REPORT

ON

ONGOING PROJECT

Title: **Evolution of superior mulberry variety suitable for temperate region through somatic hybridization**

(In Collaboration with University of Kashmir)

Project Code: **PIB-3571** **Duration:** **March, 2016- Feb, 2019**

BUDGET: **39.90 Lakhs**

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Central Silk Board.
Ministry of Textiles-Govt. of India.
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CONTENTS

GENERAL INFORMATION

1.	Name of the Institute/ University/ Organization	**Central Sericultural Research & Training Institute, Pampore, Srinagar (J&K)**
2.	State	Jammu & Kashmir
3.	Status of the Institute	Central Sericultural Research & Training Institute, Pampore caters to the development Of sericulture in North Indian states.
4.	Name and designation of the executive authority of Institute/ University	Dr. M.K.Ghosh, Director, Central Sericultural Research & Training Institute, Pampore, Srinagar-J&K
5.	Project title	**Evolution of superior mulberry variety suitable for temperate region through somatic hybridization**
6.	Category of the project	R&D
7.	Specific area	Molecular Breeding
8.	Duration (years)	03 Years
9.	Total cost (Rs.)	**39.90 Lakhs**
10.	Is the project single institutional or multiple institutional (s)	Multi Institutional : CSR & TI, CSB, Pampore, & University of Kashmir, Srinagar
11.	If the project is multi institutional, please furnish the following.	--
11.1	Name of the project co-ordinator	Dr. M.K.Ghosh
11.2	Affiliation	Director, CSR&TI, Pampore
11.3	Address	Central Sericultural Research & Training Institute, Pampore, Srinagar-J&K

12	**Scope of ongoing project and indicating anticipated product and processes**	In somatic hybridization, the diploid somatic cells (protoplasts) from any two varieties can be fused by electro fusion. Protoplasts are fused to get a tetraploid cell, from which plantlets (somatic hybrids) are raised in *in vitro* conditions. Due to fusion of protoplasts derived from different varieties, there is high possibility of genomic combinations which leads to the production of somatic hybrids with more desirable characters and fusion of protoplasts derived from same variety increases the ploidy level which is expected to enhance the expression of desirable characters. **The produced somatic hybrids with increased ploidy status can have more desirable characters such as higher leaf yield, more rooting ability, resistance towards diseases etc.** **The developed protocols and the isolated protoplasts can be utilized as raw materials for the genetic improvement of mulberry in future molecular breeding programmes.**

13	**Project Summary of Ongoing Project**	In plants, it has always not been possible to obtain full hybrids between desired individuals because of sexual incompatibility barriers. This has often proved to be a serious threat in crop improvement programs through conventional breeding programmes. Protoplasts are the naked plant cells from which cell wall has been removed, but the plasma membrane is intact. Both the isolated protoplasts but their fusion product, a somatic hybrid, can also be regenerated into whole plants. Somatic hybridization through protoplast fusion opens up possibilities for creating new genotypes which are not possible hitherto (Narayan *et.al.,* 1992). It allows us to combine entire genomes from the sexually incompatible parents and expected to result in hybrids which are superior in characters (Melchers *et.al.,* 1978). So, the current ongoing project is aimed **to produce a superior mulberry variety suitable to temperate region through somatic hybridization**.

BACKGROUND

Based on characterization and evaluation data of available 80 genotypes present in CSR&TI, Pampore (Catalogue on temperate mulberry germplasm), the varieties Goshoerami, C-4, Mandalay(s-1), Ichinose, Chinese white and Brentul Kashmir were found with most of the desirable characters but have one or two undesirable characters (Anil Dhar *et.al.,*2011). Similarly based on conclusion given in annual report (2014-2015) of CSR&TI, Pampore, PPR-1 (S-140) is found to be superior variety among the existing lines but it also has limitations such as frost damage, moderately resistant to diseases etc.

Goshoerami variety of *Morus multicaulis* has good leaf yield (4.57 kg/plant/year), moisture retention capacity (88.25%), moderately sensitive to powdery mildew, leaf spot diseases and *Glyhodes phyloalis*, but it has poor rooting ability (18.18%). C-4 variety of *M.alba* has good leaf yield (3.86 kg/plant/year), moisture retention capacity (81.38%), good rooting ability (70.00%) but it is sensitive to leaf spot disease. Mandalay (S-1) variety of *M.alba* has moderate moisture retention ability (78.34%), but it has good rooting ability (64.50%). Ichinose variety of *M.alba* also has good leaf yield ability (4.73 kg/plant/year) but it has poor rooting ability (4.67%). Chinese white variety of *M.alba* also has moderate leaf yield ability (2.29 kg/plant/year) but it has good rooting ability (62.50%). Brentul Kashmir variety of *M.alba* also has good leaf yield ability (3.37 kg/plant/year) but it has poor rooting ability (02.00%) (Anil Dhar *et.al.,* 2011). PPR-1 (S-140) variety has good leaf yield (4.329 kg/plant/year), moisture retention ability (89.64%), better rooting ability (95.00%), but the variety is moderately susceptible to frost damage and moderately susceptible to diseases.

Based on the above data, it is clear that it is **rare to find** a variety with all desirable characters. In sericulture, it is important to provide superior mulberry varieties to farmers which gives good leaf yield, resistant

to diseases & pests, having good rooting ability and resists the adverse environmental conditions.

To produce superior mulberry varieties with the above mentioned characters, usually conventional breeding is carried out by cross pollinating two varieties with desirable characters. But in certain varieties cross pollination is **not possible due to several barriers** such as timing of flowering, protoandrous or protogyny conditions, pollen incompatibility, unreceptive nature of stigma, environmental conditions (Vijayan *et.al.,*1997; Tikader *et.al.,*2014) and also sometimes species barrier.

Where as in somatic hybridization, the diploid somatic cells (protoplasts) from any two varieties can be fused by electro fusion. The electric field and PEG induced protoplast fusion can carry fine control of fusion process (Pavan *et.al.,* 2010). Fused protoplasts are regarded as tetraploid cells, from which plantlets (somatic hybrids) can be raised in *in vitro* conditions through the regeneration studies.

With this background the current ongoing project was designed and got approval in April, 2016 after clearing the Concept note by Central Office, RC meeting, Referees comments and RAC meeting.

Restricted the number of parental lines to four

Initially project was designed to carry out the somatic hybridization studies by selecting the above mentioned seven parental lines, but later on based on the recommendations of Referees and 34th RAC committee number of parental lines were restricted to four [Goshoerami, Ichinose, Chinese white, and PPR-1 (S-140)] in order to complete the project in speculated time period of 3 years (2016-2019)

The information regarding four selected mulberry varieties is summarized in the tabulated form as below

S.no	Variety	Species	Advantages	Disadvantages
1	Goshoerami	*Morus multicaulis*	➢ Leaf yield (4.57 kg/plant/year), ➢ Moisture retention capacity (88.25%), ➢ Moderately resistant to powdery mildew, leaf spot diseases and *Glyhodes phyloalis*, ➢ Frost damage (38.06%)	➢ But it has low rooting ability (18.18%).
2	Ichinose	*M. alba*	➢ Good leaf yield ability (4.73 kg/plant/year)	➢ Low rooting ability (4.67%).
3	Chinese white	*M. alba*	➢ Good rooting ability (62.50%) ➢ Early sprouter	➢ Moderate leaf yield (2.29kg/plant/year) ➢ Sensitive to powdery mildew and leaf spot diseases.
4	PPR-1 (S-140)	Interspecific Hybrid (Goshoerami **X** Chinese White)	➢ Good leaf yield (4.329 kg/plant/year), ➢ Moisture retention ability (89.64%), ➢ Better rooting ability (95.00%)	➢ Moderately susceptible to frost damage. ➢ Moderately susceptible to diseases.

Hypothesis:

The produced somatic hybrids with increased ploidy status can have more desirable characters such as higher leaf yield, more rooting ability, resistance towards diseases etc. Earlier reports are there regarding utilization of triploid (S-1635, C-1730, Tr-8, Tr-10, Tr-23 & Vishala)(Dandin *et.al.,* 1983 & Tikader *et.al.,*2014) and tetraploid mulberry varieties (Dwivedi et.al., 1983 Chakrabortiet.al., 1997) for commercial utilization. Triploid varieties Tr-8, Tr-10 and tetraploid varieties T-4 and T-10 available in germplasm bank of CSR & TI, Pampore are having good rooting abilities of 66.00%, 54.00%, 41.25.00% and 40.50% respectively (Anil Dhar *et.al.,*2011).

REVIEW OF LITERATURE

Either nationally or internationally not much work has been done on mulberry protoplast culture. Therefore progress on mulberry protoplast culture up to now is very limited. Koitz Katagiri (1989) from japan reported colony formation in a culture of mulberry mesophyll derived protoplast. Some success in fusion of mulberry protoplast with paper mulberry protoplast has been achieved by Onishi *et .al.*, in japan (1989).

Similarly, there are some reports regarding protoplast isolation (Ohyama *et.al.,* 1970; Ohnisi *et.al.,* 1987; Katagiri, 1988; Katagiri, 1989; Wei *et.al.,* 1994;Chand *et.al.,* 1996;Pavan *et.al.,* 2000_a; Pavan *et.al.,* 2000_b; Pavan *et.al.,* 2010), protoplast fusion (Ohnisi et.al., 1989; Sowers *et.al.,* 1993; Chang *et.al.,* 1992;Chang *et.al.,*1995; Chand *et.al.,* 1996 ;) and regeneration of mulberry plantlets (Adachi *et.al.,* 1999, Pavan *et.al.,* 2000_a; Bhatnagar *et.al.,* 2001,Bahu *et.al.,*2001, Bahu *et.al.,* 2003).

Not much work has been done on somatic hybridization in mulberry and till now there is no report on somatic hybridization in mulberry varieties of temperate region.

However, so far, there are some forest tree species in which plant regeneration from protoplasts has been successful reported, *viz. Liriodendron* tulipifera (Merkle *et. al.*, 1987) , *Paulownia fortune* (Wei *et. al.*, 1991a), *Picea glauca* (Attree *et. al.*, 1989), *P1atanus orientali* (Wei *et. al.*, 1991b), *Populus sp* (Russell *et. al.*, 1986,1988; Wang *et.al.,* 1991,992). *Santalum album* (Rad *et. al.*, 1985), *Solanum dulcamara*(Chand *et. al.*, 1990) and *Ulnus* (Sticklen *et. al.*, 1986).

In view of the above limitations in producing a superior variety by conventional breeding and advantages concerned with somatic hybridization, the current ongoing project (PIB-3571) was undertaken with following objectives and expected outcome:

Objectives of the ongoing project:

1. To ascertain the ploidy level of popular mulberry accessions of temperate region.
2. To produce somatic hybrids suitable to temperate climatic conditions.

Expected outcome of the project:

1. Development of superior mulberry variety suitable for temperate regions with more desirable characters in terms of leaf yield, quality of leaf, rooting ability, moisture retention ability and resistance against diseases. The produced tetraploid mulberry genotypes can also be utilized in future breeding programmes for evolving a superior triploid genotype.
2. The isolated protoplasts can be utilized in genetic transformation experiments for genetic improvement of mulberry varieties through molecular breeding in future.
3. Increase in the mulberry productivity in Jammu & Kashmir and other northwest regions, which in turn will be helpful to poor farmers for getting better livelihood and will lead to increase in silk yield of country.

PROGRESS REPORT OF ONGOING PROJECT (PIB-3571)

Work Done (Year wise)

First year (2016-17): Work Carried at CSR&TI, Pampore

1) Procured necessary chemicals, glassware, plastic ware and established plant tissue culture facilities
2) Identified the ploidy level of four selected parental mulberry lines through Flow cytometric and stomatal studies
3) Developed protocols for the in vitro clonal propagation of selected mulberry varieties

Second year (2017-18): Work Carried at University of Kashmir

1) Isolated the protoplasts from the diploid varieties.
2) Evaluated the viability of isolated protoplasts by staining procedures
3) Fused the protoplasts in different combinations by chemical method using PEG(Poly Ethylene Glycol)
4) Inducted the callus from fused protoplasts in *in vitro* conditions

Work to be Done (Year wise)

Third year (2018-19): To be carried out at University of Kashmir & CSR&TI, Pampore

1) Sub culturing of callus (Induced from fused protoplasts)(**UoK**)
2) Regeneration of shoots from callus in *in vitro* conditions (**UoK**)
3) Transfer of Micro Shoots to rooting media to produce complete plantlets (**UoK**)
4) Transfer of plantlets (Somatic Hybrids) from lab to land through green house phase.(**CSR&TI, Pampore**)

DETAILS OF THE WORK DONE DURING (2016-17)

IN VITRO CLONAL PROPAGATION OF SELECTED MULBERRY PARENTAL LINES

In vitro clonal propagation of selected mulberry parental lines was carried in 2016-17 year with the aim of having the sterile leaves of all the selected mulberry parental lines for carrying out protoplast isolation studies from the mesophyll cells of sterile leaves of *in vitro* shoot lets during the subsequent year (2017-18).

I.1 *In vitro* clonal propagation of PPR-1

A protocol was developed and standardized for the *in vitro* clonal propagation of PPR-1, a superior temperate mulberry variety using nodal explants. When the nodal explants of PPR-1 mulberry variety were inoculated onto various concentrations and combinations of cytokinins supplemented media, maximum axillary bud proliferation was obtained on combinational rather than individually supplemented hormonal media **(Fig.1)**.Over all, maximum axillary shoot length (7.2 ± 0.61 cm) and maximum no. of leaves per explant (8.1 ± 0.85) was obtained on combinational media of BAP (1.5 mg/L) and Kinetin (2.0 mg/L) after 20 days of culture **(Fig.2, Fig.4, Fig.5A & 5B)**. On individually supplemented cytokinins hormonal media maximum axillary shoot length (4.7 ± 0.61 and 3.2 ± 0.22 cm) with more no. of leaves (7.4 ± 0.16 and 6.0 ± 0.35) per explant was obtained at 1.5 mg/L and 2.0 mg/L concentration of BAP, respectively after 20 days of culture. The proliferated axillary shoots when transferred on to different concentrations of auxins containing rooting media, good response of rooting (100%) was observed on MS media supplemented with 2.0 mg/L concentration of IBA **(Fig.3, Fig.5D & 5E)**. The raised plantlets were then hardened using 1:1 ratio of vermicompost and soil **(Fig.5F, 5G)**, then gradually they were acclimatized to field conditions. The survival rate of *in vitro* raised PPR-1 plantlets in field conditions is about 70%.

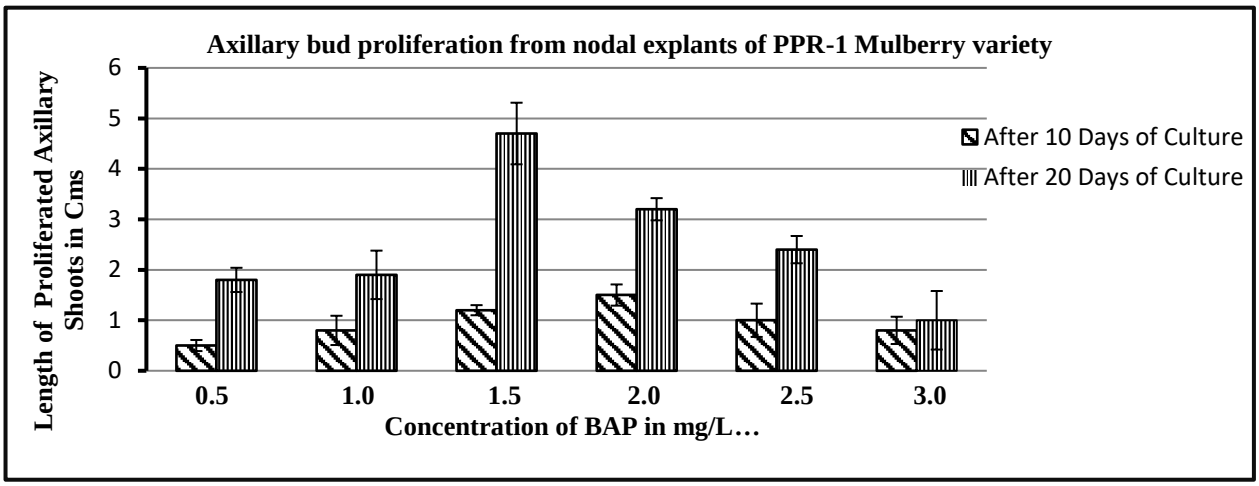


Fig.1: Axillary bud proliferation from nodal explants of PPR-1 mulberry variety on MS media supplemented with different concentrations of individual BAP hormone.

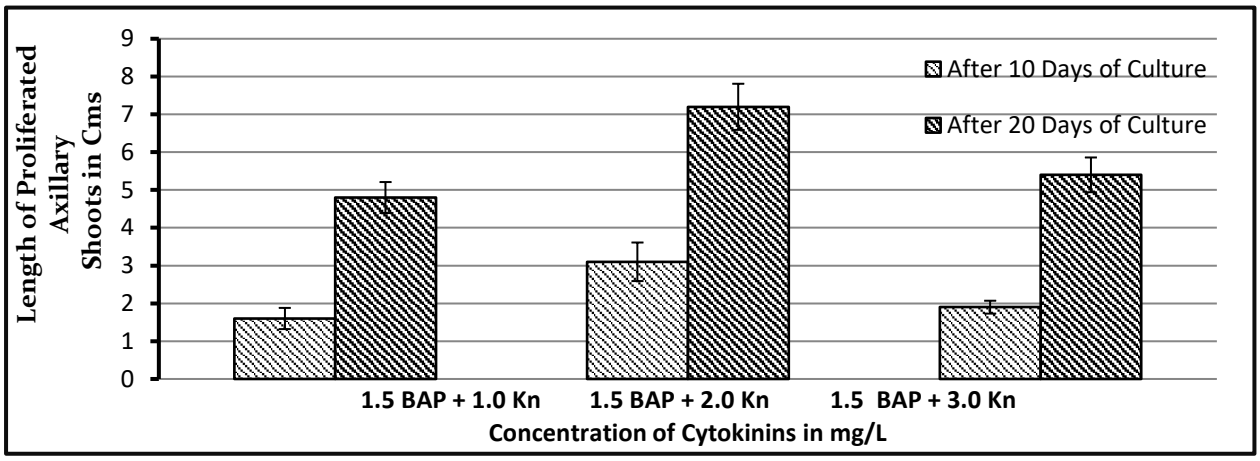


Fig.2: Axillary bud proliferation from nodal explants of PPR-1 mulberry variety on MS media supplemented with 1.5 mg/L BAP and different concentrations of Kinetin hormone in combination.

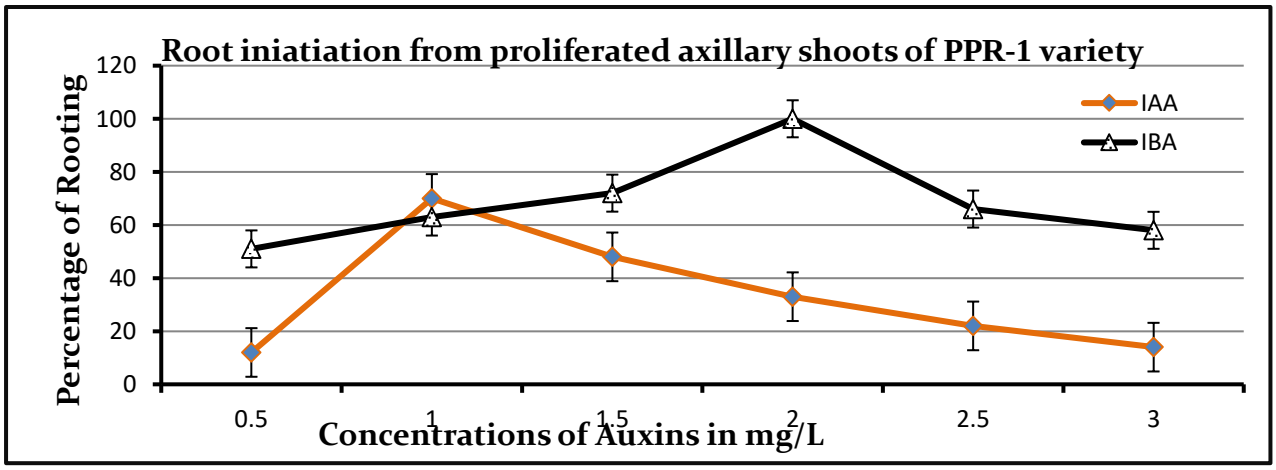


Fig.3: Initiation of roots from the proliferated axillary shoots of PPR-1 mulberry variety on MS media supplemented with different concentrations of auxins.

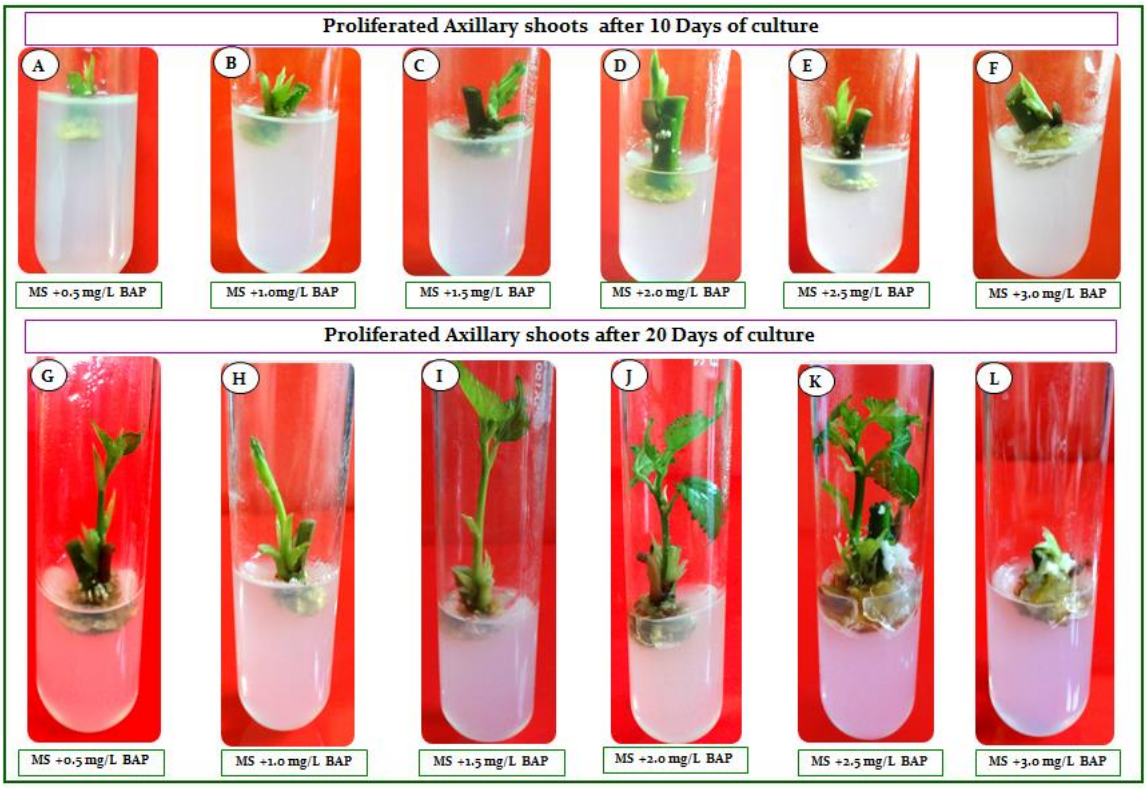


Fig 4: Proliferation of Axillary buds from the nodal segments of PPR-1 mulberry variety cultured on different concentrations of BAP supplemented MS media after 10 days (A, B, C, D, E & F) and 20 days (G, H, I, J, K & L) of culture.

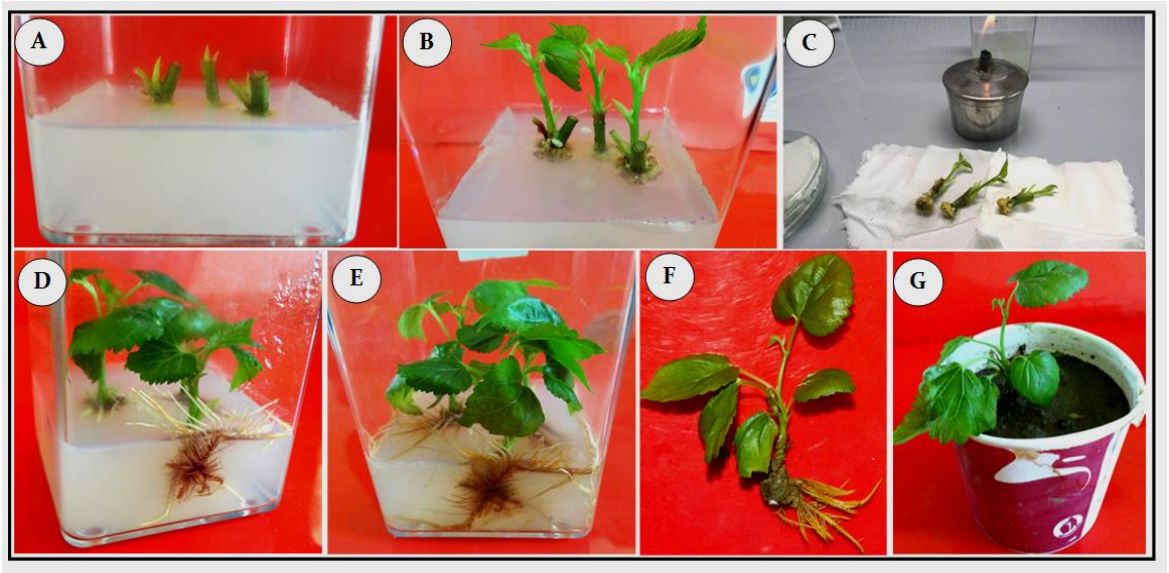


Fig 5*: In vitro* clonal propagation of PPR-1, a superior temperate mulberry variety through nodal explants.

- **A) Proliferation of axillary buds from the nodal explants on MS media supplemented with 1.5 mg/l BAP and 2.0 mg/L Kn after 3 days of culture**
- **B) Proliferation of axillary buds (3.1 ± 0.51cms) from the nodal explants on MS media supplemented with 1.5 mg/l BAP and 2.0 mg/L Kn after 10 days of culture**
- **C) Sub culturing of proliferated axillary shoots for rooting purpose**
- **D) Induction of roots from the axillary shoots cultured on 2.0 mg/L IBA supplemented Media**
- **E) The roots which were developed from axillary shoots turned brown in color after 3 weeks of culture.**
- **F) Complete PPR-1 plantlet separated from media for hardening process**
- **G) Hardening of *in vitro* raised PPR-1 plantlet**

I.2 *In vitro* clonal propagation of *Morus alba* L. Var. Chinese White

(A Research Paper on this study is accepted for publication as proceedings of One Day Hindi Seminar Organized by CSR&TI, Berhampur, in Indian Journal of Sericulture)

A protocol was developed for the *in vitro* clonal propagation of Chinese white, a temperate mulberry variety through nodal segments. When nodal explants were inoculated onto the different concentrations and combinations of plant growth regulators supplemented media, good response of shoot proliferation from axillary buds was observed on Kinetin supplemented media rather than BAP supplemented media. Maximum shoot growth in cms (6.8±0.52 & 5.3±0.41) was observed on MS + Kn (1.5 mg/L) and MS + Kn (2.0 mg/L) respectively (**Table-1**). The combinational media of Kinetin and BAP has also showed good response in terms of shoot proliferation from nodal explants, but the results were not comparable to that of individual Kinetin supplemented media (**Fig.1**). The proliferated axillary shoots when subcultured on auxins containing media, good response of rooting (95%) (**Table-2**) was observed on MS + IBA (1.5 mg/L) media (**Fig.2**, **Fig.3A, 3B** & **3C**). The hardening process is carried out by transferring the plantlets from the media to the plastic pots having sterile vermiculite, garden soil and sand in 1:2:1 ratio (**Fig.3D** & **2E**).. The hardened plantlets were initially kept in culture room at 26°C and then transferred to the field. The percent of acclimatization of *in vitro* raised Chinese white plantlets in field conditions was about 82%.

Table-1: Axillary bud proliferation from nodal explants of Chinese white mulberry variety 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

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.

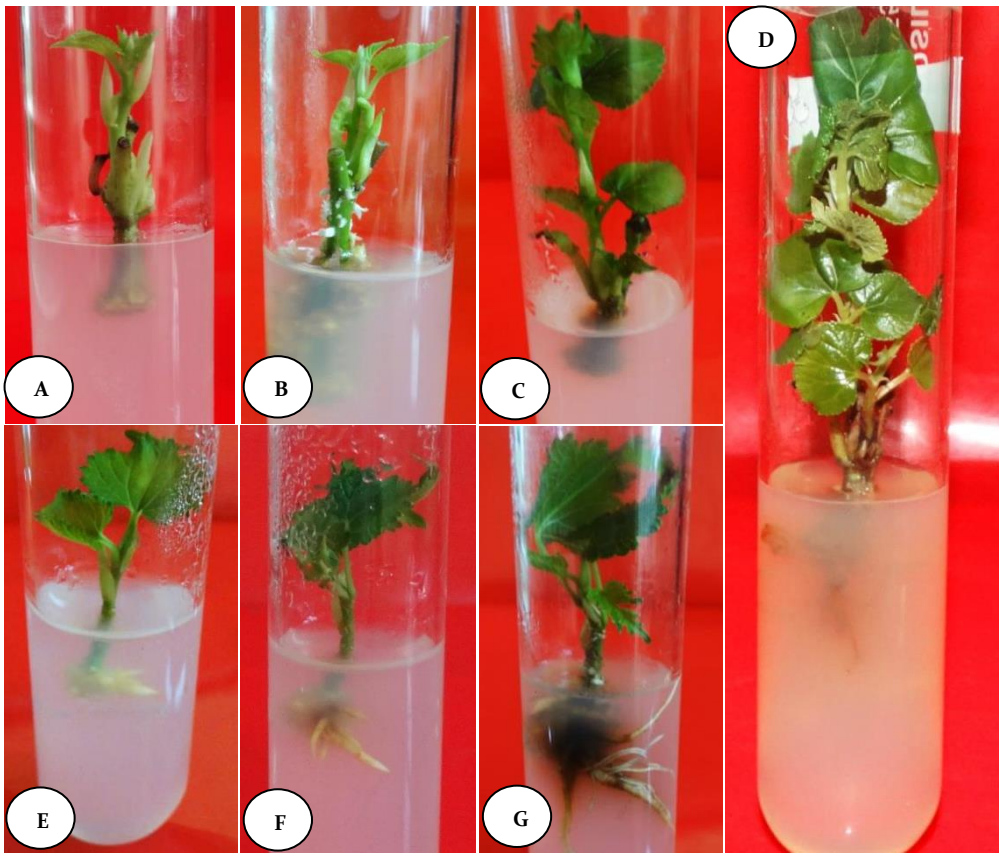


Fig 1: *In vitro* micro propagation of *Morus alba* var. Chinese white through nodal explants

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

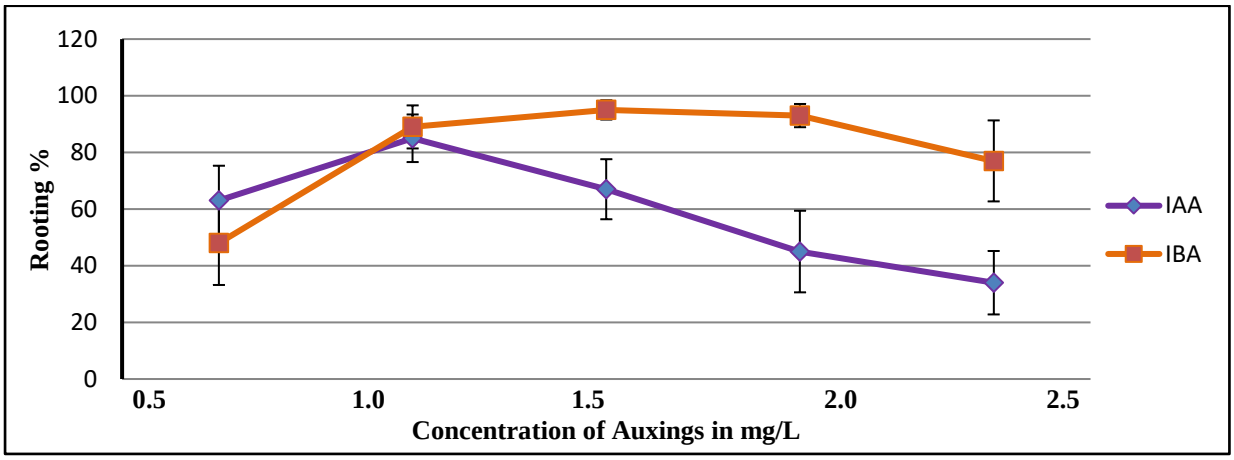


Fig.2: Root Initiation from the proliferated axillary shoots of Chinese white mulberry variety on MS media supplemented with different concentrations of auxins

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

I.3 *In vitro* clonal propagation of *Morus alba* L. Var. Ichinose

(A Research Paper on this study is Under Review in Indian Journal of Experimental Biology, SCI Indexed, Impact Factor: 1.0; NAAS Rating: 7.17)

A protocol was developed for the *in vitro* clonal propagation of Ichinose, a temperate mulberry variety through nodal segments. When nodal explants were inoculated onto the different concentrations and combinations of plant growth regulators supplemented media, good response of shoot proliferation from axillary buds in terms of number of shoots and length of proliferated shoots was observed on combinational media rather than individually supplemented cytokinins media (**Table-1 & Table-2**). Among the various combinations of cytokinins tested, the maximum number of shoots (7.7±0.46) were obtained from the nodal explants which were inoculated on to MS media supplemented with the combination TDZ (0.5 mg/L) and BAP (0.5 mg/L) after 20 days of culture (**Fig.1**). Similarly, the maximum length of proliferated shoots (4.9±0.36 cms**)** was obtained on combinational MS media supplemented with TDZ (0.5 mg/L) and BAP (2.0 mg/L) after 20 days of culture. The individual cytokinins (BAP, Kn & TDZ) supplemented media also showed the responses of shoot proliferation from nodal explants, but the results were not comparable to that of combinational cytokinins supplemented media. The proliferated axillary shoots when subcultured on individual and combinations of auxins containing media, good response of rooting (94%) (**Table-3**) was observed on MS + IBA (1.0 mg/L) media after 20 days of culture (**Fig.2 & Fig.3**). The hardening process is carried out by transferring the plantlets from the media to the plastic pots having sterile vermiculite, garden soil and sand in 1:2:1 ratio. The hardened plantlets were initially kept in culture room at 26°C and then transferred to the field. The percent of acclimatization of *in vitro* raised Ichinose plantlets in field conditions was about 82%.

Table-1: Axillary bud proliferation from nodal explants of Ichinose mulberry variety on MS media supplemented with different concentrations of individual cytokinins.

Plant Growth Regulators in mg/L			No. of shoots (X*± S.E)		Proliferated axillary shoot length in cms (X*± S.E)	
BAP	**Kn**	**TDZ**	**After 10 days of culture**	**After 20 days of culture**	**After 10 days of culture**	**After 20 days of culture**
0.5	-	-	1.0± 0.23[a]	1.2± 0.15[a]	0.2± 0.03[a]	0.5± 0.11[a]
1.0	-	-	**1.6± 0.09[d]**	**2.1± 0.19[e]**	0.4± 0.10[a]	1.0± 0.14[b]
1.5	-	-	1.2± 0.32[b]	1.6± 0.24[c]	0.8± 0.21[b]	2.1± 0.16[e]
2.0	-	-	1.2 ± 0.16[b]	1.8± 0.12[d]	**1.3± 0.11[c]**	**3.5± 0.24[e]**
2.5	-	-	1.4± 0.21[c]	1.5± 0.16[b]	1.2± 0.19[c]	1.9± 0.20[d]
3.0	-	-	1.1± 0.13[a]	1.3± 0.13[a]	1.0± 0.12[c]	1.4± 0.17[c]
-	-	-				
-	0.5	-	1.2± 0.20[a]	1.4± 0.27[a]	0.4± 0.05[a]	0.7± 0.13[a]
-	1.0	-	1.6± 0.23[c]	1.5± 0.23[a]	0.6± 0.09[a]	1.1± 0.16[b]
-	**1.5**	-	**1.8± 0.22[d]**	**2.4± 0.36[d]**	**2.4± 0.13[e]**	**3.3± 0.41[e]**
-	2.0	-	1.3± 0.31[a]	2.1± 0.29[c]	2.1± 0.26[d]	3.3± 0.36[d]
	2.5	-	1.6± 0.19[c]	1.8± 0.33[b]	1.7± 0.18[c]	2.5± 0.21[c]
-	3.0	-	1.4± 0.11[b]	1.7± 0.18[b]	0.8± 0.04[b]	1.1± 0.09[b]
-	-	-				
-	-	**0.5**	**2.3± 0.32[c]**	**4.8± 0.43[e]**	1.6± 0.21[c]	2.4± 0.21[d]
-	-	1.0	2.2± 0.35[d]	2.7± 0.26[d]	1.8± 0.14[d]	3.2± 0.38[e]
-	-	1.5	1.8± 0.24[b]	2.9± 0.32[d]	1.4± 0.20[b]	1.6± 0.14[c]
-	-	2.0	1.0± 0.08[d]	2.1± 0.37[c]	**2.9± 0.13[d]**	**3.2± 0.31[d]**
		2.5	1.0± 0.14[a]	1.4± 0.17[a]	0.4± 0.10[a]	0.7± 0.09[b]
		3.0	1.0± 0.20[a]	1.6± 0.29[b]	0.3± 0.04[a]	0.5± 0.12[a]

BAP: 6-Benzylaminopurine; Kn: Kinetin; TDZ: Thiadiazuron; *: 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

Table-2: Axillary bud proliferation from nodal explants of Ichinose mulberry variety on MS media supplemented with different concentrations and combinations of cytokinins.

Plant Growth Regulators in mg/L			No. of shoots (X*± S.E)		Proliferated axillary shoot length in cms (X*± S.E)	
TDZ+BAP	TDZ+ Kn	TDZ + BAP +Kn	After 10 days of culture	After 20 days of culture	After 10 days of culture	After 20 days of culture
0.5+0.5	**-**	**-**	**4.5± 0.51^{c}**	**7.7± 0.46^{e}**	0.4± 0.15^{a}	0.9± 0.32^{a}
0.5+1.0	-	-	3.8± 0.43^{d}	5.1± 0.64^{d}	0.7± 0.23^{b}	1.2± 0.27^{b}
0.5+1.5	-	-	2.9± 0.24^{d}	3.8± 0.34^{c}	1.4± 0.21^{c}	2.6± 0.24^{d}
0.5+2.0	-	-	1.7 ± 0.31^{b}	2.6± 0.16^{b}	**2.8± 0.33^{d}**	**4.9± 0.36^{e}**
0.5+2.5	-	-	1.2± 0.14^{a}	1.4± 0.32^{a}	1.7± 0.35^{d}	2.2± 0.16^{c}
0.5+3.0	-	-	1.0± 0.30^{a}	1.2± 0.35^{a}	1.5± 0.21^{c}	1.9± 0.11^{c}
-	0.5+0.5	-	2.3± 0.12^{b}	4.4± 0.36^{c}	0.8± 0.13^{a}	1.4 ± 0.18^{a}
-	**0.5+1.0**	-	**3.6± 0.25^{d}**	**7.2± 0.54^{e}**	0.9± 0.21^{a}	1.7± 0.24^{b}
-	0.5+1.5	-	2.7± 0.34^{c}	4.7± 0.41^{d}	**1.4± 0.25^{d}**	**3.1± 0.51^{e}**
-	0.5+2.0	-	2.3± 0.37^{b}	3.1± 0.23^{b}	1.7± 0.18^{b}	2.6± 0.40^{d}
	0.5+2.5	-	1.4± 0.21^{a}	2.8± 0.19^{a}	1.9± 0.22^{c}	2.3± 0.32^{c}
-	0.5+3.0	-	1.6± 0.24^{a}	1.8± 0.31^{a}	1.2± 0.16^{b}	1.3± 0.20^{a}
-	-	**0.5+1.0+0.5**	**2.4± 0.28^{c}**	**2.8± 0.17^{c}**	0.5± 0.08^{a}	0.9± 0.07^{c}
-	-	0.5+1.0+1.0	1.2± 0.14^{a}	1.5± 0.29^{b}	0.7± 0.06^{b}	1.1± 0.11^{d}
-	-	0.5+1.0+1.5	1.3± 0.23^{b}	1.4± 0.19^{a}	**1.4± 0.12^{d}**	**2.4± 0.32^{f}**
-	-	0.5+1.0+2.0	1.2± 0.14^{a}	1.5± 0.23^{b}	1.2± 0.22^{c}	1.7± 0.23^{e}
		0.5+1.0+2.5	1.4± 0.21^{b}	1.5± 0.16^{b}	0.5± 0.04^{a}	0.8± 0.03^{b}
		0.5+1.0+3.0	1.1± 0.10^{a}	1.3± 0.21^{a}	0.4± 0.02^{a}	0.5± 0.14^{a}

BAP: 6-Benzylaminopurine; Kn: Kinetin; TDZ: Thiadiazuron; *: 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

Table-3: Rooting induction from the proliferated axillary shoots of Ichinose on MS media supplemented with different concentrations of auxins

Plant Growth Regulators in mg/L			Rooting Percentage (X*± S.E)
IAA	IBA	IAA + IBA	After 20 days of culture
0.5	**-**	**-**	**74 ± 11.2^{b}**
1.0	-	-	51 ± 21.8^{d}
1.5	-	-	49 ± 16.4^{c}
2.0	-	-	35 ± 17.1^{a}
2.5	-	-	32 ± 14.3^{a}
-	0.5	-	57 ± 16.3^{b}
-	**1.0**	-	**94 ± 11.2 ^{e}**
-	1.5	-	72 ± 34.3^{d}
-	2.0	-	65 ± 13.6 ^{c}
-	2.5	-	42 ± 12.1^{a}
-	-	**1.0+0.5**	**76 ± 11.2^{e}**
-	-	1.0+1.0	69 ± 16.8^{d}
-	-	1.0+1.5	51 ± 21.1^{c}
-	-	1.0+2.0	36 ± 18.7 ^{b}
-	-	1.0+2.5	12 ± 14.5^{a}

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.

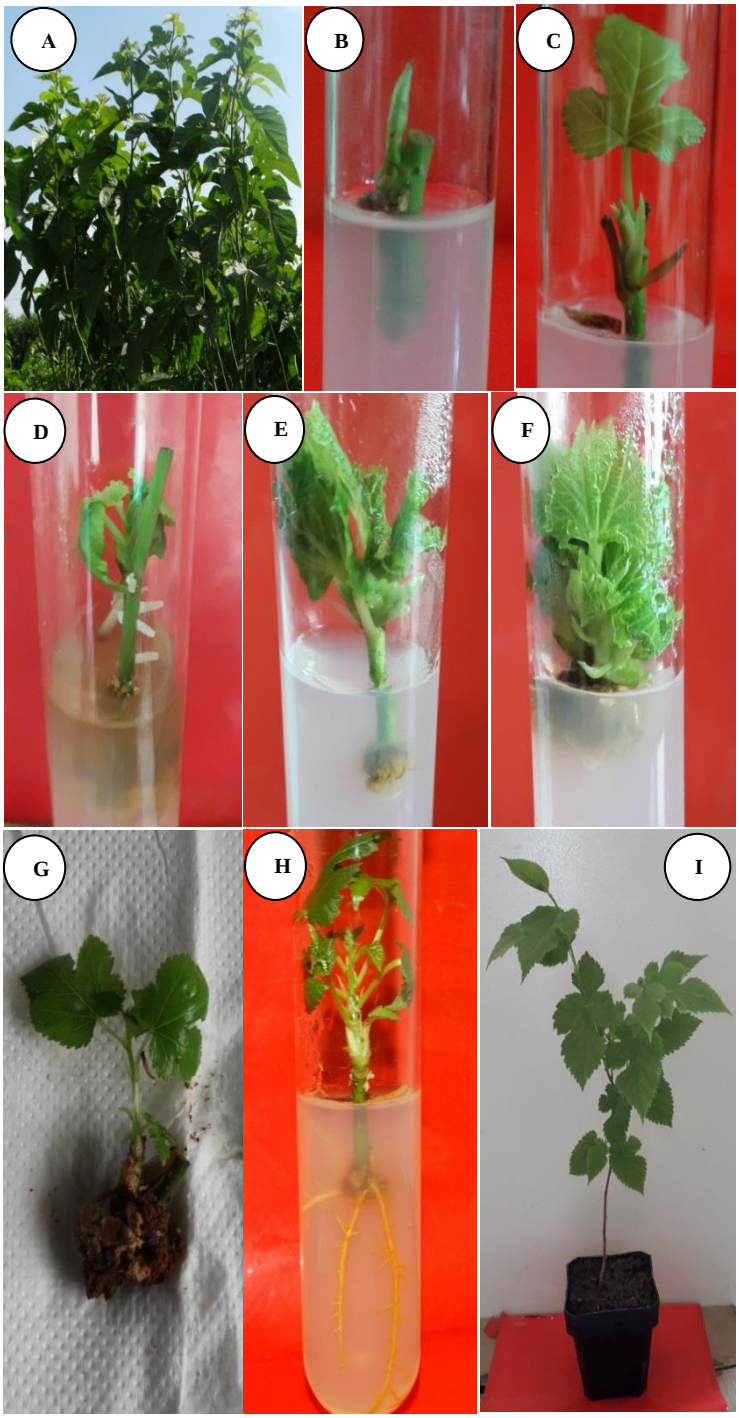


Fig 1: *In vitro* micro propagation of Ichinose through nodal explants

A) Two years old Ichinose mother plant

B) Shoot proliferation (1.3 cms) from axillary bud of nodal explant cultured on MS+ BAP (2.0 mg/L) after 10 days culture.

C) Shoot proliferation (2.4 cms) from axillary bud of nodal explant cultured on MS+ Kn (1.5 mg/L) after 10 days of culture

D) Multiple shoots (2.3) induction from nodal explants on MS + TDZ (0.5 mg/L) after 10 days of culture

E) Multiple shoots (4.8) induction from nodal explants on MS + TDZ (0.5 mg/L) after 20 days of culture

F) Multiple shoots (7.7) induction from nodal explants on combinational media of MS + TDZ (0.5 mg/L) + BAP (0.5 mg/L) after 20 days of culture

G) Separation of proliferated axillary shoot

H) Induction of roots on MS +IBA (1.0 mg/L)

I) Hardened Ichinose plantlet in a pot with sterile vermiculite, garden soil and sand in 1:2:1 ratio.

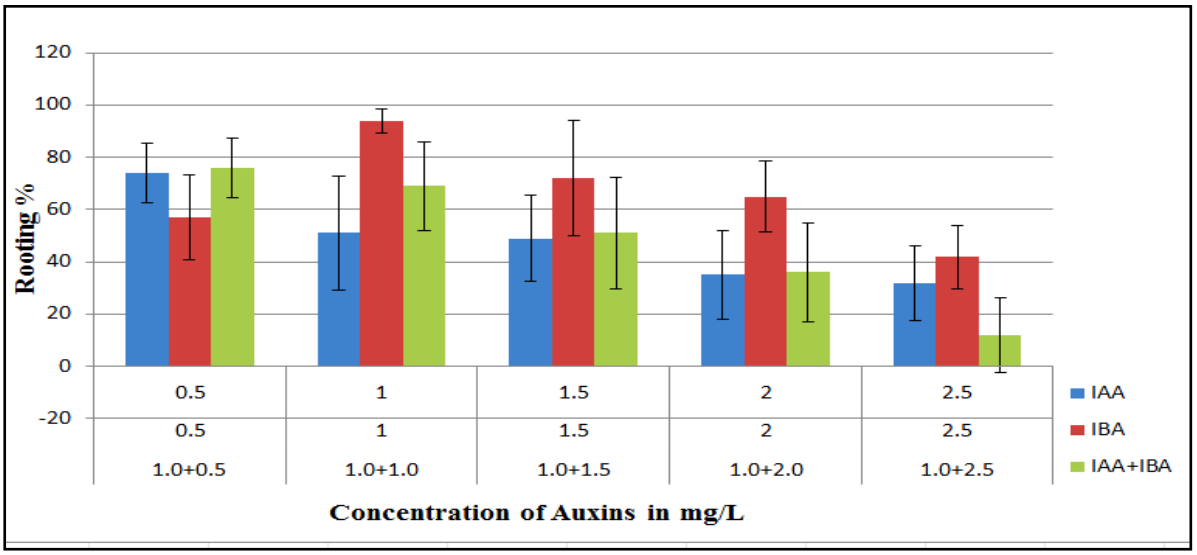


Fig.2: Root Initiation from the proliferated axillary shoots of Ichinose mulberry variety on MS media supplemented with different concentrations and combinations of auxins.

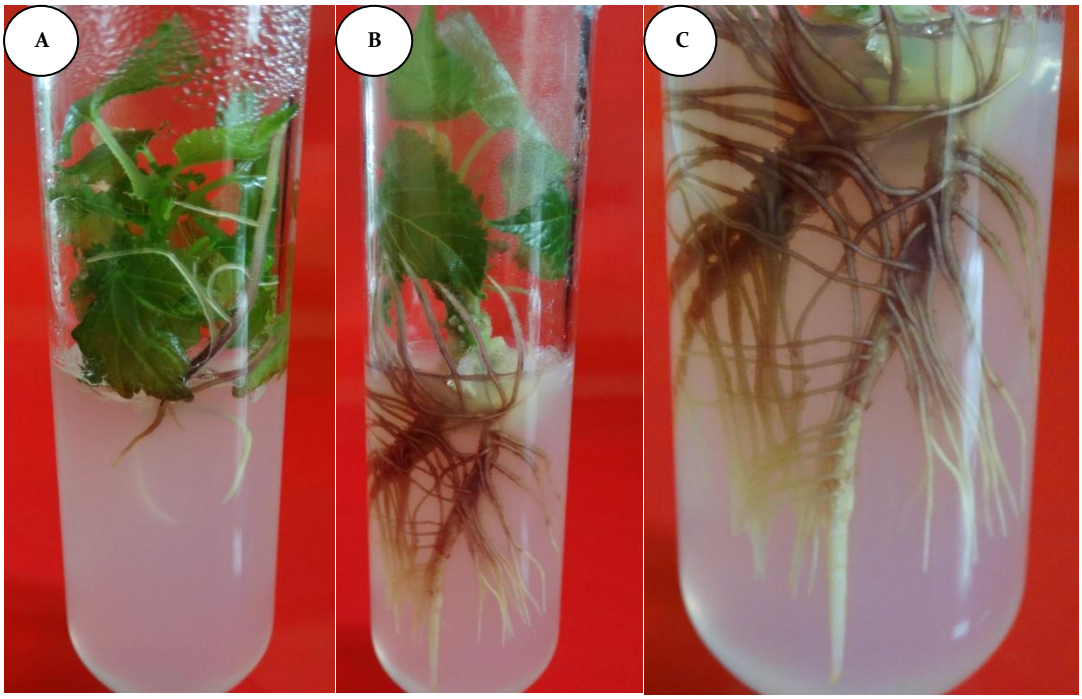


Fig 3: Turning of *in vitro* roots from white to brown color after 20 days of culture

A) Initiation of Roots (White) on MS + IBA (10 mg/L) supplemented media after 7 days of culture

B) Turning of roots into light brown in color after 10 days of culture

C) Turning of roots into dark brown color after 20 days of culture

I.4 Rapid One step protocol for the *in vitro* micro propagation of *Morus multicaulis* var. Goshoerami, an elite temperate mulberry variety through nodal segments

(Rohela et al., 2018e; Journal of Experimental Biology and Agricultural Sciences NAAS Rating: 5.07; UGC Recognized Journal)

Mulberry variety Goshoerami (*Morus multicaulis*) is the leading variety for silkworm rearing under temperate climatic conditions of Jammu and Kashmir in India. This variety was introduced from Japan during early seventies in Kashmir Valley. 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. The *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 90% 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.

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 (X*±S.E)		Root length in cms (X*±S.E)	
BAP	**IAA**	**IBA**	**After 7 days of culture**	**After 14days of culture**	**After 7 days of culture**	**After 14days 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 ± 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

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 (X*±S.E)		Root length in cms (X*±S.E)	
BAP	**IAA**	**IBA**	**After 7 days of culture**	**After 14 days 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^{e}	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.

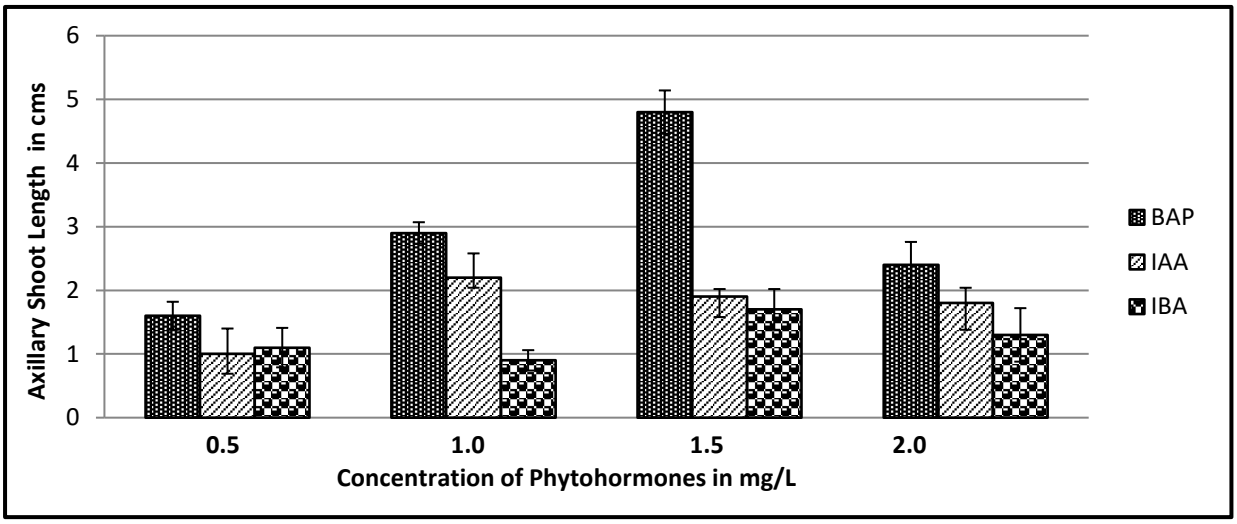


Fig.1: Effect of individual phytohormones supplemented MS media on the nodal explants of *Morus multicaulis* var. Goshoerami after 14 days of culture.

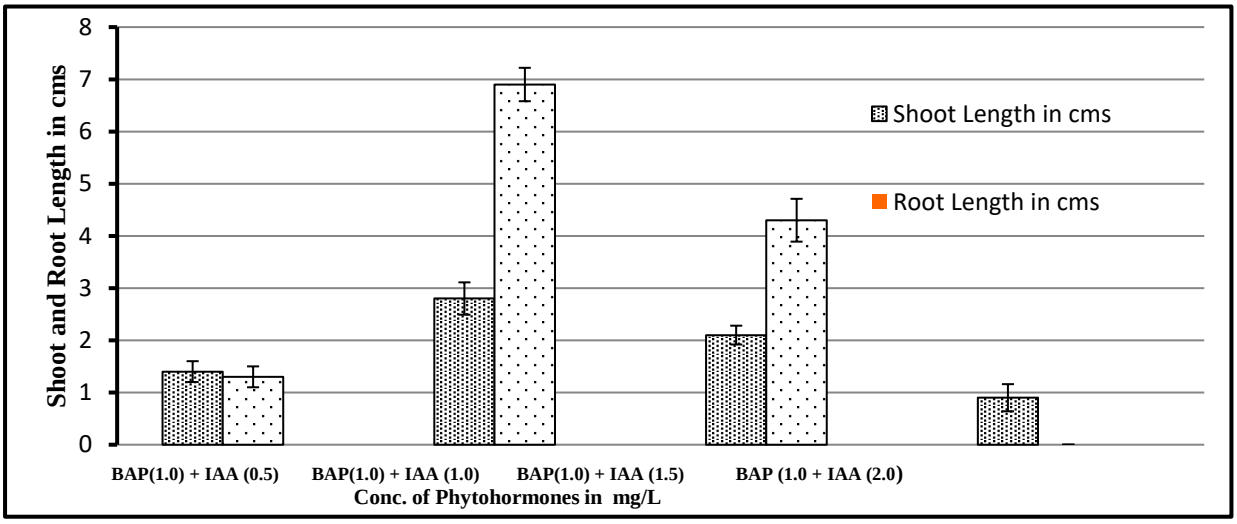


Fig.2: Effect of combination of BAP and IAA supplemented MS media on the nodal explants of *Morus multicaulis* var. Goshoerami after 14 days of culture.

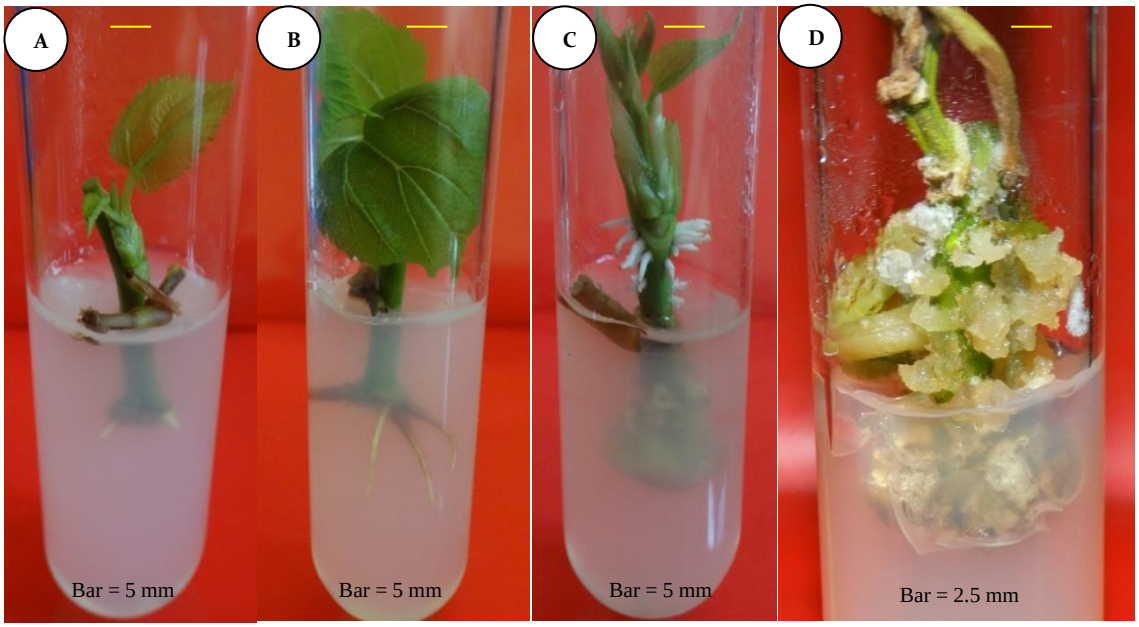


Fig.3: Micro propagation of *Morus multicaulis* var. Goshoerami by using nodal explants

A) Axillary bud proliferation (1.6 cm) and induction of roots (0.4 cm) from nodal explants of *Morus Multicaulis* var. Goshoerami on MS media supplemented with IAA (1.0 mg/L) after 7 days of culture.

B) Shoot Growth (2.2 cm) and Root Growth (2.1 cm) from nodal explants of *Morus Multicaulis* var. Goshoerami on MS media supplemented with IAA (1 mg/L) after 14 days of culture.

C) Initiation of adventitious roots from nodal segments on IAA(2.0 mg/L) supplemented media.

D) Callus induction from the internodal regions of Goshoerami on MS media supplemented with BAP (1 mg/L) and IAA (2 mg/L).

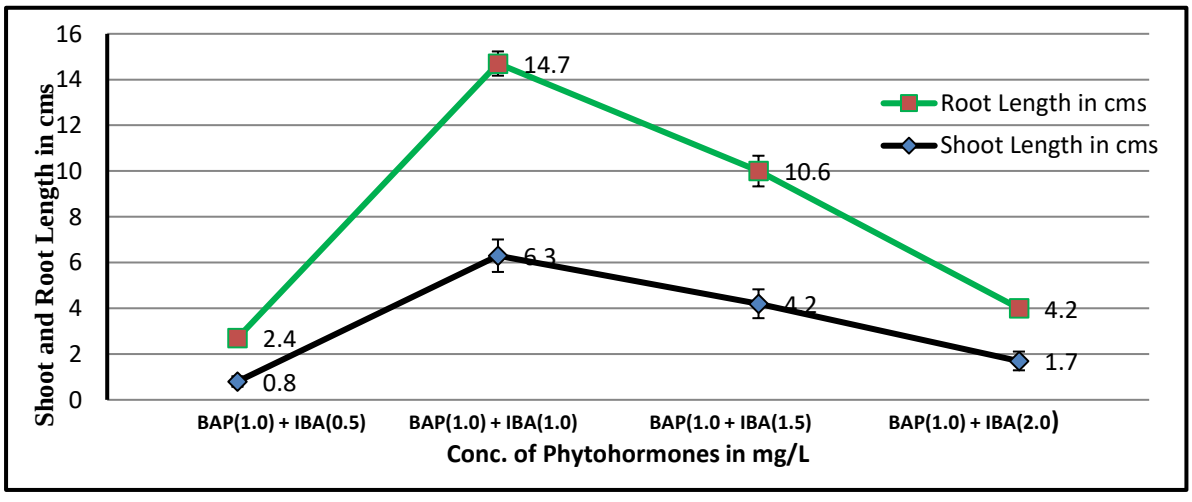


Fig.4: Effect of combination of BAP and IBA supplemented MS media on the nodal explants of *Morus multicaulis* var. Goshoerami after 14 days of culture.

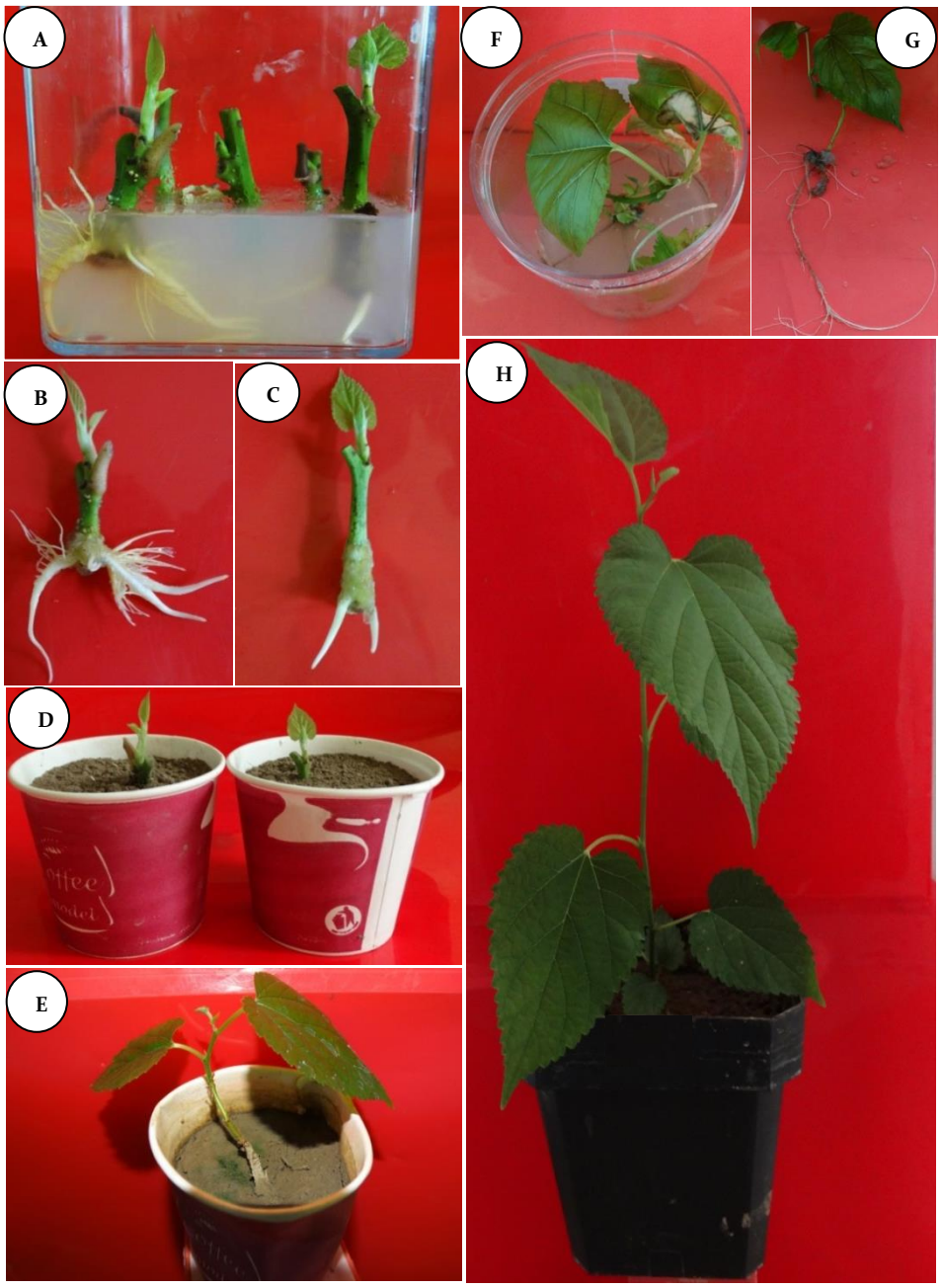


Fig.5: Micro propagation of *Morus multicaulis* var. Goshoerami by using nodal explants

A) Axillary bud proliferation (0.8 cm) and induction of roots (2.4 cm) from nodal explants of *Morus Multicaulis* var. Goshoerami on MS media supplemented with BAP (1 mg/L) and IBA (0.5 mg/L) after 14 days of culture.

B) Complete plantlet of Goshoerami with well-developed root system

C) Complete plantlet of Goshoerami with few developed roots

D) Hardening of *in vitro* raised Goshoerami plantlets

E) Hardened plantlet of Goshoerami in a poly cup by using the sterile soil and vermiculite in 2:1 ratio.

F) Complete plantlet of Goshoerami 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 Goshoerami for hardening process with well-developed root system

H) Hardened plantlet of Goshoerami (2 months old) in a plastic pot after hardening process with the sterile soil and vermiculite in 2:1 ratio.

DETAILS OF THE WORK DONE DURING (2017-18)

II. ISOLATION OF PROTOPLASTS FROM MESOPHYLL CELLS OF TEMPERATE MULBERRY (Rohela et al 2018b; International Journal of Advance Research in Science and Engineering, UGC approved Journal)

Sterile Leaves from the aseptic cultures of PPR-1, Ichinose & Chinese white & Goshoerami (which were raised during 2016-17) were utilized as raw materials for the isolation of protoplasts by enzymatic method by employing different concentrations and combinations of enzymes.

In this study, along with the appropriate combination of enzymes, other factors such as osmoticum concentration, temperature, pH of enzyme solution, duration of enzymatic treatment and RPM values of centrifugation were standardized for the maximum yield of viable protoplasts from the mesophyll cells of of four superior temperate mulberry varieties viz. *Morus alba* var. Chinese white, *Morus alba* var. Ichinose. *Morus multicaulis* var. Goshoerami and an interspecific hybrid i.e. PPR-1 (Pampore-1).

Among all the factors, concentration and combination of enzymes used, concentration of osmoticum and duration of enzymatic treatments were found to be major factors effecting the isolation of viable protoplasts in maximum number. Among the different combinations of enzyme solutions tested, the maximum yield of viable protoplasts ($5.171\pm0.354x10^6$ g^{-1} and $6.051\pm0.424x10^6$ g^{-1} fresh weight of leaf) was obtained from the enzymatic combination of 2% Cellulase, 0.5% Macerozyme and 0.2% Pectinase with the incubation period of 8 Hrs and 10 Hrs on a rotary shaker with 80 RPM (Rotations Per Minute) at 26° C from Goshoerami and PPR-1 varieties respectively **(Table-4)**.

Similarly among the different concentrations of Osmoticum (Mannitol) tested, 13% of Mannitol has proved to be good in isolating the maximum number of viable protoplasts of 72%, 77%, 88%, & 92% in Ichinose, Chinese white, Goshoerami and PPR-1 respectively. Evaluation of viability of isolated protoplasts were ascertained by using 0.2% of Evans Blue stain and 0.1% Triphenyl Tetrazolium Chloride solution.

II.1 Isolation of Protoplasts

Fully expanded leaves of 40-50 days old were excised from the in vitro shoot lets of Ichinose, Chinese white, Goshoerami and PPR-1 mulberry varieties with a sterile scalpel blade in aseptic conditions of Laminar air flow and they were cut into small pieces of 1-2 mm in size (**Fig.1**). Leaf pieces of about 1 gram (fresh weight) were incubated in sterile conical conical flask (100 ml capacity) with 15ml of filter sterilized enzyme solution (2% Cellulase, 0.5% Macerozyme & 0.25 Pectinase) which were prepared in CPW-13M solution with 5.6 pH for 6-12 Hrs on rotary shaker at 80 RPM in dark conditions at 26° C (**Table-1 & Table-2**).

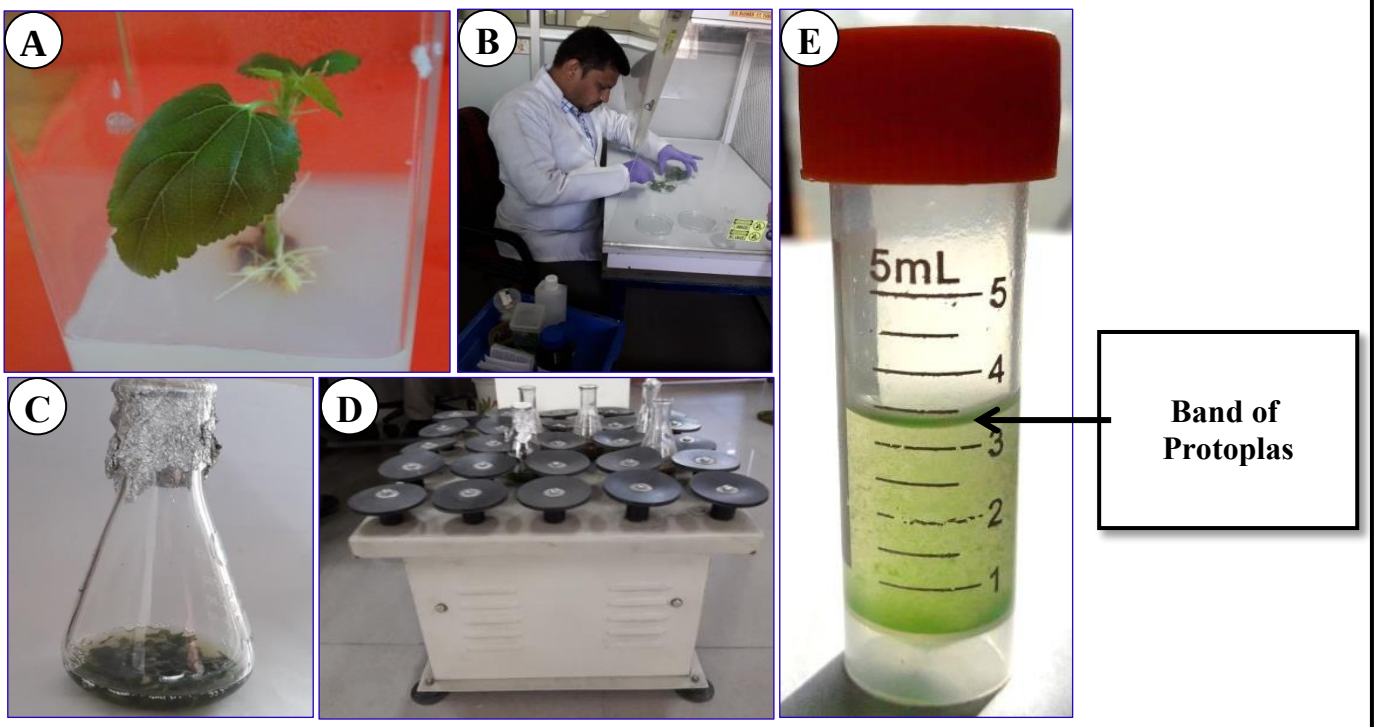


Fig.1: Isolation of protoplasts from Temperate Mulberry

A) *In vitro* Plantlet of Temperate mulberry

B) Fully expanded leaves from the *in vitro* shootlets of Temperate mulberry were cut into small pieces of 1-2 mm in size with a sterile blade under asceptic conditions of laminar air flow

C) Leaf pieces of about 1 gram fresh weight were incubated in 100 ml sterile conical flask with 15 ml of filter sterilized enzyme solution

D) Leaf pieces were incubated in enzyme solution for 8 hrs duration on rotary shaker at 80 RPM in dark conditions at 26°C

E) A distinct band of isolated protoplasts

Table-1: Composition of CPW-13M solution

Sl.No	Chemical	Quantity (mg/L)
1	KH_2P0_4	28.00
2	$KN0_3$	108.00
3	NH_4NO_3	50.00
3	$CaCl_2.2H_20$	1500.00
4	$MgSO_4.7H_20$	240.00
5	KI	0.14
6	$CuSO_4.5H_20$	0.025
7	D-Mannitol	130000
8	Sterile D.W	1000 ml
	pH	5.6

Table-2 :Composition of Standardized Enzyme Solution :

Sl.No	Enzyme	Quantity (Grams/100ml(
1	Cellulase Onozuka R-10	2.00
2	Macerozyme R-10	0.50
3	Pectinase	0.20
3	CPW-13M	100 ml
pH		5.6

II.2 Purification of Isolated Protoplasts: The isolated protoplasts were initially filtered by using steel mesh with a pore size of 45µ. The obtained filtrate was collected in a 20 ml sterile screw cap centrifuge and centrifuged at 1000 RPM for 5 minutes. After centrifugation, supernatant was discarded. Impurities like cell debris were removed from the pellet of protoplasts by carrying out centrifugation at 600 RPM for 5 minutes after the addition of 10ml of 20% sucrose solution (CPW-20S) (**Table-3**). After centrifugation, protoplasts were obtained as a distinct band, which were collected into a separate sterile screw cap centrifuge tube with the help of a pasteur pipette. The collected protoplasts were resuspended in 5 ml of CPW-13M solution to maintain them in viable state.

Table-3: Composition of CPW-20S solution

Sl.No	Chemical	Quantity (mg/L)
1	KH_2PO_4	28.00
2	KNO_3	108.00
3	NH_4NO_3	50.00
3	$CaCl_2.2H_2O$	1500.00
4	$MgSO_4.7H_2O$	240.00
5	KI	0.14
6	$CuSO_4.5H_2O$	0.025
7	Sucrose	200000 (200 Grams)
8	Sterile D.W	1000 ml
	pH	5.6

II.3 Yield and Viability of Isolated Protoplasts

To determine the yield of protoplasts, 10µl of suspended protoplast solution was taken on a clean glass slide and observed under the compound microscope (**Fig.2-5**) in different magnifiable lenses and results were expressed as number of protoplasts per gram fresh weight of leaf. For checking the viability and yield of viable protoplasts, to the suspended protoplasts solution (1ml), 20 µl of 0.2% Evans blue (20mg/10ml sterile DW) solution was added and incubated for 2 minutes. The 10µl of treated protoplasts solution was placed on a haemocytometer slide and observed under compound microscope (**Fig.6**) and the results were expressed as number of protoplasts per gram fresh weight of leaf in each mulberry variety.

II. 4 Statistical Analysis:

The data obtained from this research study was the mean of 3 replications and the data was statistically analyzed by using SPSS version 17 (SPSS Inc., Chicago, USA). The mean values were compared by Tukey's tests at the 5% level of significance. All means are represented with standard error.

Table-4: Isolation of protoplasts from three superior mulberry varieties with 2% cellulase, 0.5% macerozyme and 0.2% pectinase

Sl.No	Mulberry Variety	D-Mannitol (%)	Incubation Time (Hrs)	Total Yield of Protoplasts ($\times 10^6$ g^{-1} Fresh Weight)	Viable yield of Protoplasts ($\times 10^6$ g^{-1} Fresh Weight)	Viability (%)
01	Ichinose	12	06	1.032±0.112^{a}	0.612±0.024^{a}	59.30±1.46^{a}
			08	1.134±0.343^{a}	0.704±0.041^{a}	62.08±1.28^{b}
			10	2.212±0.154^{b}	1.230±0.212^{b}	55.61±1.89^{a}
		13	06	1.462±0.243^{a}	0.744±0.054^{a}	50.88±1.19^{a}
			08	**2.546±0.312^{b}**	**1.843±0.146^{b}**	**72.39±2.11^{c}**
			10	1.890±0.234^{a}	1.045±0.117^{b}	55.29±2.15^{a}
		14	06	1.573±0.156^{a}	1.004±0.222^{b}	63.82±1.20^{b}
			08	2.465±0.268^{b}	1.720±0.240^{b}	69.77±1.21^{b}
			10	2.664±0.342^{b}	1.584±0.326^{b}	59.45±1.45^{a}
02	Chinese White	12	06	1.824±0.282^{a}	0.982±0.045^{a}	53.94±1.38^{a}
			08	1.877±0.214^{a}	1.399±0.124^{b}	74.57±1.44^{c}
			10	1.645±0.188^{a}	1.004±0.182^{b}	61.03±1.65^{b}
		13	06	1.254±0.213^{a}	0.852±0.124^{a}	67.94±1.73^{b}
			08	**2.123±0.342^{b}**	**1.644±0.321^{b}**	**77.46±1.22^{c}**
			10	2.415±0.270^{b}	1.626±0.344^{b}	67.32±1.92^{b}
		14	06	1.845±0.208^{a}	1.204±0.206^{b}	65.25±1.52^{b}
			08	2.812±0.324^{b}	1.934±0.328^{b}	68.77±1.42^{b}
			10	2.923±0.423^{b}	2.006±0.214^{c}	68.62±1.79^{b}
03	PPR-1	12	06	1.946±0.170^{a}	1.420±0.204^{b}	72.97±1.95^{c}
			08	2.982±0.248^{b}	2.224±0.362^{c}	74.58±1.04^{c}
			10	3.385±0.426^{c}	2.795±0.188^{c}	82.58±1.53^{d}
		13	06	2.167±0.322^{a}	1.662±0.145^{b}	76.69±1.94^{c}
			08	4.148±0.184^{d}	3.024±0.266^{d}	72.90±1.26^{c}
			10	**6.862±0.508^{e}**	**6.051±0.424^{e}**	**88.19±1.04^{d}**
		14	06	2.515±0.312^{b}	1.845±0.226^{b}	73.35±1.92^{c}
			08	3.265±0.384^{c}	2.544±0.214^{c}	77.91±1.75^{c}
			10	5.864±0.422^{c}	4.811±0.326^{d}	82.05±1.39^{d}
04	Goshoerami	12	06	2.041±0.278^{c}	1.645±0.332^{b}	80.15±1.93^{f}
			08	4.462±0.120^{f}	3.775±0.409^{d}	84.62±2.44^{f}
			10	4.534±0.156^{f}	2.741±0.201^{c}	60.47±1.32^{d}
		13	06	2.864±0.218^{d}	2.381±0.123^{c}	83.16±2.02^{f}
			08	**5.867±0.237^{g}**	**5.171±0.354^{f}**	**88.14±1.38^{f}**
			10	5.963±0.125^{g}	3.246±0.312^{d}	54.45±2.35^{b}
		14	06	2.837±0.336^{d}	2.188±0.261^{c}	77.13±1.81^{e}
			08	4.082±0.165^{f}	3.257±0.108^{d}	79.81±1.42^{e}
			10	4.097±0.110^{f}	1.926±0.021^{b}	47.03±1.91^{b}

Represented Data is the mean and standard error of three replication. Mean ±s tandard errors followed by same letter is 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

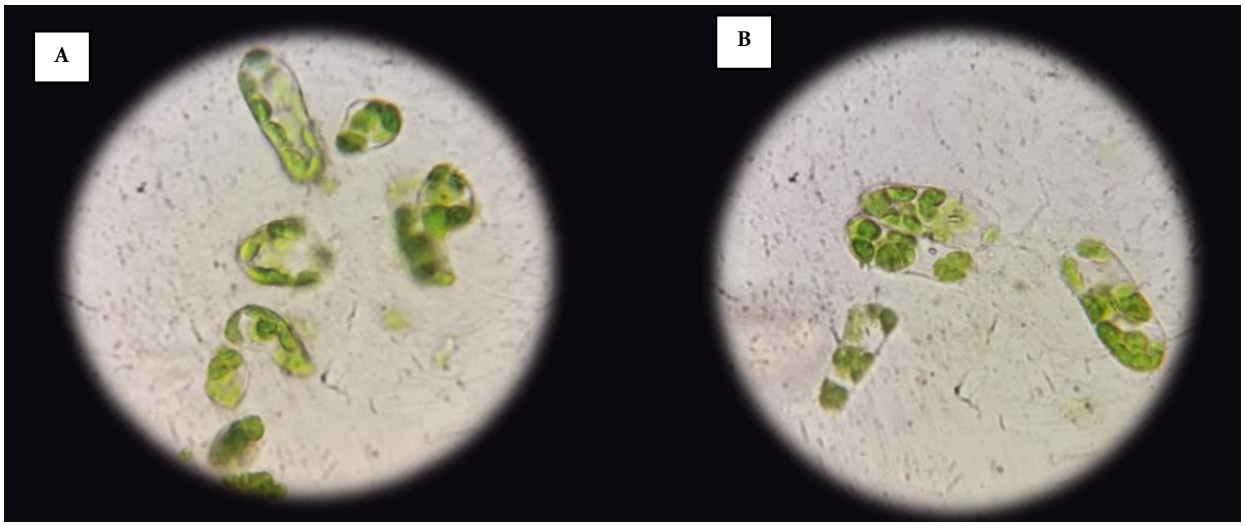


Fig.2: Microscopic view of isolated protoplasts from mesophyll cells of temperate mulberry under 40 x objective lenses
A & B) Protoplasts isolated from PPR-1.

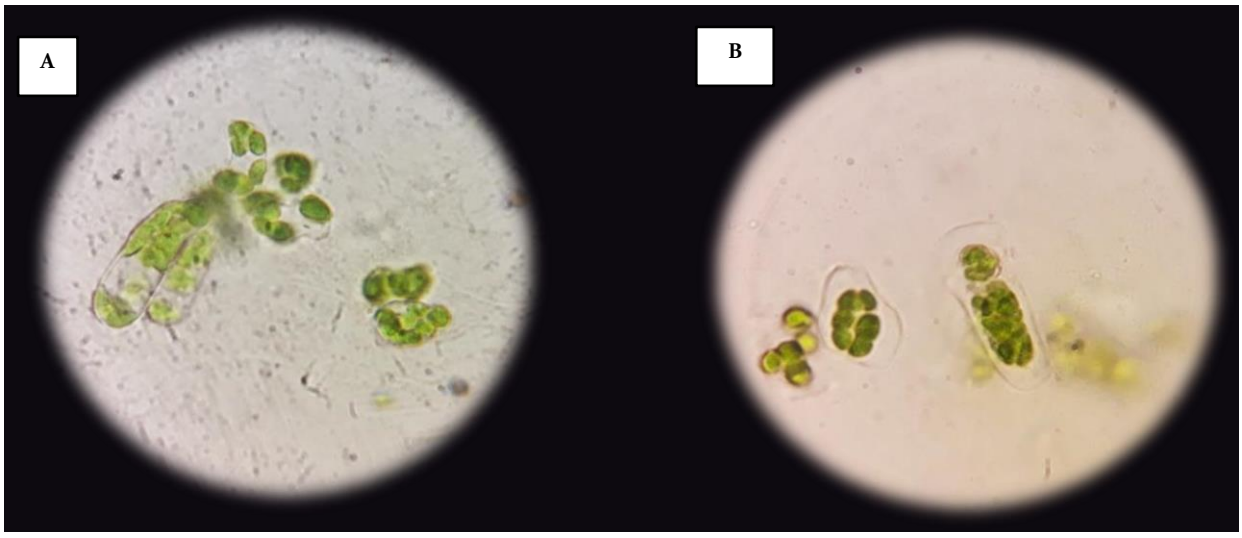


Fig.3: Microscopic view of isolated protoplasts from mesophyll cells of temperate mulberry under 40 x objective lenses
A & B) Protoplasts isolated from *Morus alba* var. Chinese White.

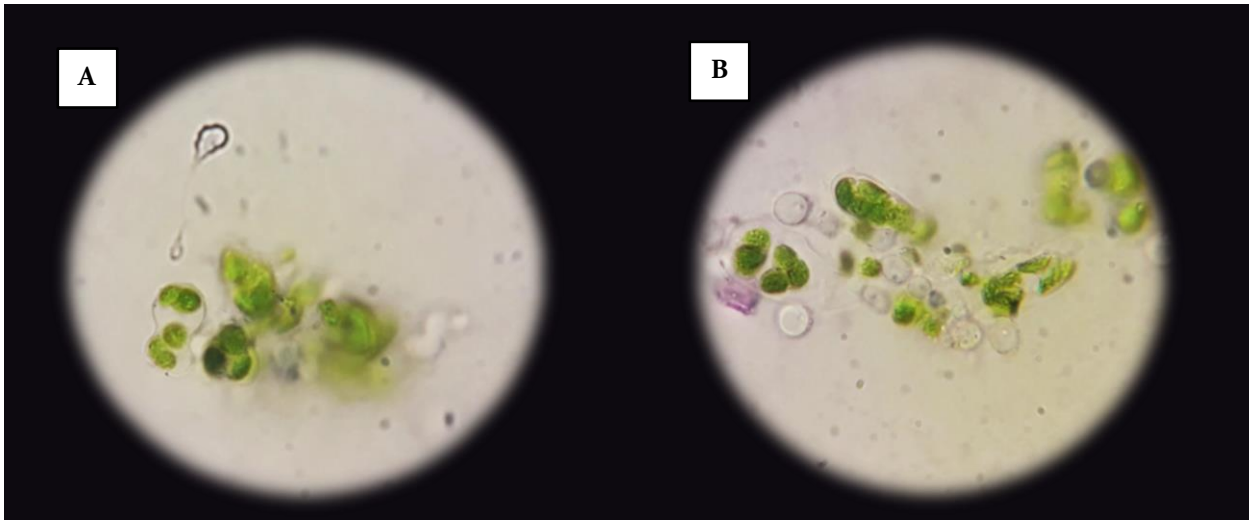


Fig.4: Microscopic view of isolated protoplasts from mesophyll cells of temperate mulberry under 40 x objective lenses

A & B) Protoplasts isolated from *Morus alba* var. Ichinose.

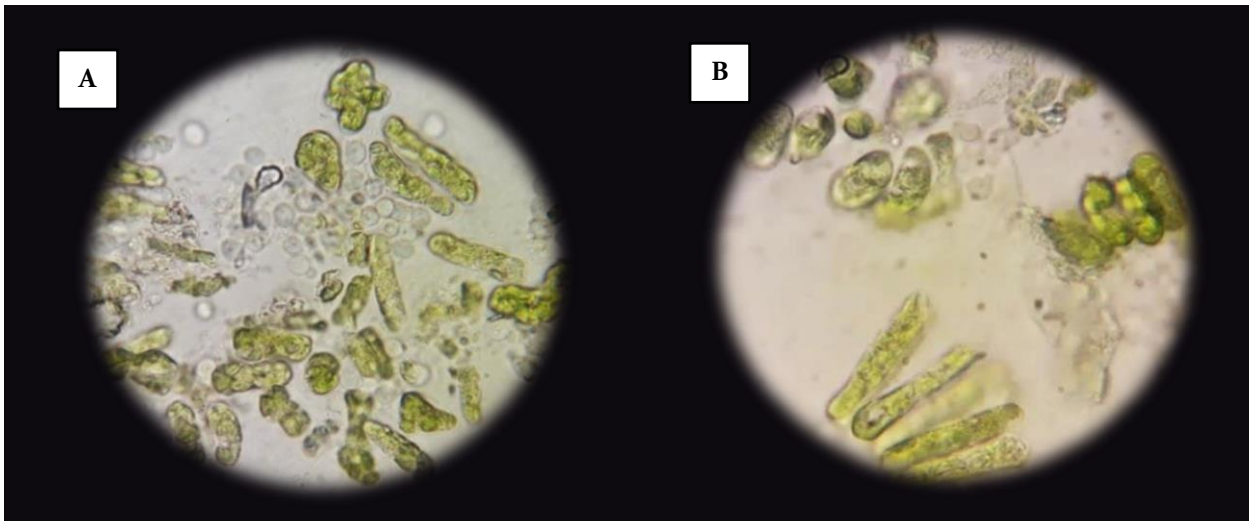


Fig.5: Microscopic view of isolated protoplasts from mesophyll cells of temperate mulberry under 40 x objective lenses

A & B) Protoplasts isolated from *Morus multicaulis* var. Goshoerami.

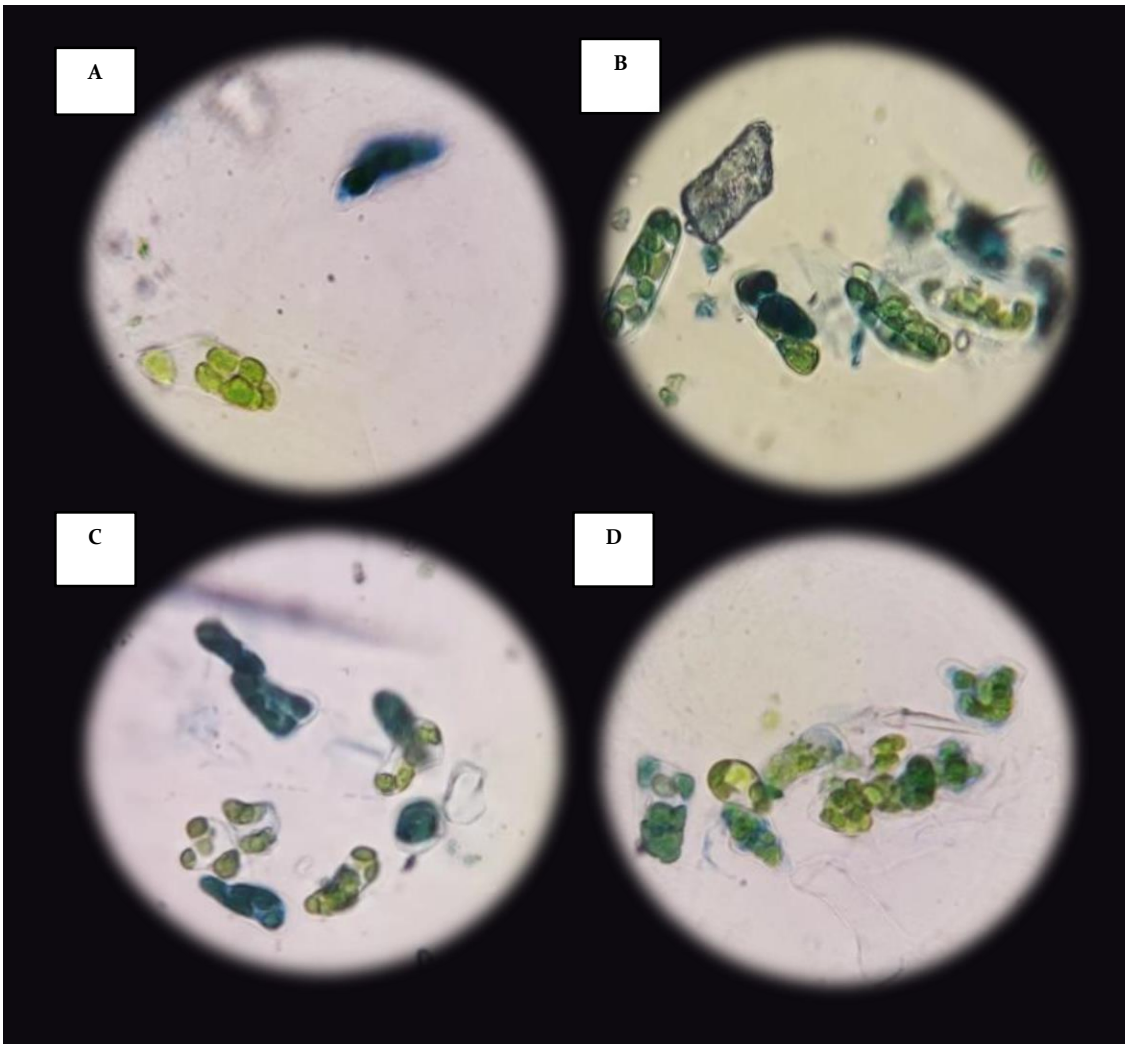


Fig.6:-Microscopic views of evaluation of viability of isolated protoplasts with evan's blue (0.2%) under 40 x objective lenses

A) Viable (unstained) and Non-viable (stained) protoplasts of PPR-1

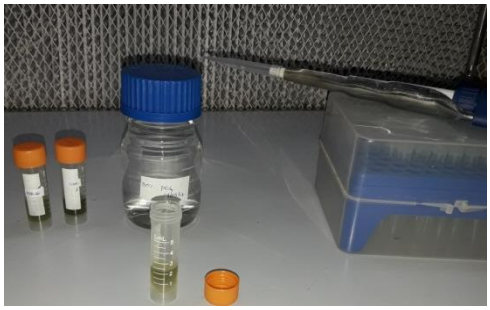
B) Viable (unstained) and Non-viable (stained) protoplasts of Chinese White

C) Viable (unstained) and Non-viable (stained) protoplasts of Ichinose

D) Viable (unstained) and Non-viable (stained) protoplasts of Goshoerami

III. FUSION OF PROTOPLASTS IN DIFFERENT COMBINATIONS

Fusion of protoplasts (of temperate mulberry) in different combinations was carried out at the collaborated institute i.e. University of Kashmir by chemical method using Polyethylene Glycol as a fusogen. Different concentrations of Fusogen was tested to fuse the protoplasts in desired combinations, among which 20 and 30% of PEG was found to be good in for the effective fusion of protoplasts (**Fig.1-4**). The following procedure is followed for fusing the protoplasts



0.5 ml of Protoplast solution Of One Mulberry Variety

+

0.5 ml of Protoplast solution of another or same variety

+

1ml PEG (10-40%) Solution

↓

Shake the tube for 5-8 Minutes

↓

Allow the tube to settle for 5-10 minutes

↓

Microscopic Examination

By following above protocol, protoplasts were fused in different combinations

1) PPR-1- Chinese White (CW)
2) PPR-1-Ichinse
3) CW-Ichinose
4) CW-CW
5) PPR-1-PPR-1

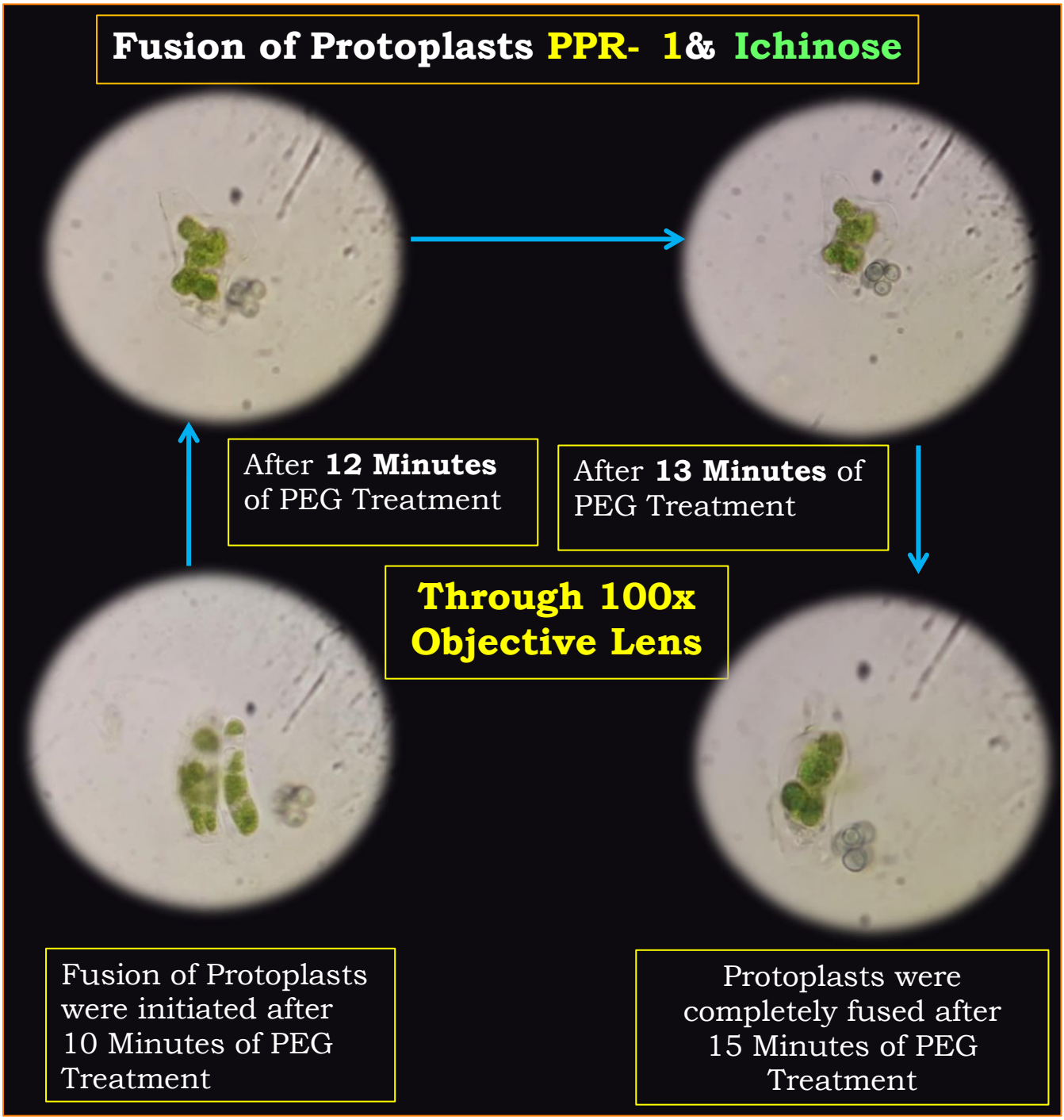


Fig.1 Fusion of protoplasts between PPR-1 & Ichinose by chemical treatment with 20% PEG for 15 Minutes duration.

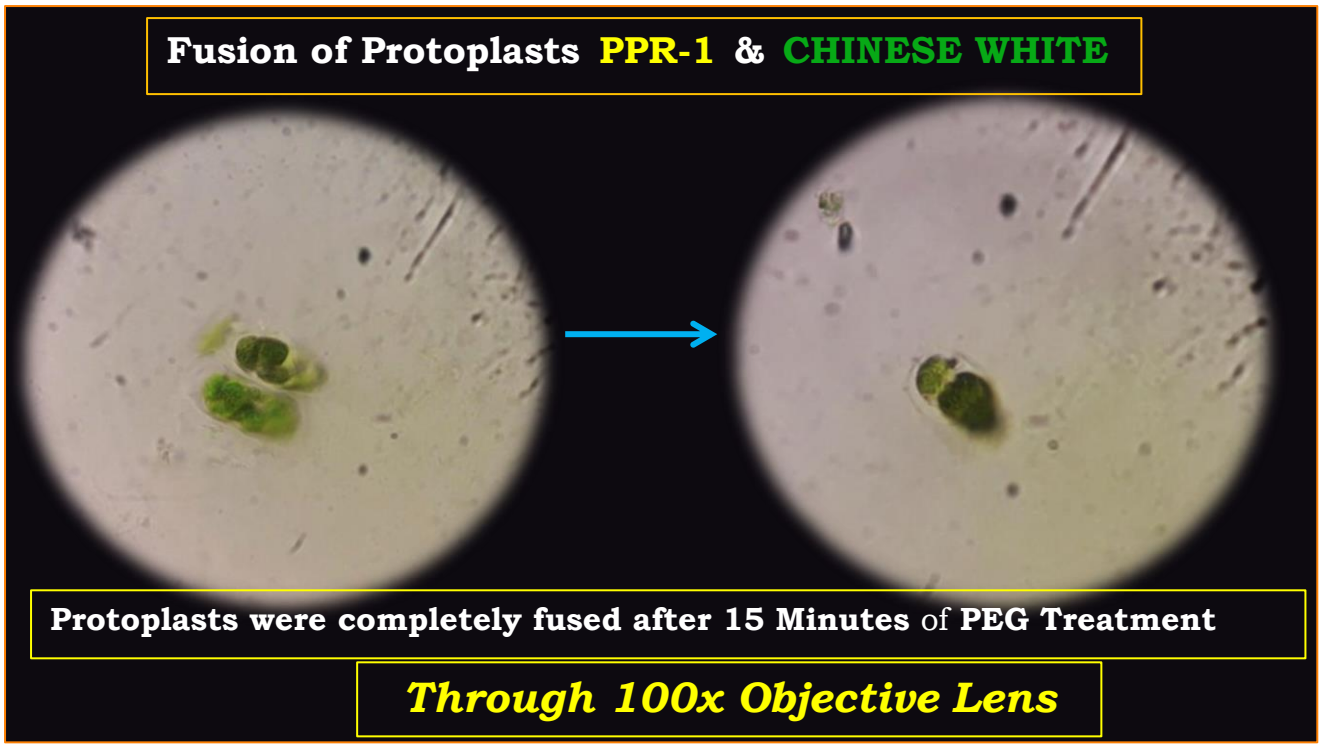


Fig.2 Fusion of protoplasts between PPR-1 & Chinese White by chemical treatment with 20% PEG for 15 Minutes duration.

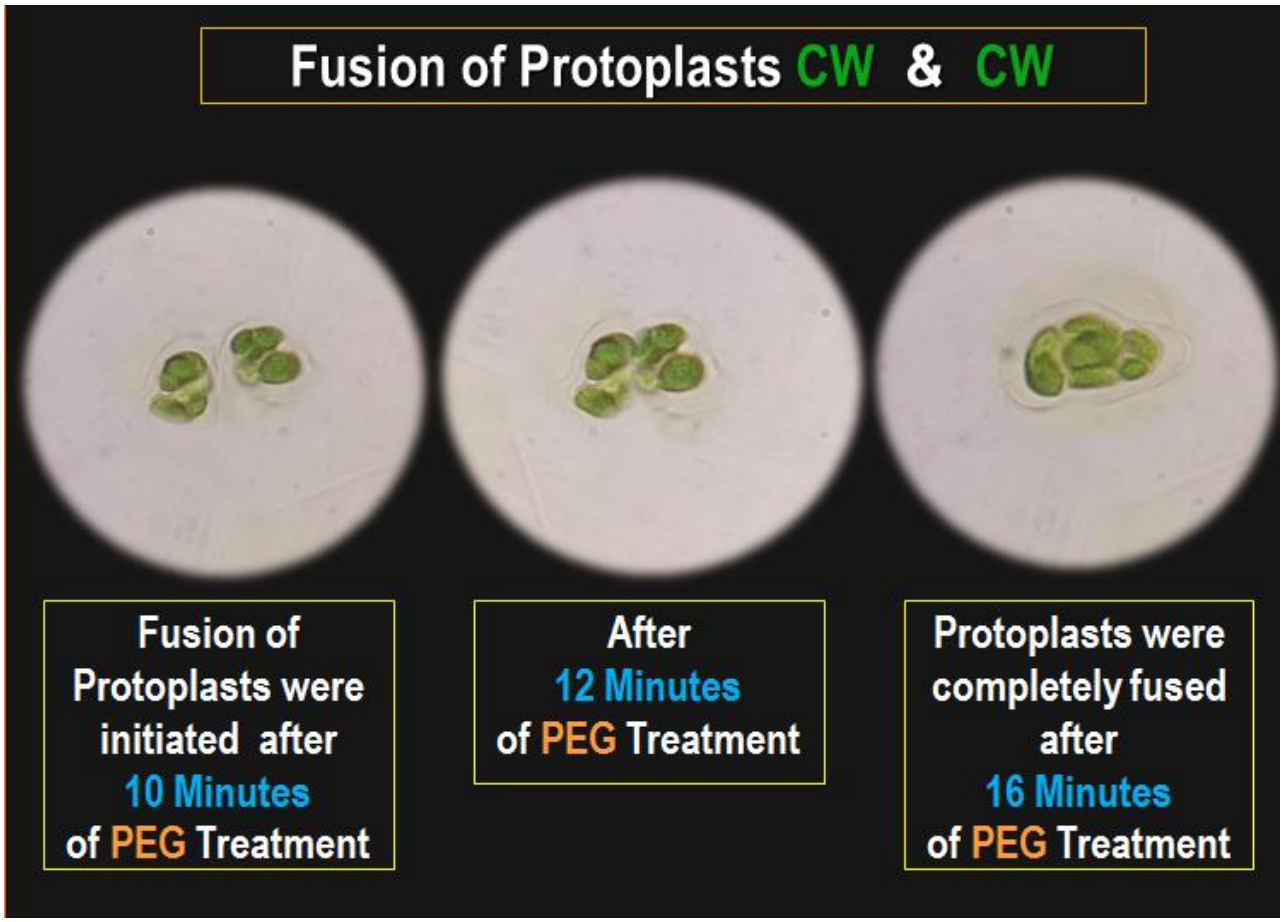


Fig.3 Fusion of protoplasts between Chinese white & Chinese white by chemical treatment with 20% PEG for 16 Minutes duration

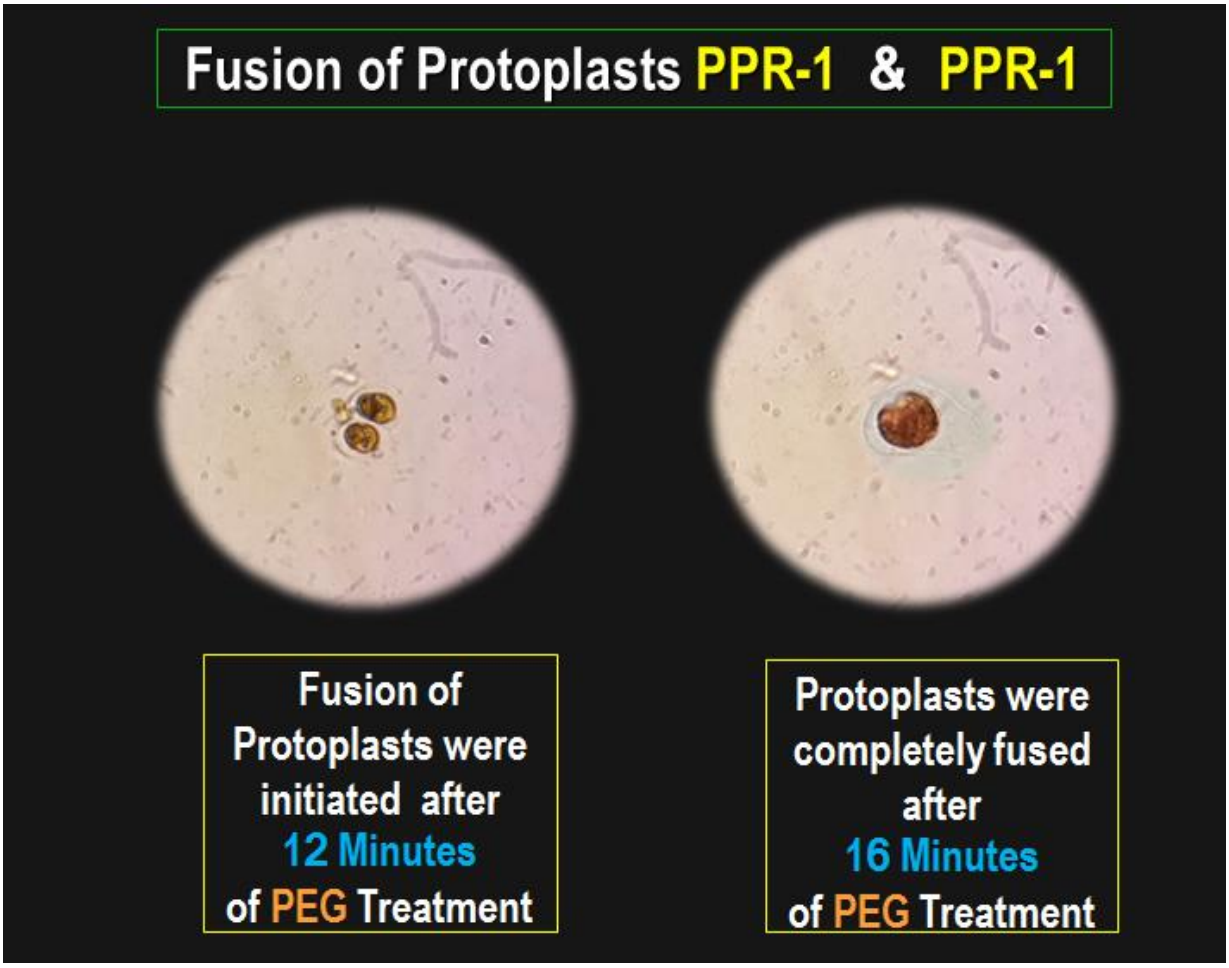
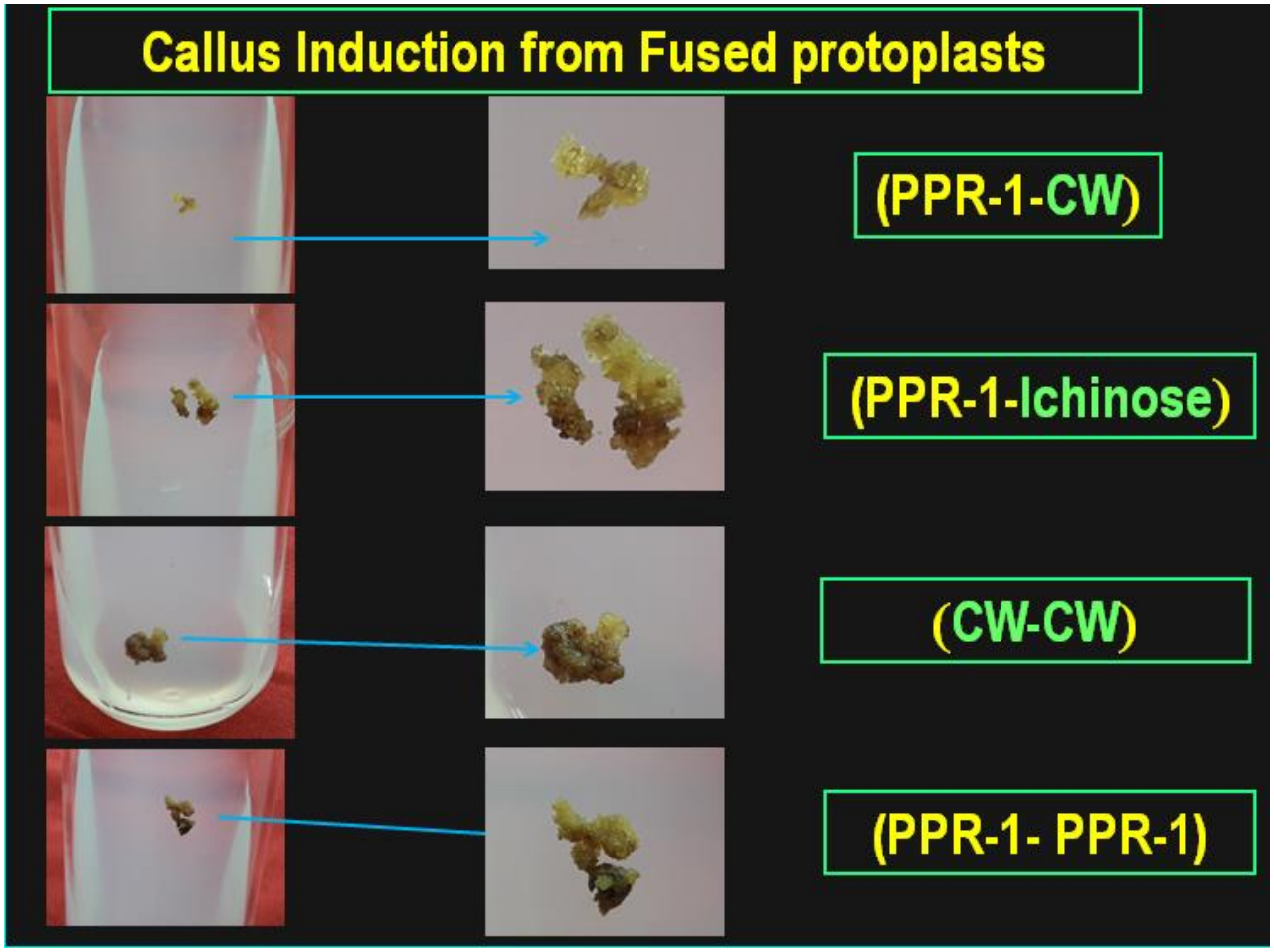


Fig.4: Fusion of protoplasts between PPR-1 & PPR-1 by chemical treatment with 20% PEG for 16 Minutes duration

III. Callus Induction from fused protoplasts

After fusion, the fused protoplasts were inoculated onto MS basal media as well as on different concentrations and combinations of hormones (auxins) supplemented media. Among the different tested hormones and concentrations, 2,4-D (2,4-Dichlorophenoxy acid acid) in lower concentrations (2µm/L) has resulted in division of fused protoplasts from most of the combinations of fused protoplasts except from the combination of Chinese white-Ichinose.



DETAILS OF THE WORK DONE DURING (2017-18)

I. SUB-CULTURING OF CALLUS

Callus induced from four combinations of fused protoplasts was successfully sub cultured on MS basal medium under 12/12 hrs photoperiod.

II. REGENERATION OF SHOOTS OF SOMATIC HYBRIDS

Shoot lets of somatic hybrids were regenerated from three combinations of callus on cytokinin supplemented media. Among the various combinations of cytokinins tested, combination of TDZ and BAP has given good results in regeneration of shoots

III. ROOTING OF SOMATIC HYBRIDS

Shoot lets of somatic hybrids of three combinations **(PPR-1-Chinese white, PPR-1-Ichinose & PPR-1-PPR-1)** were rooted successfully on IBA supplemented media

IV. HARDENING OF SOMATIC HYBRIDS

Raised somatic hybrids were gently removed from the culture tubes and were washed under running tap water to remove the adhering media and were hardened in plastic pots by using 2:1:1 ratio of garden soil, sand and FYM.

WORK TO BE DONE IN NEXT 3 MONTHS (2018-19):

1) Transfer of plantlets (Somatic Hybrids) from lab to land through green house phase to the field conditions of CSR&TI, Pampore to study the various parameters to determine their suitability to the temperate regions.

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