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IN-VITRO MICROPROPAGATIVE POTENTIAL OF *MORUS INDICA* L. CULTIVAR KAJLI FROM IMMATURE BUDS AND NODAL EXPLANTS

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ABSTRACT
In this present study, nodal explants and immature buds from mature trees of *Morus* sp var. Kajli were used for callus development and axillary shoot proliferation. Explants were cultured in MS medium supplemented with different concentration of BAP (N^6- benzyladenine) and Kinetin alone or in combination with each other. DIPA (2, 4-D) was also used to initiate callogenesis in various concentrations. In case of nodal explants, the rate of callus development was highest (60-80%) in 1.0µg/ml concentration of DIPA with media browning after 28 days of inoculation. At 2.5µg/ml concentration no callus was seen and there was no change in media also. 1.50µg/ml BAP and Kinetin and 2.00µg/ml BAP and Kinetin alone showed 50% of microshoot development with dark brown moderate callusing. This was improved by adding BAP with Kinetin. Multiple shoots produced by 1.50µg/ml BAP+ 0.5 Kinetin was vigorous after 30 days of inoculation. Rooting was highest (75%) 0.50µg/ml IAA after 20 days of sub culturing. In case of immature bud, Callogenesis is highest at 1.00µg/ml concentration of DIPA with entire surface callusing with media browning after 28 days of inoculation. The rate of bud break was increased by increasing the BAP level upto 2.00µg/ml concentration with golden brown callusing. Axillay shoots obtained from BAP subcultured in IAA and DIPA alone with BAP were tested with the highest rate in 1.00µg/ml IAA 0.9+/- 0.45 microshoots per explant followed by 1.50µg/ml IAA after 14 days of inoculation. The Four week old micropropagated plantlets were transferred to plastic pots containing a mixture of soil: sand: peat moss (1:1:2) under glasshouse condition. Hence, we can conclude that probably this is the first report of in-vitro clonal propagation of *Morus* sp var. Kajli using immature bud and node as explants through plant tissue culture technique.

KEYWORDS: Mulberry, Callogenesis, Organogenesis, PGR, BAP, Kinetin, DIPA, Hardening.

INTRODUCTION

Sericulture industry solely relies upon the very important mulberry tree (*Morus* spp. L.) It is cultivated to provide high quality nutritional leaves for feeding the silkworm *Bombyx mori* L. india is the second largest producer of silk throughout the world after China. Being a tree, the main focus of mulberry breeding is to improve leaf productivity as it alone contributes more than 38.2% to the sericulture productivity. However, improving leaf productivity is difficult being a multifactorial trait and determined by a number of associated characters such as plant height, number of branches, leaf retention capacity, nodal length, leaf size and weight, and total biomass through conventional breeding procedure only. High heterozygosity and inbreeding depression hinder the development of inbreeds failing directional breeding to make enough progress. Improvement of mulberry through conventional breeding is limited due to high heterozygosity and long generation period. Under such circumstances, the feasible means of improving specific traits without disturbing the current trait combinations is adoption of biotechnological tools. Mulberry can be vegetatively propagated through stem cuttings, grafting or budding. However, vegetative propagation of this plant is unsuccessful due to long time taken for adventitious shoot development and low rooting potential that might be due to several factors including physiological and environmental ones (Narayan *et al*., 1989). In vitro clonal propagation through axillary bud proliferation is a reliable approach for the multiplication of trees mostly within a reasonable time frame irrespective of seasonal considerations (Shirin *et al*., 2005). This is an alternative method for clonal propagation of recalcitrant tree species that do not respond well by sexual or other vegetative means (Sharma *et al*., 2000). In vitro establishment of nodal segments collected from mature trees has been reported in different species of mulberry (Tewari *et al*., 1999; Thomas, 2002; Anis *et al*., 2003).

MATERIALS & METHODS

Kajli is the main cultivated variety of mulberry found in West Bengal (CSRTI, Berhampore). Nodal explants and immature buds from Kajli were collected during April and surface sterilized. The explants (2- 3cm long) were prepared and thoroughly rinsed with running tap water for 10 minutes, 75% ethanol for 30 seconds and 15% commercial bleach (v/v) for 15 minutes and then rinsed with sterile distilled water 3 to 5 times. Dead tissues after surface-sterilization on both ends of the nodal explants were trimmed and 2cm long nodal segments were inoculated on the culture medium for bud break. MS0 (Murashige & Skoog, 1962) medium supplemented with BAP (0.25,0.5,1.0,1.5,2.0,2.53.0,3.5,4.0,4.5 µg/ml) and Kinetin (0.25,0.5,1.0,1.5,2.0,2.5,3.0,3.5,4.0,4.5 µg/ml) alone and in combination with each other for the nodal explants and MS0 (Murashige & Skoog, 1962) medium

supplemented with BAP (0.5,1.0,1.5,2.0,2.53.0,3.5,4.0,4.5 µg/ml) alone and in combination with IAA (0.5,1.0,1.5µg/ml) was used for immature buds to develop callus and for microshoot initiation. MS0 supplemented with DIPA (0.5, 1.0, 1.5, 2.0, 2.5µg/ml) was also tested for in-vitro Callogenesis. The data for bud break, shoot length, callus formation at the base as well as for surface calluses, medium browning and number of days to bud break were recorded. After 14 days, the elongating microshoots were further excised and subcultured on MS medium supplemented with IAA (0.25, 0.5, 0.75, 1.0µg/ml) for rhizogenesis. The data for in-vitro root induction (%), number of primary/secondary roots and mean root length (cm) were recorded after 20 days of culture on rooting medium. Four week old plantlets were transferred to plastic pots containing a mixture of soil: sand: peat moss (1:1:2) under glasshouse condition. The plantlets were irrigated once a day with ¼ strength of MS salts without vitamins, myoinositol and sucrose for 15 days.

RESULTS

Callogenesis and Organogenesis from Nodal explant

In-vitro callus development was highest (60-80%) in 1.0µg/ml concentration of 2,4-D/ DIPA (Fig. 1) (Table 1) on the entire surface of the nodal explants showing profuse callusing followed by 1.5µg/ml (Fig. 2) and 2.0µg/ml with moderate callusing after 28 days of inoculation. In all the cases media got browned after 28 days of inoculation. 0.5µg/ml and 2.5µg/ml concentration showed callusing at both end and single end consecutively with slight amount of callusing. The frequency of bud break was highest (50-60%) in MS medium supplemented with 1.0µg/ml DIPA. Profuse and moderate callusing was observed in 1.0µg/ml DIPA and 2.0µg/ml DIPA respectively, where 1.5µg/ml showed little callusing. Media become completely brown after 28 days of inoculation. At 2.5µg/ml concentration no callus seen and there was no change in media also. Two Plant Growth Regulators BAP and Kinetin alone or in combination was tested for microshoot proliferation (Table 2). 1.5µg/ml BAP and 2.0µg/ml BAP, as well as 1.5µg/ml Kinetin and 2.0µg/ml Kinetin alone showed 50% of microshoot development with dark brown moderate callusing (Fig.). Such shoot induction response was significantly improved by adding BAP with Kinetin. BAP was superior over Kinetin to induce such responses. Multiple shoots produced by 1.5µg/ml BAP + 0.5µg/ml Kinetin was vigorous after 30 days of inoculation (Fig. 3). Rooting was highest in 0.5µg/ml IAA with 75% rooting after 20 days of inoculation (Fig. 4) (Table 3). Micropropagated plantlet was then hardened (Fig. 5).


Figure 1.


Figure 2.


Figure 3.


Figure 4.


Figure 5.

TABLE 1: Effect of DIPA on Callus development in Kajli var. of Mulberry (after 28 days of inoculation) Explant- nodal segments. Media- MS0 (Basal MS media)

Sl No.	Concentration of DIPA[#]	Nature of Callus	Change in media	% callusing
1	0.0	No callusing	No change	0.0
2	0.5	+ at both end	No change	15-20
3	1.0	+++ entire surface of explant	Browning	60-80
4	1.5	++ entire surface of explant	Browning	50-60
5	2.0	++ entire surface of explant	Browning	50-60
6	2.5	+ at one end	No change	15-20

#Concentration of plant growth medium in µg/ml unit; + slight amount of callusing; ++ moderate callusing; +++ profuse callusing

TABLE 2: Organogenic response in Kajli var. of Mulberry (35 days of inoculation), Explant -callus induced on nodal explants, (Media- MS0+ different concentrations of BAP, Kinetin separately)

Sl No.	Media composition	change in Callus morphology	% of microshoot[$]
1	MS0	No change	0
2	MS0+ 0.25 BAP[#]	No change	0
3	MS0+ 0.5 BAP[#]	+ entire surface of explant Callus dark brown in colour	25
4	MS0+ 1.5 BAP[#]	++ entire surface of explant Callus dark brown in colour	50
5	MS0+ 2.0 BAP[#]	++ entire surface of explant Callus dark brown in colour	50
6	MS0+ 2.5 BAP[#]	++ at one end Callus pale green	25
7	MS0+ 3.0 BAP[#]	No change	0
8	MS0+ 3.5 BAP[#]	No change	0
9	MS0+ 4.0 BAP[#]	No change	0
10	MS0+ 4.5 BAP[#]	No change	0

11	MS0+ 0.25 Kin#	No change	0
12	MS0+ 0.5 Kin#	No change	0
13	MS0+ 1.5 Kin#	++ entire surface of explant Callus dark brown in colour	50
14	MS0+ 2.0 Kin#	++ entire surface of explant Callus dark brown in colour	50
15	MS0+ 2.5 Kin#	No change	0
16	MS0+ 3.0 Kin#	No change	0
17	MS0+ 3.5 Kin#	No change	0
18	MS0+ 4.0 Kin#	No change	0
19	MS0+ 4.5 Kin#	No change	0
20	MS0+ 0.5 BAP# +0.5 Kin#	No change	0
21	MS0+ 1.0 BAP# +0.5 Kin#	No change	0
22	MS0+ 0.5 BAP# +1.0 Kin#	+entire surface of explant Callus dark brown in colour	25
23	MS0+ 1.5 BAP# +1.5 Kin#	No change	0
24	MS0+ 1.5 BAP# +0.5 Kin#	++ entire surface of explant Callus dark brown in colour	75
25	MS0+ 0.5 BAP# +1.5 Kin#	No change	0
26	MS0+ 2.0 BAP# +2.0 Kin#	No change	0
27	MS0+ 2.0 BAP# +0.5 Kin#	No change	0
28	MS0+ 0.5 BAP# +2.0 Kin#	No change	0
29	MS0+ 2.0 BAP# +1.5 Kin#	No change	0
30	MS0+ 1.5 BAP# +2.0 Kin#	No change	0

concentration of PGR in µg/ml ; $ calculated from observation of 4 tubes replicated twice.

TABLE 3: Root development from regenerated microshoot (after 20 days of inoculation in rooting media) of Kajli var. of Mulberry, Media- 0.5 MS0+different doses of IAA.

Sl No.	Dose of IAA#	Rooting response	% of rooting$
1	0.25	No rooting	0
2	0.5	Profuse rooting	75
3	0.75	Limited rooting	50
4	1.0	No rooting	0

#Concentration of PGR in µg/ml; $ calculated from observation of 4 tubes replicated twice.

Callogenesis and Organogenesis from Immature Buds

After 28 days of inoculation, callogenesis was highest at 1.0 µg/ml concentration of DIPA with entire surface callusing and media browning also (fig. 6) (Table 4). Generally the rate of bud break was increased by increasing the BAP level upto 2.0µg/ml concentration (Table 5). Golden brown callusing is found in the entire surface at 1.0 µg/ml, 1.5 µg/ml and 2.0 µg/ml concentrations with brown coloured media (Fig) (Table 5). There was a sharp decrease in the higher concentration of BAP. The rate of callus formation generally increased with increase of the BAP level. Shoot growth was not affected by the development of these calluses. The axillary shoots obtained on MS media supplemented with 1.0µg/ml BAP were excised after 28 days and cut into further nodal segments for multiple shoot induction. For this purpose, IAA and DIPA alone with BAP were tested. The rate of multiple shoot induction from nodal explants was highest in MS medium supplemented with 1.0µg/ml IAA with 09 +/- 0.45 microshoots per explants followed by 1.5µg/ml IAA after 14 days of culture (Fig 7) (Table 5). Multiple shoots induced on different treatments was also different. However, very low in concentration and also the higher levels of IAA (except 1.0µg/ml and 1.5µg/ml) did not favour shoot induction under the same experimental condition. In case of DIPA, a little microshoot regeneration (05 +/- 0.35 microshoots / explant) was observed in 1.5µg/ml concentration of DIPA with profuse golden brown callus along with rhizogenesis (Table 5). But interestingly, the lower concentration of DIPA (0.5µg/ml) exhibited profuse rooting rather than shooting with 03 +/- 0.15 microshoots/ explants (Fig. 8). The number of primary as well as secondary roots was also highest in those media. Development of secondary roots was significantly improved by the use of DIPA (Table 5). After successful clonal propagation, the rooted microshoots (Fig. 9) were subsequently hardened following proper methods of hardening.


Figure 6.


Figure 7.


Figure 8.


Figure 9.


Figure 10

TABLE 4: Effect of DIPA on Callus development in Kajli var. of Mulberry (after 28 days of inoculation) Explant- immature bud. Media- MS0 (Basal MS media)

Sl No.	Concentration of DIPA#	Nature of Callus	Change in media	% callusing
1	0.0	No change	No change	0.0
2	0.5	No change	No change	10-15
3	1.0	+++ entire surface of explant	Browning	50-60
4	1.5	+ entire surface of explant	Browning	25-40
5	2.0	++ entire surface of explant	Browning	20-30
6	2.5	No callusing	No change	0-10

+ slight amount of callusing; ++ moderate callusing; +++ profuse callusing; # concentration of PGR in µg/ml; calculated from observation of 4 tubes replicated twice.

TABLE 5: Callogenesis & other Organogenic responses in Kajli var. of Mulberry (after 28 days of inoculation) Explant- Immature bud. Media- MS0 (Basal MS media)

Sl No.	Media composition	Nature of Callus/ organogenic responses	Change in media
1	MS0	No callusing	No change
2	MS0+ 0.5 BAP#	++ at both end	No change
3	MS0+ 1.0 BAP#	+++ entire surface of explant Callus golden brown in colour	Browning
4	MS0+ 1.5 BAP#	++ entire surface of explant Callus golden brown in colour	Browning
5	MS0+ 2.0 BAP#	++ entire surface of explant Callus golden brown in colour	Browning
6	MS0+ 2.5 BAP#	+ at one end Callus pale green	Browning
7	MS0+ 3.0 BAP#	+ at one end Callus pale yellow	Slight Browning
8	MS0+ 3.5 BAP#	+ at one end	No change
9	MS0+ 4.0 BAP#	No change	No change
10	MS0+ 4.5 BAP#	No change	No change
11	MS0+ 1.0 IAA+0.5 BAP#	+++ entire surface of explant; Callus golden brown in colour; Highly regenerative; 09±0.45 microshoots per explant	Browning
12	MS0+ 1.5 IAA+0.5 BAP#	++ entire surface of explant; Callus golden brown in colour; Highly regenerative; 06±0.55 microshoots per explant	Slight Browning
13	MS0+ 1.5 DIPA+0.5 BAP#	+++ entire surface of explant; Callus golden brown in colour; Highly regenerative; 05±0.35 microshoots per explant; Rooting	Slight Browning
14	MS0+ 0.5 DIPA+0.5 BAP#	+++ entire surface of explant; Callus golden brown in colour; Highly regenerative; 03±0.15 microshoots per explant; Profuse rooting	Slight Browning

#Concentration of plant growth medium in µg/ml unit.+ slight amount of callusing; ++ moderate callusing; +++ profuse callusing; calculated from observation of 4 tubes replicated twice.

DISCUSSION

Clonal propagation via in vitro establishment of nodal explants is quite a reliable method for the maintenance of clonal fidelity, high proliferation rate and easy manipulation (Hossain *et al*., 1992; Katase, 1993; Pattnaik *et al*., 1996; Pattnaik & Cland, 1997; Chitra & Padmaja, 1999; Lu, 2002; Akram & Aftab, 2009; Memon *et al*., 2010). It has been observed in earlier studies that BA was quite effective for shoot development than other purine- based cytokinins (Yadav *et al*., 1990; Pattnaik & Cland, 1997; Shirin *et al*, 2005; Akram & Aftab, 2009). BA played significant role for in vitro bud break and shoot multiplication with vigorous shoot growth in *Morus* sp. (Yadav *et al*., 1990). These results agree with the present investigation. The callus development was prominent and golden brown in appearance(Figure-10).The concentration of BA also affected shoot growth. BA higher than 4.4µM suppressed shoot growth in different *Morus* species (Hossain *et al*., 1992; Pattnaik and Cland, 1997). Positive effect of BA for shoot growth has been documented in other tree species of tropical origin (Shirin *et al*., 2005; Akram & Aftab, 2009). The optimal endogenous and exogenous levels of cytokinins and auxins might control the balance for in vitro development and multiplication of axillary shoots (Shirin *et al*., 2005). The development of callus is a common phenomenon during shoot induction in *Morus* species (Sharma & Thorpe, 1990; Pattnaik *et al*., 1996). Callus growth at the shoot base makes rooting difficult by interfering with the connection between shoot and root growth (Williams & Taji, 1989; Jain *et al*., 1990; Quraishi *et al*., 1996). Auxin

concentrations (Kim *et al*., 1985) and nodal explants maturity (Sharma & Thorpe, 1990) were the determining factors for callus development at the cut basal ends in different *Morus* species. Kim *et al*., (1985) reported considerable amount of callus on NAA (0.5-2mg/l) containing medium in *M. alba*. So, it is evident that mulberry can be propagated through nodal segments using as explants through plant tissue culture technique and this is our immense effort to micropropagate successfully var. Kajli using a nodal explant for the very first time. Though, in- vitro clonal propagation of mulberry through immature buds is quite uncommon till now, probably this is the first report of propagating a whole plantlet from immature buds of *Morus* sp var. Kajli in anexic condition and successful hardening in the field.

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