were utilized as explants for the *in vitro* culture.

Culture medium and conditions

Consistently throughout the experiments, a basal medium comprising MS medium, supplemented with 3% (w/v) sucrose and 8 g/L agar, was employed (Murashige and Skoog, 1962). Prior to autoclaving at 121 °C and 15 psi for 20 minutes, the pH of the medium was adjusted to 5.8. For the study, an MS medium containing various concentrations and combinations of plant growth regulators 6-Benzylaminopurine (BAP), Kinetin (KIN), Indole-3 butyric acid (IBA), and 1-Naphthalene acetic acid (NAA) was employed. The selected runner tips, serving as explants, underwent surface sterilization in a solution of 0.1% $HgCl_2$ with 2 drops of Tween 20 for five minutes. Subsequently, the sterilized explants were rinsed three times with sterile distilled water, each rinse lasting for 4 minutes. The sterilized explants were then directly inoculated into vials containing MS medium supplemented with different concentrations of BAP and KIN (Table 1).

Table 1. Different concentrations and combinations of growth regulators used in the experiment for *in vitro* initiation of shoot and root in BARI Strawberry-1

Treatment code	Treatments for Shoot Regeneration	Treatments for Rooting
T0	MS medium without Plant Growth Regulators (PGRs)	MS medium without Plant Growth Regulators (PGRs)
T1	MS + BAP 1.0 mg/L	MS + IBA 1.0 mg/L + NAA 0.0 mg/L
T2	MS + BAP 1.0 mg/L + KIN 0.5 mg/L	MS + IBA 0.0 mg/L + NAA 1.0 mg/L
T3	MS + BAP 1.0 mg/L + KIN 1.0 mg/L	MS + IBA 1.5 mg/L + NAA 1.0 mg/L
T4	MS + BAP 1.5 mg/L + KIN 1.0 mg/L	MS + IBA 1.5 mg/L + NAA 1.5 mg/L
T5	MS + BAP 1.5 mg/L + KIN 1.5 mg/L	-

The cultures were nurtured in a growth room under meticulously controlled conditions, maintaining a temperature of 25.0 ±1.0°C, with fluorescent tubes providing a light intensity ranging from 2000-3000 lux. The photoperiod followed a cycle of 16 hours of light and 8 hours of darkness (16L/8D), while the relative humidity was maintained within the range of 60-70%. For the purpose of rooting, regenerated shoots were transferred to solidified MS medium supplemented with varying concentrations of IBA (1.0 mg/L and 1.5 mg/L) and NAA (1.0 mg/L and 1.5 mg/L).

Data collection

Throughout the experimental duration, we thoroughly monitored the growth responses of the inoculated explants, ensuring comprehensive data collection on various parameters of shoot and root growth. Systematic recording included data on the number of shoots per explant, shoot length in centimeters, number of leaves per shoot, and percentage of shoot regeneration. Additionally, we gathered data on the number of roots per shoot, root length in centimeters, and percentage of root regeneration.

Statistical analysis

The data collected from experimental observations were subjected to thorough statistical analysis using the Microsoft Statistical (MSTATC) program, supplemented by Microsoft Excel where appropriate. We employed a one-way analysis of variance (ANOVA) methodology, followed by Duncan's multiple range tests, facilitating robust statistical comparisons. All findings were effectively presented as mean values accompanied by the standard error (SE). Furthermore, significance levels were determined using P-values, with values at the 1% level considered indicative of statistical significance. Our analysis offers valuable insights into the parameters of shoot and root growth, potentially paving the way for significant advancements in this research field.

Results

Effect of different concentrations and combinations of cytokinins (BAP and KIN) on shoot regeneration

Days to shoot initiation

The duration needed for shoot initiation was notably affected by varying concentrations and combinations of cytokinins (BAP and KIN) in the MS medium. The shortest time for shoot initiation (9.00 days) was recorded in treatment T4. Conversely, the longest time for shoot induction was observed in the treatment lacking plant growth regulators (Table 2).

Table 2. Effect of different concentrations of BAP and Kinetin on shoot initiation from runner tip of BARI Strawberry-1

Treatments (MS +BAP mg/L+ KIN mg/L)	DSI	NS/E	LS (cm)	NL/S
T0	16.00±1.00^{a}	1.60±0.00^{b}	0.757±0.06^{e}	1.00±1.00^{c}
T1	13.00±1.00^{b}	4.00±0.58^{b}	1.27±0.12^{d}	2.00±1.00^{bc}
T2	12.00±1.00^{b}	2.30±1.00^{ab}	1.92±0.08^{c}	3.33±0.58^{ab}
T3	9.00±1.00^{c}	4.40±1.00^{a}	2.47±0.06^{b}	3.67±1.15^{ab}
T4	9.00±1.00^{c}	6.33±0.58^{a}	2.87±0.12^{a}	4.00±1.00^{a}
T5	12.00±1.00^{b}	6.00±0.58^{ab}	2.00±0.10^{c}	3.33±0.58^{ab}
$LSD_{0.05}$	1.78	1.26	0.159	1.62
Level of significance	**	**	**	**
CV (%)	8.45	32.64	4.83	31.60

** = Indicates significant at 1% level of probability. The data in the table with similar letters are not statistically different. DSI=Days to shoot initiation; NS/E=No. of shoots/explant; LS=Length of shoots (cm); NL/S=Number of leaves/ shoot

Number of shoots per explant

Treatment T4 yielded the highest number of shoots per explant (6.33) followed by explants cultured in MS medium with T5 (6.00 shoots per explant) and T3 (4.4 shoots per explant) treatments, respectively. In contrast, the control treatment (T0) exhibited the lowest number of shoots per explant (1.60). Importantly, the incorporation of cytokinins (BAP and KIN) in the culture medium led to a significant enhancement in shoot proliferation compared to the control (Table 2).

Number of leaves per shoot

Treatment T4 produced the highest number of leaves per shoot (4.00), followed by T3 with 3.67 leaves per shoot. Treatments T2 and T5 showed the same number of leaves per shoot (3.33). In contrast, the control treatment exhibited the lowest number of leaves per shoot (1.00). These findings indicated that the culture medium supplemented with 1.5 mg/L BAP + 1.0 mg/L KIN was optimal for shoot proliferation (Table 2).

Length of shoots (cm)

The length of shoots exhibited significant variation across different concentrations of growth regulators. The longest shoot length (2.87 cm) was Treatment T4 recorded in T4 treatment, whereas the control treatment displayed the shortest shoot length (0.757 cm) (Table 2).

Per cent shoot regeneration

Treatment T4 demonstrated the highest percentage of shoot regeneration (70.33%), followed by a slight decrease to 67.5% in treatment T5. Treatment T3 yielded a lower percentage of shoot regeneration (48.8%) compared to treatment T4. These results highlighted the substantial influence of cytokinins, especially BAP and KIN, on the *in vitro* growth and development of strawberry plantlets (Figure 1).

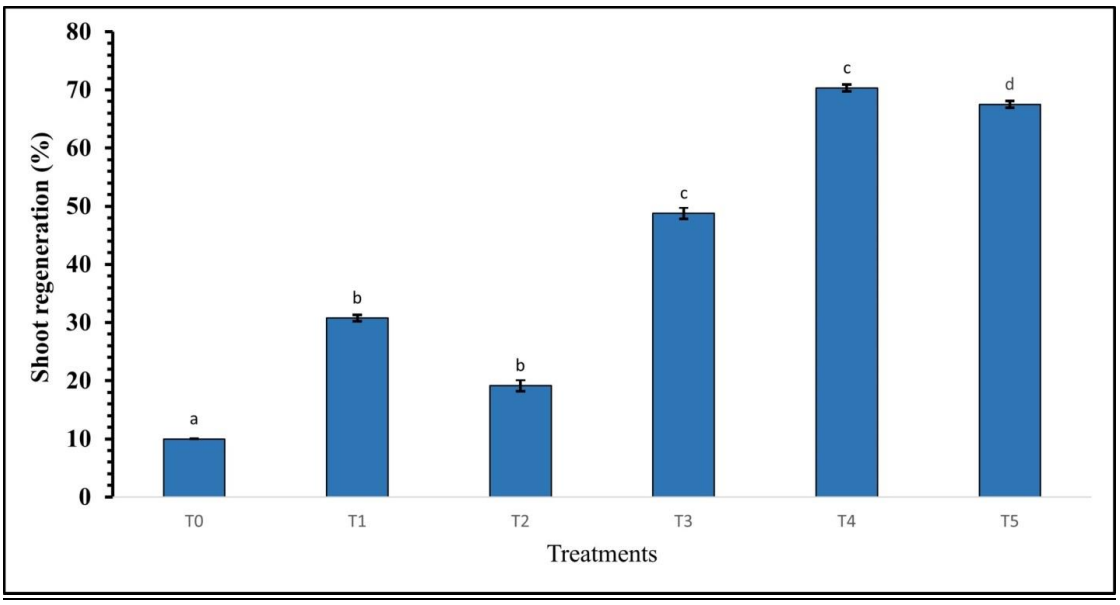

Figure 1. Effect of different concentrations and combinations of BAP and KIN on percent shoot regeneration of BARI strawberry-1. Values are presented as mean ± SD, (n=3)

Effect of different concentrations and combinations of auxins (IBA and NAA) on root formation

Root initiation in regenerated shoots

The duration necessary for rooting was significantly affected by different concentrations and combinations of auxins in the MS medium. Specifically, the supplementation of 1.5 mg/L IBA + 1.00 mg/L NAA in the MS medium led to the shortest rooting duration (8.00 days) (Table 3).

Table 3. Effects of different concentrations and combinations of auxins on rooting of *in vitro* regenerated shoots of BARI Strawberry-1

Treatments (MS + IBA mg/L + NAA mg/L)	DRI	NR/S	LR (cm)
T0	16.00±1.00^{a}	1.33±0.58^{b}	0.917±0.10^{c}
T1	12.00±2.00^{b}	2.33±0.58^{b}	2.00±0.10^{b}
T2	12.00±1.00^{b}	2.00±1.00^{b}	2.07±0.25^{b}
T3	8.00±0.00^{c}	4.00±1.00^{a}	2.77±0.21^{a}
T4	9.33±0.58^{c}	2.67±0.58^{ab}	2.60±0.17^{a}
$LSD_{0.05}$	2.04	1.41	0.320
Level of significance	**	**	**
CV (%)	9.82	31.40	8.57

** = Indicates significant at 1% level of probability. The data in the table with similar letters are not statistically different. DRI=Days to root initiation; NRS=Number of roots/ shoot; LR=Length of root (cm).

Number of roots per shoot

Treatment T3 exhibited the highest number of roots, significantly surpassing other treatments. The maximum number of roots (4.00 roots/shoot) was observed in treatment T3, followed by 2.67 roots/shoot in treatment T4. Conversely, MS medium containing only 1.0 mg/L NAA (T2) produced the lowest number of roots (2.00 roots). The control treatment (without IBA and NAA) resulted in the lowest number of shoots (1.33), significantly different from all other treatments (Table 3).

Length of roots (cm)

Root length typically increased with varying concentrations of IBA and NAA. The longest root length (2.77 cm) was observed in treatment T3, whereas the shortest root length (0.917 cm) was noted in the control treatment (Table 3).

Per cent root regeneration

The highest rooting percentage (40%) was obtained at T3 treatment followed by T4 treatment (26.7%) and T1 treatment (23.33%) and T2 treatment (20%). IBA at higher concentration increased percent root regeneration as compared to lower ones (Figure 2).

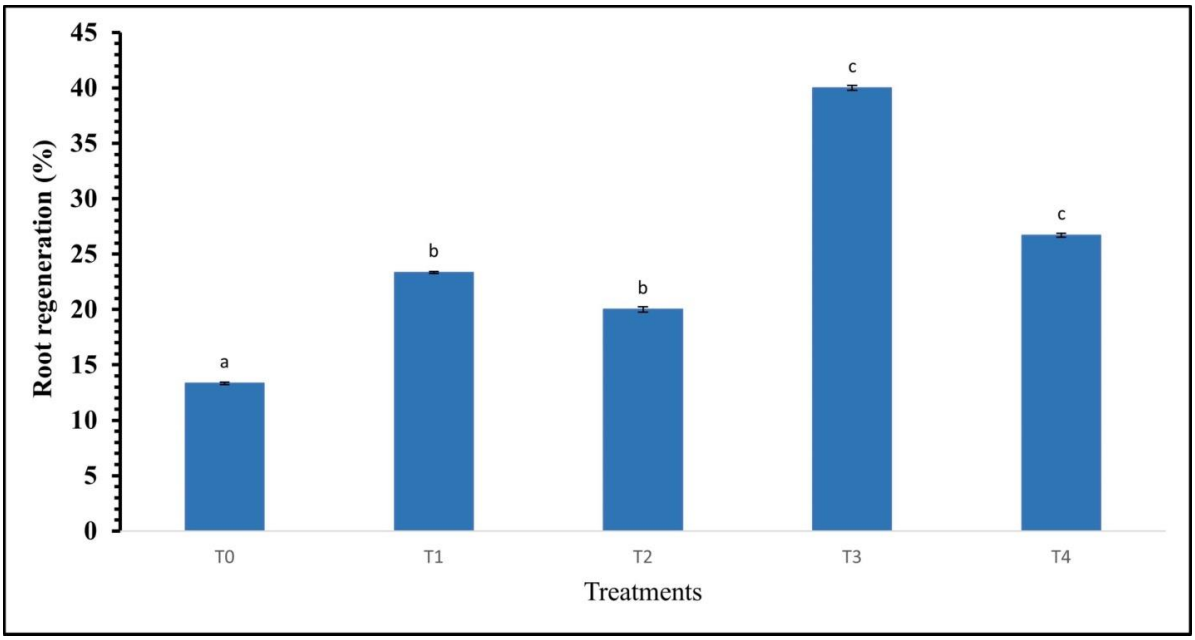

Figure 2. Effect of different concentrations and combinations of IBA and NAA on per cent root regeneration of BARI strawberry-1. Values are presented as mean ± SD, (n=3).

Finally, healthy and vigorous *in vitro*-grown strawberry plantlets were transferred from culture vials to small pots containing a mixture of coco peat, sand, and garden soil in a 1:2:1 ratio. The survival rate of the plantlets was 60%, with initial slow growth followed by vigorous growth after acclimatization in field conditions (Figure 3 and 4).

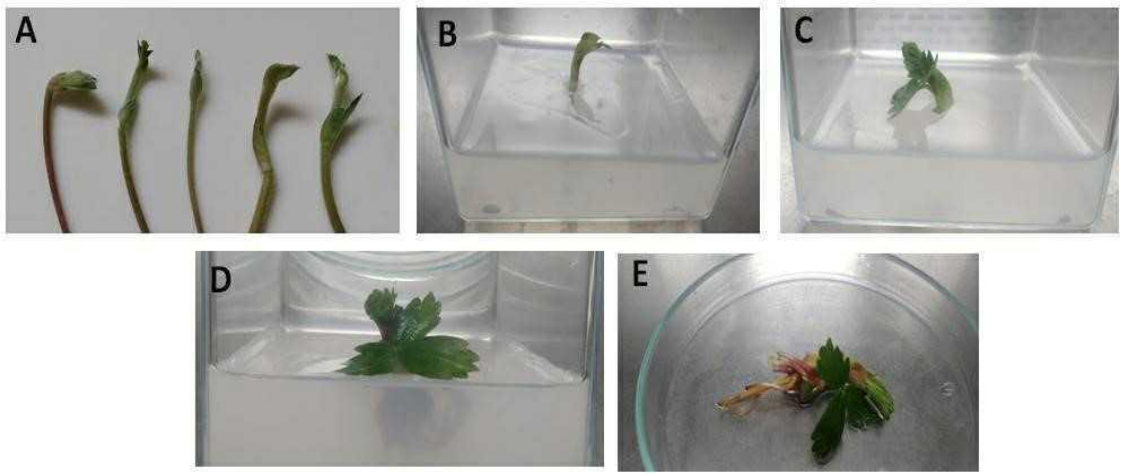

Figure 3. *In vitro* regeneration of BARI Strawberry-1. A. Runner tips used as explants. B. Explants inoculated in MS medium supplemented with cytokinins for shoot initiation C. Regenerated shoots transferred in root initiation medium containing auxins. D. Root formation *in vitro* regenerated shoot E. Strawberry plantlet ready to transfer for acclimatization.

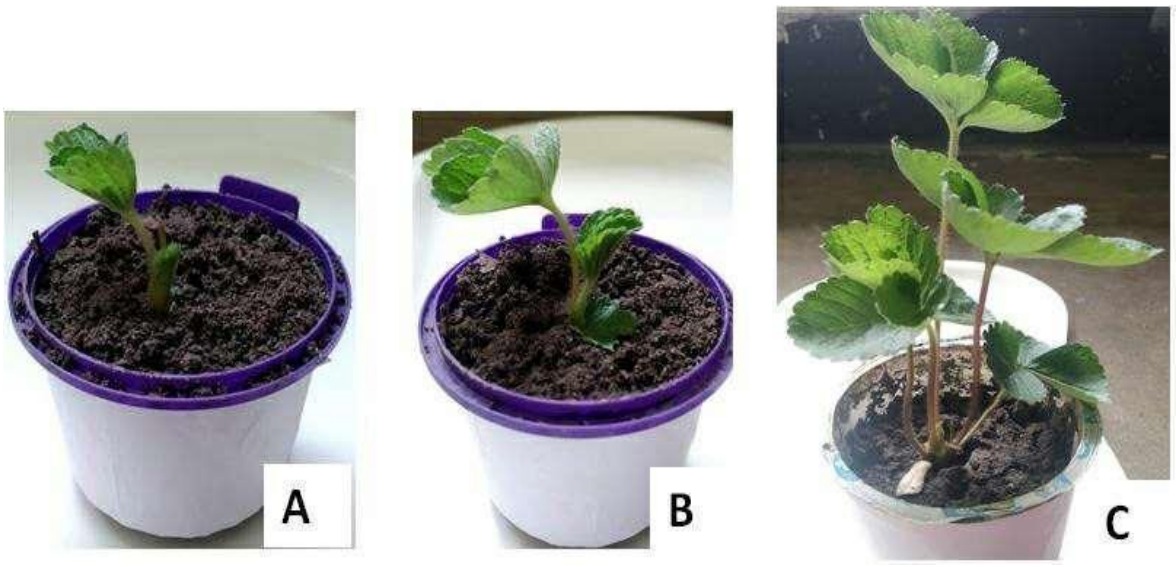

Figure 4. Acclimatization of *in vitro* regenerated strawberry plantlets under *ex vitro* condition. A. Transfer of *in vitro* grown strawberry plantlets in the potting mixture. B. Strawberry plantlet after 3 weeks of culture. C. Strawberry plantlet after 8 weeks of culture.

Discussion

The research investigated how different concentrations and combinations of cytokinins (BAP and KIN) affect *in vitro* shoot regeneration of strawberry runner tips, along with the effects of various concentrations and combinations of auxins (IBA and NAA) on *in vitro* root regeneration of BARI Strawberry-1. This study provides valuable insights into refining tissue culture protocols to enhance the efficiency of strawberry micropropagation. The results demonstrated notable effects of BAP and KIN combinations on shoot initiation time, with treatments T4 and T3 showing the shortest initiation duration. This highlights the significance of employing suitable cytokinin concentrations, aligning with previous research findings (Diengngan *et al*., 2014). Furthermore, the combination featured in T4 yielded the highest number of shoots per explants, suggesting a synergistic effect of BAP and KIN in enhancing shoot proliferation, consistent with findings from prior studies (Sakila *et al*., 2007; Moradi *et al*., 2011; Bhat *et al*., 2012). In addition, the addition of BAP significantly boosted shoot multiplication compared to the control, underscoring its crucial role in enhancing shoot proliferation. Moreover, the length of shoots was positively affected by varying concentrations of BAP and KIN, with treatment T3 displaying the longest shoot length. This underscores the involvement of cytokinins in governing shoot elongation during *in vitro* culture, aligning with earlier research findings (Hasan *et al*., 2010; Mahmoud and Kosar, 2013; Sakila *et al*., 2007). Likewise, the cytokinin concentrations notably influenced the number of leaves per shoot, with treatment T4 showing the highest number of leaves. This observation supports the involvement of cytokinins in leaf development and differentiation.

Furthermore, concerning the effects of auxins on root regeneration, our findings revealed that the time required for root initiation was notably affected by the concentrations and combinations of IBA and NAA in the MS medium. The shortest duration for root initiation was noted in treatment T3, which aligns with previous studies emphasizing the role of auxins in stimulating root development (Biswas *et al*., 2008). Moreover, the combination featured in T3 yielded the highest number of roots per regenerated shoot, suggesting a synergistic effect of IBA and NAA in enhancing root proliferation, consistent with previous research (Sakila *et al*., 2007; Haddadi *et al*., 2010; Diengngan *et al*., 2014). Furthermore, the length of roots was positively influenced by varying concentrations of IBA and NAA, with treatment T3 exhibiting the longest roots. This underscores the role of auxins in regulating root elongation during *in vitro* culture.

Conclusion

The study has shown that cytokinin and auxin concentrations, as well as their combinations, have a significant impact on *in vitro* shoot and root regeneration of strawberry runner tips of BARI Strawberry-1. Optimal concentrations of these growth regulators led to shortened initiation times, increased proliferation of shoots and roots, elongation, and enhanced regeneration of both shoots and roots. These findings are crucial in refining tissue culture protocols for strawberry micropropagation, which is essential for

producing robust and healthy plants for commercial cultivation. To further improve micropropagation protocols, future research could focus on uncovering the molecular mechanisms that underlie the synergistic effects of cytokinins and auxins on shoot and root regeneration in strawberries. This understanding would help to develop more targeted approaches to improve micropropagation protocols. Additionally, field studies can be conducted to evaluate the performance of tissue-culture-derived plants under various environmental conditions, ensuring their successful establishment and growth in agricultural settings. Such efforts are essential for advancing strawberry cultivation practices and meeting sustainable agriculture demands.

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