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Plantlet Reproduction of King White Mulberry (*Morus macroura* Miq) via Direct Organogenesis

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Abstract: *In vitro* propagation is apromising method in regeneration instead of difficult-to-produced plants through seeds or stem cuttings (traditional propagation methods). The starting explants were both shoot tips and nodal cuttings explants taken from mature trees of king white mulberry (*Morus macroura* miq). Explants were cultured on Murashige and Skoog (MS) medium supplemented with both BA and Kin in various concentrations (1.0, 2.0, 4.0 and 6.0 mg/l) for each in a combination with IBA (0.1 mg/l). A significant induction of direct organogensis with notable appearance of callus were observed at the high cytokinins concentrations. Nodal explants were more superior than shoot tips in proliferation producing more number of shoots. Results indicated that BA was the most suitable source of cytokinins for shoot multiplication at various comparable rates with Kin. Rooting formation was performed on MS medium amended with 2.0 mg/l of IBA plus 1.0 g/l activated charcoal, which was sufficient to encourage the capability of roots formation. The acclimatized plants with 75% survival rate were shifted to the field conditions with notable success.

Key words: *In vitro* propagation · *Morus macroura* · MS · Shoot tip explant · Nodal explant · BA · Kin · IBA and Activated charcoal (AC)

INTRODUCTION

The genus *Morus* belongs to the family *Moraceae* that comprises nearly 35 species. *Morus alba*, *Mours macroura*. and *Morus multicauli*. are promising species for sericulture industry [1]. Mulberry fruit is characerized by having medicinal components. It can be used to nourish different human organs, treats exhaustion, anemia and hair. It is also used utilized to treat urinary incontinence, tinnitus, dizziness and constipation in the elderly and the anemic [2]. Composition of mulberry fruit is about 85-88% of water, 7.8-9.2% of sugars mainly as glucose and fructose, 0.4-1.5% of protein, 0.4-0.5% of fatty acids such as linoleic, stearic and oleic acids in the seeds, 1.1-1.9% of free acids mainly malic acid, producing the sour taste, 0.9-1.4% of fibers and 0.7-0.9% of minerals. Mulberry wood is used in handicrafts, cabinet work and a major source for making field-hockey sticks and tennis rackets [1].

Morus macroura, commonly known as 'king white' is a medium-sized spreading tree with weeping habit. It is an important tree species in the sericulture industry. The leaves are the sole food material available for rearing silkworm (*Bombyx mori* L.) [3]. Yield of leaves is limited to varieties, plant shape, age, number and length of branches and shoots and fertilizers [4]. It produces long catkins which when fully ripened become honey-sweet eaten as fresh but can also be sun-dried and eaten as sweets. As known in most of plants, propagation through seed is undesirable due to variability resulting from crossing among genotypes. The vegetative propagation of *Morus macroura* through cuttings or grafting is not economically viable because it involves lot of skilled manpower, expensive nursery facilities and due to long time for adventitious shoots to develop and low rooting potential that might be due to several factors including physiological and environmental ones and take a long wait of 4 to 5 years to obtain plants ready for harvest [5].

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Also, the percentage of cuttings success varied within a range of 65-80% [6, 7]. Thus, mulberry micropropagation offers unconventional quick methods with less cost, effiecient proliferation rate and high number of plants formed in a relatively short period and space. Explants have a key role in the success of micropropagation in the initial establishment. The quickness of successive reproduction steps under *in vitro* situation are significantly reliant on the genetic make-up, stage, source, physiological and pathological status of the mother plant [8]. Both shoot tip and nodal segments of juvenile or adult mulberry shoots mictopropagation were found to be suitable for micropropagation [9, 10]. As mentioned by Vijayan *et al*. [7] who demonestrated different protocolos for morus species and non of them dealt with king white mulberry.

Acclimitization of plants established *in vitro* is one of the critical phases in the production of micropropagated plants. Realization of identical plant with high survival rate requires prepared controlled environments and also adjustment of the interior microclimate to contest with the local envirnoment [7, 11].

Therefore, the main objective of the present study was to establish an efficient micropropagation protocol for *Morus macroura*.

MATERIALS AND METHODS

Experiments were performed in the *in vitro* culture lab at the Applied Research Center of Medicinal Plants and Natural Products, Ministry of Health, Egypt, during the period from 2012 to 2017.

Plant Material and Culture Conditions: Emerged twigs during spring of two weeks-old green shoots raised on five years old white king mulberry trees were collecte during spring. Shoots were collected and washed several times with running tap water. Two types of explants were used, shoot tips (1- 2 cm long) and stem nodes (2-3 cm long) which were prepared and surface sterilized. Both explants were immersed in 70% Ethanol for 20 Sec., followed by 1% sodium hypochloride for 5 min. Afterward, explants were washed several times by sterilized distilled water after each treatment. The explants were cultured on the surface of the medium inside the laminar air flow cabinet. Murashige and Skoog (MS) basal medium was used during varied steps of *in vitro* propagation [12]. At the beginning, MS supplemented with BA or Kin at (1.0, 2.0, 4.0 and 6.0 mg/l) were used in a combination with IBA (0.1 mg/l) for axillary shoot bud initiation. Ascorbic acid at 150 mg/l and citric acid 150 mg/l were used to control the browning according to Torres [13]. The pH of MS medium was adjusted at 5.7±0.1 prior to supplementation of the agar. The media were distributed into jars where each jar (600 cm^3) contained 50 ml nuterient medium and sterilized by autoclaving at 121 °C and pressure 15.2 Kg/cm^2 for 20 min. Data registered included shoots number/explant, shoot generation percentage (%) and shoot length (cm) after 30 days of culture induction.

Generated shoots of about 6.5 - 8.0 cm length were rooted on half-strenth MS medium with IBA at 0.0, 2.0 and 4.0 mg/l in combination with 0.0 or 1.0 g/l activated charcoal (AC). Data were recorderd as the number of root/shoot, root generation percentage (%) and root length (cm) after 45 days of culture on rooting media. Plantlets were transferred into plastic pots (8 cm) containing vermiculite: peat moss (1:1 v/v) for acclimatization under glasshouse conditions. Each pot contained one plantlet. During hardening procedure, plants were irrigated on regular basis and sheltered with polyethylene sheets to conserve humidity around plants, polyethylene sheets were gradually removed through 15 days. Surviving plants were recorded after 6 weeks from transplanting.

Experiments were designed in a completly randomized design. Each treatment was formed of 3 replicates of 4 jars each. Data were subjected to analysis of variance procedure for statistical analysis. Multicomparison test was performed using LSD method at 5% level of significance [14].

RESULTS AND DISCUSSION

Morphogenesis: Data illustrated in Table (1) show that type of explants morphogenetic differentiation depends on the effects of various concentrations of cytokinin applications. Culturing of both shoot tip and nodal explants on MS media gave the maximum percentage 100% of shoots at various cytokinins concentrations with relative suppression of any root development. Meanwhile, notable shoot and callus induction were observed from both explants at high level of both cytokinins (6 mg/l). Also, nodal explants formed shoot and callus together with the media containing both cytokinins at 4.0 mg/l. We can assume that, the ability of both explants in the direct organogenesis levels with absence of root formation was noted due to presence of cytokinin even at lower concentration. Similar results were obtained on other *Morus* species [15-17]. Inserted cytokinins in media is well recognized to enhance shoot induction rate, improve cell division and in other instances suppress rooting [18].

Table 1: Effect of cytokinins types and concentrations plus 0.1 mg of IBA on the morphogenetic characters of shoot tips and nodal explants of king white Mulberry

Cytokinin		Shoot formation		Root formation		Callus formation	
Type	Conc. (mg/l)	Shoot tip	Nodal	Shoot tip	Nodal	Shoot tip	Nodal
BA	1.0	+	+	-	-	-	-
	2.0	+	+	-	-	-	-
	4.0	+	+	-	-	-	+
	6.0	+	+	-	-	+	+
Kin	1.0	+	+	-	-	-	-
	2.0	+	+	-	-	-	-
	4.0	+	+	-	-	-	+
	6.0	+	+	-	-	+	+

• (+) Positive responce
• (-) Negative responce

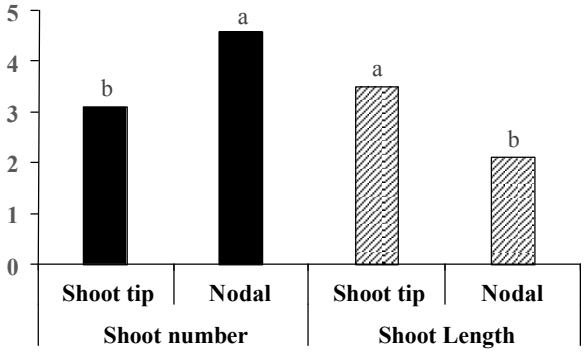

Fig. 1: Effect of explants type on number of shoots/ explant and shoot length (cm) of king white mulberry.

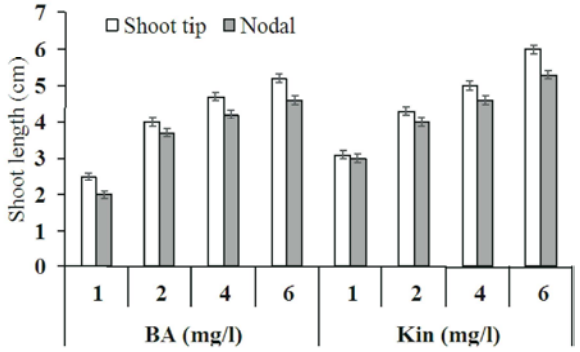

Fig. 2: Response of shoot tips and nodal explants as affected by type and concentration of cytokinins on shoot length of King White Mulberry.

***In vitro* Shoot Proliferation:** The ability of both explants on regeneration is illustrated in Figure (1). Nodal explants gave a higher number of shoots (4.5) with a notable lower shoot length (2.1 cm) when compared to shoot tips (3.1 and 3.5 cm). The superiority effect of nodal explant in terms of the number of shoots formed per explant has also been reported in five cultivars of mulberry, i.e. *Morus alba*, cultivars Chinese White, Kokiso-27 and Ichinose and *M. multicaulis* cultivars Goshoerami and Rokokuyaso as reported by Bhau and Wakhlu [15]. Also, a similar results was observed by Kozak [19] and Ananthi *et al*. [20] who suggested that nodal explants is considered the best source of multiple shoot induction. The differences in response of nodal and shoot tip explants has been attributed to the differences between the physiological status of the buds on different regions of stem and shoot tips exert strong apical dominance even in the presence of cytokinins. Regarding to the number of available shoot tips in each plant when compared to stem node mostly we may go for stem node.

The use of both cytokinin affected positively the length of regenerated shoots along with the increase of their concentration (Figure 2). Stem nodal augmented shoot length in all concentrations of both cytokinins when compared to shoot tips. This result was confirmed for both types of explants. The tallest shoots per explant was produced on a medium supplemented with 6 mg/l of either BA or Kin treatments. Also, for both explant, Kin showed superior results than BA application at various concentrations. Shoots resulted from shoot tips were taller than nodal explants at all concentration of both cytokinins. Also, Kin treatment gave taller shoot length when compared to BA application at each concentration.

Shoot number was affected by the increasing in high cytokinins concentrations as shown in (Figure 3). The highest shoots number were observed at 4 mg/l of both cytokinin applications with notable reduction at 6 mg/l. Higher level of both cytokinins beyond (4 mg/l) reduced shoot number. Also, for both explant, BA showed superior results than Kin application at various concentrations. This result is in agreement with result obtained by Fatima and Anis [21] and the earlier report on multiple shoot formation of mulberry cultivars as described by Bhau and Wakhlu [15]. The ability of BA on

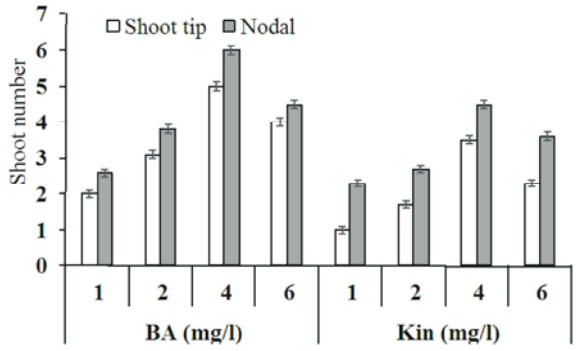

Fig. 3: Response of shoot tips and nodal explants as affected by type and concentration of cytokinins on the number of shoot/explant of King White Mulberry.

Table 2: Effect of IBA concentrations in presence or absence of and activated charcoal (AC) on root induction of King White Mulberry shoots after 4 weeks of culture

Treatments				
IBA (mg/l)	AC (g/l)	Root formation (%)	Root number/ plantlet	Root length (cm)
0.0	0.0	0.0	0.0	0.0
0.0	1.0	20	3.5	1.5
2.0	0.0	75	8.0	2.3
2.0	1.0	85	8.5	3.6
4.0	0.0	65	8.3	4.5
4.0	1.0	73	9.5	5.1
L.S.D at 5%		2.0	0.5	0.2

shoot intiation and shoot proliferatiion was obseved also by Fatima and Anis [21] and Chen *et al*. [22]. The advantage of BA in shoot induction may be related to the capability of plant tissues to metabolize BA more readily than other synthetic growth regulators or the interference of BA in the prompt effect on the production of natural hormones such as zeatin within the tissue. Akram and Aftab [23] reported that, in several mulberry species, BA was effective for shoot development than other purine-based cytokinins. BA is highly stable when compared to other cytokinins which is considered an explanation for the noticed action realized [18].

***In vitro* Rooting:** Rooting was observed after 28 days from culturing on the rooting medium. The highest rooting percentage (85%) was obtained on MS medium supplemented with IBA (2.0 mg/l) and in presence of AC (1.0 g/l). Whereas, the highest root number/ plantlet (9.5) and root length (5.1 cm) was observed on MS medium supplemented with IBA (4.0 mg/l) and AC (1.0 g/l). Significant differences were observed for root production on basal MS medium alone or when supplemented with 1.0 g/l activated charcoal. Rooting was much better when activated charcoal was added to the MS medium supplemented with IBA as shown in Table (2). Auxin is well known to promote cell elongation via increment wall extensibility. Auxin cooperate in the organization of cell wall characteristics by inducing wall loosening [24].

Activated charcoal clearly had a significant effect on improving growth of. It can improve development and growth of many plant species *in vitro* [25-27]. The beneficial effect of AC is thought to be attributed to its adsorption of inhibitory substances in the culture medium [28]. The influence of AC on growth regulator adsorption is remain unknown but some workers believe that AC may gradually release certain adsorbed products, such as nutrients and growth regulators which become available to plants [29].

Acclimatization: Resulted plantlets were transferred into controlled condition inside a greenhouse. From this investigation the acclimatized plants were then shifted to the field conditions with 75% survival rate. Acclimatized plants were morphologically uniform with fairly good growth.

CONCLUSION

The present study demonstrates an efficient micropropagation protocol for shoot tips and nodal explants of mature King White Mulberry tree. This is a rapid and reproducible method as compared to those traditionally used methods (viz. seeds and cuttings) for propagation approaches.

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