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Short note

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Efficient and easy micropropagation of *Morus nigra* and the influence of natural light on its acclimatization

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Abstract: Micropropagation, which employs various plant growth regulators (PGRs) in the culture medium for the induction of multiple shoots as well as adventitious roots, is a widely-used technique for the propagation of the genus *Morus*. The main objective of the present study was to evaluate the effect of PGR-free culture medium on the quantitative and qualitative characteristics of the micropropagation of *Morus nigra* under the growth room and greenhouse environmental conditions. Although a higher rate of multiplication (4.7–5.2 shoots/explant) was obtained from the treatments using Benzyladenine (BA) as the PGR, the PGR-free culture medium also exhibited comparable multiplication rate (4.1 shoots/explant) with a higher quality of shoots and without any symptoms of hyperhydricity. Furthermore, the use of PGR-free culture of *M. nigra* for *in vitro* propagation combined the steps of shoot multiplication and rooting phase using the same culture medium, further simplifying the process. In this study, the micro-shoots were also assessed for their *in-vitro* rooting and acclimatization potential. The incubation of an *in-vitro* culture of nodal explants in a controlled growth room for 28 days exhibited optimum response with about 90% rooting and 100% plantlet survival during the acclimatization phase. These results were better than those incubated under greenhouse conditions.

1. Introduction

Morus nigra is an exotic plant of unknown origin, with wild populations occurring in the Aegean Region of Turkey (Browicz, 2000). Its fruits are a rich source of phenolic compounds such as flavonoids (Arfan *et al.,* 2012).

Its propagation via seeds is limited due to a high degree of heterozygosity and a long period of sexual immaturity to reach fruit production (Vijayan, 2014). Mulberry (*Morus* sp.) is commercially propagated via the rooting of stem cuttings. However, *in vitro* clonal production of plantlets via micropropagation could also be achieved using MS medium supple-

mented with benzyladenine (BA) at concentrations of 0.5 to 2.5 mg L^{-1} for multiple shoot induction and indole butyric acid (IBA) or naphthalene acetic acid (NAA) at concentrations of 0.25 to 2.0 mg L^{-1} for root induction, in different species of this genus (Chitra and Padmaja, 2005; Ahmad *et al.,* 2007; Ji *et al.,* 2008; Chattopadhyay *et al.,* 2011). Some protocols have also used gibberellic acid (GA_3) for the elongation of shoots (Gogoi *et al.,* 2017).

Although plant growth regulators (PGRs) such as BA, IBA, and NAA promote and regulate the *in vitro* development and improve the efficiency of micropropagation, the addition of PGRs to the culture medium may also result in undesirable effects, varying from the loss of quality of generated plantlets and phenotypic variations, to certain undesirable genetic mutations called somaclonal variations (Bairu *et al.,* 2011).

The control of environmental conditions in a growth chamber or culture room for *in vitro* propagation is another requirement of commercial micropropagation, which increases the costs of micropropagated plantlets (Chen, 2016). Cardoso *et al.* (2013) suggested that the use of photoautotrophic micropropagation following pre-acclimatization in a greenhouse using natural light could reduce the costs of this technique, along with enhanced *in vitro* plantlet quality.

This study aimed to develop a simple protocol for the micropropagation of black mulberry (*M. nigra*) without the use of plant growth regulators (PGR-free micropropagation). The study also evaluated the use of *in vitro* pre-acclimatization under greenhouse conditions for the acclimatization of generated *M. nigra* explants.

2. Materials and Methods

Establishment of in vitro cultures of Morus nigra

Apical and axillary buds procured from mature (4-year-old) field-grown plants of *M. nigra* cv. 'Portuguesa' were used for the establishment of *in vitro* cultures. Young shoots of 1 to 2 cm length were immersed in 70% (v/v) ethyl alcohol for 30 sec, followed by surface sterilization in 40% sodium hypochlorite solution (containing 2.0-2.5% of active chlorine) for 15 min. After asepsis, the explants were thoroughly washed with sterile distilled water and trimmed further to a length of 3-5 mm using a scalpel blade, under a laminar airflow hood. The explants were then inoculated in flasks containing 30 mL MS medium (Murashige and Skoog, 1962) with half strength of macronutrients (½ MS) and supplemented with 30 g L^{-1} sucrose, 0.1 mg L^{-1} myo-inositol, 1.0 mg L^{-1} BA, and 0.1 mg L^{-1} NAA. The pH of the culture medium was adjusted to 5.8±0.05, after which 6.4 g L^{-1} agar-agar was added and the medium was autoclaved at 121°C and pressure of 1 kgf/cm² for 20 min. The culture flasks were incubated in a growth room with a light intensity of 2,500 lux (cold fluorescent lamps of 20 watts), 16-h photoperiod, and temperature of 25±2°C.

After *in vitro* establishment, the explants were maintained every 30 days by sub-culturing the nodal segments in ½MS culture medium without the addition of plant growth regulators, until the numbers of segments obtained were sufficient to execute the experiments.

Effect of different PGRs on in vitro shoot multiplication

The basal culture medium described for the *in vitro* establishment (½MS medium supplemented with 30 g L^{-1} sucrose, 0.1 mg L^{-1} myo-inositol) was used throughout the study, with variations in the types and concentrations of the most commonly used PGRs for the genus, *viz.,* PGR-free; BA at 0.25, 0.50, and 1.0 mg L^{-1}; Thidiazuron (TDZ) at 0.10 mg L^{-1}; and GA_3 at 3.0 mg L^{-1} (Vijayan, 2014).

For the experiment, the nodal explants (1.0±0.2 cm) with one axillary bud were obtained from *in vitro* cultures previously grown on PGR-free ½MS medium for 30 days. The growth conditions were identical to those described previously.

The experiments were conducted using a completely randomized design (CRD), with five replicates (flasks) containing four nodal explants in each. After 30 days of incubation of the culture, growth parameters such as shoot length, number, and width of leaves, and multiplication rate (number of new nodal segments obtained from each inoculated explant) were evaluated for each treatment. All the measurements were carried out using a digital caliper.

In vitro rooting and acclimatization

The nodal explants (1.0±0.2 cm) containing one axillary bud were inoculated in flasks containing ½MS basal medium without PGRs. Eight treatments in a 2×4 factorial design, with two environmental conditions, i.e., culture room and greenhouse, and four different durations of *in vitro* culture (7, 14, 21, and 28 days) were used. The culture room conditions were identical to those described in the establishment phase, while the greenhouse was covered with

diffusion agricultural plastic sheets of 150 µm thickness, with a light intensity of 65000±3000 lux, and average temperature ranging from 28.1 to 29.5°C during the period of *in vitro* rooting and acclimatization phase. Each treatment comprised of four flasks containing five nodal segments in each. The *in vitro* assessments were conducted after each period of growth (7, 14, 21, or 28 days). The number of nodal segments with new sprouts and roots was the parameter used for the evaluation. The field-based assessments were also conducted after 30 days of plant growth under greenhouse conditions on a commercial substrate consisting of peat (Pindstrup®, Denmark). The evaluation parameters included the height of plantlets, and the number and width of leaves. All the measurements were performed using a digital caliper.

The obtained data were analyzed using analysis of variance (ANOVA) and the means were subjected to Tukey's comparison post-test at 5% probability. The software ASSISTAT version 7.7 beta was used for the statistical analysis (Silva and Azevedo, 2006).

3. Results and Discussion

Successful establishment of Morus nigra

The development was initiated through the growth of shoots with fresh leaves after 14 days of inoculation, without any contamination or oxidation of explants. The first subculture was carried out after 30 days of inoculation and the shoots were multiplied until sufficient quantity of the explants was obtained for the ensuing micropropagation experiments.

PGR-free culture medium resulted in single-step micropropagation and higher quality of plantlets

The nodal segments cultured on PGR-free ½MS medium showed the best average height of plants (6.4 cm), with a good number (4.4) and width of leaves (1.8 cm). The addition of 0.25 mg L^{-1} and 1.0 mg L^{-1} BA increased the multiplication rate from 4.1 (PGR-free) to 4.7 and 5.24, respectively (Table 1).

However, the addition of BA, even at the lowest concentration used in culture media (0.25 mg L^{-1}), negatively affected the quantitative (height of plants and number and width of leaves) (Table 1) as well as qualitative parameters of regenerated shoots, showing callus formation at the basal cut ends and yellowish leaves (Fig. 1 A-C), compared with PGR-free medium (Fig. 1 F). These symptoms were more intense (yellowish-brown leaves) in case of TDZ, even at a low concentration of 0.1 mg L^{-1} (Fig. 1 D). The addition of GA_3 resulted in a reduction in the number of leaves and axillary buds, leading to a lower multiplication rate (Table 1; Fig. 1 E) than the PGR-free medium.

In most of the protocols related to *in vitro* micropropagation of *Morus* sp., the addition of cytokinins

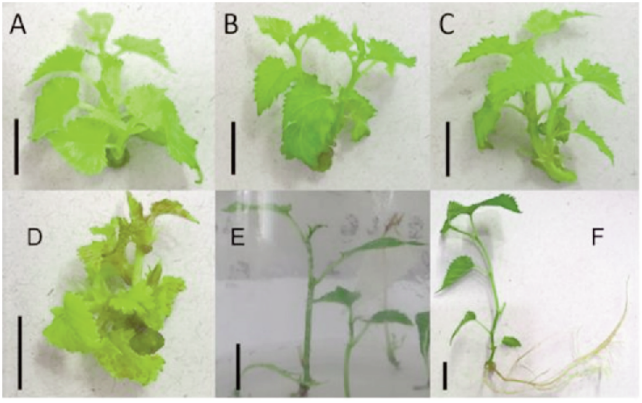

Fig. 1 - Shoots of *Morus nigra* in different types and concentrations of plant growth regulators (PGRs): (A) 0.25 mg L^{-1} BA; (B) 0.50 mg L^{-1} BA; (C) 1.0 mg L^{-1} BA; (D) 0.10 mg L^{-1} TDZ; (E) 3.0 mg L^{-1} GA_3. (F) PGRs-free MS½ medium. Bar = 1 cm.

Table 1 - Effects of culture medium with different PGRs on the *in vitro* development and multiplication of nodal segments of *Morus nigra*

Treatment	Plantlet		Leaves	
	Height (cm)	Multiplication rate*	Number	Width (cm)
PGR-free	6.4 A	4.1 BC	4.5 A	1.8 A
BA 0.25	3.5 B	4.7 AB	3.7 B	1.2 B
BA 0.50	3.5 B	5.0 A	3.7 B	1.1 B
BA 1.00	3.3 B	5.2 A	3.6 B	1.1 B
GA_3	3.6 B	2.2 D	2.4 C	1.2 B
TDZ	2.3 C	3.6 C	3.4 B	1.1 B
F value (PGRs)	18.24	22.66	7.86	16.59
Coefficient of variation (%)	38.3	26.4	30.8	26.6

* Multiplication rate referring to the mean number of nodal segments obtained by each segment inoculated *in vitro*.
Average values followed by the same letter in the column do not differ from each other by Tukey's test at 5% probability.

to the culture medium had shown positive effects on the multiple shoot induction (Chattopadhyay *et al.,* 2011; Lalitha Natarajan *et al.,* 2013). Until now, no report was available on the use of PGR-free culture medium for the *in vitro* multiplication of *Morus* sp. Yadav *et al.* (1990) observed a high shoot multiplication rate from shoot apex (4.2) and nodal explants (11.3) in *M. nigra* using MS medium supplemented with 1.0 mg L^{-1} BA. They also recorded a high shoot multiplication rate (6.3) with nodal explants using kinetin (KIN). Anis *et al.* (2003) recorded *in vitro* multiplication rates ranging from 2.4 to 5.2 using 2.0 mg L^{-1} BA and 0.2 mg L^{-1} NAA in *M. alba*. On the other hand, although Akram and Aftab (2012) did not observe multiple shoot induction in *M. macroura* using either BA or KIN singly in MS medium, the supplementation of BA with IBA in the culture medium resulted in a high multiplication rate of 4.7 shoots/explant.

In the current study, the excision of shoot apical meristem (SAM) of *M. nigra* helped in breaking the dormancy of axillary buds in nodal explants cultured *in vitro* and increased the level of cytokinin. Excision of the SAM also caused a reduction in the endogenous levels of auxins and enhanced the expression of genes encoding Isopentenyl transferase (IPT), a key precursor enzyme for the biosynthesis of endogenous cytokinin. This increased the level of endogenous cytokinin in the axillary buds, resulting in stimulation of cell division and shoot development (Tanaka *et al.,* 2006). The highly resistant nature of BA to the degradation activity of cytokinin oxidases explained the largely improved *in vitro* shoot productivity when this PGR was added to the culture medium (Ashikari *et al.,* 2005).

The use of PGR-free medium resulted in the production of shoots and roots without any supplementation. However, Yadav *et al.* (1990) observed that IBA was more effective in the promotion of rooting in *M. nigra* as compared to IAA and NAA, and increased the number of roots from 1.2 (auxin-free) to 6.1 (0.25 mg L^{-1}). These results are strikingly similar to our findings, as we also obtained only 1-2 roots per stem nodal segment without using auxins (data not shown). Nevertheless, although a lower number of roots were produced, even a single root was enough for the acclimatization of *Morus nigra* plantlets.

The plants obtained from the culture medium containing BA exhibited some morphological alterations compared with those developed in the PGR-free medium. The abnormalities included difficulty in *in vitro* manipulation during subculture due to the breakage of brittle tissues, reduced internodal length, and yellowish leaf appearance. These symptoms of hyperhydricity were observed irrespective of the BA concentration (Fig. 1A-C). The repeated subcultures on BA containing medium led to somaclonal variations in the micropropagated plants (Nasser and Mahmood, 2014). Nanism, hyperhydricity, and chlorosis were some of the abnormalities reported in the *in vitro*-raised plants with continuous use of high concentrations of BA (Israeli *et al.,* 1991; Kumar *et al.,* 1999). In the current study, no morphological abnormality was observed in the obtained plantlets, even after 24 months with mensal subcultures, when PGR-free culture medium and nodal explants of 1.0±0.2 cm were used.

Greenhouse pre-acclimatization did not show optimal development of plantlets

A notable difference was observed in the shoot development from *in vitro* nodal segments of mulberry after seven days of inoculation and 21-28 days of growth in growth room and under greenhouse conditions, with about 100% of nodal explants producing new shoots in the growth room compared to only 80% under greenhouse conditions (Fig. 2 A). However, the highest impact of environmental conditions was observed on the rooting, where only 40% of the nodal segments produced roots in the greenhouse compared to 90% in growth room conditions (Fig. 2 B). Although up to 80% shoot regeneration from the nodal explants was recorded after 14 days of culture in greenhouse conditions, the rate later reduced to 70% and 40% after 21 and 28 days, respectively. This could be attributed to the death of plantlets after cultivation for 14 days in greenhouse conditions, possibly due to excessive ambient light intensity (above 68,000 lux), which caused maximum daytime temperatures reaching between 35.1 to 36.7°C. This temperature was around 10°C higher in comparison to the standard growth room conditions, i.e., 25±2°C temperature at a light intensity of 2,500 lux.

High temperatures may lead to the alteration of cellular processes, such as protein expression (Koini *et al.,* 2009; Stavang *et al.,* 2009) and the hormonal regulation of development by, for example, cytokinin (Macková *et al.,* 2013). The acquisition of thermotolerance (acclimatization) requires the expression of heat shock proteins (HSPs) in order to avoid oxidative stress and involves the synthesis of abscisic acid (ABA) (Penfield, 2008). ABA is a well-known phytohormone, which inhibits the growth of plants under

environmental stress or pathogen attack (Sharp and LeNoble, 2002). Therefore, it could result in the reduced shoot and root formation in the nodal segments of *M. nigra* under the extreme light and temperature in greenhouse conditions. Future experiments should use covers to reduce the light intensity and extreme temperatures in the flasks, similar to those used for the gerbera pre-acclimatization tests (Cardoso *et al.*, 2013).

The *in vitro* culture of micropropagated plantlets during elongation and rooting phase under greenhouse conditions is called as pre-acclimatization. This has been successfully executed in gerbera (*Gerbera jamesonii*) and was reported to promote the growth and acclimatization rate of gerbera plantlets and reduce the costs of micropropagation (Cardoso *et al.,* 2013). The major differences between the previous and present studies lie within the partial control of environmental factors, which in the previous study were maintained as 25±5°C, PPFD 100 µmol m^{-2} s^{-1} and a 12-h photoperiod, i.e., natural growing conditions for gerbera. In contrast, in our study with *M. nigra*, the incidence of high light intensity (900-1800 µmol m^{-2} s^{-1}), a temperature exceeding 30°C, and longer photoperiod (12:30 h) proved detrimental to *in vitro* as well as field-based development of plantlets.

Growth room environment for 28-days of in vitro cultivation provided optimum conditions for in vitro rooting and acclimatization

As shown in Table 2, the previously observed effect of *in vitro* conditions on the development of plantlets was also reflected during the acclimatization stage under greenhouse conditions. The nodal explants maintained *in vitro* under greenhouse conditions also resulted in reduced development of plantlets in the acclimatization stage.

The duration of *in vitro* culture incubation also affected the development of acclimatized plantlets of *M. nigra*. Although a 7-day-incubation period resulted in 96% survival, plantlets were better developed with a 28-day-incubation period before acclimatization (Table 2). The plants maintained for different durations under *in vitro* greenhouse conditions showed improvement only in leaf width (0.57 cm at 7-d versus 1.79 cm at 28-d), with no gain in terms of shoot length and number of leaves (Table 2; Fig. 2C). On the other hand, plants grown under culture room conditions showed better *ex vitro* acclimatization of *M. nigra* plantlets, as the shoots obtained after 28 days of *in vitro* incubation were longer (9.2 cm) and had greater leaf width (3.2 cm) compared to those incubated *in vitro* in the greenhouse (3.8 cm and 1.8 cm, respectively).

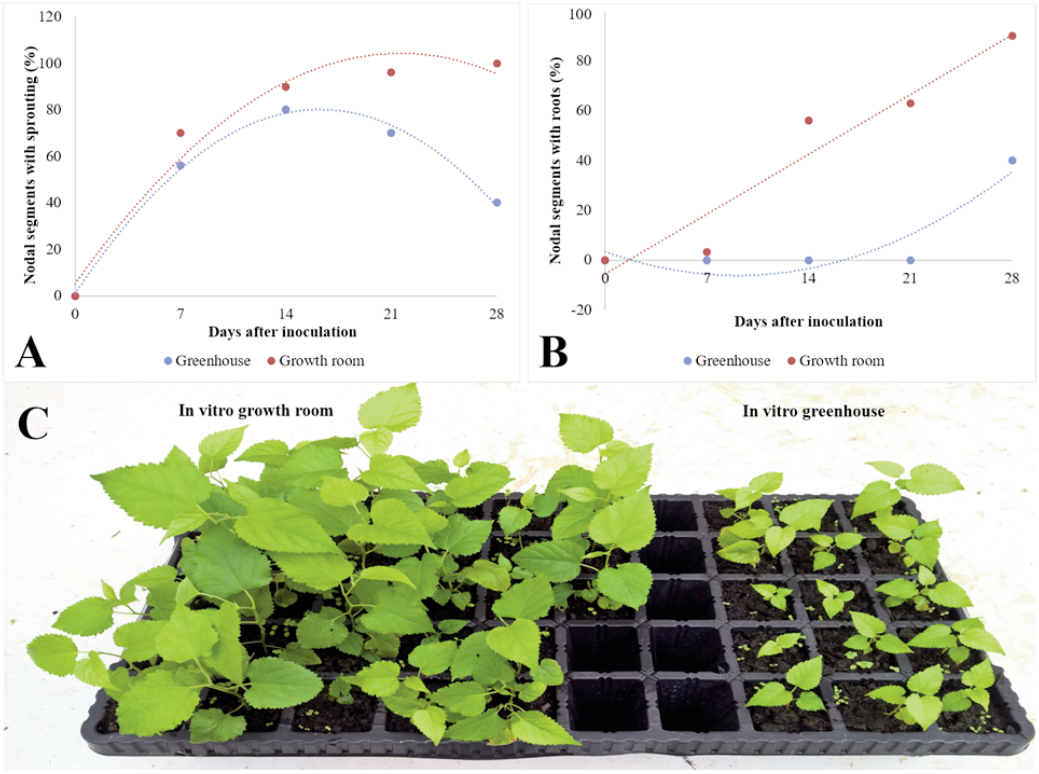

Fig. 2 - Number of nodal segments with new shoots (A) and roots (B) along the period of 28-days of in vitro cultivation. Plants after 30 days from ex vitro acclimatization (C) and previous *in vitro* cultivated under growth room (left) and greenhouse (right).

Table 2 - Influence of the *in vitro* cultivation period (7, 14, 21 and 28-days) and environmental conditions (growth room and greenhouse) on the acclimatization stage of plantlets of *M. nigra*

Environmental conditions	Time of *in vitro* cultivation (days)			
	7	14	21	28
Survival of plants (%)				
Growthroom	96	86	73	86
Greenhouse	73	83	53	50
Length of shoots (cm)				
Growthroom	2.8 bA	4.6 bA	4.3 bA	9.2 aA
Greenhouse	2.2 aA	1.9 aB	2.1 aB	3.8 aB
Number of leaves				
Growthroom	3.6 A	4.5 A	4.5 A	4.8 A
Greenhouse	3.3 A	2.7 B	3.7 B	3.5 B
Leaf width (cm)				
Growthroom	0.78 cA	1.20 bcA	1.42 bA	3.23 aA
Greenhouse	0.57 bA	0.75 bA	0.97 bA	1.79 aB
F test	LS	NL	DL	
Environment	**	*	**	
Period	**	**	**	
Environ x Period	**	NS	**	
Coefficient of variation (%)	30.7	16.9	27.0	

Values with the same letters - upper case between cultivation ambient and lowercase between conduction period - do not differ from one another by Tukey's test. Significant at 1 (**) and 5 (*) of probability. NS - not significant.

The culture room conditions of programmed and controlled temperature with low light intensity were conducive enough for the production of plantlets with healthy shoots and roots, and consequent successful acclimatization (100%) of *Morus nigra*.

4. Conclusions

A single-step protocol using PGR-free culture medium for the *in-vitro* shoot induction, multiplication, and rooting was optimized for efficient large-scale production of *M. nigra* plantlets. The micropropagated shoots of *M. nigra* were maintained in PGR-free culture medium for two years without any symptoms of morphological abnormalities or somaclonal variations. The *in vitro* rooting under culture room conditions after 28 days presented better response during the acclimatization phase, with the production of plantlets of superior quality. Since pre-acclimatization in the greenhouse did not result in enhanced adaptation of plantlets, further extensive experiments with more variations in the controlled conditions of temperature and light intensity should be undertaken in order to optimize a viable and efficient method for the cost-effective micropropagation of *Morus* sp.

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