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RAPID ONE STEP PROTOCOL FOR THE *in vitro* MICRO PROPAGATION OF *Morus multicaulis* VAR. GOSHOERAMI, AN ELITE MULBERRY VARIETY OF TEMPERATE REGION

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Nodal explants

Goshoerami

Micro propagation

Rooting

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ABSTRACT

Morus multicaulis cv. Goshoerami is the leading mulberry variety for silkworm rearing under temperate climatic conditions of Jammu and Kashmir, India. However, the propagation of this popular mulberry variety has always remained a point of contention due to its poor rooting response through stem cuttings. It normally takes 4 to 5 years for raising the saplings of this variety through conventional root grafting techniques. Therefore, for quick propagation of this poor rooting popular mulberry variety, a one step *in vitro* protocol was developed by culturing nodal explants from 2 year old plants on Murashige & Skoog (MS) media supplemented with individual as well as combination of phytohormones. The maximum shoot bud proliferation (6.3 ± 0.71 in cm) and rooting (14.7 ± 0.53 in cm) was observed when nodal explants were cultured on the combinational media of BAP (1 mg/L) and IBA (1 mg/L) after 14 days of culture. These *in vitro* raised plantlets were hardened by using the sterile soil and vermiculite in 2:1 ratio. Only 25 days were required for the micro propagation and hardening of raised plantlets of Goshoerami through this single step protocol. The hardened plantlets were successfully established in the field with 83% survival rate. The developed one step protocol can be used efficiently for the mass propagation of this elite mulberry variety throughout the year with in short span of 25 days.

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1 Introduction

Sericulture is a remunerative industry with most of the benefits going to the rural and poor people across the country i.e. silkworm rearers. Silk is commonly called as the queen of textiles due to its shiny luster, softness, being pleasingly in nature, long durability and having good tensile properties (Rahmathulla, 2012). Among the five types of silks produced in India, the mori silk has a major share in sericulture industry (Datta, 1994). Several factors influence the end product in sericulture i.e. quality cocoon characters. The major factors are quality mulberry leaf, rearing seasons or environmental conditions such as suitable temperature and genetic constitution of silkworm hybrids used (Rahmathulla, 2012).

Mori silkworm is monophagous in nature and feeds only on mulberry leaves (Meneguim et al., 2010) which accounts as the major factor (38%) in mori silk production (Vijayan et al., 1997a; Vijayan et al., 1997b; Shankar et al., 2001; Srivastava et al., 2006; Vijayan, 2010; Vineet et al., 2012; Gandhi et al., 2012).

Mulberry leaf is not only being used for silk production but it also has several other applications such as feed supplement to several animals (Phinye et al., 2003; Anbarasu et al., 2004; Ba et al., 2005; Martinez et al., 2005; Bakshi & Wadhwa, 2007; Kandylis et al., 2009), several medicinal values, as a protein source (Butt et al., 2008), neuroprotective (Niidome et al., 2007), reduce the symptoms of diabetes (Ewelina et al., 2016), lowering the blood glucose, triglycerides levels in body (Andallu et al., 2001) improvement of skin (Lee et al., 2002; Fang et al., 2005) and for neutralizing the free radicals (Naowaratwattana et al., 2010) etc. Hence Sanchez (2000 & 2002) described mulberry as exceptional foliage available worldwide and in use for feeding several animal species.

For sericulture, across the country several mulberry varieties were used as superior types in producing quality cocoons. So now-a-days, lot of importance is being given to raise and multiply the region and climate wise most suitable mulberry varieties for expanding the sericulture and attracting the farmers in the present dominated era of cereals, pulses, fruits and horticulture crops.

Temperate regions of India with varied agroclimatic conditions (Gani et al., 2016; Aftab et al., 2018) is most suitable for bivoltine type of sericultival practices. In Kashmir region, among the various varieties of mulberry *Morus multicaulis* var. Goshorami is presently used as one of the elite genotypes in silk production by mori silk worms (*Bombyx mori* L.) (Aftab et al., 2012). *M. multicaulis* var. Goshorami has large sized leaves, good canopy, good leaf yield, moisture retention capacity and has the ability to develop into any type of plantation like bush, dwarf or tree type based on requirements. Kour et al. (2015) has recorded the maximum leaf yield of 4.8 tons/ha during the autumn season from the Goshorami, further they have concluded to utilize

Goshorami variety as one of the important genotype for gaining maximum benefit at farmer's level in temperate regions of India. Similarly Shabir et al. (2014) has recorded the maximum leaf lamina length in Goshorami during spring (19.75 cm) and autumn (22.25 cm) seasons, when they evaluated the seventeen temperate mulberry varieties during spring and autumn seasons. They also recorded the highest leaf weight in Goshorami, out of 17 selected mulberry varieties during spring (3.11 g) as well as in autumn (5.03 g) seasons.

The major limiting factor in multiplying the Goshorami by conventional stem cuttings technique is its poor rooting ability (Aftab et al., 2012; Rohela et al., 2016b). This is mainly due to prevailing cold conditions (Shukla et al., 2016) which in turn decreasing soil temperature and effecting the root formation from the stem cuttings of Goshorami. In order to induce the roots, the soil temperature has to be increased by using any of the advanced techniques or plantlets have to be produced in *in-vitro* and controlled conditions.

In vitro propagation techniques have been used extensively since long time for the multiplication of important plant varieties across the world. During the last forty years, several researchers have reported the success in micro propagation of several mulberry varieties from different explants (Thomas, 2002). So, in this present research we are attempting to develop an *in vitro* micro propagation protocol for propagating the Goshorami mulberry variety throughout the year in proportionate manner through the rapid and single step based *in vitro* culture technique with short span of period, instead of multiple steps based *in vitro* propagation techniques.

2.1 Materials and methods

2.1 Plant Material

Three years old Goshorami mulberry variety available in the CSR&TI, Pampore institute was used as parent plant material in this *in vitro* propagation studies.

2.2 Surface sterilization

Nodal segments of 2-3 cm length were collected from the 3 years old *M. multicaulis* var. Goshorami plants present in the mulberry fields of CSR&TI, Pampore institute, Jammu and Kashmir, INDIA. The explants were initially washed for 4-6 times under running tap water followed by washing with tween-20 solution for 3-5 minutes, then treated with 0.5% Sodium hypochlorite (NaOCl) solution for 3 minutes, 60% ethanol for 2 minutes and at last treated with 0.1% Mercuric Chloride (HgCl₂) for 2-3 minutes duration. Then the traces of HgCl₂ present on surface of explants were removed by rinsing the treated nodal explants in sterile distilled water.

2.3 Media preparation and sterilization

Readymade MS media powder (Murashige & Skoog 1962) of about 4.42 grams was weighed and dissolved in 1 liter of distilled water, later 30 grams of sucrose (3%) was dissolved and the pH of the MS media was adjusted to 5.5 ± 0.3 . After adjusting the pH, 0.8% of agar-agar (8 grams /1000ml), a solidifying agent was added and dissolved by slow heating. Now before dispensing the prepared basal MS media into the required culture vessels, appropriate concentrations (0.5-2.0 mg) and combinations of phytohormones (BAP, IAA & IBA) were added to the prepared MS media. Culture tubes and other culture vessels with prepared MS media containing different concentrations and combinations of phytohormones were sterilized in an autoclave at 121°C and 15 lbs pressure for 15 to 20 minutes.

2.4 Inoculation

Laminar air flow cabinet is used for providing the aseptic conditions during the inoculation of surface sterilized nodal segments onto MS media with the help of sterile forceps, spirit lamp and sterile tissue paper.

2.5 Incubation

The nodal segments inoculated culture tubes and phyta jars were kept in a culture room at $26 \pm 2^\circ\text{C}$ under 16/8 hours of light period. Cultures were maintained with the 3000 Lux light intensity under the florescent lamps.

2.6 Statistical Analysis

The data obtained during this research study was statistically analyzed by using SPSS Version 17 (SPSS Inc., Chicago, USA) and Tukey's tests at the 5% level of significance was carried for comparing the mean values. All means are represented with mean \pm SE.

3 Results

When the nodal explants of *M. multicaulis* var. Goshoerami were inoculated onto different concentrations and combinations of phytohormones containing MS media, good responses of complete plantlet formation by simultaneous shoot and root development was observed on combinational media of cytokinins and auxins rather than individual hormones supplemented media. Among different combinations tested, BAP and IBA supplemented MS media has recorded the maximum shoot and root lengths (cm) from the formed complete plantlets.

3.1 Effect of individual phytohormones

Initially when nodal explants were inoculated onto individual phytohormones such as BAP, IAA and IBA containing MS media, responses in terms of proliferated shoot length (cm) and root length (cm) were recorded after 7 and 14 days of culture duration (Table 1 & Figure 1). On individual phytohormones supplemented media, maximum axillary bud proliferation (2.9 ± 0.17 cm) was

Table 1 Effect of individual phytohormones supplemented MS media on the nodal explants of *Morus multicaulis* var. Goshoerami

Plant Growth Regulators in mg/L			Proliferated axillary shoot length in cms ($\bar{X} \pm \text{S.E}$)		Root length in cms ($\bar{X} \pm \text{S.E}$)	
BAP	IAA	IBA	After 7 days of culture	After 14 days of culture	After 7 days of culture	After 14 days of culture
0.5	-	-	1.0 ± 0.16^c	1.6 ± 0.22^b	-	-
1.0	-	-	2.4 ± 0.31^e	2.9 ± 0.17^d	-	-
1.5	-	-	2.6 ± 0.17^f	4.8 ± 0.34^e	-	-
2.0	-	-	1.2 ± 0.46^c	2.4 ± 0.36^d	-	-
-	0.5	-	0.7 ± 0.10^b	1.0 ± 0.40^b	-	0.3 ± 0.02^a
-	1.0	-	1.6 ± 0.06^a	2.2 ± 0.38^c	0.4 ± 0.16^b	2.1 ± 0.85^d
-	1.5	-	0.4 ± 0.09^a	1.9 ± 0.12^b	-	1.3 ± 0.63^c
-	2.0	-	0.6 ± 0.19^a	1.8 ± 0.24^b	-	-
-	-	0.5	0.3 ± 0.11^a	1.1 ± 0.31^b	-	0.6 ± 0.13^b
-	-	1.0	0.6 ± 0.43^a	0.9 ± 0.16^b	0.3 ± 0.03^b	7.2 ± 0.68^e
-	-	1.5	1.0 ± 0.23^b	1.7 ± 0.32^d	0.1 ± 0.15^a	1.8 ± 0.64^c
-	-	2.0	0.5 ± 0.08^a	1.3 ± 0.46^b	-	0.2 ± 0.07^a

BAP: 6-Benzylaminopurine; IAA: Indole-3-Acetic Acid; IBA: Indole-3-Butyric Acid;

*: Mean of 10 replications and SE: Standard Error

Means \pm 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

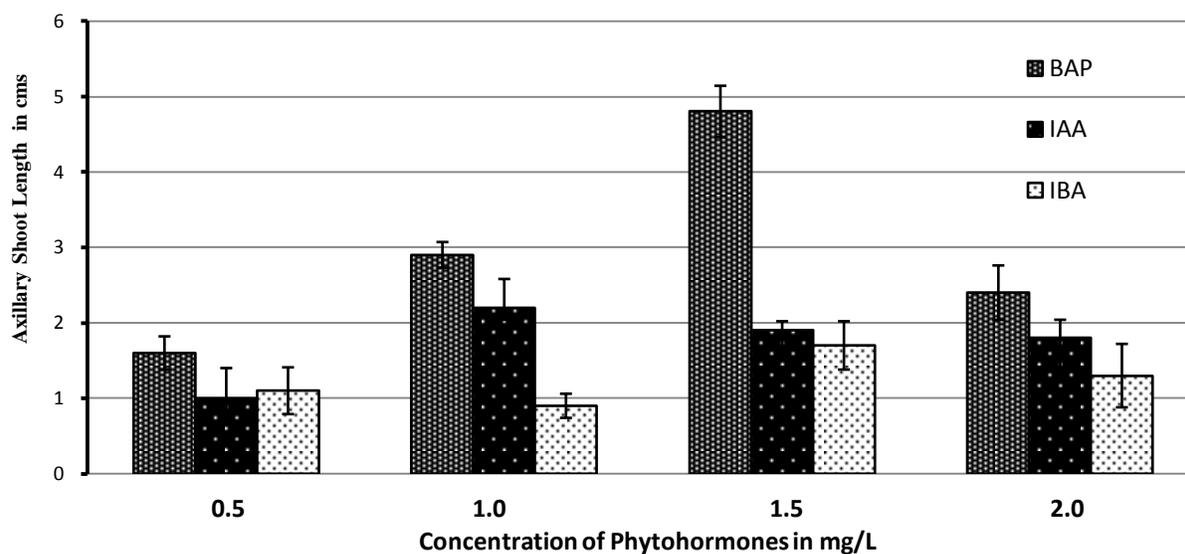


Figure 1 Effect of individual phytohormones supplemented MS media on the nodal explants of *Morus multicaulis* var. Goshierami after 14 days of culture.

observed from nodal explants cultured on MS media supplemented with 1.0 mg/L concentration of BAP (Table 1).

On different concentrations of BAP (0.5, 1.0, 1.5 & 2.0 mg/L) supplemented media, axillary bud proliferation responses were recorded after 14 days of culture. On IAA supplemented media, even though both shoot and root developments were observed from nodal explants, the response of shoot development is slow whereas root development is fast. At lower concentrations of IAA (1.0 mg/L), minimal shoot growth (1.6 ± 0.06 cm) and root growth (0.4 ± 0.16 cm) responses was observed after 7 days of culture (Figure 3A), but when results were recorded after 14 days of culture duration, slight increase in the shoot growth (2.2 ± 0.38) and enhanced root growth (2.1 ± 0.85) responses (Figure 3B) were observed (Table 1). The above results were clearly indicating that on IAA (1.0 mg/L) supplemented media, the shoot growth is slow and root growth is faster.

At higher concentrations of IAA (2.0 mg/L), along with axillary bud proliferation adventitious roots were formed from the nodal explants exactly at the place where the earlier petiole has fallen off from the inoculated nodal explants (Figure 3C). This result was observed repeatedly from nodal explants on IAA (2.0 mg/L) supplemented media. Further, in case of root formation, at individual phytohormones supplemented media, maximum root growth (7.2 ± 0.68 cm) was observed on IBA (1.0 mg/L) supplemented media after 14 days of culture (Table 1).

3.2 Effect of combinational phytohormones

On individual phytohormones supplemented media, as maximum shoot growth (2.9 ± 0.17 cm) was observed on MS media supplemented with 1.0 mg/L BAP, this concentration of BAP was taken as constant and combined with different concentration of either IAA or IBA while preparing combinations of phytohormones supplemented media (Table 2). Slow growth was recorded on the combinational media of BAP (1.0) and IBA at lower concentrations. Minimal axillary bud proliferation (0.8 ± 0.23 cm) and root growth (2.4 ± 0.15 cm) was observed from nodal explants of Goshierami on MS media supplemented with BAP (1 mg/L) and IBA (0.5 mg/L) after 14 days of culture (Figure 5A, 5B and 5C).

Overall, when nodal explants were cultured on different concentrations and combinations of phytohormones supplemented media, the maximum axillary bud proliferation or shoot growth (6.3 ± 0.71) (Figure 5F) and root growth (14.7 ± 0.53) (Figure 5G) was observed on combinational media of BAP (1.0 mg/L) and IBA (1.0 mg/L) (Figure 4) after 14 days of culture. Initially after 7 days of culture the initiated roots were white, but after 14 days of culture, the developed roots turned brown in color, similar observation was also made by Sajeevan et al., (2011) for *in vitro* raised V1 mulberry plantlets. The turning of *in vitro* induced roots into brown color is majorly due to the nature of phenolic compounds produced by the root system of tree species (Rohela et al., 2016a).

Table 2 Effect of Combination of phytohormones supplemented MS media on the nodal explants of *Morus multicaulis* var. Goshoerami

Plant Growth Regulators in mg/L			Proliferated axillary shoot length in cms ($\bar{X} \pm \text{S.E}$)		Root length in cms ($\bar{X} \pm \text{S.E}$)	
BAP	IAA	IBA	After 7 days of culture	After 14days of culture	After 7 days of culture	After 14days of culture
1.0	0.5	-	0.8± 0.13 ^b	1.4± 0.20 ^b	0.6± 0.13 ^a	1.3± 0.20 ^b
1.0	1.0	-	2.2± 0.26 ^c	2.8± 0.31 ^d	1.2± 0.58 ^b	6.9± 0.32 ^g
1.0	1.5	-	1.6± 0.14 ^d	2.1± 0.18 ^c	1.8± 0.64 ^c	4.3± 0.41 ^e
1.0	2.0	-	0.7± 0.31 ^b	0.9± 0.26 ^a	-	-
1.0	-	0.5	0.4± 0.33 ^a	0.8± 0.23 ^a	0.7± 0.16 ^a	2.4± 0.15 ^c
1.0	-	1.0	2.8± 0.34 ^d	6.3± 0.71 ^c	2.1± 0.49 ^d	14.7± 0.53 ^h
1.0	-	1.5	0.9± 0.46 ^b	4.2± 0.63 ^b	2.0± 0.45 ^d	10.6± 0.67 ^f
1.0	-	2.0	1.2± 0.17 ^c	1.7± 0.41 ^b	0.6± 0.31 ^a	4.2± 0.19 ^d

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.

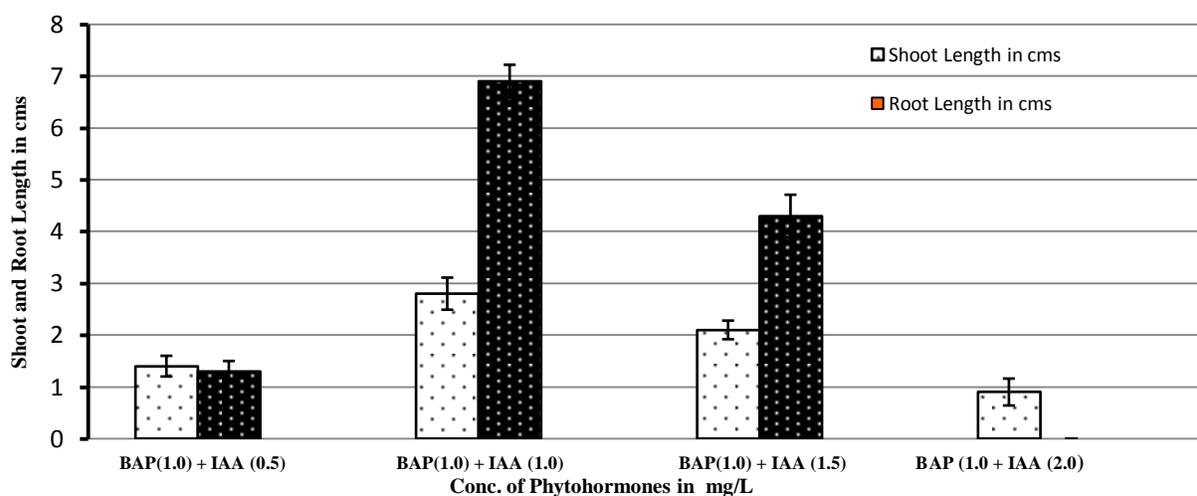


Figure 2 Effect of combination of BAP and IAA supplemented MS media on the nodal explants of *Morus multicaulis* var. Goshoerami after 14 days of culture.

On another combination of BAP(1.0) and IAA (0.5, 1.0, 1.5 & 2.0 mg/L) supplemented media, even though both the axillary bud proliferation and root initiation was observed after 14 days of culture (Figure 2), but the results were not comparable to that of BAP and IBA supplemented media. On combination of BAP (1 mg/L) and IAA (2 mg/L) supplemented media along with axillary bud proliferation (0.9 ± 0.26), adventitious callus was also formed from the internodal regions of nodal explants after 14 days of culture (Figure 3D).

3.3 Hardening and acclimatization

Developed *in vitro* plantlets were hardened by using the sterile soil and vermiculite in 2:1 ratio (Figure 5D). Totally 25 days were required for the micro propagation and hardening of raised plantlets of *M. multicaulis* var. Goshoerami through this single step protocol. The hardened plants were initially kept in cups (Figure 5E) and pots (Figure 5H) for two months duration and maintained in culture room and then transferred into the field

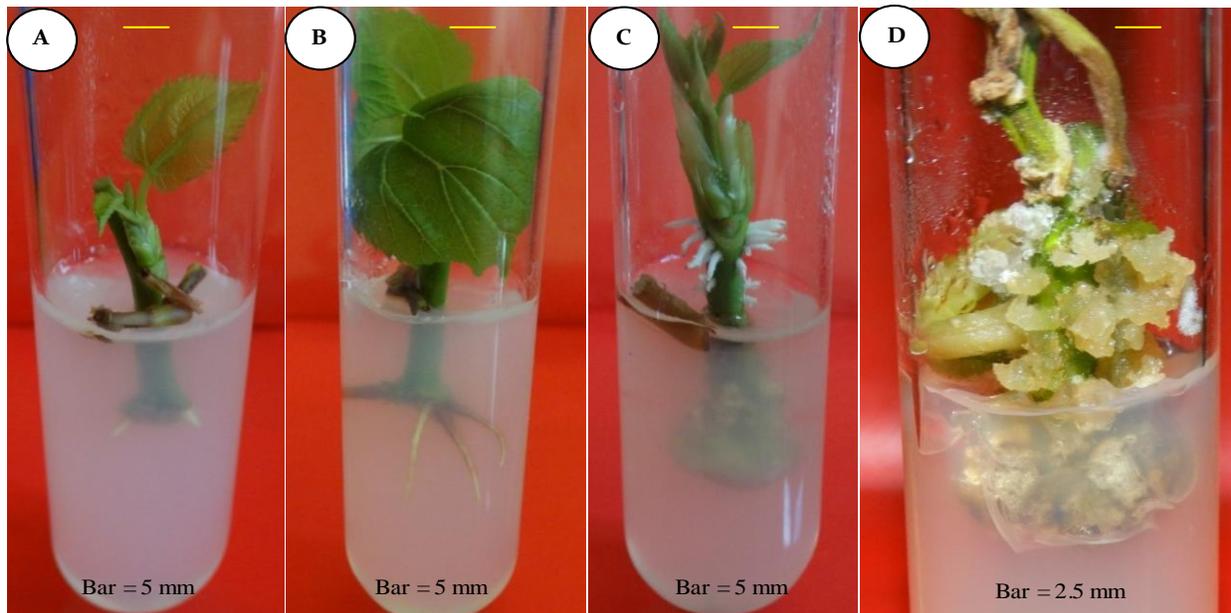


Figure 3 Micro propagation of *Morus multicaulis* var. Goshokerami by using nodal explants

- Axillary bud proliferation (1.6 cm) and induction of roots (0.4 cm) from nodal explants of *Morus Multicaulis* var. Goshokerami on MS media supplemented with IAA (1.0 mg/L) after 7 days of culture.
- Shoot Growth (2.2 cm) and Root Growth (2.1 cm) from nodal explants of *Morus Multicaulis* var. Goshokerami on MS media supplemented with IAA (1 mg/L) after 14 days of culture.
- Initiation of adventitious roots from nodal segments on IAA(2.0 mg/L) supplemented media.
- Callus induction from the internodal regions of Goshokerami on MS media supplemented with BAP (1 mg/L) and IAA (2 mg/L).

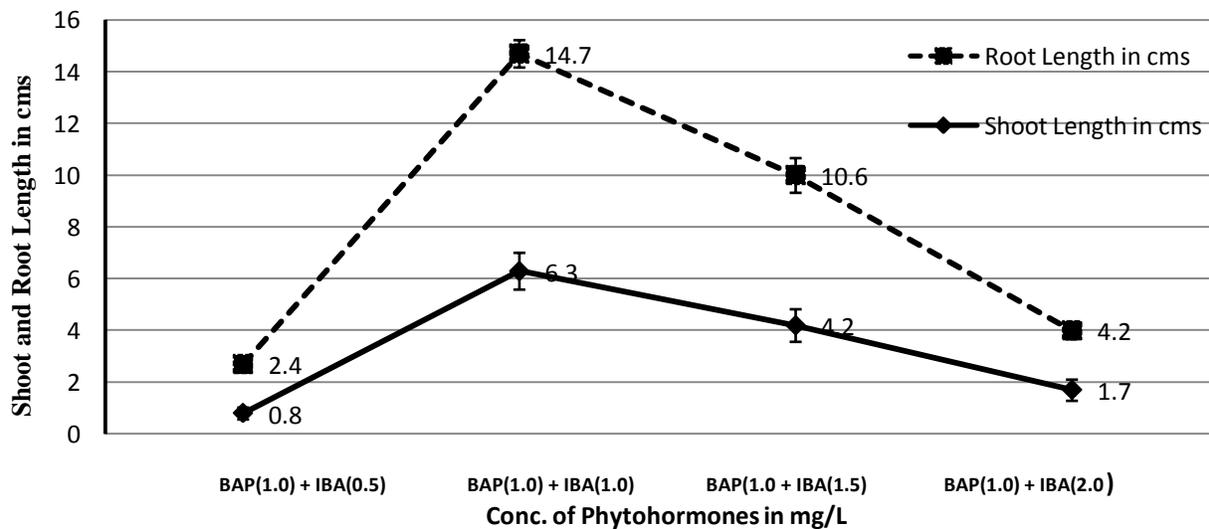


Figure 4 Effect of combination of BAP and IBA supplemented MS media on the nodal explants of *Morus multicaulis* var. Goshokerami after 14 days of culture.

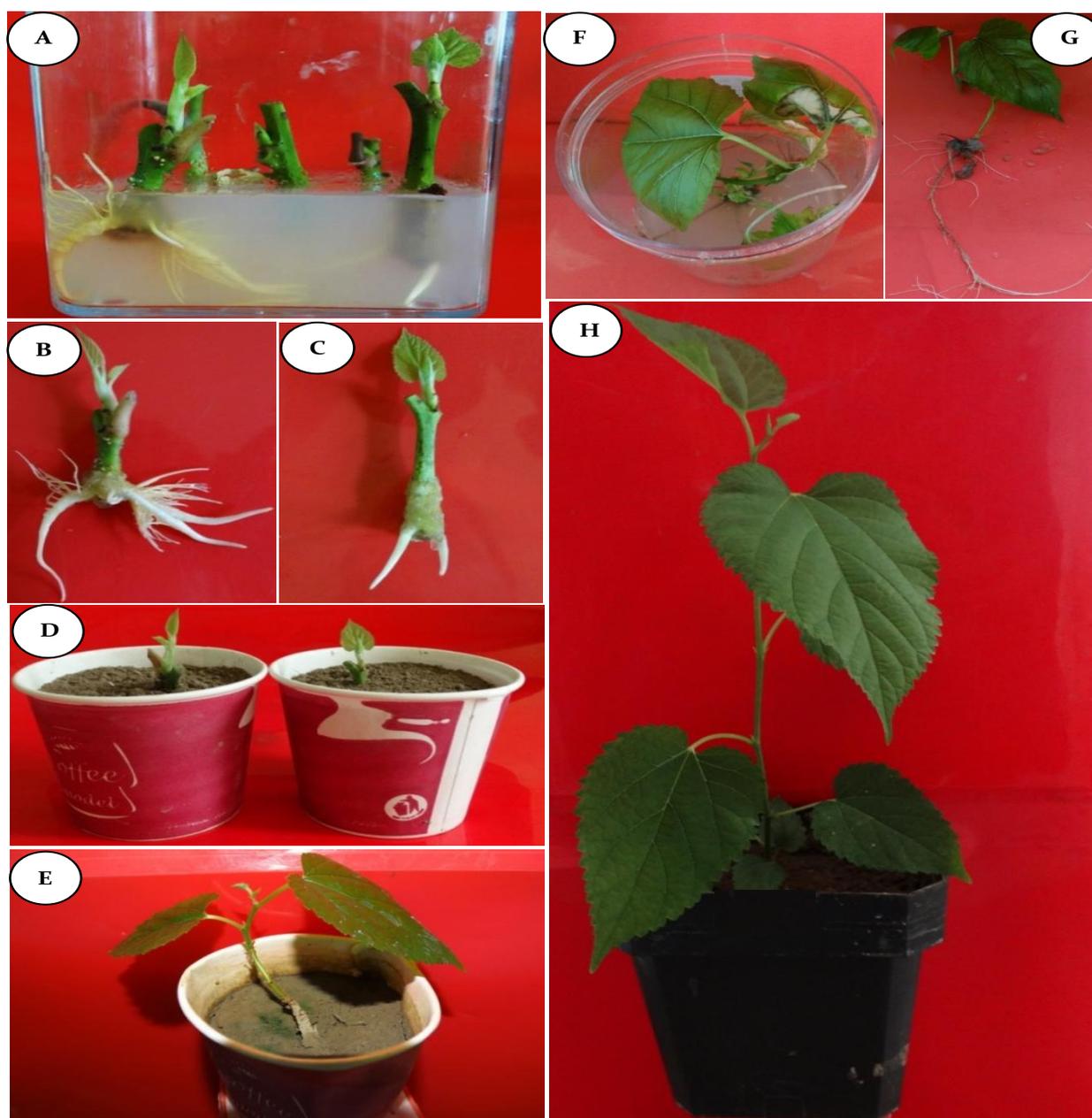


Figure 5 Micro propagation of *Morus multicaulis* var. Goshorami by using nodal explants

- A) Axillary bud proliferation (0.8 cm) and induction of roots (2.4 cm) from nodal explants of *Morus Multicaulis* var. Goshorami on MS media supplemented with BAP (1 mg/L) and IBA (0.5 mg/L) after 14 days of culture.
- B) Complete plantlet of Goshorami with well-developed root system
- C) Complete plantlet of Goshorami with few developed roots
- D) Hardening of *in vitro* raised Goshorami plantlets
- E) Hardened plantlet of Goshorami in a poly cup by using the sterile soil and vermiculite in 2:1 ratio.
- F) Complete plantlet of Goshorami with well-formed axillary shoot (6.3 cm) and root system (14.7 cm) developed from nodal explants cultured on the combinational media of BAP (1 mg/L) and IBA (1 mg/L) after 14 days of culture
- G) Separated plantlet of Goshorami for hardening process with well-developed root system
- H) Hardened plantlet of Goshorami (2 months old) in a plastic pot after hardening process with the sterile soil and vermiculite in 2:1 ratio

conditions after gradually increasing the temperature from 27° C to as per the field conditions. The hardened plantlets were successfully established in the field with 83% survival rate.

4 Discussions

In present study, good response in terms of axillary bud proliferation and shoot growth from nodal explants of *M. multicaulis* var. Goshorami was obtained on MS media supplemented with BAP hormone. Chitra & Padmaja (1999) also reported the similar results; they achieved maximum shoot growth from nodal explants of *M. indica* cultivar M5 by using BAP (0.5 - 1.0 mg/L) as the main phytohormone supplemented with MS media. Raghunath et al. (2013) and Vijayan et al. (2000) has reported mulberry leaf as explant and BAP as the hormone for micro propagation of *M. indica* cv. V1. Cytokinin, BAP (6-Benzylaminopurine) was also reported as the main plant growth regulator in micro propagation of several other plants species. Sujatha et al. (2013) obtained maximum number of plantlets in sponge gourd (*Luffacy lindrica*) by using leaf and nodal explants at 1.5 mg/L concentration of BAP. Similarly on individually supplemented BAP (Rohela et al., 2018b) and in combinations of BAP with TDZ (Thiadiazuron) (Rohela et al., 2013; Rohela et al., 2015; Rohela et al., 2016c; Korra et al., 2017; Rohela et al., 2018a) of multiple shoots were regenerated from nodal explants, shoot tip explants, leaf and stem based calluses of *Rauwolfia tetraphylla* and *Morus* Sps.

In mulberry, several researchers have reported the clonal propagation of mulberry plantlets by using nodal segments (Bapat & Rao, 1990; Pattnaik & Chand, 1997; Chitra & Padmaja, 2001; Anis et al., 2003) as explants and BAP as the plant growth regulator. But, all these earlier reporters have used nodal explants and carried out micro propagation of mulberry plantlets by two step protocol, where initially axillary bud proliferation was carried on a shoot medium and later the proliferated axillary shoots were excised and transferred onto rooting media for root initiation. Whereas in present study, we have successfully carried out *in vitro* micro propagation of *M. multicaulis* var. Goshorami by a rapid and single step protocol. Through this research study, we reported the simultaneous shoot and root development from the nodal segments of *M. multicaulis* var. Goshorami in a single step on the MS media supplemented with different combinations and concentrations of cytokinins and auxins.

Similarly, one step protocol was also reported for the micro propagation of several other plant species, such as *Vaccinium macrocarpon* (Debnath & Mc Rae, 2005; Debnath, 2008), *Bacopa monnieri* (Naik et al., 2014), *Stevia rebaudiana* bertonii (Peixe et al., 2015), *Lilium martagon* var. *cattaniae* (Marijana et al., 2012), *Bambusa balcooa* Roxb. (Beena et al., 2015), *Cicer arietinum*

(Sujatha et al., 2007), *Malus domestica* (Bommineni et al., 2001), and *Aloe vera* (Dwivedi et al., 2014).

Out of several concentrations and combinations of phytohormones tested in this study, the combination of BAP and IBA has given better results. Overall the maximum shoot bud proliferation (6.3± 0.71 in cm) and best rooting (14.7± 0.53 in cm) was observed when nodal explants were cultured on the combinational media of BAP (1 mg/L) and IBA (1 mg/L) after 14 days of culture. Cytokinin and auxin combination of BAP and IBA were used earlier for the micro propagation of several other plant species such as *Musa sp.* (Ikram-ul-haq & Dahot, 2007; Buah et al., 2010), *Strelitzia reginae* (North et al., 2012) and *Melia azedarach* (Silvia et al., 2002).

Conclusion

The developed rapid one step protocol could be used efficiently for the *in vitro* micro propagation of an elite temperate mulberry variety *M. multicaulis* var. Goshorami within the short duration of 25- 40 days to that of 60-80 days duration of routinely carried multiple steps based *in vitro* culture techniques. Also the long duration of raising the Goshorami mulberry saplings through conventional techniques can be drastically reduced if the winter dormancy period of temperate region can be utilized for the developed rapid one step *in vitro* propagation protocol along with green house phase of acclimatizing the raised plantlets to field conditions.

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Conflict of interest

All the authors declare that there is no conflict of interest

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