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PRELIMINARY RESEARCH ON *IN VITRO* PROPAGATION OF *ZIZIPHUS JUJUBA* MILL.

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Abstract

In the present study, shoots of Ziziphus jujuba Mill. cv. 'Huping Zao' were used to identify an in vitro propagation protocol. Murashige and Skoog medium was used for this purpose. In the initiation and establishment stage, the best variant in terms of explant growth proved to be the one to which 2 mg L^{-1} IBA + 0.1 mg L^{-1} GA_3 + 1.5 mg L^{-1} NAA were added. Regarding the explant multiplication stage, the best results were obtained using 2 mg L^{-1} IBA, respectively 2 mg L^{-1} IBA + 0.5 mg L^{-1} NAA. The obtained results confirm the role and importance of different concentrations of hormones on the growth and development of explants.

Key words*: in vitro, jujube, tissue culture, shoots.*

INTRODUCTION

Ziziphus jujuba Mill. (jujube, Chinese date) belongs to the *Rhamnaceae* family, it is a fruit tree species originating in China for more than 7000 years and the place where most varieties are found (Liu et al., 2020; Jin, 2018). Jujube is intensively cultivated mainly in China, USA, India, Middle East, Australia, Italy (Liu et al., 2020) due to its beneficial properties on health (Stan et al., 2021; Chen et al., 2019; Cosmulescu et al., 2018), used in traditional medicine as anti-inflammatory, detoxifying, antioxidant, antimicrobial, gastrointestinal protective, cardiovascular, anticancer (Liu et al., 2021). It is a species that is quite difficult to multiply by generative methods (Karimpour et al., 2013; Sapkota et al., 2020; Rahaman et al., 2018) and if this is possible, the percentage of germination is very low (Stănică, 2019). The vegetative propagation methods among the most used are grafting, propagation by cuttings and shoots (Stănică, 2019; Yao, 2016), but if the procedure is not carried out correctly, the chances of success are non-existent and also the lack of rootstocks makes this aspect very difficult. A rapid propagation method is tissue culture (buds, shoots, leaves, callus) or *in vitro* micropropagation (Dai et al., 2009; Gu & Zhang, 2005; Khazaei et al., 2015). According to Hussain et al. (2012) plant tissue culture allows the growth of whole plants, organs, tissues or cells under controlled aseptic conditions in the laboratory, provides all the nutrients, energy and water necessary for explant growth through the used culture medium, and the explants development can then be manipulated by adding growth hormones (Phillips & Garda, 2019) depending on the pursued objectives (callusogenesis, organogenesis, rhizogenesis). This method has advantages and disadvantages. Among the advantages we can mention the rapid micropropagation (Iliev et al., 2010), in a relatively short time, of a large number of plants identical to the donor plant (the mother plant from which the plant material is harvested), obtaining clones, viruse free plants, grown under aseptic conditions and controlled environmental factors (George et al., 2008). The process is laborious, expensive (Liu et al., 2015), it is carried out in sterile laboratory conditions, and special attention must be paid to the preparation of the plant material in order

to have a chance of success (Rahaman et al., 2018). Of course, the most common culture medium used by researchers for *in vitro* propagation of jujube is the Murashige and Skoog culture medium (Yıldırım et al., 2015; San et al., 2014; Zhou & Liu, 2009; Goyal et al., 2006; Jian et al., 2006; Gu & Zhang, 2005) supplemented with different growth hormones in different concentrations. Due to climate changes, i.e. increasing temperatures, lengthening of the growing seasons, increasing periods of drought on the territory of Romania, as well as the strategic position on the globe, the Chinese jujube can be considered a suitable species for future orchards or as individual plants in private gardens (Stănică, 2019). Recent studies have demonstrated the adaptability of this species to the climate of our country, in teaching nurseries, research stations, private gardens, but especially due to the existence of the spontaneous jujube ("Dobrogea olive") present in Dobrogea area, Southeast part of Romania (Stănică, 2019). The purpose of this research paper was to establish a proper sterilization protocol for jujube explants, i.e. to find an appropriate growth medium and hormonal balance for their successful micropropagation.

MATERIALS AND METHODS

Plant material and sterilisation

The biological material was represented by *Ziziphus jujuba* Mill. cv. 'Huping Zao'. Jujube branches were harvested from the collection orchard of USAMV of Bucharest during dormancy (BBCH 00), transported to the laboratory and subjected to a fungicidal treatment by surface sterilization using 0.1% Thiophanate methyl 70% and 0.2% Mancozeb 80%. The branches were kept in water at room temperature (24 ± 2°C) until the time of onset of vegetation (BBCH 10-11/31), when the shoots were detached and subjected to two methods of sterilization under aseptic conditions. The first method was the one described by Soliman and Hegazi (2013), namely washing the explants with detergent for 5 minutes, then rinsing them with an antioxidant solution (1 g of ascorbic acid + 5 g of citric acid dissolved in 200 mL of sterile distilled water), followed by washing for 15 minutes with a 20% sodium hypochlorite solution, then one minute with a disinfectant solution (2 mL disinfectant + 200 mL sterile distilled water) and finally 6 rinses with sterile distilled water. The second sterilization method consisted of washing the explants for 20 minutes with water and detergent, followed by 20 minutes with 40% sodium hypochlorite solution, a rinse with alcohol (70%) for 10 seconds, respectively 6 rinses with sterile distilled water.

Culture medium and conditions

Explants were grown on MS culture medium (M5519, Sigma-Aldrich) supplemented with 3% sucrose (w/v) and gelled with 7 g L^{-1} agar. Different concentrations of growth hormones (Sigma-Aldrich) on 4 variants in the case of the initiation and establishment stage, respectively 3 variants in the case of the multiplication and transfer stage were used (Table 1). The pH of the initial medium was 4.6 after preparation, and adjusted to 5.8 before autoclaving (Raypa AES-8 for 20 minutes at 121°C) by adding 1M NaOH. After sterilization, the culture medium was distributed in sterile Erlenmeyer flasks in a laminar flow hood. After inoculation, the explants were incubated in a climatic chamber (Sanyo MLR351) at a temperature of 24 ± 2°C, 3000 lux light intensity and 70% humidity, with a photoperiod of 16 hours. After 40 days on initiation medium, explants were multiplied and transferred to MS culture medium using 3 variants of hormones (Table 1). The explants were carefully cut, brown spots and yellowed leaves were carefully removed, so that only perfectly healthy explants were inoculated on the new culture medium.

Table 1. The used growth regulators

Stage	Variant	Growth hormones (mg L^{-1})*				
		TDZ	BAP	IBA	GA_3	NAA
Initiation and establishment	A_1	0.25		0.1		
	A_2		0.5	0.1		
	A_3			1.5	0.1	
	A_4			2.0	0.1	1.5
Multiplication and transfer	V_1			2.0		
	V_2			2.0		0.5
	V_3			1.0		

*TDZ-thidiazuron; BAP–benzyl amino purine; IBA-indole-3-butyric acid; GA3-Gibberellic acid; NAA-1-Naphthylacetic acid

Statistical analysis

Measurements of shoot height (starting from the surface of the culture medium), shoot

diameter (expansion at the widest points taking into account the tips of the leaves) and number of leaves per shoot were made. The obtained data were processed in the IBM SPSS Statistics 26 program and represent the mean, standard deviation, the limits of variation and the coefficient of variability.

RESULTS AND DISCUSSIONS

Important in the case of disinfection of plant material from the natural environment, consists in the application of an appropriate fungicidal treatment. Hansika et al. (2017) used Captan 50%, 1.2 g L^{-1}, Thiophanate methyl 70%, 2 g L^{-1} and Chlorothalonil, 1.8 mL L^{-1} as a disinfectant for jujube explants, the best results being obtained by immersing shoots in Captan for 20 minutes with a 79.9% success rate. The treatment applied by surface sterilization in this study (0.1% Thiophanate methyl 70% and 0.2% Mancozeb 80%) had a success rate of 87.3%. Regarding the sterilization of explants, both used variants gave promising results with their survival percentage of 77% in the case of the first sterilization variant, while Soliman and Hegazi (2013) had an explant survival rate of 86% using the same protocol, respectively 92% in the case of sterilization option number two. Safarnejad (2015) mentions in his study the sterilization of jujube buds using 0.02% $HgCl_2$ for 3 minutes, 70% ethanol for 2 minutes and 30% NaOCl for 15 minutes. Also Yıldırım et al. (2015) sterilized jujube shoots by immersion in 3% NaOCl (v/v) for 18 minutes, followed by three rinses in sterile distilled water for 5 minutes. Another sterilization option with a yield of 65.5% is that described by Melyan et al. (2014) using 2% $Ca(ClO)_2$ for 15 min and 70% ethanol for 3 min. Khazaei et al. (2015) mention the use of 70% ethanol for 1 minute and 2% sodium hypoclorite for 25 minutes, followed by rinsing with distilled water for 25 minutes for jujube buds disinfection protocol.

The best variant in the initiation and establishment stage of the *in vitro* culture was the A_4 variant in terms of all three determined morphological characteristics, namely the height of the explants (with an average of 1.77 cm), the diameter of the shoots (with a value average of 1.58 cm) and the number of leaves (with an average of 5.67 leaves/shoot) (Table 2). This demonstrates the role of growth hormones used 2 mg L^{-1} IBA + 0.1 mg L^{-1} GA3 + 1.5 mg L^{-1} NAA compared to the other variants. Yildirim et al. (2015) had the best results using the combination of 0.1 mg L^{-1} TDZ + 0.5 mg L^{-1} BAP + 0.1 mg L^{-1} IBA + 0.3 mg L^{-1} GA_3 in the production of new shoots in *Ziziphus jujuba* multiplied *in vitro*. At the same time, Ma et al. (2012) confirmed the use of thidiazuron (along with $AgNO_3$ and NAA) as beneficial in the shoot regeneration process and Wang et al. (2013) the use of thidiazuron in combination with IBA in the process of new shoot emergence. Huo et al. (2007) mention IBA in a concentration of 0.2 mg L^{-1} along with 5 mg L^{-1} BA as being beneficial for the proliferation of jujube explants in the variety 'Gagazao'. The high coefficient of variability indicates large differences between the inoculated Erlenmeyer flasks within each variant. Herman (2015) mentions IBA and NAA as being among the most used auxins in tissue culture for cell division, callus formation, shoot growth and rooting. Thidiazuron is considered a frequently used cytokinin in the case of woody species (Huetteman & Preece, 1993) but with a lower incidence compared to BAP. Gibberellic acid (GA_3) was successfully used for jujube shoot elongation along with benzylaminopurine by Melyan et al. (2014).

Table 2. Results obtained during the initiation and establishment phase

Var	Descriptive statistics*	Shoot height (cm)	Shoot diameter (cm)	Number of leaves
A_1	Mean±SD	1.24 ± 0.63	1.48 ± 0.93	4.21 ± 1.55
	Range	0.30-3.00	0.10-3.80	1.00-7.00
	CV%	50.80	62.83	36.81
A_2	Mean±SD	1.28 ± 0.45	1.54 ± 0.97	3.83 ± 2.41
	Range	0.40-2.10	0.20-3.60	0.00-1.00
	CV%	35.15	59.74	62.92
A_3	Mean±SD	1.30 ± 0.76	1.15 ± 0.66	4.29 ± 1.81
	Range	0.30-2.70	0.50-2.30	1.00-7.00
	CV%	58.46	57.39	42.19
A_4	Mean±SD	1.77 ± 0.23	1.58 ± 0.72	5.67 ± 0.52
	Range	1.40-2.00	0.80-2.40	5.00-6.00
	CV%	12.99	45.56	9.17

*SD = Standard Deviation; CV% = Coefficient of variation

Hao et al. (2013) successfully used a hormone balance composed of thidiazuron and NAA in MS medium for callus culture and subsequent shoot emergence of jujube. The hormonal variant with thidiazuron in this study had the lowest values in terms of shoot elongation

(with an average of 1.24 cm) followed by the variant with BAP (with an average of 1.28 cm) in terms of the same characteristic using 0.25 mg L^{-1}. Zhou and Liu (2009) confirm the use of gibberellic acid as having an important role in the elongation of the shoots, respectively 0.5 mg L^{-1} in the case of 'Dongzao' shoots, results consistent with those obtained in this work, the two variants with GA_3 having the higher results regarding this characteristic (but in a lower concentration 0.1 mg L^{-1}).

Regarding the multiplication and transfer stage, although no root emergence was observed, the best effect on shoot height growth and leaf emergence and formation was 2 mg L^{-1} IBA, with mean values of 2.01 cm, respectively 5.30 leaves/shoot. V_2 was beneficial to shoot diameter expansion, respectively 2 mg L^{-1} IBA + 0.5 mg L^{-1} NAA, with an average of 1.71 cm. Yıldırım et al. (2015) suggest the use of 2 mg L^{-1} IBA as beneficial to root formation (with a percentage of 76.7% root formation in their study) (Table 3). In the present study the same concentration of IBA used had no impact on rhizogenesis formation. The high coefficient of variation indicates a high degree of variability within the determinations performed and between variants.

Table 3. The results obtained in the multiplication and transfer stage

Var	Descriptive statistics*	Shoot height (cm)	Shoot diameter (cm)	Number of leaves
V_1	Mean±SD	2.01±0.31	1.51±0.42	5.30±0.73
	Range	1.10-2.60	0.80-2.50	4-7
	CV%	15.42	27.81	13.77
V_2	Mean±SD	1.91 ± 0.37	1.71 ± 0.62	5.00±1.64
	Range	1.40-2.50	1.00-3.30	3-8
	CV%	19.37	36.25	32.80
V_3	Mean±SD	1.30 ± 0.55	0.64 ± 0.47	2.00±2.74
	Range	0.80-1.90	0.30-1.20	0-5
	CV%	42.30	73.43	137

*SD = Standard Deviation; CV% = Coefficient of variation

Hansika et al. (2017) used MS culture medium supplemented with 1.5 mg L^{-1} BAP and obtained a percentage of 96.66% in terms of shoot elongation, and the lowest values using TDZ 0.2 mg L^{-1} (3.33%), but with callus production. They also mention the lack of formation of new shoots in any of the two variants used and the lack of root formation on medium supplemented with IBA, results consistent with those obtained in this paper. Future research on other hormone combinations will be conducted to identify the best variant in terms of emergence and formation of new shoots and root formation respectively. The obtained results confirm the role and influence of hormones on the growth and development of jujube explants. Compliance with the work protocol, starting from the choice of explants, their disinfection, sterilization, inoculation and ensuring the environmental factors necessary for development, is the key to the success of the culture initiation and establishment stage. Aseptic conditions are the most important aspect of tissue cultures to avoid unwanted infections. The culture medium and the hormonal balance are the decisive factors in the appearance and development of certain morphological processes (differentiation, dedifferentiation, callus formation, shoot elongation, leaf growth, new shoot formation).

CONCLUSIONS

Growth hormones influence the growth and development processes of explants. MS culture medium using the combination of IBA, GA_3 and NAA was found to be beneficial during the initiation and establishment stage of the culture. Special attention must also be paid to the sterilization of the explants in order to have as high a percentage of success as possible. None of the hormonal variants caused the emergence of new shoots and the formation of roots. Further research on increasing the concentration of hormones but also the use of others will be carried out to establish a suitable protocol for the *in vitro* propagation of the Chinese jujube.

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