EFFECTS OF DIFFERENT MEDIA ON CALLUS INDUCTION OF *JUNIPERUS PHOENICEA* L .

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ABSTRACT

The present study was undertaken to develop a reproducible protocol for efficient in vitro callus initiation of *Juniperus phoenicea* L. Optimal growth and morphogenesis of tissues may vary for different plants according to their nutritional requirements. Used three types of half strength of basal media MS (Murashige and Skoog), ROM (Rugini Olive Medium) and WPM "Woody Plant Medium" (Lloyd and McCown,) modified with different type and concentration of plant growth regulators (PGR's) The MS, ROM and WPM media supplemented with 2,4- D only were the best treatment for calli induction within 10-22 days of cultivation with shoot tips explants of *J. phoenicea*. In contrast , presence (2,4-D) with other growth regulators did not give any response. ROM medium gave the best result of callus formation (70%), which was initiated after ten days of cultivation.

Keywords:- Callus initiation, *Juniperus phoenicea* L ; Lloyd and McCown; Rugini Olive Medium.

INTRODUCTION

Juniperus phoenicea is a shrub from the family Cupressaceae that grows in arid regions with low winter temperatures and high irradiation in summer (El-Bana *et al*., 2010). It is endemic to regions of the Mediterranean basin and populates North Africa and Canary Islands. Species from the genus Juniperus can be found in single or mixed stands in Portugal, Morocco, Algeria, Tunisia, Egypt, Saudi Arabia and Jordan (Al-Ramamneh *et al*., 2012 and Medini *et al*., 2013). In Libya, J. phoenicea is abundant in the Al Akhdar mountain region (Hegazy *et al*., 2013).

Junipers play an important ecological, landscape and economic roles: they increase water resources, prevent soil erosion (Rahmonov *et al*., 2017). Juniper

forests are exploited for timber, while berries are used as a spice for flavoring food (Kentelky, 2011). Their hardy nature and drought tolerance make junipers a good choice ornamental plants (Loureiro *et al*., 2007 and Kentelky, 2011). The pharmaceutical attributes of *J. phoenicea* and its use in traditional medicine are well documented. Extracts from the leaves and berries are used to treat diarrhea, rheumatism, bronco-pulmonary symptoms and diabetes (Allali *et al*., 2008). Also can improve liver and kidney functions, and may be useful in healing hepatotoxicity (Ali *et al*., 2010). Antioxidant activity was also reported in extracts of J. phoenicea, possibly owing to its content of phenols, sterols and flavonoids (Medini *et al*., 2013).

This plant species expose to danger of extinction due to different factors ,including its low regeneration capacity (Khater and Benbouza 2019). The effect of climatic changes in the area, especially increasing temperature and decreasing rainfalls were noticed, in addition, human impacts such as over grazing, agricultural expansion, medical plants collection, fires, etc. (Medini *et al*., 2013).

Therefore, attempts have been made to restore juniper forests based on the different techniques of sexual and asexual propagation (Garcia *et al*., 2010). However, the sexual propagation of juniper is very often limited mainly due to their low seed production, low germination rate, deep physiological dormancy and decreased viability of the seed embryos (Baravardi *et al*., 2014). Also lower seed quality results from insufficient pollination and from infections of cones by pests (Garcia *et al*., 2010). Another problem for this propagation strategy is the elevated level of heterozygosity and the extended cycle of seed production, which in juniper is up to eight years (El-Darier *et al*., 2009).Generally, conventional breeding of woody trees is a slow and difficult process (Mamo *et al*., 2011).

The world has a very rich biodiversity of plant species. Many of which are herbaceous and many others are woody in nature. In the view of propagation, woody plants are difficult to propagate than herbaceous species. The difficulty in propagation is due to their poor seed germination capacity, as seeds are not viable in most of the time. In this case the favorable season is a very important criterion for the successful germination. Moreover, the slow growth is also a barrier, because apical and

axillary buds become dormant during specific time periods(Baravardi *et al*., 2014). Therefore, woody plants require favorable season for the germination of seeds and buds. Further, some more conventional methods of propagation such as cuttings and gratings' are also used for woody plants. But these are not much effective methods for their large scale production. As, for a wide population, woody plants are important source of timber, medicines, fruits, dyes etc. Therefore, there is a need to propagate them wisely as well as in large amount to fulfill the requirements of the population. The possible approach to overcome the problem is micropropagation.

Micropropagation is the technique of growing the plants from seeds or small pieces of tissues under sterile condition in a laboratory on a specially selected medium. It allows mass multiplication of a species from a small piece of tissue. One of the important aspects of this technique is that it is not dependent on the season for the propagation. Through micropropagation, a number of woody plant species have been propagated successfully during past years. But, the success of micropropagation technique depends upon the use of plant growth regulators in the culture medium. Growth regulators regulate the growth and developmental processes, which are present in various concentrations in different plant parts.

Tissue culture can be a promising alternative for propagating recalcitrant plant species. *J. phoenicea* was micropropagated *in vitro* using explants prepared from adult trees as well as young seedlings and it had emerged as a tool to improve the propagation of many plants (Loureiro *et al*., 2007; AlRamamneh *et al.,* 2012).

Callus induction contained a cluster of undifferentiated cells. It could be initiated with a small part of plant tissue or explants that was induced using phytohormones to induce the production of calli. Generally, an equal amount of auxins and cytokinins gave the desired effect, but different species responsed differently to different levels of plant growth regulators. Dahleen *et al*.,(2002) reported that, large size of explant usually promoted better callus induction mainly because of the larger wound size and also higher level of endogenous plant growth regulator’s level. It was also reported that explants was aseptically transferred in

semi-solid medium and incubated in light environment for 3 – 8 weeks and subcultured at forth week interval for callus induction. (Gamborg and Philips 1999) studied the organogenesis took longer in callus culture than in direct plants.

Javeed *et al*., (1980) established The first callus cultures in juniper plant by tissue culture technique using different type of media supplemented with different concentration of plant growth regulators. Ilashi (1986) obtained a soft, friable callus of a creamy color from 2-year-old shoot cuttings of *J. polycarpos* was obtained on MS medium supplemented with 0.5 mg l^{-1} 2,4-D and 2 mg l^{-1} Kin, but no organogenesis was observed.

The biotechnology approach of this present study aiming to production of calli cultures from shoot tips of *J. phoenice using* different types of media contain different combination and concentration of auxin and cytokinin as plant growth regulators (PGRs) (Establishment of *in vitro* indirect plant regeneration protocol of *J. phoenice*).

MATERIAL AND METHODS

The healthy shoot tip (as explnt) of *Juniperus phoenicea* L. was collected from the parent plants shrubs which were located in the Al-Jabal Al-Akhdhar region, Libya in August 2019.

Media preparation

Optimal growth and morphogenesis of tissues may vary for different plants according to their nutritional requirements. Moreover, (Sharma et al.,2014) found that, in certain circumstances tissues from different parts of plants may be had different requirements for satisfactory growth . As shown in Tables 1 Three types of full strength of basal media MS (Murashige and Skoog 1962), ROM (Rugini Olive Medium 1984) and WPM “Woody Plant Medium” (Lloyd and McCown, 1980) modified as follow in table (2) for callus induction Medium (CIM).

Surface sterilization of *J. phoenicea* explants

Explants of *J. phoenicea* were soaked in Topsin fungicide (5g l^{-1}) for a completely day, and then they were washed well with sterile double distilled water .

On the 2nd day, the seeds were soaked in copper oxychloride (1 g l^{-1}) for 24 hrs. The 3rd day, they were treated with a sterile solution of mercury chloride at a concentration of 0.1 g l^{-1} for a quarter of an hour, then they were washed 3 times with sterile water. Then the seeds were placed in a solution of ascorbic acid with citric acid (50 mg g l^{-1}) for an hour and at the last steps the surface of explant were sterilized by dipping in 70% (v/v) ethanol for 2 and 4 min and then they were immediately rinsed with a sterile double distilled water to remove ethanol traces.

Table 1. Composition of three different types of media used for *J. phoenicea* callus induction

Ingredients	Amount (mg/l)		
Macronutrients			
	MS medium	**ROM medium**	**WPM medium**
Ammonium nitrate	**1650.00**	**412.000**	**400.000**
Potassium nitrate	**1900.00**	**1100.000**	**-**
Calcium chloride	**440.00**	**332.200**	**72.500**
Magnesium sulphate	**370.00**	**732.600**	**180.690**
Potassium phosphate monobasic	**170.00**	**340.000**	**170.000**
Potassium sulphate	**-**	**-**	**990.000**
Calcium nitrate	**-**	**416.920**	**386.340**
Potassium chloride	**-**	**500.000**	**-**
Micronutrients			
Potassium Iodide	**0.830**	**0.830**	**-**
Boric acid	**6.200**	**12.400**	**6.200**
Manganese sulphate monohydrate	**22.300**	**16.00**	**22.300**
Zinc sulphate heptahydrate	**8.600**	**-**	**8.600**
Molybdic acid (sodium salt)	**0.250**	**0.213**	**0.213**
Copper sulphate pentahydrate	**0.025**	**0.025**	**0.250**
Cobalt chloride hexahydrate	**0.025**	**0.025**	**-**
Iron stock			
Ferrous sulphate heptahydrate	**27.800**	**27.800**	**27.800**
EDTA disodium salt dehydrate	**37.300**	**37.300**	**37.300**
Vitamins			
Myo-inositol	**100.00**	**100.00**	**100.00**
Nicotinic acid	**1.000**	**5.000**	**0.500**
Pyridoxine HCl	**1.000**	**0.500**	**0.500**
Folic acid	**-**	**0.500**	
Thiamine hydrochloride	**10.000**	**0.500**	**1.000**
D-biotin	**-**	**0.050**	**-**
Amino acid			
Glycine	**2.000**	**2.000**	**2.000**
Total (mg/l)	**4.1**		

Table(2). Composition of different three type of media used for callus induction

Eighteen types of modified media (treatments) were utilized as shown a above. The experiment was consisted of forty jars per treatment with three explants per jar. Each type of treatment were divided in to two groups, 1st group was maintained in a growth room 25 ±2 ºC with a daily 16hrs photoperiod under standard cool white fluorescent tubes and the 2nd group maintained in complete darkness. Jars were checked regularly for callus induction. After five weeks from culture, the frequency of explants (shoot tip) producing calli (%) and calli character (type and color) were recorded and callus growth score was assessed by visual rating into four categories as

treatment	Media composition	treatment	Media composition
CIM$_1$	MS+3 mg l^{-1} 2, 4-D	**C**IM $_{10}$	ROM + 3 mg l^{-1} 2, 4-D+ mg l^{-1} BA
CIM $_2$	MS +2 mg l^{-1} BA	**C**IM $_{11}$	ROM + 3 mg l^{-1} 2, 4-D+ 2 mg l^{-1} NAA
CIM $_3$	MS + 2 mg l^{-1} NAA	**C**IM $_{12}$	ROM + 2 mg l^{-1} BA + 3 mg l^{-1} NAA
CIM $_4$	MS + 3 mg l^{-1} 2, 4-D+ 2 mg l^{-1} BA	**C**IM $_{13}$	WPM +3 mg l^{-1} 2, 4-D
CIM $_5$	MS + 3 mg l^{-1} 2, 4-D+ 2 mg l^{-1} NAA	**C**IM $_{14}$	WPM +2 mg l^{-1} BA
CIM $_6$	MS + 2 mg l^{-1} BA + 3 mg L^{-1} NAA	**C**IM $_{15}$	WPM + 2 mg l^{-1} NAA
CIM $_7$	ROM +3 mg l^{-1} 2, 4-D	**C**IM $_{16}$	WPM + 3 mg l^{-1} 2, 4-D+ 2 mg l^{-1} BA
CIM $_8$	ROM +2 mg l^{-1} BA	**C**IM $_{17}$	WPM + 3 mg l^{-1} 2, 4-D+ 2 mg l^{-1} NAA
CIM $_9$	ROM + 2 mg l^{-1} NAA	**C**IM $_{18}$	WPM + 2 mg l^{-1} BA + 3 mg l^{-1} NAA

prolific (4), good (3), medium (2) and poor (1). All obtained calli cultures were subcultured on the same fresh callus media every 3 weeks from incubation to obtain callus stock, while non-responded treatments were excluded.

Callus frequency

The frequency of developing calli was calculated according to Sah *et al*., (2014) as follows:

$$\textbf{Calli (\%)} = \frac{\textbf{Number of explants exhibiting calli formation}}{\textbf{Number of explants cultured}} \times 100$$

RESULT

The MS, ROM and WPM media supplemented with 2,4- D only were the best treatment for calli induction within 10-22 days of cultivation with shoot tips explants of *J. phoenicea* . However, no callus formation was observed as a result of culturing on the other different plant growth regulators (Table 3). Callus proliferation was

better under normal condition compared to complete darkness, for each the same medium.

ROM medium gave the best result of callus formation (70%), which was initiated after ten days of cultivation. Several types of calli cultures were distinguishable based on their appearance (Table 3). Adding 3 mg l^{-1} 2, 4-D to MS and WPM (C_1 and C_{13}) medium were induced callus after 22 days. Among all the applied plant growth regulators , the results were showed that adding 2,4-D separately at 3 mg l^{-1}, was found to be the only effective one for J. *phoenicea* callus induction cultivated on all media used In contrast , presence (2,4-D) with other growth regulators did not give any response. However, no callus formation was observed as a result of culturing on other used media **(**Table 3). The ROM medium when supplemented with 2,4-D at 3 mg l^{-1} for a long time (C_7) was found to be effective for *J. phoenicea* callus induction and cells dedifferentiation (Fig. 2). Cultured shoot tip explants on ROM and WPM media produced generally whitish calli, while root explants gave rise to brownish-white, nodular, friable callus.

Table (3). Effect of different media composition and plant growth regulators on various morphological responses and callus character derived from shoot tips of *J. phoenicea*. Data were recorded after 4 weeks of culture at normal condition.

Morphological response and characterization of callus		
Explants type / Media composition	**Callus frequency (%)**	**shoot tips**
C_1	20^c	Callus$^+$ - Brownish white- Nodular and Friable
C_2		No response
C_3		No response
C_4		No response
C_4		No response
C_5		No response
C_6		No response
C_7	70^a	Callus^{+++} - whitish - Nodular and Friable
C_8		No response
C_9		No response
C_{10}		No response
C_{11}		No response
C_{12}		No response
C_{13}	50b	Callus^{+++} - whitish - Nodular and Friable
C_{14}		No response
C_{15}		No response
C_{16}		No response
C_{17}		No response
C_{18}		No response

Where: $^+$ means low; $^{++}$ Moderate and $^{+++}$ High callusing.

Note: Cultivation in complete darkness did not give a response regarding callus formation, so it was omitted from the table for simplicity.

DISCUSSION

The world has a very rich biodiversity of plant species. Many of which are herbaceous and many others are woody in nature. In the view of propagation, woody plants are difficult to propagate than herbaceous species. The difficulty in propagation is due to their poor seed germination capacity, as seeds are not viable in most of the time. In this case, the favorable season is a very important criterion for the successful germination. Moreover, the slow growth is also a barrier because apical and

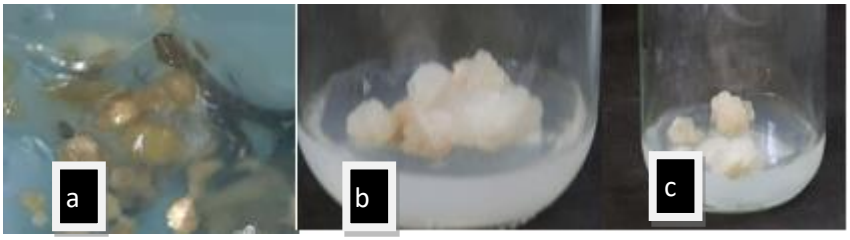

Fig. 1. Morphology of calli derived from shoot tip explants on MS (a), ROM (b) and MPW (c) media containing 3 mg l^{-1} 2, 4-D (C_1,C_7 and C_{13}) incubated under normal condition (light –dark cycle 16/8 h) after one month.

axillary buds become dormant during specific time periods. Therefore, woody plants require favorable season for the germination of seeds and buds.

The first work on the reproduction of juniper by in vitro cultures was performed by Javeed et al., in 1980. Since then, few studies have been published on this subject (Loureiro *et al*.,2007; Zaidi *et al*.,2012; Ahani *et al*.,2013 and Kaviani *et al.*, 2017) and only single publications have reported the success of this method.

Callus formation is frequently considered the manifestation of the dedifferentiated cellular state. Second, calli can regenerate somatic embryos and new plants by dedifferentiation. Dedifferentiation is frequently considered the manifestation of the stem cell-like state to switch fate preceding the commitment for proliferation (Xing *et al*., 2010).

For callus induction, shoot tip explants were cultured on nutrient media supplemented with different concentrations of auxins individually or in combinations with cytokinins. Basal medium without growth regulator was failed to induce callus from most of woody species (Sharma and Vashistha, 2011; 2014). This was

probably due to the insufficient level of endogenous growth regulators in explants to induce callus and therefore it requires an exogenous supply. In woody plants, commonly auxins were used IAA, NAA, IBA and 2,4-D. In some investigations, 2,4-D has been essential for callus formation, The development of callus from immature leaf explants is directly related to the presence of 2,4-D which is a suitable growth hormone responsible for callus induction in most plant Species in plant tissue culture work Mamun *et al.* (2004) and Baskaran *et al.* (2006). The production of a yellowish, compact and nodular callus at cut edge of explant may be due to the wound caused during the process of cutting which resulted in a synchronous cell division. This is considered as a process of de-differentiation of organized tissue and is similar to the work of Qin *et al.* (2005) and Xing *et al.*. (2010). Thus, there was optimum Callus induction with increase in 2,4-D concentration. Among the previous factors, plant growth regulators play a key role in cell division and differentiation callus induction (Lee and Huang, 2013). Research revelation pointed to lower recalcitrance in monocots when auxins and cytokinins are used in the culture media (Ikeuchi *et al.*, 2013) as 2, 4-D along with BAP (Sahoo *et al*., 2011) or NAA (Bano *et al.,* 2005).

In addition to 2,4-D was effective for inducing callus in Moringa oleifera (Kurokawa *et al*., 2016), *Citrus jambhiri* (Savita *et al*., 2011) and *Simmondsia chinensis* (Ball ,2015). According to Murashige (2004) 2,4-D was a most potent auxin and stimulated callus. All mentioned a above were agreement with the obtained results which were shown that adding 2,4-D separately at 3 mg/ l^{-1} on all used media used (MS, ROM and WPM) was the only effective way for J. *phoenicea* callus induction cultivated. Callus proliferation was better under normal condition compared to complete darkness, for each the same medium. The highest callus frequency growth score (70 %) was attained accompanied with obvious callus growth increment when ROM medium modified with 2, 4-D. In contrast, NAA played an important role in callus formation in Cinnamomum camphora (Sharma and Vashistha, 2015), Pseudarthria viscid (Cheruvathur and Thomas, 2011) and Tinospora cordifolia (Sharma and Vashistha, 2011;2014). Similarly, IAA

has been used in some in vitro culture studies to initiate callus (Sahoo *et al*., 2011; Kurokawa *et al*., 2016).

The same composition of plant growth regulators is not suitable for all Juniperus varieties, the modifying media are diversified to overcome the genotypic influence for particular Juniperus varieties. Among the previous factors, plant growth regulators play a key role in cell division and differentiation during somatic embryogenesis (Lee and Huang, 2013). Research revelation pointed to lower recalcitrance in monocots when auxins and cytokinins were added in the culture media (Ikeuchi *et al*., 2013) as 2, 4-D plus BA (Sahoo *et al*., 2011) or NAA (Bano *et al*., 2005). In addition to Panjaitan et al. (2009) observed that, addition of 2, 4-D alone was induced calli upto 80 % in MR219, whereas the mixture of 2, 4-D with kin was induced lower percentage of calli (61 %) in the same variety (Kurokawa *et al*., 2016). However Trejo-Tapia *et al.* (2002) suggested that a combination of auxin (NAA and 2, 4 -D) was better than using the single auxin.

Tissue culture can be a promising alternative for propagating recalcitrant plant species. *J. phoenicea* was micropropagated *in vitro* using explants prepared from adult trees as well as young seedlings (AlRamamneh *et al.*, 2012). These studies have shown that free MS medium was inefficient for the *in vitro*

propagation of *J. phoenicea*, resulting in browning and necrotic areas of the explants. In contrast to MS medium, OM medium alone or supplemented with cytokinins (0.2 to 0.5 mg/ l^{-1}kin, 0.1 to 0.2 mg/ l^{-1}BA, and 0.5mg/l^{-1}TDZ) and/or auxin (0.5 mg/ l^{-1}NAA), was stimulated axillary bud differentiation and development of new branches (Loureiro *et al*.,2007; Al-Ramamneh *et al.*, 2012). Moreover Loureiro *et al.* (2007) reported that 40% of in vitro shoots of J. *phoenicea* was rooted when exposed to 0.5 mg/ l^{-1}IBA for 5 min and then transferred to free OM medium.

Doerner and Celenza (2000) found that stimulation in cell cycle was resumed by auxin (2.4-D) addition to the culture medium and was lost when depleted from it. In auxin-free medium (totipotent lost), cells accumulate in the G1, phase, and addition of auxin releases the G1, arrest, cells enter the S phase and, thereafter, they

divide (totipotent acquired). In other words auxin is necessary for competent cells to express their competency.

CONCLUSIONS

This the study established a promising protocol for callus induction of J. *phoenicea* . Whereas callus was induced on shoot tips explants cultured on ROM medium fortified with 3 mg L^{-1} 2, 4-D showed the best result. Research on the micropropagation of junipers should be intensified because in many cases, the in vitro method may be the only alternative allowing the cloning of increasingly rare and shrinking populations of these woody plants. The development of new strategies for the reproduction of junipers based on in vitro techniques may be particularly important today in the face of climate change, an additional threat to natural forest ecosystems(Kaviani *et al.*, 2017) .

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