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Short communication

Application of Embryo Rescue Technique in *Juglans regia* L. x *J. nigra* L. Hybridization

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Abstract

Embryo culture were used to produce inter specific walnut hybrid plants between *Juglans nigra* L. and *J. regia* L. Developed ovaries were collected from *J. rejia* female flowers which were pollinated with *J. nigra* pollen grain by high dusting on a mature tree. In order to isolate embryos, exocarps were aseptically removed. Isolated embryos from ovules at different developmental stages were transferred to the different kind of growth-regulators hormone free media (MS, Half-MS, MS and Half-MS supplemented with 1 mg/l BA +0.1 mg/l NAA) for embryo development and germination. There was a significant difference between media for embryo germination α= 0.05 level. Highest percentage of embryo germination was observed in MS hormone free medium (13.7%). Because of long period required for embryo development, isolation of embryo less than 45 days old embryos were impractical. There were a highly significant difference between age of embryos for embryo germination α= 0.01 level. Embryo with more than 45 days old germinated and produced plants on all applied media. Highest germination rate were observed on cross-pollinated embryos which was isolated from 90 old ovaries. Before being transferred to the potting soil and green-house, plantlets sub cultured in the same medium within jars. Thirteen successful acclimatized plantlets were transferred to the field.

Key words: *Juglans nigra*, *Juglans regia*, *In vitro* hybridization, Embryo rescue, Embryo maturation

Introduction

Walnuts are very valuable trees for nuts and wood production but we still needed to produce available logs for veneer industry. It is also used in the pharmaceutical and food industries in the country. Different breeding approach has been used for wood and nut improvement. Inter specific hybridization can be used as a solution to meet high wood quality and high yield of edible nuts. Despite of some adverse effects, inter specific hybridization still remains a powerful tool to generate novel genetic combinations in plant breeding programs [1]. These concerns have been particularly relevant for a group of economically important *Juglans* L. species for which inter specific hybridization events are possible but uncommon and hybrids are known to vary in their vegetative vigor and fertility [2]. The Juglans genus consists of 21 species traditionally divided into four distinct sections: (1) Sect. *Dioscaryon* Dode ex W.E.Manning consisting solely of the highly valuable commercial Persian walnut species (*J. regia*) which is native to the temperate regions of the world for its high quality wood and edible nuts, (2 Sect. *Rhysocaryon* Dode ex W.E.Manning including 16 North and South American Juglans species such as the valuable hardwood species *J. nigra* (black walnut), (3) Sect. *Cardiocaryon* Dode ex W.E.Manning including three species all native to East Asia, *J. ailantifolia* Carrière, *J. cathayensis* Dode, and *J. mandshurica* Maxim., and (4) Sect. *Trachycaryon* Dode ex W.E. Manning consisting only of the North American species *J. cinera* L. [3]. Many of these species are capable for hybridizing, but the hybridization rate seems to reflect the phyllogenetic relationships, with intra-sectional crosses more successful than inter-sectional crosses [4]. In

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general, the black walnuts (*Rhysocaryon*) are unable to hybridize with species of sections *Trachycaryon* and *Cardiocaryon* with the exception of *J. nigra* and *J. ailantifolia* [3]. In particular, in the last two decades renewed interest from both industry and academic researchers in the use of *J.×intermedia* Jacques (*J. nigra×J. regia*) for rootstock and/or timber production has been observed in many countries [5,6]. *J. nigra* and *J. regia* are two phyllogenetically divergent walnut species. Nowadays, both of species are widely cultivated throughout the temperate regions of the world. [3]. They are wind-pollinated, monoecious, and dichogamous species with the same number of chromosomes (2n=32).

Although hybridization between Persian walnut (*J. regia*) and black walnut (*J. nigra*) can be used and hybrids between these two species have better growth and wider adaptability but due to post-zygotic barriers which are expressed in the endosperm and embryo abortion has been often failed [6]. Many attempts have been done to interbreed the two species producing populations with characteristics intermediate between the two species. But there are some reproductive mechanisms that may prevent successful natural hybridization. *In vitro* embryo rescue techniques has been effectively used to overcome post zygotic barriers and developing inter-specific hybrids in many trees for breeding programs [7-9]. Therefore we used the embryo culture technique as an auxiliary method in the inter-specific hybridization of *J. nigra*. and *J .regia* to rescue hybrids..

Material and Methods

Artificial pollination was carried out between *J. regia* and *J. nigra* in one direction. Pollen grains of *J. nigra* were collected in March from the Forest research station of shast-kola belong to University of Agriculture and Natural Resource science of Golestan. The female flower of *J. regia* tree covered with transparent paper before artificial pollination with *J .nigra* pollen grain.

Artificial pollination of *J. regia* was carried out using high dusting pollen grain on stigma of selected female flower in a mature tree. A control pollination was made on stigma of covered female flower on the same tree. Immature fruits (intact with endocarp,) still attached to the branches, were collected and surface disinfected under tape water running for 12 hours and 12 min in a 2.6% solution of calcium hypochlorite followed rinses in sterile de ionized water. To rescue fertilized embryos, exo carp was removed with a knife and embryos isolated aseptically. Embryos at the different developmental stages{(45, 60, 70 and 90 days after pollination (DAP)}, transferred to growth-regulators hormone free media (MS, Half-MS, MS and Half-MS supplemented with 1 mg/l BA plus 0.5 mg/l NAA growth regulator hormone) supplemented with activated charcoal in 10*6 mm jar dishes. Collected data were analyzed according to the Factorial RCBD (randomized complete block design) experimental design using SAS System. The tested medium for the embryo germination and plantlet development contained a 50% concentration of MS inorganic salts with Fe-EDTA 10-M and PH adjusted to 5.8. Two concentrations of sucrose (0.06M and 0.17M) were used. Media was autoclaved for 20 min at 120C and then dispensed 40 ml in each sterile jar. Cultures were incubated at 24 °C under a 16 h photoperiod with light provided by cool white 40-watt fluorescent lamps (4000-5000 lux) .plantlets 1-2 cm in height were transferred to jars containing the same medium and kept for two months under the same conditions before being acclimatized.

Results and Discussion

Germination of embryos was observed when isolated embryos were placed on the surface of all applied agar medium (MS hormone free, Half-MS hormone free, MS and Half-MS media supplemented with 1 mg/l BA plus 0.5 mg/l NAA growth regulator hormone). Analysis of collected data indicated that significant differences were observed between age of isolated embryo and between four different used culture media at α=0.01 and 0.05 level respectively (Table 1). The highest embryo germination appeared on MS medium supplemented with 1 mg/l BA plus 0.5 mg/l NAA growth regulator hormone (21.111%) and had a significant differences with others nutritive treatments at α=0.05 level using Duncan multiple range test (table 1 and 2). Simplify MS hormone free medium were used for embryo rescue in poplar and willows intra specific and inter specific hybridization [1, 7-9]. Different kinds of media and their complement were used by several authors for intra, inter -specific tree hybridization [1, 10-13] and [7]. In contrast to the report of Payghamzadeh [14], which white- cream, loose and friable callus were induced on walnut embryo culture using BAP and indole-3-butyric-acid (IBA),

Table 1 Analysis of variance for the effect of culture media and age of embryo on number of germinated embryos

Source of variation	DF	SS Sum of Square	MS Mean Square	F value	Pr>F
Block	4	1.44014534	0.36003633	27.51	<.0001
A(age of embryo)	3	0.18973350	0.06324450	4.83**	0.0044
B(culture media)	3	0.10564525	0.03521508	2.69*	0.0542
A*B	9	0.10939987	0.01215554	0.93	0.5071
Error	60	0.78524025	0.01308734		
Total	79	2.63016420			

**= Highly significant difference at 0.01% level
*= significant differences 0.05% level

Table 2 Mean effect of independent variables (media) on *Juglans nigra* x *J. regia* embryo germination and significant differences

Duncan Grouping		Mean Germinated embryo (%)	Culture Media	Treatment (B)
-	A	21.111	MS+H	4
B	A	20.403	Half-MS+H	3
B	-	19.567	MS	2
B	-	19.239	Half-MS	1

The different letters are significant at α=0.05 level

Table 3 Mean effect of independent variables (age of embryo) on *Juglans* and *nigra* x *J. regia* embryo germination and significant differences

Duncan Grouping		Mean Germinated embryo (%)	Age of embryos DAP(Day after pollination)	Treatment (A)
-	A	21.162	90	4
B	A	20.751	65	3
B	C	19.359	55	2
-	C	18.787	45	1

The different letters are significant at α=0.05 level

in our study, auxins and cytokinins at proper combination and concentration have been caused direct embryo germination instead of callus induction. In spite of few authors who used hormone growth regulator free media, for embryo germination, media supplemented with different complement such as vitamin, hormone growth regulator and natural carbohydrate sources was necessary to embryo development and germination [10, 11]. Inorganic elements of MS and WPM medium did not have a significant effect on the *in vitro* development of isolated germs in cherries and plums, but MS and WPM supplemented organic components (6-BA at either 0.5 or 1.0 mg/l) supplemented in MS and WPM media caused successful indirect embryogenesis [14].The age of embryo (number of days post pollination) affected the ability of the embryos to germinate in culture media. Due to different period require for embryo development in different species, exact date of embryo isolation is necessary to capture mature embryo. Compare to mature embryo, immature embryo for embryo development and germination need culture media with more complete supplementation of vitamin and hormones. Different embryo culture approaches were used in *Juglans* species for different purposes such as: micro propagation through mature embryo [14] and immature embryo [15], inter-specific hybridization through immature cotyledons [16] and embryo multiplication of (*J. nigra* × *J. regia*) for micropropagation [16]. Due to long term embryo development, embryo less than 45 days old embryo did not possible to isolated and germinated. There were highly significant differences between 45, 60, 70 and 90 days old embryo (days after pollination) at 0.01% level. Ninety days old embryo produced highest germination (21.126%) and showed

significant differences with other age treatment using Duncan Multiple Range Test (Table 3).

In spite of adding activated charcoal to the media, an average of 33.7% cultured embryos became necrotic and discarded. The same observation was reported by Payghamzadeh [14], for immature cotyledons of walnut (*Juglans regia* L.) culture. Malformed ovules with abnormal plantlets were observed in 15 days cultured and died later. The same results were observed in ovary, ovule and embryo culture of many species such as poplar [1,9], cherry [17] and peach [18].

References

1. Jafari MA, Modir-rahmati A. Production of *Populus euphratica* Oliv. × *P. alba* L. hybrid poplars through ovary and ovule cultures. Plant Genetic Newsletter. 2000:122:13-15.
2. Dandekar AM, Martin LA, McGranahan GH. Genetic transformation and Foreign gene expression in walnut tissue. J. of American society for Horticultural Scince 1988:113;945-949.
3. Manning, WE. The classification within the Juglandaceae. Annals of Missouri Botanical Garden. 1978:65: 1058-1087.
4. McGranahan GH, Tulecke W, Arulsekar S. and Hansen JJ. Intergeneric hybridization in Juglandaceae: *Petrocarya sp*. x *Juglans regia*. J of American society for Horticultural Scince. 1986:111;627-630.
5. Clark J, Hemery G. and Savill P. Early growth and form of common walnut (*Juglans regia* L.) in mixture with tree and shrub nurse species in southern England. Forestry. 2008:81:631-644.
6. Woeste K. Heartwood production in a 35-year old black walnut progeny test. Can J Forest Res. 2002:32:177-181.
7. Jafari MA, Modir-rahmati A, Tavesoli A. Application of ovary and ovule culture in *P. alba* L x *Populus euphratica* OLIV hybridization. Silvae Genet. 1998:47:5 -6.
8. Ramming DW. The use of embryo culture in fruit breeding. *HortScience* 1990:25:393-398.
9. Kalagari M, Jafari MA. Tabari M, Hosseini : Intraspecific hybridization of *Populus euphratica* Oliv. Using in vitro technique. J of Sci Islamic Republic of Iran. 2004:15:109-122.
10. Li W, Wang R, Zhu X. On the embryo development and ovule culture of interspecific hybrids between *Populus simonii* and *P. pyramidalis* Barkh. Acta Bot Sin. 1983:25:409-417.
11. Kouider M, Skirvin R M and Saladin K P. Culture immature embryo of *Populus deltoides In Vitro*. Can J For Res. 1984:14:965-958.
12. Li W, and Li J. *In vitro* culture of hybrid ovules in *Populus*. Sci. Sliv. 21:339-346.
13. Raqiun C, and Trousard L. *In vitro* ovary embryo culture as a tool for poplar hybridization Can J Bot, 1993:71:1271-1275
14. Payghamzadeh K. Somatic embryogenesis from immature cotyledons and meristemic culture of walnut (*Juglans regia* L.). The MSc thesis. College of agriculture, Dep of Plant breeding and Biotechnology, University of Agricultural and Natural Resources of Sari, Iran. 2008:pp 48-77.
15. Dumanoglu H. Dessiccation using saturated salt solutions and improvement germination rate of walnut (*Juglans regia* L.) somatic embryos. Turk J Agric For. 2000:24:491-498.
16. Cornu D, Jay-Allemand C. Micropropagation of hybrid walnut trees (*Juglans nigra* × *Juglans regia*) through culture and multiplication of embryos. Ann. Sci. For 1988:46:113-116.
17. Kukharchyka N, Kastrickaya M. Embryo rescue techniques in *Prunus* L. breeding. Journal of Fruit and Ornamental Plant Research Vol. 14 (Suppl. 1), 2006.
18. Pinto ACQ, Rogers SMD, Byrne DH. Growth of immature peach embryos in response to media, ovule support method, and ovule perforation. Hort Science. 1994:29:1081-1083.