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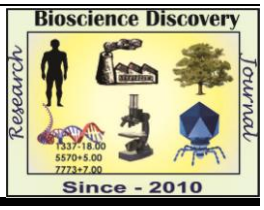

Effect of different sugar sources during *in vitro* sprouting efficiency of Indian mulberry (*Morus spp.*) genotypes using shoot apex explants

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Abstract

In vitro sprouting efficiencies of two indian mulberry genotypes *AGB24* and *AGB43* were tested providing different forms and combination of sugar in the MS basal medium using shoot apex explant. Three different sugar sources like, glucose, fructose and sucrose were used at 1%, 2% & 3% level for each and even the 1.5%+1.5% combination also with each other. Among all the sugars tested, sucrose at 3% level showed the optimum result in *in vitro* sprouting traits of both the genotypes. The resulted optimum sprouting% and shoot lengths were 87.50±10.61% and 5.71±0.59 cm in genotype *AGB24* and 87.50±3.54%, 2.36±0.33 cm in genotype *AGB43*. In both the genotypes, fructose showed the lowest values in sprouting traits in this case. It was also noted that the 120 days old sub cultures of both the genotypes are optimum to have maximum shoot length and numbers. However, the combination of sand, soil and FYM at 2:1:1 ratio showed optimum survival% i.e., upto 70% during the field evaluation. All the data were analyzed by variance test ANOVA and found statistically significant.

INTRODUCTION

Mulberry (*Morus spp*.) is the sole food plant of silkworm *Bombyz mori* L. due to the presence of protein '*Morine'* which is ultimately converted into silk proteins. Mulberry is also considered as nutritive, medicinal and economically important crop plant cultivated worldwide (Koyuncu, 2004; Srivastava *et al*., 2006; Ercisli and Orhan, 2007). Development of new mulberry verities through modern breeding approaches always plays a vital role in generation of transgenic plant with desired characters. However, *in vitro* plant regeneration offered a new avenue to raise new varieties of mulberry through different body parts like internodal callus (Raghunath *et al*., 1992), leaf explant (Raghunath *et al*., 2013), immature embryo, cotyledons (Kim *et al*., 1985), anther (Shoukang *et al*., 1987), stem cutting (Chitra *et al*., 2016) and even shoot apex cultures of *Morus ihou* (Chang, 1985), *M. nigra* (Ivanicka, 1987) and *M. alba* (Aroonpong & Chang, 2015). The different forms of carbon sources like glucose, fructose and sucrose in the media supplemented with growth responsive

hormones for mulberry plant regeneration showed significant effects on growth and development during *in vitro* conditions (Oka & Ohyama, 1978; Katagiri & Venkateswaralu, 1991; Pradhan *et al*., 2010; Aroonpong & Chang, 2015).

There are several reports on mulberry micro-propagation and the effect of different sugar sources with varied composition and concentrations on growth and development is well established. The percentage of shooting and rooting is also depends on the concentration of growth promoting hormones used in MS medium (Sengupta *et al*., 2016). However, in this present article that report the effect of different carbon sources with different concentrations along with different percentage of hormones in MS basal medium during *in vitro* culture of shoot apex of two Indian mulberry genotypes *AGB24* & *AGB43*. The protocol for shoot and root induction in different sugar concentration during shoot apex culture is already established in different plants (Ugandhar *et al*., 2012, 2014) and here we have successfully established the same in two mulberry genotypes.

MATERIALS AND METHODS

Preparation of plant material

The actively growing shoot tips of 45 days old mulberry genotypes *AGB24* and *AGB43* were used and collected from the Experimental Plot, Advanced Generation Breeding Programme, Central Sericultural Research & Training Institute, Mysore, India. Pre-treatment was carried out through thorough washing the sample with running tap water for 45 minutes followed by 0.2% bavistin treatment for 1 hour, 0.05% mercuric chloride treatment for 6 min, 70% ethanol wash for 30 sec, liquid bleach treatment for 2 min and final wash by sterile water for 20 min. The sterilized shoot apex portion of about 1cm size was cut with a sharp sterile blade inside the laminar air flow chamber.

Culture media and conditions

For sprouting, MS basal medium (Murashige and Skoog 1962) supplemented with cytokinin (BAP 2.0 mg/L) and agar (0.8%) was used. Three variable sources of sugar were used in the basal medium with different concentrations and combinations. Glucose (1%-3%), fructose (1%-3%), sucrose (1%-3%) and combinations of glucose + fructose (1.5%+1.5%), fructose + sucrose (1.5%+1.5%), sucrose + glucose (1.5%+1.5%) were also tested in two genotypes. Rooting of sprouted cultures was induced in the ½MS medium supplemented with hormones IBA & NAA 1.0 mg/L & 0.5 mg/L for *AGB24* & *AGB43*, respectively (Sengupta et al. 2016). The pH of all medium were adjusted to 5.8 with 1N NaOH/HCl and sterilized by autoclaving at 15 psi for 15 min. The cultures were maintained at adjusted temperature (25±1°C) with white fluorescent light at of 30-50 µE m^{-5} s^{-1} photon flux density under a photoperiod regime of 16/8 hrs light/dark conditions.

Hardening and data recording

The well rooted plantlets were carefully transferred into the pots containing Soil:Sand:FYM (2:1:1) and maintained in green house room allowing the essential environmental conditions. Initially, all the pots were covered with transparent plastic sheet for 20 days and later shifted the plants into the field for further growth. Sprouting percentage of 20 days old cultures and shoot length, shoot number of 40 days old plants was recorded with three repeated experiments. All data were statistically analyzed by analysis of variance test (ANOVA).

RESULTS DISCUSSION

Effect of different sugars in sprouting

Successfully sterilized shoot apex is cultured on MS medium supplemented with different sources of sugars, agar and respective hormones (Fig.1a). Different sugar sources and combinations showed significant effects in morphological traits of two different mulberry genotypes (Table1). For genotype *AGB24* each sugar sources individually showed the optimum sprouting% (Fig.1b) at 3% level i.e., 86.00±7.07, 80.00±14.41 and 87.50±10.61 for glucose, fructose and sucrose, respectively. But the combination of sucrose and glucose resulted the maximum sprouting% at 1.5% level each compared to other. We also noticed that the individual sugar source is better for sprouting rather using the combinations. In case of genotype *AGB43* only glucose and sucrose individually showed the optimum sprouting% at 3% level i.e., 75.00±6.11 and 87.50±3.54, respectively whereas fructose showed it at 2% level i.e., 40.00±14.20 which is comparatively much lower. However, the combination of sucrose and glucose resulted the maximum sprouting% at 1.5% level each like genotype *AGB24*. The sprouted shoot length was optimum i.e., 5.71±0.59 and 2.36±0.33 for sucrose at 3% level in genotype *AGB24* (Fig.1c) and *AGB43*, respectively. It was also observed that the individual sugar is comparatively effective than the combinations in two genotypes.

Table 1: Effect of different concentration and combinations of sugars in shooting of mulberry genotypes

Genotypes	Form of sugar	Concentration (%) and combination	Sprouting % (±SD)	Shoot length in cm (±SD)
AGB24	Glucose	1	70.00±14.14	1.49±0.01
	Glucose	2	75.00±7.07	3.52±0.17
	Glucose	3	86.00±7.07	3.04±0.21
	Fructose	1	55.00±7.88	2.14±0.16
	Fructose	2	70.00±7.10	1.43±0.49
	Fructose	3	80.00±14.41	2.58±0.67
	Sucrose	1	30.00±3.89	1.09±0.22
	Sucrose	2	84.00±6.50	2.21±0.09
	Sucrose	3	87.50±10.61	5.71±0.59
	Sucrose + Glucose	1.5 + 1.5	75.00±7.07	2.46±0.09
	Fructose + Sucrose	1.5 + 1.5	47.50±3.54	1.45±0.38
	Glucose + Fructose	1.5 + 1.5	37.50±10.68	1.60±0.56
AGB43	Glucose	1	50.00±4.50	1.48±0.67
	Glucose	2	35.00±6.22	1.59±0.39
	Glucose	3	75.00±6.11	2.31±0.16
	Fructose	1	20.00±7.70	0.44±0.16
	Fructose	2	40.00±14.20	1.56±0.40
	Fructose	3	25.00±7.04	1.34±0.03
	Sucrose	1	25.00±9.89	1.03±0.21
	Sucrose	2	82.50±10.60	1.81±0.28
	Sucrose	3	87.50±3.54	2.36±0.33
	Sucrose + Glucose	1.5 + 1.5	67.50±10.61	2.19±0.09
	Fructose + Sucrose	1.5 + 1.5	12.50±9.74	0.27±0.35
	Glucose + Fructose	1.5 + 1.5	36.00±6.22	0.55±0.37
CV @ 5%			5.56	0.21

Effects of time period of shooting:

We have also investigated the time period required for optimum shoot length and shoot numbers in two genotypes. It is necessary and so-called mandatory issue in plant tissue culture to establish a stable time period for sub-culture or multiplication with respect to its maximum number of growing shoots *i.e.*, multiple shoots. Here, in this case both the genotypes *AGB24* and *AGB43* showed a common and stable time period for optimum shoot length and number of multiple shoots regeneration. We recorded the data for 30, 60, 90, 120 & 150 days of sub-culture and found 120 days is the optimum time period for maximum shoot length and shoot numbers in both the genotypes. The recorded values of shoot length and number are 2.58±0.10 cm, 11.0±0.28 cm of *AGB24* and 2.54±0.13 cm, 10.15±0.35 cm of *AGB43*, respectively (Table2). We claim that 120 days is the optimum time for growth log phase because after that the plantlet showed comparatively lower values of shoot length and numbers.

Table 2: Effect of subcultures on shooting from shoot apex cultures

Genotypes	Duration of subculture (days)	Shoot length in cm (±SD)	No. of multiple shoots (±SD)
AGB24	30	1.45±0.26	2.10±0.42
	60	1.89±0.13	4.90±0.70
	90	2.26±0.05	7.30±0.42
	120	2.58±0.10	11.00±0.28
	150	2.06±0.06	7.75±0.35
AGB43	30	1.31±0.04	2.15±0.07
	60	1.97±0.13	4.40±0.07
	90	2.30±0.14	6.90±0.14
	120	2.54±0.13	10.15±0.35
	150	2.08±0.07	6.90±0.28
CV @ 5%		0.19	0.57

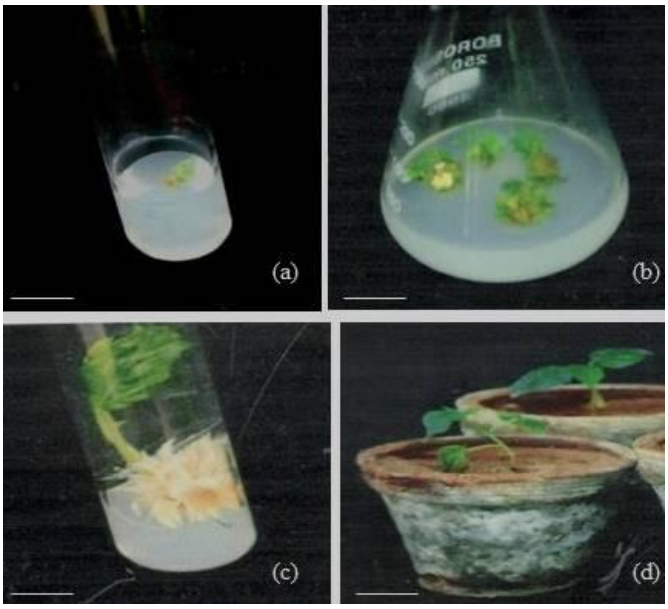

Figure1: (a) Sterile shoot apex is inoculated in MS medium of mulberry genotype *AGB43*. (b) Two weeks old sprouted culture of genotype AGB24. (c) Three weeks old genotype *AGB24* on MS medium supplemented with 1.0 mg/l IBA. (d) Hardening of genotype *AGB43* is ready for evaluation. Size bar: 3.5 cm for (a), 3 cm for (b), 2.8 cm for (c) and 10 cm for (d).

Hardening of plants:

Young plantlets of shoot apex explants were sub-cultured on ½ MS medium supplemented with rooting hormone and the rooting was initiated after two weeks of sub-culture. However, the root lengths were also varied in both the genotypes. The combination of soil, sand and FYM was appropriate for hardening of new plants of both the genotypes (Fig.1d) and they showed upto 70% survival ability. The hardened plants were finally transferred into the field for trial maintaining the recommended agronomical package & practices.

However, in this study we were interested to check the effects of different sugar sources and combinations in two Indian mulberry genotypes during *in vitro* multiplication using shoot apex explant. However, shoot apex is previously used for multiplication of different mulberry species (Tewary & Subha Rao, 1990; Aroonpong & Chang, 2015). Different sugar sources in MS basal medium showed significant morphological variations in shoot length, leaf width and even root system in *Morus alba* L. (Pradhan *et al*., 2010) and in shoot

regeneration from leaf explant (Vijayan *et al*., 2000). However, in general sucrose is the principal product of carbon fixation in plants (Avigad and Dey, 1997). Other sources of sugars have to be converted into simple glucose which is the starting point of carbohydrate metabolism for glycolysis followed by energy production (Brownleader *et al*., 1997). In the past, it is reported that the sucrose containing media elicit the best response in *in vitro* mulberry culture (Enomoto, 1987). In this report, we have also got almost the similar results in both the genotypes *AGB24* & *AGB43* among the three sugar sources. It is also noted that the combination of two sugars not worked well as individual. Some researchers also reported that fructose is also worked well in mulberry and other plant during *in vitro* regeneration (Ohyama and Oka, 1976; Chitra and Padmaja, 2002; Ratnadewi *et al*., 2012; Jacygrad *et al*., 2013). This variation occurs due to the ability of glucose metabolism in different genotypes. Moreover, it is proved that all the derivatives of simple glucose play the role in *in vitro* growth and development of mulberry plants. But on comparative analysis sucrose is reported as more favourable sugar source at 3% level. We have also reported that the 120 days of sub-cultures are best to get optimum morphological traits like shoot length and size at commercial point of view for the sericulture industry.

Mulberry is the major food plant for silkworm *Bombyx mori* L. and the leaf production is directly related to production of good quality of silk. So, it is necessary to produce qualitative and quantitative mulberry leaf for the sericulture industry. Keeping in mind these objectives, researchers are working to multiply the mulberry genotypes *in vitro* having superior quality of commercial traits. For quick multiplication *in vitro* using shoot apex is used here with different sugars to test the best performance of each. So, in this study we showed that the individual sucrose at 3% level is suitable for *in vitro* growth and development in this two mulberry genotypes.

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