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In vitro Clonal Propagation of Chinese White (*Morus alba* L.) : A Temperate Mulberry Variety through Nodal Segments

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ABSTRACT

In temperate regions; due to predominant cold conditions, it is hard to propagate the mulberry varieties through stem cuttings based vegetative propagation. In view of this limitation, a protocol was developed for *in vitro* clonal propagation of *Morus alba* L. cv. Chinese white, a temperate mulberry variety through nodal segments. When nodal explants were inoculated on to the different concentrations and combinations of plant growth regulators supplemented media, good response of shoot proliferation from axillary buds of nodal explants was observed on Kinetin (Kn) supplemented media rather than 6-benzylaminopurine (BAP) supplemented media. Maximum response in terms of shoot length (cm) (6.8 ± 0.52 and 5.3 ± 0.41) was observed on MS media supplemented with Kn at concentrations of 1.5 mg/L and 2.0 mg/L, respectively. The combinational media of Kn and BAP has also showed good response of shoot proliferation from nodal explants, but the results were not comparable to that of individual Kinetin supplemented media. The proliferated axillary shoots when subcultured on auxins amended media, good rooting response of (95%) was observed on MS media fortified with 1.5 mg/L of indole-3-butyric acid (IBA). The hardening process is carried out by transferring the plantlets from the media to the plastic pots containing sterile vermiculite, garden soil and sand in 1:2:1 ratio. The hardened plantlets were transferred and acclimatized to field conditions with survival rate of 82 per cent. The developed protocol could be utilized for large scale multiplication of *Morus alba* L. cv. Chinese white.

Keywords: Nodal explants, Clonal propagation, Plant growth regulators, Rooting, Hardening, Acclimatization

SERICULTURE is an important agroindustry where silk worms are reared by feeding with mulberry leaves to produce commercial silk (Datta, 2007 and Rohela *et al.,* 2016b). Silk production has been empowering the rural and poor people across the world since long time (Dewangan *et al.,* 2012 and Shukla *et al.,* 2016). Today more than 30 countries of Asia, Europe, Australia, America and African continents are practicing sericulture for providing employment to rural people and for economy generation (Rohela *et al.,* 2020b). According to western historians, sericulture has spread to Indian sub-continent from Sub Himalayan regions (Datta, 2007) and Aryans have cultivated the mulberry plants along the banks of Brahmaputra and Ganges rivers. In the beginning, the silk has been produced from Kashmir region of India and quality of silk produced from this region has become very famous across the world.

Jammu & Kashmir is one of the traditional sericulture regions of India and this region is most suitable for bivoltine sericulture (Maqbool *et al.,* 2015; Gani *et al.,* 2018 and Shukla *et al.,* 2019), where some of the mulberry varieties such as Goshoerami, Ichinose and Chinese white are in practice as superior varieties for rearing the silk worms (Shabir *et al.,* 2013 and Rohela *et al.,* 2020a). These varieties are giving good leaf yield and moderately resistant to cold but all these varieties have poor/low rooting ability in this cold prevalent (October to March) area; because of which their multiplication by stem cuttings is restricted to large extent in this temperate regions of North West India. This is one of the reasons where sericulture sector has not expanded in proportionate ratio in this region when compared to Southern and North Eastern parts of India (Rohela *et al.,* 2018a and Shukla *et al.,* 2018).

Although the saplings of these varieties are raised by other conventional techniques such as root grafting and bag grafting but these techniques have their limitations due to low survival rate and slow growth rate. Moreover, saplings through these techniques cannot be raised during winter months due to low atmospheric and soil temperature. So an alternate strategy is needed to multiply the mulberry plantlets and to expand the sericulture sector in North West parts of India. *In vitro* propagation, green house and bud grafting techniques can be proved to be vital and if these techniques are exploited, one can utilize the long winter period (6 months) for the growth of plantlets in laboratory conditions and also overcome the burden of short supply of mulberry sapling to sericultural farmers of this region to expand the sericulture sector.

There are several reports regarding the raising of genetically identical mulberry plantlets through the *in vitro* culture of leaf (Vijayan *et al.,* 2000 and Yamanouchi *et al.,* 1999), petiole (Bhatnagar *et al.,* 2001 and Bhau & Wakhlu *et al.,* 2003), shoot tip (Yadav *et al.,* 1990 and Pattnaik & Chand, 1997), axillary buds (Patel *et al.,* 1983 and Jain *et al.,* 1990) and nodal explants (Sharma & Thrope, 1990; Yadav *et al.,* 1990; Pattnaik & Chand, 1997; Chitra & Padmaja, 1999 and Zaki *et al.,* 2011). But most of these reports are regarding the clonal propagation of tropical and sub-tropical mulberry varieties. And there is scanty information about the *in vitro* micro propagation of temperate mulberry varieties (Rohela *et al.,* 2021).

In the view of above limitations, the present study has initiated the tissue culture work on temperate mulberry varieties from CSR&TI, Pampore (J&K) and through this studies earlier the *in vitro* clonal propagation of other superior temperate mulberry varieties of Goshoerami (Rohela *et al.,* 2018b) and PPR-1 (Rohela *et al.,* 2018c) were also reported. Now through this research paper; an efficient protocol for the micro propagation of Chinese white mulberry variety through nodal explants was reported.

Material and Methods

Explants: Nodal segments were collected from 3 years old Chinese white mother plants which are available in the mulberry field of CSR & TI, Pampore in the month of April and used in this research study.

Media Preparation: Readymade MS medium powder (Himedia, Mumbai) of about 4.41 grams were weighed and dissolved initially in 300 ml distilled water taken in a conical flask, 25 grams of sucrose (2.5%) was also added to it and final volume was made up to 1 Liter by adding distilled water. The pH of the medium was adjusted to 5.5 ± 03 by using 0.1 N NaOH / 0.1 N HCl. After adjusting the pH, 8 grams of agar (0.8%), a solidifying agent was added and dissolved it by heating. The prepared medium was allowed to cool down to room temperature. Before dispensing the prepared medium in to required vessels such as culture tubes, culture bottles and phytajars, required concentrations and combinations of plant growth regulators were added to it from the stock solutions.

Sterilization of Media: Culture tubes and phytajars with the prepared MS medium were closed tightly with autoclavable caps and sterilized in an autoclave at 121°C, 15 lbs pressure for 15-20 minutes duration. This moist heat sterilization procedure with combined effect of high pressure and heat will eliminate all the form of microbes from the media.

Explant Surface Sterilization: Nodal segments were collected from 3 years old Chinese white (*Morus alba* L.) mother plants available in the mulberry field of CSR&TI, Pampore in the month of April. Initially nodal explants were washed under running tap water for 3-5 times for the removal of surface adhered soil and dust particles. Then the explants were treated with Tween-20 solution for 2 minutes followed by NaOCl for 2 minutes and 60 per cent ethanol for 2 minutes. After every treatment explants were rinsed in sterile distilled water to remove the adhering chemical sterilizing agents. Finally, the explants were taken into the aseptic conditions of laminar air flow cabinet and treated with 0.1 per cent $HgCl_2$ solution for 2-3 minutes. The surface adhered traces of $HgCl_2$ were removed by rinsing the explants in sterile distilled

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water. With this protocol approximately up to 98 per cent of nodal explants sterilization was carried out.

Inoculation: The surface sterilized nodal explants of Chinese white (*Morus alba* L.) were inoculated onto surface of MS medium (Murashige and Skoog, 1962) present in culture tubes and phytajars under the aseptic conditions of laminar air flow cabinet with the help of sterile forceps, sterile scalpel blades etc.

Incubation: Culture tubes and phytajars with inoculated nodal explants were kept in culture room under controlled conditions of 16/8 hours of 3000 lux intensity light at 26 ± 2°C.

Statistical Analysis: The data obtained from the research was analyzed statistically by using ANOVA & SPSS Version 17 and means were compared using Tukey's tests at the 5 per cent level of significance. All means are presented with ± SE.

Results and Discussion

Effect of Cytokinins on Axillary Bud Proliferation from Nodal Explants

Nodal explants of Chinese white (*Morus alba* L.), when inoculated onto different concentrations and combination of cytokinins supplemented MS media, good response in terms of shoot proliferation from the axillary buds was observed on individual concentrations of Kinetin supplemented media rather than individual BAP and combinational media of Kinetin and BAP. On individual BAP supplemented media the highest shoot growth (3.3 ± 0.34 cms) (Fig. 1A) was observed on MS media amended with BAP (1.5 mg/L) after 20 days of culture (Table 1). Overall, the maximum shoot proliferation in cms (6.8 ± 0.52 and 5.3 ± 0.41) was observed on MS supplemented with Kn (1.5 mg/L) and Kn (2.0 mg/L) (Fig. 1B-D) respectively after 20

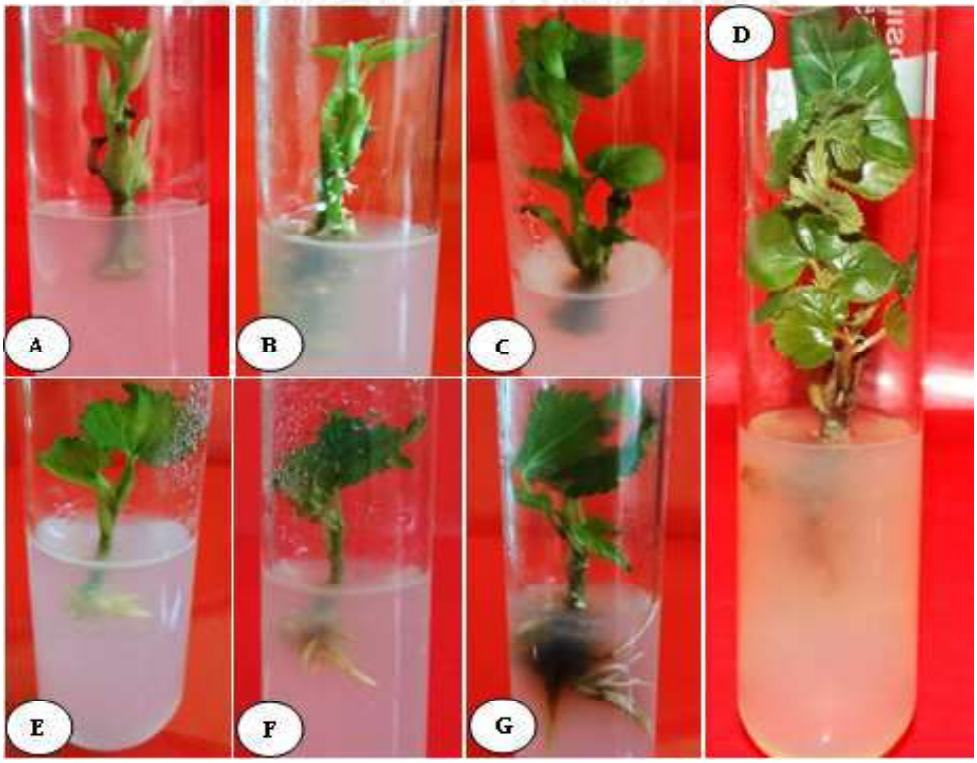

Fig. 1: *In vitro* micro propagation of Chinese white through nodal explants

A) Shoot proliferation (3.3 cms) from axillary bud of nodal explant cultured on MS+ BAP (1.5 mg/L) after 10 days culture
B) Shoot proliferation (5.3 cms) from axillary bud of nodal explant cultured on MS+ Kn (2.0 mg/L) after 20 days of culture
C) Shoot proliferation (5.6 cms) from axillary bud of nodal explant cultured on MS+ Kn (1.5 mg/L) after 10 days of culture
D) Shoot proliferation (6.8) from axillary bud of nodal explant cultured on MS+ Kn (1.5 mg/L) after 20 days of culture
E) Initiation of Roots (White) on MS + IBA (1.5 mg/L) supplemented media after 4 days of culture
F) Turning of roots into light brown in color after 7 days of culture; G) Turning of roots into black color after 10 days of culture

TABLE 1

Axillary bud proliferation from nodal explants of *Morus alba* var. Chinese white on MS media supplemented with different concentrations of cytokinins.

Plant Growth Regulators in mg/L			Proliferated axillary shoot length in cms (X*± S.E)	
BAP	Kn	BAP + Kn	After 10 days of culture	After 20 days of culture
0.5	-	-	0.2± 0.02[a]	0.4± 0.12[a]
1.0	-	-	0.7± 0.11[b]	1.9± 0.21[b]
1.5	-	-	1.9± 0.18[d]	**3.3± 0.34[e]**
2.0	-	-	1.4± 0.42[c]	2.2± 0.31[c]
2.5	-		1.6± 0.30[c]	1.7± 0.22[b]
3.0	-	-	1.1± 0.19[c]	1.3± 0.14[b]
-	-	-		
-	0.5	-	0.6± 0.10[b]	0.8± 0.16[a]
-	1.0	-	0.9± 0.21[b]	1.2± 0.24[b]
-	**1.5**	-	5.6± 0.23[e]	**6.8± 0.52[g]**
-	**2.0**	-	3.5± 0.39[e]	**5.3± 0.41[f]**
	2.5	-	1.2± 0.21[c]	2.7± 0.16[c]
-	3.0	-	0.5± 0.09[a]	1.6± 0. 22[b]
-	-	-		
-	-	**0.5 +1.5**	1.4± 0.26[c]	**3.6± 0.31[d]**
-	-	**1.0 +1.5**	1.8± 0.16[d]	**4.1± 0.18[e]**
-	-	1.5 +1.5	1.6± 0.32[d]	1.7± 0.24[b]
-	-	2.0+ 1.5	1.9± 0.11[d]	2.8± 0.60[c]
		2.5 +1.5	0.8± 0.18[b]	1.3± 0.37[b]
		3.0 +1.5	0.2± 0.03[a]	0.5± 0.02[a]

BAP: 6-Benzylaminopurine; IAA: Indole-3-Acetic Acid; IBA: Indole-3-Butyric Acid; *: Mean of 10 replications and SE: Standard Error; Means ± SE followed by same letters are not significantly different at P=0.05 according to SPSS Version 17 (SPSS Inc., Chicago, USA) and means were compared using Tukey's tests at the 5% level of significance

days of culture (Table 1). These results are in accordance with Attia-O-Attia *et al.* (2014) who has also used Kinetin (1mg/L) as the main cytokinin for shoot multiplication from the axillary buds of Al-Taify mulberry variety (*Morus alba* L.). Similarly Seki *et al.* (1971) used Kinetin hormone for the callus induction from different explants of mulberry.

There are several recent reports regarding usage of Kinetin as the main cytokinin hormone for the *in vitro* micro propagation of several other plant species such as *Anacyclus pyrethrum* (Singh *et al.,* 2012), *Luffa cylindrica* (Sujatha *et al.,* 2013), *Phoenix dactylifera* L. (Mazri *et al.,* 2013), *Phoenix dactylifera* L. (Meziani *et al.,* 2015). As the best responses were observed on MS media with Kn (1.5 mg/L), this concentration was kept as constant and

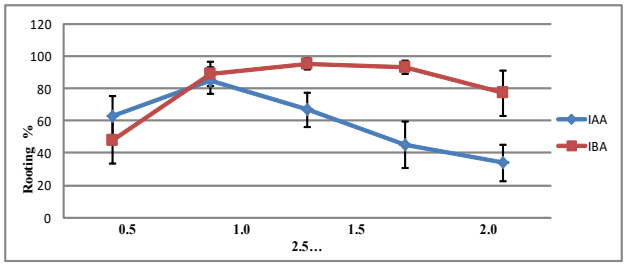

Fig. 2: Root Initiation from the proliferated axillary shoots of Chinese white mulberry variety on MS media supplemented with different concentrations of auxins

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TABLE 2

Rooting induction from the proliferated axillary shoots of Chinese white mulberry variety on MS media supplemented with different concentrations of auxins

Plant Growth Regulators in mg/L		Rooting Percentage (X*± S.E)
IAA	IBA	After 20 days of culture
0.5	-	63 ± 12.3 [a]
1.0	-	**85 ± 08.4** [b]
1.5	-	67 ± 10.6 [c]
2.0	-	45 ± 14.4 [b]
2.5	-	34 ± 11.2 [c]
-	0.5	48 ± 14.8 [a]
-	1.0	89 ± 07.6 [b]
-	**1.5**	**95 ± 03.4** [c]
-	2.0	93 ± 01.2 [b]
-	2.5	77 ± 14.3 [c]

IAA: Indole-3-Acetic Acid; IBA: Indole-3-Butyric Acid; *: Mean of 10 replications and SE: Standard Error
Means ± SE followed by same letters are not significantly different at P=0.05 according to SPSS Version 17 (SPSS Inc., Chicago, USA) and means were compared using Tukey's tests at the 5% level of significance.

Fig. 3: Hardening and acclimatization of *in vitro* raised Chinese white plantlets

A) Proliferated axillary shoot (9.2 cms) with well-developed roots after 20 days of culture on MS+IBA (1.5 mg/L)
B) Complete plantlet with well-developed roots was separated from media for hardening process
C) Complete plantlet with well-developed roots was separated from media for hardening process
D) Hardening of plantlets in plastic pots having sterile vermiculite, soil and sand in 1:2:1 ratio
E) Hardened plantlet was initially grown in culture room at 26° C.

different concentrations of BAP were taken while preparing the combinational media of cytokinins. Even though the combinational media of BAP and Kn also showed the responses of shoot proliferation from the nodal explants, but the responses were not comparable to that of individual Kinetin supplemented media.

Effect of Auxins on Rooting of Auxillary Shoots

The proliferated axillary shoots when transferred on to different concentrations of auxins containing media, complete plantlets were formed with the best rooting (95 %) on MS+ IBA (1.5 mg/L) media (Fig. 2) after 20 days of culture (Table 2). As soon as roots were initiated the shoot length has drastically increased (from 5.6 cms to 9.8 cms) with in duration of 2-3 days. Initially the developed roots are white in color (Fig. 1E), but the later turned to light brown (Fig. 1F) and black color after 20 days of culture (Fig. 1G). The turning of roots into brown color is majorly due to the production of secondary metabolites by the root system of plants (Rohela *et al.,* 2016a).

Hardening and Acclimatization

Hardening was carried out by transferring the plantlets from media (Fig. 3A-C) to plastic pots (Fig. 3D) having sterile vermiculite, soil and sand in 1:2:1 ratio. Initially the hardened plantlets were kept in culture room (Fig. 3E) at 26°C and later shifted to field conditions. The per cent of acclimatization of *in vitro* raised Chinese white mulberry plantlets in field conditions was about 82 per cent.

Among the various concentrations and combination of plant growth regulators used in the present study; good response of shoot proliferation from axillary buds of nodal explants of Chinese white mulberry variety was observed on Kinetin (Kn) supplemented media in terms of shoot length (cm) (6.8 ± 0.52 and 5.3 ± 0.41) at concentrations of 1.5 mg/L and 2.0 mg/L, respectively. The proliferated axillary shoots was rooted (95%) successfully on MS media fortified with 1.5 mg/L of indole-3-butyric acid (IBA). The *in vitro* raised plantlets were hardened in plastic pots containing 1:2:1 ratio of sterile vermiculite, garden soil and sand. The hardened plantlets were successfully acclimatized to field conditions with survival rate of 82 per cent. The developed protocol could be utilized for large scale clonal multiplication of *Morus alba* L. cv. Chinese white.

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