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Effects of phytohormones on the growth and development of *in vitro* microplants of Mulberry (*Morus alba* L.)

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Abstract
The ancient Mulberry (*Morus alba* L.) plant serves as a popular silkworm feed, as well as a source of food for animals and medication. Vegetative propagation can result in reduced yields of virus-infected materials. This study aimed to evaluate the efficacy of a disease-free *in vitro* microplant culture technique by testing several combinations of MS media supplemented with BA, KIN alone, BA with NAA, and KIN with NAA. The experiment required the use of explants obtained from fully developed plants cultivated in the field, which were subjected to surface sterilization. After several attempts, it was determined that the sterilization method employing a 0.1% HgCl2 solution was effective. Nodal explants performed better than alternative techniques for axillary shoot proliferation. The results of this experiment showed that BA (cytokinin) was superior to KIN (cytokinin) in terms of axillary shoot production, and roots were well formed in KIN and NAA. Auxillary shoot growth was shown to be tremendous on MS medium supplemented with 2.5 mg/l BA (T4) and a combined hormone of 2.0 mg/l BA with 0.25 mg/l NAA (T8), 1.5 mg/l KIN with 0.25 mg/l NAA (T11), and 1.0 mg/l KIN with 0.5 mg/l NAA. Moreover, significant root formation was found in KIN 2.5 mg/l in collaboration with 0.5 mg/l NAA (T13). Consequently, it should be concluded that the development and progression of the mulberry microplants had an impact on the hormonal action.

Keywords: Mulberry, microplant, 6-benzyl amino purine, kinetin, naphthaleneacetic acid

Introduction
Mulberry (*Morus alba* L.) is a hardy, fast-growing woody perennial tree from the genus Morus in the Moraceae family [1, 2]. It is economically significant in the silkworm (*Bombyx mori*) industry because caterpillars feed on its leaves. Mulberry silk is considered to account for 90% of global raw silk production, significantly improving the lives of countless people worldwide. As a result, great leaf yield is required for sericulture to remain profitable. Furthermore, mulberry fruits are plump, succulent, and tasty berries that are suitable for human consumption. It has been observed that the leaves of *Morus alba*, which are rich in important phytochemicals such as phenols, flavonoids, and coumarins, can lower people's blood pressure and cholesterol levels [3, 4]. Typically, the genus consists of over 15 species that are distributed in temperate, subtropical, and tropical regions in Asia, Africa, and North America [5]. The heterozygous nature of mulberry trees makes it impractical to upgrade specific features by conventional breeding [6, 7]. The economic feasibility of propagating mulberry through grafting is not viable [8]. A significant challenge for the widespread replication of promising genotypes is their limited ability to root effectively, despite the current usage of stem cutting propagation methods [9]. And only 30-40% of stem cuttings successfully survive the process of trimming, transit, and ultimate implantation [10], for transgenic approaches in crops, it is crucial to have an efficient regeneration procedure *in vitro*. Tissue culture techniques, such as micropropagation, offer a rapid and reliable approach to produce a substantial number of identical plantlets quickly and consistently, regardless of the season. Mulberry is a resistant species in terms of tissue culture, and shoot regeneration is heavily reliant on the genotype, kind of explant, and mix of growth regulators utilized in the culture media [11, 12, 13].
The primary purpose of the study was to evaluate *in vitro* growth responses from the shoot tip and nodal segments of the experimental plant by selecting and standardizing media composition, hormonal treatment, and culture environment to ensure constant high production of disease-free plantlets.

Materials and methods
Plant material and working space
The plant materials or explants employed for microplant development in this study were directly collected from the mulberry tree at the botanical garden of the University of Rajshahi. The *in vitro* culture methods were conducted at the "Plant Breeding and Gene Engineering Laboratory" located in the Department of Botany at the University of Rajshahi, Rajshahi 6205, Bangladesh.

Surface sterilization and aseptic culture establishment
For the purpose of explant inoculation, disease-free, vigorously growing shoots with tips and nodal regions containing auxiliary buds (4-5 cm in length) were collected from fully developed plants cultivated in the garden. Afterwards, the plant components underwent meticulous cleaning in the laboratory using running tap water to minimize the presence of dust and surface contaminants. Subsequently, the sample was divided into nodal and shoot segments and placed in separate flasks for the purpose of surface sterilization.
As part of the surface sterilization process, the shoot and nodal segments were individually treated with 1% savlon for 10 minutes, while being continuously shaken. Following a thorough washing of the materials (shoot and nodal segments) using distilled water and a laminar airflow cabinet, we next moved them to a sterilized 250 ml conical flask. They were immersed for different lengths of time in a solution containing 0.1% $HgCl_2$ [14]. The sample was subsequently washed with sterile distilled water, repeating the process at least four or five times to remove any residual sterilant. The explants used in this study were segments (1.0–1.5 cm) containing nodes or shoot tips, which had undergone surface sterilization.

Culture conditions

The sterilized nodal explants were cultured on MS [15], base medium supplemented with 30 gm/l sucrose and 6-benzyl aminopurine (BAP) and kinetin (KIN) separately and in combination with naphthalene acetic acid (NAA) to cultivate the explants in culture bottles (Table 1). A 20-ml medium solidified with 7 g/l Nobel agar (Merck, India) and pH adjusted to 5.7 is contained in each culture bottle.

Polypropylene caps were used to close culture bottles and parafilm M (Laboratory Film) (Chicago, II. 60631, USA). Following autoclaving at 121 °C and 15 psi for 20 minutes, the samples were incubated in the tissue culture growth chamber for 24 hours under a 16-hour photoperiod (using cool white fluorescent lamps with about 20 µmol/m2/s of light intensity) at 24 ± 1 °C.

Table 1: Media composition of different treatments

Treatment code	MS media with			
	MS (g/l)	BA (mg/l)	KIN (mg/l)	NAA (mg/l)
T-1 (Control)	30	0	0	0
T-2	30	0.5	0	0
T-3	30	1.5	0	0
T-4	30	2.5	0	0
T-5	30	0	0.5	0
T-6	30	0	1.5	0
T-7	30	0	2.5	0
T-8	30	2.0	0	0.25
T-9	30	0.5	0	0.5
T-10	30	2.5	0	0.5
T-11	30	0	1.5	0.25
T-12	30	0	1.0	0.5
T-13	30	0	2.5	0.5

Statistical analysis

At 15 and 30 days after inoculation (DAI), morphological values were obtained for the shoot number, leaf number, root number, and plant height (cm). For all treatments, mathematical mean, standard errors (SE) were computed.

Substantial variations between means were evaluated by utilizing Duncan's multiple-range test (DMRT) in accordance with the given instructions by IBM SPSS software version 20 (SPSS Inc. USA).

Table 2: Mean values with standard error of different characters of shoot number, leaf number, root number, plant height (cm) at 15 and 30 days after inoculation (DAI), and also the results of days to shoot initiation

Treatments	Shoot number		Leaf number		Root number		Plant height (cm)		
	15 DAI	30 DAI	15 DAI	30 DAI	15 DAI	30 DAI	15 DAI	30 DAI	Days to shoot initiation
T-1 (Cont.)	2.333±0.333^{a}	3.333±0.882^{a}	3.667±0.882^{a}	6.667±1.856^{a}	0.667±0.667^{a}	17.667±1.453^{a}	0.7±0.115^{a}	2.267±0.484^{a}	6
T-2	2.333±0.333^{b}	4.667±1.202^{a}	3.333±0.882^{b}	9±2.309^{a}	0±0.000^{a}	1±1.000^{a}	1.06±0.186^{a}	2.333±0.384^{a}	6
T-3	1.667±0.333^{b}	5±1.528^{a}	2.667±0.667^{b}	10±3.055^{a}	0±0.000^{a}	0±0.000^{a}	1.1±0.173^{a}	2±0.321^{a}	7
T-4	3.667±0.333^{a}	5.333±0.882^{a}	6±0.577^{a}	11±1.856^{a}	0±0.000^{a}	0±0.000^{a}	1.1±0.231^{a}	1.867±0.384^{a}	6
T-5	2.333±0.333^{a}	2.667±0.333^{a}	3±1.000^{a}	5.333±1.202^{a}	0±0.000^{a}	6±2.082^{a}	1±0.153^{a}	1.667±0.233^{a}	7
T-6	2±0.577^{a}	2.667±0.333^{a}	2.667±0.667^{a}	5±0.577^{a}	0±0.000^{a}	3±2.517^{b}	0.833±0.120^{a}	1.5±0.231^{a}	7
T-7	1.667±0.333^{a}	3.333±0.333^{a}	2.667±0.333^{a}	6.333±0.882^{a}	0±0.000^{a}	2.333±1.333^{b}	0.833±0.088^{a}	1.333±0.186^{a}	9
T-8	3±0.577^{a}	6.333±1.202^{a}	4.667±0.333^{a}	9.333±1.202^{a}	0±0.000^{a}	0±0.000^{a}	0.733±0.088^{a}	1.5±0.351^{a}	8
T-9	1.667±0.333^{b}	6.667±0.882^{a}	3.333±0.882^{a}	9.667±1.764^{a}	0±0.000^{a}	0±0.000^{a}	0.6±0.153^{a}	1.467±0.145^{a}	9
T-10	2±0.000^{ab}	3.667±1.202^{b}	3.333±0.333^{a}	6±1.528^{b}	0±0.000^{a}	0±0.000^{a}	0.5±0.153^{a}	1.133±0.338^{a}	8
T-11	3±0.577^{a}	4.667±0.667^{a}	5±0.577^{a}	7.333±0.882^{a}	0±0.000^{a}	0.667±0.667^{b}	0.867±0.120^{a}	1.433±0.273^{a}	10
T-12	2.667±0.667^{a}	3.333±0.882^{a}	4.667±0.667^{ab}	5.333±0.882^{b}	0±0.000^{a}	3.667±2.333^{b}	0.6±0.153^{a}	0.9±0.458^{a}	9
T-13	1.667±0.333^{a}	3.333±0.882^{a}	3±0.577^{b}	4.667±1.202^{b}	0.333±0.333^{a}	9±4.583^{a}	0.567±0.120^{a}	1.4±0.702^{a}	10
CV%	6.25	5.47	7.14	6.26	4.25	6.36	5.25	7.47	

Means with the same letter in the same column are non-significant at 5% significance level

Results and discussion

The single nodal explants of Mulberry plant (*Morus alba* L.) from *in vitro* generated shoot cultures were excised and sub-cultured on MS medium solidified with agar and supplemented with different concentrations of BA, KIN alone, BA with NAA, and KIN with NAA combination in order to find out the most suitable culture media formulation. Four parameters such as shoot number, leaf number, root number, and plant height (cm) were considered to evaluate the superior microplants. The results obtained in this study are discussed under the following paragraph.

There was a noticeable change in the shoot number's pattern at 15 DAI. The highest shoot number was figoured out in T4 (3.667) and it was succeeded by T8 (3.00), T11 (3.00), T12 (2.667) and T5 (2.333) at 15 DAI. This section has no substantial change between T8 and T11 based on DMRT. The lowest shoot number was observed in T3 (1.667), T6 (2.00), T9 (1.667), T10 (2.00) and T13 (1.667). Based on DMRT, there is no notable difference between T3 and T13 in this instance. Additionally, DMRT indicates that T6 and

T10 have no significant variations. The difference of shoot numbers had changed considerably at 30 DAI. The highest shoot number was found in T9 (6.667) and it was followed by T8 (6.333), T4 (5.333), T3 (5.00), T2 (4.667). Likewise, T2 and T12 have no substantial difference according to DMRT. The lowest shoot number was found in T1 (3.333), T5 (2.667), T6 (2.667), T12 (3.333). T1, T12, and T13 have no notable dissimilarity based on DMRT. Also, T5 and T6 have no significant difference.

There was a considerable variation in plant height at 15 DAI. The highest plant height was found in T3 (1.100) and T4 (1.100). It was succeeded by T2 (1.06), T5 (1.00), T11 (0.867), T6 (8.833), T7 (8.833), T1 (0.700). According to DMRT, T6 and T7 have no significant difference. T10 had the lowest plant height and it was followed by T9 (0.600), T12 (0.600), and T13 (0.567). However, T9 and T12 have no significant difference based on DMRT.

At 30 DAI, plant height showed considerable variation. The longest plant height was noticed in T2 (2.333) and it was succeeded by T1 (2.267), T3 (2.00), T4 (1.867), T5 (1.667), T8 (1.500), T11 (1.433). The shortest plant height was found in T12 (0.900) and it was followed by T13 (1.400), T10 (1.333), T7 (1.333). Based on DMRT, T10, and T7 have no substantial changes.

The current research present that, nodal explants can produce multiple shoots when placed in a medium with the right amount of BA and KIN for the length of the shoots. Because inapropriate concentration of growth regulators can decrease the growth of plantlets. The promotive activity of BAP for producing multiple shoots has been earlier described in several mulberry species [16]. Among the examined growth regulators showed that T4 (2.5 mg/l BA) has best result for shoot initiation and shoot length. In *Morus alba*, low BAP concentrations have also promoted shoot induction [17]. As like this studies has shown T2 (0.5 mg/l BA) has good number of shoot proliferation. Shoot development was inhibited by an increase in BAP concentration (>2.5 mg/l) which has been reported [18].

There was a noticeable change in the character of the root at 15 DAI. T1 and T13 had shown the presence of roots. All the other concentrated growth regulators did not show any root formation. In the presence of auxin nodal explants were able to form root formation. At 30 DAI there was found differences in the number of roots. T13 (9.00) had the most number of roots and it was followed by T5 (6.00), T12 (3.667), and T6 (3.00). Here, the most effective root proliferation was reported in KIN combined with NAA (2.5 mg/l KIN and 0.5 mg/l NAA). According to [19], NAA functions as a better rooting agent.

The current study project aimed to develop a dependable technique for disease-free sustainable mulberry micropropagation. At last, a workable method for large-scale mulberry propagation was created using different concentrations of growth regulators. To compare the results with DMRT, the data was examined using the mean, standard error, coefficient of variability percentage (C V%), and analysis of variance. At 15 days after inoculation, differences were noted between hormonal treatments in terms of days to shoot initiation, shoot number, leaf number, number of roots, and height of plants.

There was comparable variations in the number of leaves. T4 had the highest number of leaves at 15 DAI and it was succeeded by T11 (5.00), T12 (4.667), T8 (4.667), T1 (3.667), T2 (3.333), T9 (3.333), T10 (3.333). There are no remarkable differences among T2, T9, and T10 following DMRT. Moreover, T3 (2.667), T6 (2.667), and T7 (2.667) had the lowest number of leaves.

Conclusion

Through many micropropagation tests, an achievable research procedure for massive cultivation of mulberries using nodal segments which is disease-free was proven. The number of shoots, leaves, plant height, and number of roots varied throughout hormonal treatments. According to the study, tissue culture can be utilized to create disease-free plantlets by combining different hormones as the outcomes evidenced it.

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Conflict of interest

Neither to be declared.

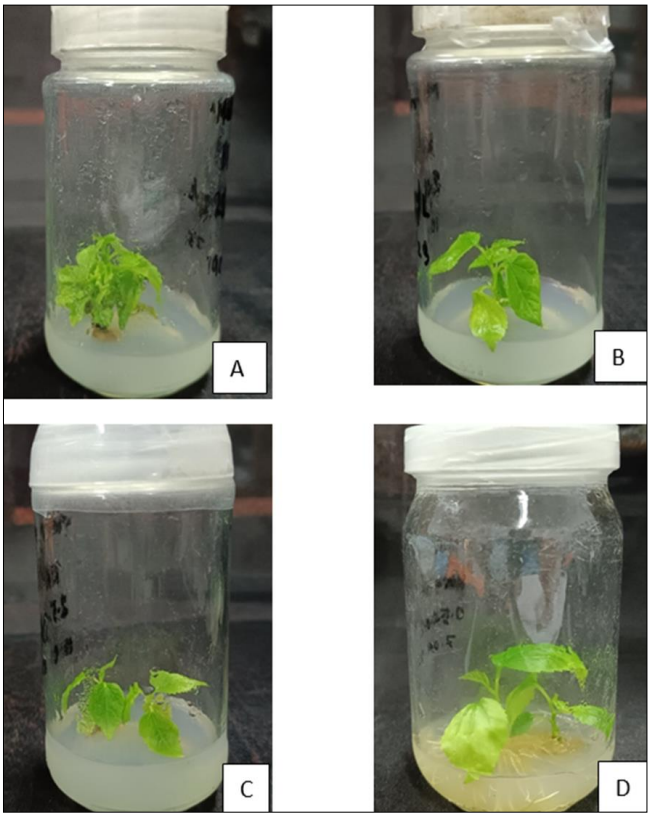

Fig 1: Various stages of *in vitro* micropropagation: **A-** Beginning of shoot multiplication on MS medium combined with 0.5 mg/l BA. **B**-Regenerated plantlets with fully established leaf cultured on MS medium containing BA 2.5 mg/l. **C-** A suitable height of plantlet after 30 days, in which KIN is 0.5 mg/l with MS medium. **D-** Regenerated microplants with substantial roots were stimulated with MS medium with KIN 2.5mg/l and NAA 0.5 mg/l

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