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Proliferation of *Juglans regia* L. by *In vitro* Embryo Culture

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Abstract: One of the new and important ways of plant propagation is micropropagation or tissue culture technique. Using this technique is preferred due to its high propagation of plants in short periods. In this research tried to find the optimal culture media for the *in vitro* embryo growth and proliferation of Persian walnut (*Juglans regia* L.). In the first stage of this study, the effects of MS, DKW, NGE, WPM culture media without growth regulators were studied and compared. In second stage, five concentrations of BAP (0, 0.1, 0.5, 1, 2 mg/l), in combination with five concentrations of IBA (0, 0.1, 0.2, 0.4, 0.5 mg/l) were applied. Experiments were carried out under conditions of 25°C, light/dark period of 16 and 8 h and a light intensity of 3000 lux. Results showed that the best growth percentage was obtained in NGE (61.1%) and the optimal treatment was (0.5 mg/l BAP, 0 mg/l IBA).

Key words: Proliferation · Embryo culture · *In vitro* · Walnut · Juglans

INTRODUCTION

Embryo culture is the sterile isolation and growth of an immature or mature embryo *in vitro*, with the goal of obtaining a viable plant. The first attempt to grow embryo of angiosperms was made by Hanning in 1904, who obtained viable plants from *in vitro* isolated embryos of two crucifers Cochleria and Raphanus. Embryo culture is now a well established branch of plant tissue culture. The underlying principle of embryo rescue technique is the aseptic isolation of embryo and its transfer to a suitable medium of development under optimum culture conditions. Indeed the most important of medium aspect of embryo culture work is the selection of medium necessary to sustain continued growth of the embryo.

Walnut (*Juglans regia* L. Juglandaceae) is an important temperate nut crop. It is rich source of proteins, fats, minerals and is a concentrated source of energy. Low percentage of seed germination and long propagation cycle are the main cause in application of the methods of plant regeneration from *in vitro* cultured embryos [1-3].

Because woody plants are still often very difficult to culture; many different types of media have been employed. We hope that by providing information regarding the physiological functions of the various media components, we will help the reader to design media more effectively. In particular, we wish to point out the importance of chemical interactions [4].

Embryo culture, in which embryos are excised aseptically and cultured on the medium, helps in overcoming the post fertilization barriers of hybridization and enables faster multiplication of plants. In addition, it also reduces the generation cycle in woody plants like walnut where seeds require stratification period of 2-3 months for germination [4, 5, 6].

The most problems of walnut micropropagation are: pollution inside explants, sensitivity to disinfectants, oxidation of phenolic component in explants, adapting to culture medium, rooting and difficulties related to the transfer to soil.

For micropropagation of Juglans spp. different culture media have been used, such as Driver and Kuniyuki, [1] (DKW), Murashige and Skoog (MS), Cheng (1975), Gamborg *et al.* (1968) and Lloyd and McCown (1981) (WPM), and NGE medium developed by Zamora S.A. (2006) with varying success [7].

In germination studies of *J. regia* L. RodrIguez used the culture medium K (h) with different benzylaminopurine (BAP) (0, 4, 20 and 40 mM) and indole butyric acid (IBA) (0, 0.4, 4 and 20 mM) concentrations for 5 and 7 weeks. The best treatment was 40 μM BAP without IBA, resulting in a germination percentage of 80% [8]. In other studies, such as Cossio and Minotta [9],

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different culture media and different BAP concentrations were compared, the most appropriate medium was MS [9]. With reference to the different BAP treatments, some morphological anomalies different to those found by Rodriguez [10] were discovered. These were thought to be due to the presence of the cotyledon [10]. However, subsequently, Rodriguez *et al.* [8], following new *in vitro* germination studies, found that these anomalies referred to by Cossio and Minotta [9] could not have been due to the presence or absence of cotyledons, but rather to other factors [8].

Different media have been used for the proliferation stage of the explants as indicated by Jay-Allemand [11], using a primary culture in half strength Knop's medium and a secondary one in Miller medium with 1 mg/l BAP [12] Revilla *et al.* [12] reported that the best growth regulator treatment is 1 mg/l BAP and 0.1 mg/l IBA [1], but Rodriguez *et al.* [13] suggested the addition of 2 mg/l kinetin to this treatment [13]. Some authors, like Saadat and Hennerty [14], analysed the factors which affect multiplication, comparing culture media, growth regulators and gelling agents and concluded that the optimal medium was DKW with 2.2 g/l of phytagel, 1 mg/l BAP and 0.01 mg/l IBA [14]. In view of all these references, it is necessary to determine the optimal *in vitro* conditions for our particular plant material, determining the optimum media for *in vitro* germination and the growth regulator concentration for the proliferation stage of the obtained explants.

MATERIALS AND METHODS

The nuts of two Persian walnut (*Juglans regia* L.), (walnut A and walnut B) (Table 1), were harvested on 15 September 2009 and washed in running tap water. Epicarp was removed and the remaining part of the fruit (nut) was sterilized by treating with 70% of ethanol for 5 min followed by washing twice with distilled water. Thereafter the disinfected stones were cracked by piercing a pointed forceps through the suture at the pedicel end and mature embryos were carefully excised and put in medium culture.

Experiment 1: Optimum Culture Medium for *in vitro* Growth of Embryo: The studied culture media (Table 2) were MS, NGE, DKW and WPM medium. All the media were prepared without growth regulators. Sucrose was used at 30 g/l as a carbon source and the gelling agent was 9 g/l of agar. The final pH of the solution was adjusted to 5.7 with 0.1 N NaOH. The culture conditions were 25 ±1°C and 3000 lux with a photoperiod of 16 h light and 8 h darkness. The duration of the study was 60 days cultivation. At the end of experiment the following parameters were measured.

Table 1: Walnuts Identification used in this research

	Location	Ground elevation(m)	Flowering	Shape of fruit
Walnut A	Around Ajabshir City Garden	1347	Fast Growing	Big and Cycle
Walnut B	Valley Kabodan-Ajabshir	2106	Late Growing	Small and Ellipse

Table 2: Salt composition (mg/l) of each culture medium

	MS	NGE	WPM	DKW
NH_4NO_3	1650	908	908	1416
KNO_3	1900	723	723	-
$CA(NO_3)_2.4H_2O$	_	2248	2248	1968
$CaCl_2.2H_2O$	440	699	699	149
K_2SO_4	-	-	-	1559
$MgSO_4.7H_2O$	370	2053	2053	740
KH_2SO_4	170	155	155	265
$MnSO_4.4H_2O$	22.3	22.3	22.3	33.5
$Na_2MoO_4.2H_2O$	0.25	0.25	0.25	0.39
$ZnSO_4.7H_2O$	8.6	8.6	8.6	-
$Zn(NO_3)_2.6H_2O$	-	-	-	17
KI	0.83	0.83	-	-
H_3BO_3	6.2	6.2	6.2	4.8
$CuSO_4.5H_2O$	0.025	0.025	0.25	0.25
$CoCl.6H_2O$	0.025	0.025	-	-
$NiSO_4.6H_2O$	-	-	-	0.005
$FeSO_4.7H_2O$	27.8	27.8	27.8	33.8
$Na_2EDTA.2H_2O$	37.3	37.3	37.3	45.4
Myo-inositol	100	100	100	100
Thiamino-HCl	0.1	0.1	1	2
Nicotinic acid	0.5	0.5	0.5	1
Pyridoxine-HCl	0.5	0.5	0.5	-
Glicine	2	2	2	2
Glutamine	-	-	2	-

- Percentage of root and leaf creator embryos,
- Root and no leaf creator embryos
- Leaf and no root creator embryos.

There were 6 replicates for each culture medium and for each replicates three embryos has been cultured in each Petri dish. Variance analysis and means comparison was carried out by Duncan's test at P=0.01 and 0.05.

Experiment 2: Optimum Growth Regulators Concentrations for the Embryos Growth: The best culture medium from experiment 1 was used for walnut A and added five concentrations of BAP 0, 0.1, 0.5, 1, 2 mg/l in combination with five concentrations of IBA 0, 0.1, 0.2, 0.4, 0.5 mg/l. The growth conditions were the same as

the previous experiment. The duration of the experiment was 30 days. At the end of experiment the percentage of the following parameters was measured:

- 1) Root and leaf creator embryos, 2) Root and no the leaf creator embryos, 3) Leaf and no the root creator embryos, 4) Root and callus creator embryos.

There were 5 replicates for each treatment and in each replicates three embryos has been cultured. Variance analysis and means comparison was carried out by Duncan's test at P=0.01 and 0.05.

RESULT AND DISCUSSION

Optimum Culture Medium for Growth of Walnut Embryos: The results showed that genotype influenced optimal culture medium for growth walnut embryo from different trees. The results are shown in Tables 3, 4 and Figs 1 and 2. Mean values followed by different letters are significantly different at 5%; Duncan's multiple range tests by using SPSS software.

The results of embryo culture on media DWK, NGE, MS and WPM without growth regulators to determine the optimal media for growth using Walnut embryo from two walnuts of trees(Table 3 and Fig 2a,b) showed that the best medium for the embryos from walnut A was NGE medium with

- 61.1 percent of embryos creating root and leaf,
- 22.22 percent of embryos creating root and no leaf,
- 16.66 percent of embryos creating leaf no root.

While the best medium for the growth of embryos for another tree (B) was DKW medium (Table 4 and Figs 1, 2) with

- 52.78 percent of embryos creating root and leaf,
- 41.67 percent of embryos creating root and no leaf,
- 5.56 percent of embryos creating leaf no root.

Also, Tables 3 and 4 showed that the response of embryos to different culture medium for root and leaf creating embryos, the responsibility percentage was reached to 100% with the two types of Walnut.

The effect of the culture medium on germination was marked. Looking at the results we can see the deep influence of the culture medium on the *in vitro* germination of embryos.

Table 3: Mean values comparison of embryos growth from walnut A by using SPSS software

Medium	Root and leaf %		Root and no leaf %		Leaf and no root %	
DKW	22.22±7.02	b	22.22±7.02	b	33.33±0	a
WPM	0±0	c	58.33±10.31	a	0±0	c
NGE	61.1± 10.24	a	22.22±7.02	b	16.66±7.45	b
MS	22.22± 7.02	b	61.11±10.24	a	0.00	c

Table 4: Mean values compare of embryos growth from walnut B by using SPSS software

Medium	Root and leaf %		Root and no leaf %		Leaf and no root %	
DKW	52.78±6.69	a	41.67±5.69	c	5.56±0.55	a
WPM	0±0	c	61.11±5.55	b	5.56±0.55	a
NGE	22.22±7.02	b	0±0	d	5.56±0.55	a
MS	0±0	c	94.45±5.55	a	5.56±0.55	a

The genetic basis of variability in tissue culture response and morphogeneis is most likely due to differences in hormone metabolism within the explant and endogenous hormone levels that is established by the level of gene expression for individual hormones by the genotype.

The release of phenolic component into the culture medium and the embryo browning appeared in the WPM media. This problem did not exist with the other media. These embryos did not germinate during the period of the study. Development of the embryos classified as ‘‘whole plant’’, ‘‘stem’’, ‘‘root’’, was variable depending on the culture medium used. For walnut A, Figure 1 and Figure 2 shows how a large majority of the embryos evolved into complete plants, For walnut A, NGE being the medium where the highest number of plants was obtained (61.1%), followed by MS and DKW, with 22.2% and 22.2% respectively. For walnut B, DKW being the medium where the highest number of plants was obtained (52.78%), followed by NGE with 22.2%. The greatest number of leave was found in both the NGE and DKW media, for walnut A and B respectively.

Taking into account all the variables studied, the most appropriate culture medium for the *in vitro* germination of the seedlings was the NGE medium, For walnut A and the DKW medium For walnut B.

Different ions are active agents in the culture medium not their combination. So to compare the two medium, review of total ions concentration in two different medium is appropriate. To compare two different medium, number of mg/l is required to be calculated in mM and then determine the final concentration of each ion [15].

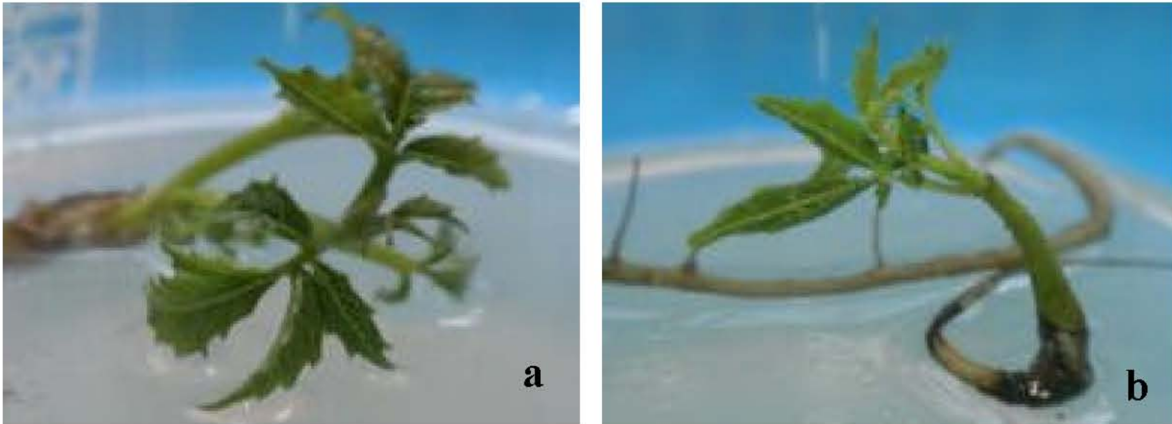

Fig. 1a and b: Embryos Growth for walnut A in NGE medium

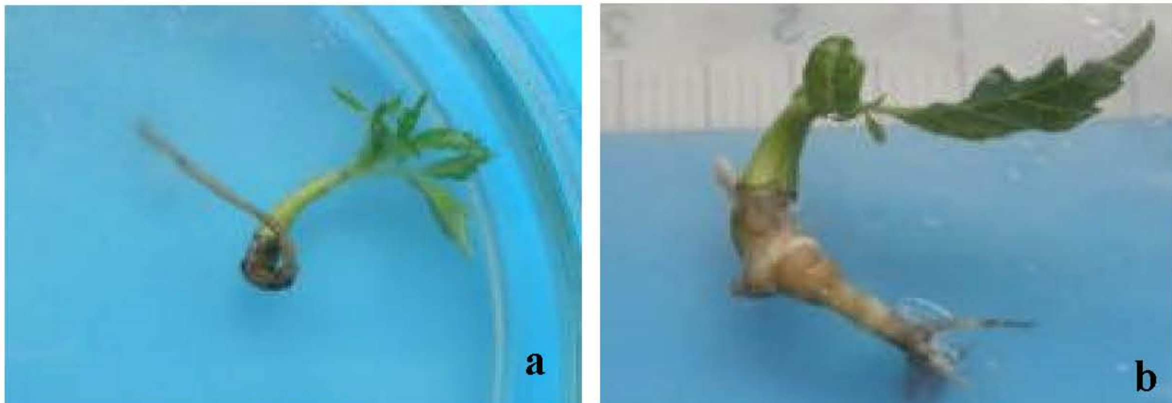

Fig. 2a and b: Embryos Growth for walnut B in DKW medium

Considering that minerals macroelements can be a limit factor for growth of walnut explants [16], comparing the amounts of macroelements in culture medium shows that WPM medium is infirm medium than other mediums. Medium NGE have Ca^{2+}, Cl^{-}, Mg^{2+} and So_4^{2-} more than MS medium, but less K^{+}, NH_4^{+} and NO_3^{2-} and comparing to DKW medium, NGE have more Ca^{2+}, Cl^{-}, Mg^{2+} and NO_3^{-} and K^{+}, NH_4^{+} and So_4^{2-} is less. Considering the results obtained by Bosela, M.J. and Michler [17], Vitrification in medium with low salt concentration (½ DKW, WPM) with a higher frequency observed comparing to medium with high salt concentration culture (MS, DKW) [17]. In medium NGE for walnut A and DKW for walnut B which are medium culture with high salt concentration, Vitrification not observed. But, in the second stage of experiments IBA and BAP treatments were applied, Vitrification caused death of some embryos which can be cause of Cytokinin. Researches of Amiri [16] Showed that the best response with concentrations of 1.5 times of macroelement minerals. Thus, DKW and NGE medium with respect to the high amount of macroelements were not faced Vitrification and can give enough mineral to embryos and not create growth restriction. Similar results by Sanchez *et al.* [7], Bourrain *et al.* [18], Tetsumura *et al.* [19], Bosela and Michler, [5], Roschke [20] for reproduction walnut *J. regia* by using DKW medium has been reported [4, 19, 14].

The results of embryo of walnut A and B in four medium showed that growth and organogenesis walnut embryos cultured are different. It means the type of embryo cultivated is affect. This difference can be due to the difference between endogenous hormone levels and their ratio and also can be due to physiologically and different genotype of embryos. Genotype effects on proliferation walnut (*J. regia*) researched by Scaltsoyiannes *et al.* [21]. It was observed that different genotypes have different needs for growth and there were significant differences in proliferation rate of different 12 genotype Persian walnut. Effect of genotype in determining the medium was observed as well [21].

Fig. 3: Embryos growth in concentrations (a) 0 mg/l IBA and 0.5 mg/l BAP, (b) 0.1 mg/l IBA and 0.5 mg/l BAP, (c) 0.1 mg/l IBA and 2 mg/l BAP, (d) 0.4 mg/l IBA and 2 mg/l BAP.

Table 5: Effect of various concentrations of IBA in combination with BAP on growth of Walnut cultured two months on NEG medium

Hormone					
IBA (mg/l)	BAP (mg/l)	Root and leaf %	Leaf and Callus %	Root and no leaf %	Leaf and no root %
0	0	50± 0 a	0 ± 0 e	30±12.24 d	20±12.24 de
0.1	0	30±12.24 b	0 ± 0 e	0 ± 0 e	0 ± 0 e
0.2	0	20±12.24 c	0 ± 0 e	60±10 c	0 ± 0 e
0.4	0	0± 0 d	0 ± 0 e	100±0 a	0 ± 0 e
0.5	0	0± 0 d	0 ± 0 e	80±12.24 b	0 ± 0 e
0	0.1	0± 0 d	0 ± 0 e	0 ± 0 e	40±10 cd
0.1	0.1	0± 0 d	0 ± 0 e	0 ± 0 e	30±12.24 d
0.2	0.1	0± 0 d	0 ± 0 e	0 ± 0 e	0 ± 0 e
0.4	0.1	0± 0 d	0 ± 0 e	0 ± 0 e	0 ± 0 e
0.5	0.1	0± 0 d	0 ± 0 e	0 ± 0 e	0 ± 0 e
0	0.5	0± 0 d	0 ± 0 e	0 ± 0 e	100±0 a
0.1	0.5	0± 0 d	20±12.24 de	0 ± 0 e	80±12.24 b
0.2	0.5	0± 0 d	0 ± 0 e	0 ± 0 e	60±10 bc
0.4	0.5	0± 0 d	0 ± 0 e	0 ± 0 e	40±10 cd
0.5	0.5	0± 0 d	0 ± 0 e	0 ± 0 e	0 ± 0 e
0	1	0± 0 d	20±12.24 de	0 ± 0 e	60±10 bc
0.1	1	0± 0 d	60±10 bc	0 ± 0 e	30±12.24 d
0.2	1	0± 0 d	40±10 cd	0 ± 0 e	0 ± 0 e
0.4	1	0± 0 d	60±10 bc	0 ± 0 e	0 ± 0 e
0.5	1	0± 0 d	20±12.24 de	0 ± 0 e	0 ± 0 e
0	2	0± 0 d	20±12.24 de	0 ± 0 e	0 ± 0 e
0.1	2	0± 0 d	80±12.24 a b	0 ± 0 e	0 ± 0 e
0.2	2	0± 0 d	60±10 bc	0 ± 0 e	30±12.24 d
0.4	2	0± 0 d	90±10 ab	0 ± 0 e	0 ± 0 e
0.5	2	0± 0 d	20±12.24 de	0 ± 0 e	60±10 bc

Optimum Growth Regulator Concentrations for the Embryos Growth: The effect of growth regulator concentrations on the embryos growth is shown in Fig. 3. The results of analysis variance embryo growing percent in hormone treatment with different concentration are shown in Table 5. Mean values followed by different letters are significantly different at 5%; Duncan's multiple range tests.

The results of embryo culture in optimum culture medium (NGE) containing indole butyric acid (IBA) and benzylaminopurine (BAP) with different concentrations to obtain the optimum growth regulator concentrations for the embryos growth (Table 5) showed that the best concentration for growth of cultured embryo are: concentrations (0 mg/l IBA and 0.5 mg/l BAP) with 100 percentage of leaf and no root creating embryos (Fig 3a). Meanwhile concentrations (0.1 mg/l IBA and 0.5 mg/l BAP) gave 80 percentage (Fig 3b) with 20 percentage of leaf and callus creating embryos, concentrations (0.1 mg/l IBA and 2 mg/l BAP) with 80% of leaf and callus (Fig 3c). On the other hand concentrations (0.4 mg/l IBA and 2 mg/l BAP) gave 90% of leaf and callus (Fig. 3d).

Also, when cultured the embryos on NEG - free growth regulators, it gaves 50% of root and leaf creating embryos. Similar results by Sanchez-Zamora [7] using the WPM medium and hormone treatment 0 mg/l IBA and 0.5 mg/l BAP (81%) and by Kaur *et al.* [2] using MS medium and hormone treatment 0.5 mg/l BAP and 2 mg/l giberellic acid (GA3) with (6.66%) [2, 7].

The results of embryo walnut in different hormonal treatments show that the presence of BAP and IBA are essential in the medium for growth and organogenesis. It shows the importance of hormones and the interaction of on growth and organogenesis of cultivated embryos. high auxin:cytokinin ratios stimulated the formation of roots, low auxin:cytokinin ratios led to the formation of shoots. At intermediate levels the tissue grew as an undifferentiated callus [22].

In current research, embryos cultivated in NGE medium without growth regulators, after two months and frequent subculture only few leave were growth. So it seems likely endogenous hormone levels are less and application of these hormones for the induction of rapid growth and organogenesis is essential. Accordingly, we used different concentrations of IBA and BAP in experiments.

In this study, lack of BAP hormones and existing of IBA causes embryos rooting and the presence of Cytokinin and auxin cause shoot and stem for embryos. This result shows that each hormone specifically with regard to its special structure affects on growth and organogenesis and concentration and ratio of concentration effect in determining growth and the presence of all compounds of hormones by other hormones control.

The results obtained regarding hormone treatments in this research same as Sanchez-Zamora *et al.* [7] results.

CONCLUSION

Walnut is considered to be one of the most recalcitrant species *in vitro*. It is necessary to determine the optimal culture conditions to establish it in a culture medium and proliferate. The effect of the saline composition of the culture medium on *in vitro* germination was remarked. NGE is the most appropriate culture medium. These Results are necessary in order to approach the programs of genetic breeding of this species successfully. With respect to the proliferation stage of the cluster, differences have also been observed between the treatment studies with 0.5 mg/l BAP is the optimum treatment.

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