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ORIGINAL ARTICLE

Investigation of the Effects of Growth Regulators on Callus induction in *Juniperus excels* L.

Hassan Baravardi[1], Gholam Ali Ranjbar[2], Sodeh Kamali Farah Abadi[3]
[1]MSc Student of Plant Breeding, Sari Agriculture Sciences and Natural Resources University (SANRU), Sari, Iran
[2]Associated Professor, Sari Agriculture Sciences and Natural Resources University (SANRU), Sari, Iran
[3]Cytogeneticlabratoary staff, Sari Agriculture Sciences and Natural Resources University (SANRU), Sari, Iran
[1*]*Corresponding Author*, Hassan Baravardi
Email: h.baravardi@yahoo.com

ABSTRACT
Juniperus L. (Cupressaceae) is a genus of evergreen shrubs or trees and the second most diverse of the conifers, with some 67 species in the world occurring from seaside level to above the tree-line (Adams 2004). In recent years, plant tissue culture technology has become a powerful tool for in vitro propagation of many plant species. In present research diameter of induced callus were analyzed to determine the appropriate culture medium. This study was performed on fall of 2014 in a complete randomized design with three replication. The MS culture medium containing NAA with Kin and 2.4-D with Kin hormones. ANOVA statistical analysis showed significant differences at 1% level. Highest callus diameter belong to medium containing 4 mgl^{-1} 2.4-D and 0.2 mgl^{-1} Kin, also maximum callus diameter was belonged to 4 mgl^{-1} NAA and 0.2 mgl^{-1} Kin. The highest percentage of callus induction (73%) was belonged to medium containing 3 mgl^{-1} 2.4-D and 0.2 mgl^{-1} Kin. Therefore, hormones concentrations used are able to induce callus in Juniperus species.
Keywords: *callus, diameter, hormones, Juniperus excelsa L.*

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INTRODUCTION

Juniperus L. (Cupressaceae) is a genus of evergreen shrubs or trees and the second most diverse of the conifers, with some 67 species in the world occurring from seaside level to above the tree-line [1]. Junipers are long-lived trees which sometimes live up to 2000 years. This genus is represented in Iran by five species, namely, *Juniperuscommunis* L., *Juniperusexcelsa*M. Bieb., *Juniperus foetidissima* Willd. ,*Juniperus oblonga* M. Bieb and *Juniperussabina*L. *Juniperusexcelsa* occurs in the Mediterranean region, SE Europe, Caucasus, Iran, Iraq and the Arabian Peninsula. It is taxonomically the most difficult and the most common juniper species in Iran. It can be found almost in all alpine regions of Iran including the Alborz and Zagros mountain chains and higher elevations of SE Iran [2,3]. It survives under harsh climatic conditions of mountain rocky escarpments, and is considered as a unique precursor tree species in such growth habitats. Members of local communities in Iran have likely been using *J. excesla* for various purposes from long times ago. However, traditional knowledge regarding the use of *J. excels* by indigenous communities in the country has not yet been fully documented. The present study aims to obtain better understanding of the traditional knowledge of rural people of Iran about *J. excelsa*.

Juniperus excelsa(syn. *Juniperuspolycarpos*K. Koch & *Juniperus macropoda* Boiss. var. *polycarpos*) is an evergreen tree or occasionally a shrub, up to 20-25 m tall, with a pyramidal or broad crown. Juvenile leaves are ternate, acicular, while mature leaves are scale-like, ovate rhombic, light green or yellowish-green. Male strobili are solitary, terminally located on ultimate branch lets. Female cones are mostly solitary and auxiliary, sub-terminally located on ultimate branch lets, surrounded by green leaves or bracts, purplish-green to blue colored.

Geographical Distribution and Habitat of J. excelsa in Iran

Juniperus excels is generally a higher altitude taxon. It occurs throughout the hills and mountains of remote areas in Iran. The Alborz mountain chain in north, the Kopet-daghmountains in the northeast, the Zagros mountain chain in the west and southwest, the Jebale e Barez, and Makran Mountains in south and southeast of Iran comprise its natural habitats in the country. It becomes increasingly rare southwards, along the latter mentioned Mountains. The Juniper forest is the only sources of fuel and timber for the local residents of the area due to which extensive areas have been cut over. The forest is threatened due to die back disease, dwarf mistletoe and a large number of other biotic and abiotic factors [4]. Its berries and oil are used for medicinal purposes. These forests are also heavily used for grazing of sheep and goats, especially during the summer. The growth rate of tree is very slow, seeds have a very low potential of regeneration, if saved from people and predators. Khattak and Sheikh [5] worked on dry-zone a forestation in the Juniper forests of Baluchistan. Javed *et al*., [6] first reported *in vitro* cultivation of *J. excelsa,* however, they were unable to achieve much success. Negussie [7] successfully tried *in vitro* induction of multiple buds on excised cotyledons of *J. excelsa*. Conventional breeding of woody trees is a slow and difficult process due to elevated levels of heterozygosity and extended regeneration cycles [8]. Micropropagation is an alternative method of vegetative reproduction. *In vitro* propagation techniques in forestry are useful for mass reproduction of superior trees found in nature, various methods of micropropagation, auxiliary shoot proliferation has been found to be the most successful for achieving plantlet regeneration from mature forest trees [9]. Therefore, to overcome the problems of deforestation and for conservation and rehabilitation of Juniperu species, present study was conducted to develop a method for rapidly multiplying protocol for large scale micro-propagation.

MATERIALS AND METHODS

Callus cultures were initiated from lateral buds cuttings obtained from 8-year-old plants. The lateral buds were surface sterilized as shoots were put for (i) 10 min in runner water; (ii) 5 min in washing liquid solution, (iii) 30 min in sterile water; (iv) 15 min in fungicide, Benomil 2% followed by washing with sterile water, (v) 10 min in 25% sodium hypochlorite (NaOCl) detergent, followed by four times washing with sterile water, and finally, (vi) 30 sec in 70% ethanol followed by washing with sterile water.

Culture Condition

MS basal medium [10] supplemented with 3 % (w/v) sucrose and 0.7 % (w/v) agar was used for subsequent experiments. The pH of the medium (supplemented with respective growth regulators) was adjusted to 5.7-5.8 with 1 N NaOH or 1 N HCL before galled with 0.7 % (w/v) agar (Merck, German). The medium was dispensed into autoclaved at 105 kPa and 121ºC for 20 minutes. The lateral bud explants were inoculated on culture medium in 8 cm petry dishes, containing 20 ml of medium. All cultures were incubated at 25 ± 2ºC under dark conditions and subcultures were done within 30 days.

Organogenic Callus Induction

Lateral buds segments from shoots of 8-year-old plants were used as explants and placed on callus initiation medium which contained MS salts [10], vitamins supplemented with diverse concentrations of 2,4-D (0, 2, 3, 4 mgl^{-1}) and Kin (0, 0.1, 0.2 mgl^{-1}) alone or in combination and also medium which supplemented with diverse concentrations of NAA (0- 2- 3- 4 mgl^{-1}) and Kin (0- 0.1- 0.2 mgl^{-1}) alone or in different combinations of Kin and NAA for callus induction.

RESULTS AND DISCUSSION

Percentage of callus induction

In vitro Callus formation depended on both amounts and types of hormones added to medium. Analysis of variance of treatments between 2,-D and Kin showed that medium treated with 2,4-D and Kin had significant effects on callus induction of the used explants in 1% and 5% probability levels, respectively (table 1). However, interactions of these hormones were not significant. Mean comparison of data indicated that treatment containing 3 mgl^{-1} 2,4-D and 0.2 mgl^{-1} Kin had the highest percentage of callus induction (73%). There is no significant difference between concentrations 0.2 and 0.1 $mgl^{-1}$of Kin. Analysis of variance of treatments between NAA and K in demonstrated that use of Kin and NAA and interaction of them have significant effects on callus induction in 1% probability level (table 2). In this hormonal treatment like 2,4-D the greatest amount of calls formation occurred in medium containing 3 mgl^{-1} NAA and the lowest callus formation occurred on free hormone medium treatment. Explants treated with 0.2 and 0.1 mgl^{-1} Kin showed significant differences of callus formation, as treatment 0.2mgl^{-1} produced the most callus mass. According to mean comparisons results, in interaction NAA with Kin, the highest callus formation rate (60%) was achieved on 3 mgl^{-1} NAA with 0.2 mgl^{-1} Kin. Also, results indicated that by increasing in concentrations of growth regulators up to 3 mgl^{-1} callus formations were increased, however, higher concentrations callus formation showed reduction. Auxin is discussed as one of the most important growth regulators to start distinction or lack of distinction. Researchers believed

that increasing in hormonal concentrations higher than optimal *in vitro* have inhibitory effects on internal hormone explants and may decrease callus production [11,12].

Callus diameter

Analysis of variance of treatments 2,4D and Kin showed that separate use of 2,4D and Kin had significant effects on 1% and 5% probability level, respectively. However, interaction between these two hormones was not significant. Mean comparison of data indicated that treatment containing 4 mgl^{-1} 2,4D with 0.2 mgl^{-1} Kin had the highest diameter of callus induction. Analysis of variance of treatments NAA and Kin showed that use of both growth regulators Kin and NAA had significant effects on diameter of callus induction, but interaction of NAA and Kin was not significant. Treatment 4 mgl^{-1} NAA and 0.1 mgl^{-1} Kin produced the highest diameter of callus, while, there was no significant effect with treatment 3 mgl^{-1} NAA and 0.1 mgl^{-1} Kin . The lowest diameter of callus occurred on free of hormone medium.

Table 1) Analysis of variance 2,4-D with Kin effects on **traits**

Source	df	Mean **square**	
		Diameter	Percentage of callus induction
2,4-D	1	92.779**	2322.29 **
Kin	2	0.148*	279.13 *
Kin×2,4-D	3	10.077n.s	70.5 ns
Error	6	0.083	78.98

*, ** significant at the 0.05 and 0.01 probability levels, respectively. ns, not significant.

Table 2) Analysis of variance NAA with Kin effects on **traits**

Source	df	Mean **square**	
		Diameter	Percentage of callus induction
NAA	1	0.248**	1513.49 **
Kin	2	8.993**	492.66 **
Kin×NAA	3	0.083n.s	118.43 **
Error	6	0.040	14.52

*, ** significant at the 0.05 and 0.01 probability levels, respectively. ns, not significant.

Table3. The mean 2,4-D and Kin effects on trait.

Plant Growth Regulators (mg/l)	Diameter of callus induction	Percentage of organic callus induction	Type and nature of callus
2,4-D			
0	0 d	0 c	—
2	2.874 c	30 b	white
3	4.593 b	61 a	white
4	6.310 a	45 b	white
Kin			
0	2.365 b	26.7 b	—
0.1	2.470 b	35.4 ab	—
0.2	3.039 a	41.5 a	—
2,4-D+Kin			
2+0.1	2.308 d	37 f	Brown
2+0.2	3.738 c	42 def	Brown
3+0.1	4.040 c	57 b	Brown
3+0.2	4.964 b	73 a	Brown
4+0.1	6.959 a	46 de	Brown
4+0.2	7.255 a	49 cd	

For a given means within each Column followed by the same letter are not significantly differences (P < 0.05).

Table4. The mean NAA and Kin effects on traite.

Plant Growth Regulators (mg/l)	Diameter of callus induction	Percentage of organogenic callus induction	Type and nature of callus
NAA			
0	0 c	0 c	—
2	3.588 b	36 b	white
3	4.528 a	51 a	white

4	5.023 [a]	37 [b]	white
Kin			
0	2.067 [b]	21 [c]	—
0.1	2.681 [a]	32 [b]	—
0.2	2.938 [a]	41 [a]	—
NAA+Kin			
2+0.1	4.074 [e]	42 [d]	Brown
2+0.2	4.644 [cd]	55 [b]	Brown
3+0.1	5.355 [ab]	46 [c]	Dark Brown
3+0.2	4.287 [de]	60 [a]	Brown
4+0.1	5.775 [a]	39 [d]	Brown
4+0.2	4.851 [bc]	49 [c]	Dark Brown

For a given means within each column followed by the same letter are not significantly differences ($P < 0.05$).

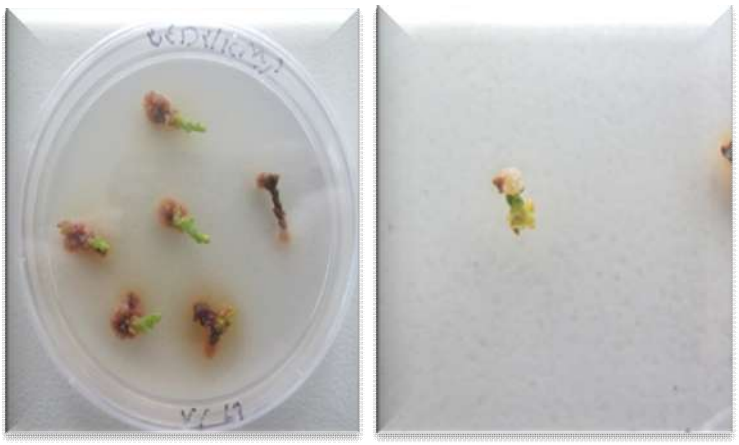

Figure1 callus induction of Juniperus by 2,4-D and kin hormones

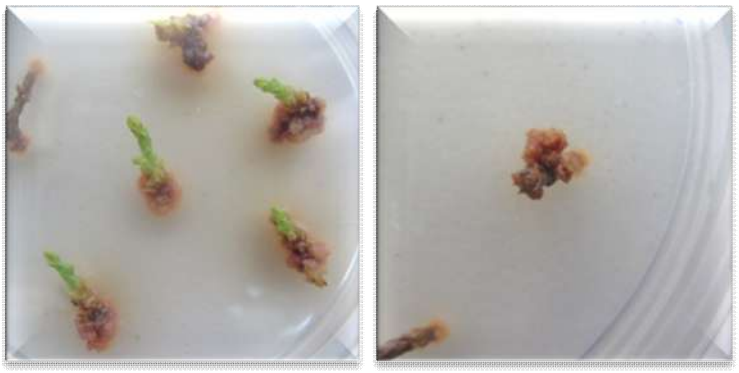

Figure2 callus induction of Juniperus by NAA and kin hormones

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