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Effect of Phyto-hormones on Superior Growth using Nodal Explants of Mulberry (*Morus indica* L.)

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Key words: Shoot initiation, Rooting, MS medium, Commercial propagation

Abstract

Efficient micro-propagation of mulberry (*Morus indica* L.) plays a crucial rolefor its agricultural and commercial applications. This study focuses on optimizing *in vitro* micro-propagation including rooting using nodal explantson MS basal medium, evaluating the effects of phyto-hormones e.g. BAP (6-Benzylaminopurine) and NAA (α-Naphthalene acetic acid) concentrations on shoot and root development. Various concentrations of BAP (0.5-3.0 mg/l) were tested for shoot initiation, in addition with 0.3 mg/l NAA kept constant for shoot development. Rooting was assessed using NAA concentrations ranging from 0.5 to 3.0 mg/l. The highest mean number of shoots (6.75 shoots/ explants) was achieved at 8.0 mg/l BAP, while 2.5 mg/l NAA resulted in the highest rooting percentage (85%) and root length (4.50 cm). Significant effects of BAP and NAA on shoot elongation, leaf production and rooting characteristics were observed. This study demonstrates that optimizing BAP and NAA concentrations significantly enhances the *in vitro* regeneration of mulberry, providing a reliable method for clonal propagation. These findings hold potential for commercial propagation and breeding programs for the improvement of mulberry.

Introduction

Mulberry (*Morus indica* L.), a cornerstone of global sericulture, is a fast-growing, deciduous woody perennial native to China and widely cultivated across Asia, Africa, and the Americas (Das et al. 2012). Its foliage serves as the exclusive feed for silkworms (*Bombyxmori* L.), underpinning a multi-billion-dollar silk industry spanning over 40 countries. Despite its economic significance, conventional propagation methods face critical limitations. Seed-based propagation introduces genetic heterogeneity due to cross-pollination and polyploidy, while cuttings exhibit poor rooting efficiency and

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seasonal dependency (Kavyashree 2007, Chattopadhyay et al. 2012). These challenges are compounded by the dioecious nature of the plant and heterozygosity, which obstruct genetic improvement via traditional breeding (Datta et al. 2000).

In vitro micro-propagation offers a robust alternative, enabling rapid, year-round production of clonal plants. However, existing protocols for nodal explants preferred for their meristem tic vigor-remain inconsistent, particularly for commercial cultivars like S_{13} and V_1 (Chattopadhyay et al. 2012). Shoot initiation and root induction in *Morus* spp. are highly contingent on growth regulators: cytokinins like 6-benzylaminopurine (BAP) drive shoot proliferation, while auxins like α-naphthalene acetic acid (NAA) regulate root morphogenesis (Parveen and Shahzad 2010, Rout et al. 2008). Yet, suboptimal hormone ratios often result in stunted shoots, delayed rooting or hormonal toxicity (George et al. 2008, Sharma and Kapoor 2015). For instance, excessive BAP disrupts shoot-to-root equilibrium, while supraoptimal NAA suppresses root elongation (Ali and Kumar 2011, Singh and Tiwari 2014).

While studies on *Morus alba* and *M. nigra* highlight species-specific responses to BAP and NAA (Hosseinpour and Salehi 2020, Bhattacharyya and Kumaria 2010), standardized protocols for *M. indica* nodal explants remain scarce. Prior efforts focused on nodal buds (Kavyashree 2007) lack scalability, as nodal segments offer higher proliferation potential. This study addresses these gaps by systematically optimizing BAP (0.5-3.0 mg/l) and NAA (0.5-3.0 mg/l) concentrations for *M. indica* cv. V_1, aiming to establish a reproducible protocol for commercial sericulture. By harmonizing cytokinin-auxin ratios with morphogenetic requirements, enhance shoot multiplication, root induction, and acclimatization efficiency-critical for meeting the escalating demands of silk production and genetic conservation.

Materials and Methods

Plant Material and Explant Preparation: Nodal segments were collected from mature *Morus indica* (cv. V1) plants (3 years old) maintained at the Institute of Biosciences and Technology, MGM University, MS, India experimental farm. The explants were cut into 1.5-2.0 cm pieces, each containing a single nodal bud. The explants were washed under running tap water for 5 min to remove external dust and contaminants, followed by treatment with 2% Tween 20 detergent. They were then surface sterilized using 70% ethanol for 30 sec, followed by immersion in 0.1% mercury chloride ($HgCl_2$) for 3 min. The explants were rinsed three times with sterile distilled water to remove any traces of $HgCl_2$.

Culture Medium and Experimental Design: The medium was supplemented with varying concentrations of 6-Benzylaminopurine (BAP) ranging from 0.5 to 3.0 mg/l, while the concentrations of NAA (0.3 mg/l), asparagine (25 mg/l) and glutamine (1 mg/l) were kept constant across all treatments. The experiment was designed as a Completely Randomized Design (CRD) with six treatments and four replications. The treatments for

BAP supplementation were as follows- T_1: 0.5 mg/l BAP, T_2: 1.0 mg/l BAP, T_3: 1.5 mg/l BAP, T_4: 2.0 mg/l BAP, T_5: 2.5 mg/l BAP, T_6: 3.0 mg/l BAP. Each treatment received 20 ml of MS medium supplemented with the specified additives.

Medium Preparation and Sterilization: The pH of the medium was adjusted to 5.8 using 1NHCl or 1NNaOH. The medium was supplemented with 3% (w/v) sucrose and solidified with 0.8% agar. The final volume was adjusted to 1000 ml with distilled water and sterilized by autoclaving at 121°C for 20 min at 15 psi. The medium was allowed to cool to 45°C before adding heat-labile supplements, which were filter-sterilized through a 0.22 µm membrane filter.

Inoculation and Incubation: Each test tube contained 20 ml of MS medium supplemented with BAP at the specified concentrations. The sterilized nodal explants (2.0-2.5 cm) were transferred vertically into the test tubes. Cultures were incubated at 25 ± 2°C under a 16 hrs photoperiod with a light intensity of 3000 lux provided by cool white fluorescent lamps. Sub-culturing was performed at 28-day intervals on fresh MS medium.

Data Collection and Statistical Analysis: The following biometric parameters were recorded at 27 Days after Inoculation (DAI): Days to shoot initiation, Number of shoots per explants, Shoot length (cm), Number of leaves per explants.

Shoot length was measured from the base to the tip of the plantlet at the time of sub-culture and the number of shoots and leaves was recorded per explant. The data were analyzed using analysis of variance (ANOVA) at a 5% level of significance. Statistical measures such as standard error (S.E.) and critical difference (C.D.) were calculated to assess treatment effects.

Effect of NAA on Root Induction: A separate set of experiment was conducted to evaluate the effects of varying NAA concentrations (0.5-3.0 mg/l) on the rooting efficiency of *Morus indica* nodal segments. The treatments for NAA supplementation were: T_1: 0.5 mg/l NAA, T_2: 1.0 mg/l NAA, T_3: 1.5 mg/l NAA, T_4: 2.0 mg/l NAA, T_5: 2.5 mg/l NAA, T_6: 3.0 mg/l NAA. Each treatment received 20 ml of MS medium. Cultures were maintained at 25 ± 2°C under a 16 hrs light/8 hrs dark cycle with a light intensity of 2000-3000 lux provided by cool white fluorescent lamps. Explants were sub-cultured every 15 days.

Rooting Data Collection and Statistical Analysis: The following biometric parameters were recorded at 27 DAI: days required for root initiation, number of roots per explants, root length (cm), rooting percentage (%).

Statistical analyses were conducted using ANOVA at a 5% significance level. Standard error (S.E.) and critical difference (C.D.) values were calculated to determine the significance of differences between treatments. This methodology ensures reproducibility and provides a clear framework for assessing the impact of NAA concentrations on the rooting performance of *Morus indica* nodal segments.

Results and Discussion

The results of the present study on "Effect of Phyto-hormones on superior growth using Nodal Explants of Mulberry (*Morus indica* L.)" are presented below.

The mean number of days required for shoot initiation was 7.37 (Table 1, Fig. 1). The number of days required for shoot initiation was significantly influenced by different levels of BAP combined with a constant level of NAA. Treatment T_5 (BAP 2.5 mg/l) was significantly superior to the other treatments, recording the minimum number of days for shoot initiation (6.50). The days required for shoot initiation by treatments T_1, T_4, T_2, T_6 and T_3 were 7.00, 7.00, 7.75, 8.00 and 8.00, respectively.

The observations reveal that the mean number of shoots produced per explant of *Morus indica* L. at 27 DAI (Days after Inoculation) was 3.66. The number of shoots was significantly influenced by different levels of BAP in combination with a constant level of NAA. Treatment T_5 (BAP @ 2.5 mg/l) was significantly more effective than the other treatments, producing the maximum number of shoots (6.75). Treatments T_6, T_4, T_3, T_2 and T_1 produced mean shoot numbers of 4.75, 4.50, 4.50, 4.20 and 4.20, respectively (Table 1).

Table 1. Response of different levels of BAP on nodal segment of mulberry (27 DAI).

Treatments (T)	Biometric observation of shoot proliferation (27 DAI)			
	Number of Days for Shoot initiation	Shoots/ explant	Shoot elongation (cm)	Leaves/ explant
T_1	6.5	4.25	3.27	3.0
T_2	7.0	4.25	3.7	3.0
T_3	8.0	4.5	4.4	2.5
T_4	7.0	4.5	4.0	3.5
T_5	8.0	6.75	4.6	5.0
T_6	7.75	4.75	3.4	2.25
S.E. ±	0.36	0.36	0.31	0.33
C.D. 1%	1.19	1.26	0.79	1.57
Mean	7.37	3.66	1.15	3.84

Treatments different levels -BAP + NAA Constant 0.3 mg/l.

The mean length (cm) of the main shoot, as influenced by various treatments of BAP and NAA levels at 27 DAI, is presented in Table 1. The mean shoot length of *Morus indica* L. was 4.6 cm at 27 DAI. Shoot elongation was significantly influenced by different levels of BAP in combination with constant NAA. Treatment T_5 (BAP @ 2.5 mg/l) was significantly superior to the other treatments, producing the highest shoot length (4.6 cm). Similarly, treatment T_4 (BAP @ 2.0 mg/l) and treatment T_3 (BAP @ 1.5 mg/l) were significantly more effective than treatment T_2 (BAP @ 1.0 mg/l) and T_6 (BAP @ 3.0 mg/l),

except for treatment T_1 (BAP @ 0.5 mg/l). The shoot elongation (cm) recorded for treatments T_3, T_4, T_2, T_6, and T_1 were 4.4, 4.0, 3.4, 3.7 and 3.27, respectively. Data presented in Table 1 shows that the mean number of leaves per explant of *Morus indica* L. at 27 DAI was 3.84. The number of leaves per explant was significantly influenced by different levels of BAP and NAA. Treatment T_5 (BAP @ 2.5 mg/l) produced the highest number of leaves (5.0) and was at par with treatment T_4 (3.5). Both treatments were significantly superior to the rest. Treatment T_6 recorded the minimum mean number of leaves per explant (2.25). The mean number of leaves produced by treatments T_4, T_2, T_1, T_3 and T_6 were 3.5, 3.0, 3.0, 2.5 and 2.25, respectively.

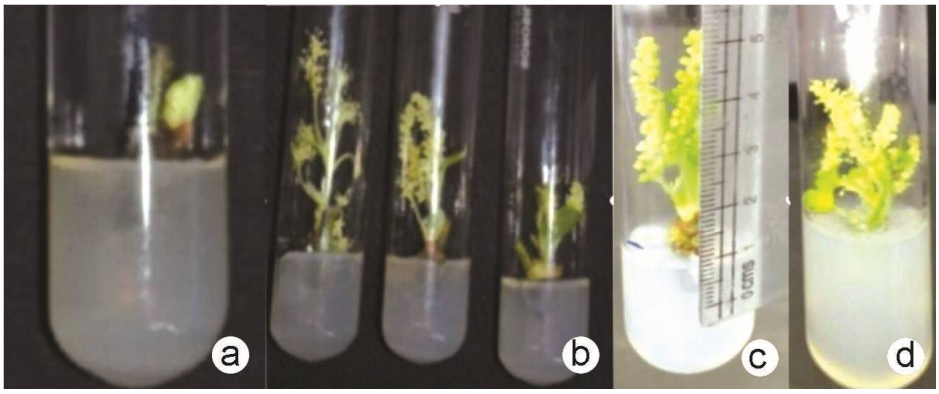

Fig. 1. (a) Shoot initiation, (b) Shoot multiplication, (c) Elongation of shoots, (d) Leaves proliferated.

The present study demonstrates that 6-Benzylaminopurine (BAP) concentration critically regulates *in vitro* shoot induction, elongation and leaf proliferation in mulberry (*Morus* spp.) nodal segments. T_5 (8.0 mg/l BAP) outperformed other treatments, yielding the highest shoot proliferation (6.75 shoots/explant), maximum shoot elongation (4.6 cm) and greatest leaf count (5.0 leaves/explant). These results align with Parveen and Shahzad (2010), who reported enhanced shoot multiplication in *Morus indica* at optimal cytokinin levels. Lower BAP concentrations (T_1-T_3) resulted in fewer shoots and reduced elongation, likely due to insufficient meristematic activation, a trend also noted in guava micro-propagation (George et al. 2008). While T_5excelled, slight declines in leaf production at higher BAP levels (e.g., T_3, T_6) suggest cytokinin-induced hormonal imbalances, as excessive BAP may disrupt shoot-to-root ratios (George et al. 2008). Statistical significance (C.D. at 5% level; (Panse and Sukhatme 1967) confirms BAP's dose-dependent role in shoot development.

Table 2 and Fig. 2 are presented to evaluate the influence of varying NAA concentrations on rooting parameters in *Morus indica* nodal segments. Statistical analysis revealed significant differences ($p < 0.05$) across treatments for all parameters, as indicated by standard error (S.E.) and critical difference (C.D.) values. Root

initiation (Table 2) occurred fastest in T_1 (0.5 mg/l NAA) at 7.50 days, while T_6 (3.0 mg/l NAA) required the longest duration (9.25 days). The mean time for root initiation across treatments was 8.33 days, with statistically significant variation (S.E. ± 0.42; C.D. = 1.32). This suggests that lower NAA concentrations accelerate root emergence, whereas higher doses delay it.

Table 2. Response of different levels of NAA on rooting of mulberry nodal segments (27 DAI).

Treatments NAA (mg/l)	Biometric observations of rooting (27 DAI)			
	Days for root initiation	Roots per explant	Root length (cm)	Rooting (%)
T_1 (0.5)	7.5	3.25	2.5	45
T_2 (1)	8.0	4.0	3.0	55
T_3(1.5)	8.5	4.5	3.75	60
T_4(2)	7.75	5.0	4.0	70
T_5(2.5)	9.0	6.25	4.5	85
T_6(3)	9.25	4.75	3.25	50
S.E.±	0.42	0.35	0.31	3.67
C.D.1%	1.32	1.12	0.96	11.42
MEAN	8.33	4.63	3.5	60.83

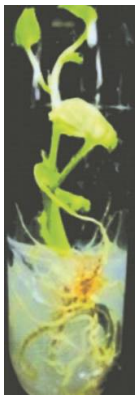

Fig. 2. Response of different levels of NAA on rooting of mulberry nodal segments (27 DAI).

Root proliferation (Table 2) peaked at 6.25 roots/explant in T_5 (2.5 mg/l NAA), declining sharply to 4.20 roots/explant in T_6. The mean root count (4.63 roots/ explant; S.E. ± 0.35; C.D. = 1.12) underscores a hormetic response: moderate NAA concentrations enhance root induction, while excessive levels inhibit growth. Root elongation followed a similar trend, with maximum length (4.50 cm) observed in T_5, compared to 2.50 cm in T_1. The mean root length (3.50 cm; S.E. ± 0.31; C.D. = 0.96) highlights T_5's superiority, whereas T_6's reduced elongation (3.20 cm) implies inhibitory effects at 3.0 mg/l NAA (Table 2).

Rooting efficiency (Table 2) further corroborated T_5's efficacy, achieving 85% success compared to 45-50% in T_1 and T_6. The mean rooting percentage (60.83%; S.E. ± 3.67; C.D. = 11.42) confirms that 2.0-2.5 mg/l NAA optimizes rooting, while deviations reduce efficiency. T_5 (2.5 mg/l NAA) emerged as the optimal treatment, excelling in all metrics: rapid initiation, maximal root count, elongation and 85% rooting success. Conversely, T_6 (3.0 mg/l NAA) exhibited reduced performance, likely due to auxin toxicity suppressing cellular elongation and differentiation. These findings align with studies demonstrating dose-dependent auxin effects, where supraoptimal concentrations disrupt root meristem activity. The statistical significance (C.D.> S.E. across parameters) reinforces that NAA concentration critically regulates rooting in *Morus indica*.

Effect of NAA on Rooting Parameters: The study further evaluated NAA's impact on rooting in *Morus indica* nodal explants. Root initiation varied significantly across treatments, ranging from 7.50 days (T_1: 0.5 mg/l) to 9.25 days (T_6: 3.0 mg/l). Faster initiation at lower NAA concentrations (0.5-2.0 mg/l) corroborates Kavyashree (2007), who linked reduced root initiation time to moderate auxin levels in mulberry. Conversely, delayed initiation at T_6 aligns with inhibitory effects observed in *Azadirachta indica* (Sharma and Kapoor 2015). Root proliferation peaked at 6.25 roots/ explant (T_5: 2.5 mg/l NAA), declining to 4.20 roots at T_6. This hormetic response mirrors findings in *Morus alba* (Rout et al. 2008), where intermediate auxin levels maximized root formation. Higher concentrations likely induced toxicity, as reported in *Camellia sinensis* (Bhattacharyya and Kumaria 2010).Root elongation followed a similar trend, with T_5 achieving the longest roots (4.50 cm) compared to T_1 (2.50 cm). Moderate NAA concentrations enhance cellular division (Vijaya and Padmaja 2016), while excess auxin (T_6) stunts growth, as seen in *Stevia rebaudiana* (Singh and Tiwari 2014). Rooting percentage was highest at T_5 (85.0%), consistent with *Morus nigra* studies (Hosseinpour and Salehi 2020). Reduced efficiency at T_6 (50.0%) reflects auxin's inhibitory effects at supraoptimal doses, noted in *Psidium guajava* (Ali and Kumar 2011, Anis et al. 2003).

The effect of combines 2.5 mg/l NAA (for root induction, elongation, and 85% rooting) with 8.0 mg/l BAP (for shoot proliferation and leaf development). This aligns with auxin-cytokinin synergy principles (Taiz et al. 2015, Das and Biswas 2012), where BAP promotes shoot meristem activity while NAA stimulates root differentiation. The findings provide a scalable method for mulberry propagation, vital for conservation and horticulture.

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