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Assessment of *Juniperus Phoenicea* L. Seedling Produced via Tissue Culture Using RAPD Analysis

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A B S T R A C T

Woody plants are more difficult to reproduce than other plant species found in nature. In addition to seed dormancy, which is caused by inhibitors in the embryo, endosperm, and/or testicle, Phoenicia, like other juniper species, does not exhibit a high rate of plant production by germination of seeds.

This research's primary goals were to propagate *Juniperus phoenicea* L. (*J. phoenicea*) using tissue culture by identifying a promising protocol for utilizing kinetin (0.5 mg/l^{-1} with Murashige and Skoog 1962, MS), Rugini Olive Medium 1984, ROM, and Lloyd and McCown 1980, WPM to break the secondary dormancy of seeds. Additionally, RAPD fingerprints were used for molecular analysis to determine the total genomic DNA of the parent plant compared to the in vitro plant obtained.

The RAPD-PCR products they obtained demonstrated a high percentage of polymorphism resulting from using BA-K5 as a primer compared to BA-K2. The results showed that the medium (WPM) supplemented with 0.5 g/l^{-1} kin gave the highest significant increment for germination percentage (60%), while MS gave the lowest germination rate (8%). The study concludes that the *in vitro* culture method for zygotic embryo germination may be a viable way to overcome the challenges posed by poor juniper germination rates and boost seed propagation.

1 Introduction

The genus Juniperus. which belongs to the Cupressaceae family is the greatest recognizable group of trees and plants that are evergreen on Earth after the tree of pines, comprises approximately 80 species (Zaidi et al., 2012). Juniperus trees are very diverse in terms of geological, geomorphological, and climatic periods; they can be found growing in temperate and subpolar regions of North America and Eurasia, as well as in tropical highlands (Rahmonov et al., 2017 and Castro et al., 2011). In many countries, juniper wood is used to make lead pencils, installations, and other outdoor structures, making it economically valuable (Mamo et al., 2011). Additionally, juniper berries provide oil for several pharmaceutical products (Zaidi et al., 2012). According to Allen (2017), certain extracts are used to treat infections, fungal diseases, communicable conditions,

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and communicable diseases such as colds, gynecological conditions, malignancies, and diabetes.

In vitro biotechnology is a collection of methods for aseptically cultivating various cultures that encourage cell proliferation and genetic reprogramming. It is regarded as a crucial instrument in both fundamental and practical research (Balaraju et al.,2016). Plant tissue culture has a limited application in forestry but is widely employed in horticulture and agriculture (Balaraju et al.,2016). Transgenic plant technology is just one of the many uses for plant tissue culture, which is a very versatile instrument that can help with other issues that humans face, not just those that are related to food supply. According to research in this area, the leaf, apical meristem, bud, and root of the J. phoenicea plant can all be utilized as sources of explants in the first stage of micropropagation (Murashige, 1974). the likelihood that an in vitro culture using a selected ex-vitro mother plant under ideal conditions to reduce contamination will be successful. This phase was considered the first step to prepare the donor plant as a source of different explants. The first work on in vitro juniper reproduction was done by Javeed et al. in 1980. There have been a few publications on the topic since then (Ahani et al., 2013; Kaviani et al., 2017), and one article has just reported the effective technique. Because in vitro tissue culture is often one of the alternative techniques that enable the cloning of rare residents of these woody plants, research on juniper micropropagation should be expanded (Kaviani et al., 2017). According to Ahani et al. (2013), the PCR-based RAPD method with arbitrary primers requires just nanogram amounts of template DNA, doesn't require radioactive probes, and is a rather easy procedure in comparison to other approaches. In their brief use, RAPD markers have amply demonstrated their value for studying plant genomes (Ahani et al., 2013). Genome mapping, gene tagging, genetic diversity research, cultivar identification, and parentage analysis are among the applications of RAPD markers. Additionally, molecular markers offer many benefits, such as polymorphism abundance, lack of pleiotropic effect, minor environmental influence, and quick detection. Joshi et al. (2008) considered DNA-based molecular techniques to be an alternative to chemical and morphological markers. Nonetheless, it has been proposed as a great tool for determining genetic diversity, phylogenetic linkages, pharmacogenetic description, plant species authentication, genetic mapping, and species characterization in a variety of plants. The polymerase chain reaction (PCR) technology known as randomly amplified polymorphic DNA (RAPD) makes it possible to develop identification assays without knowing the target DNA sequence beforehand. This is the primary benefit of RAPD, which eliminates the need for time-consuming and costly sequencing efforts (Kuang et al., 2018). To determine whether any genetically random variations would manifest, the RAPD technique was applied using an extract from the leaves of an in vivo J. phoenicea plant as a parent control and three in vitro germinated seedlings of the same plant. Wesche et al. (2005) found that a single gene was responsible for the prostrate shrub Juniperus sabina patch RAPD fingerprinting. Recent growth rates ranged from 1.8 to 6.8 cm annually on average. The study area's greatest patches had minimum years ranging from 770 to 2940. As a result, seedlings have been planted during times when the climate has been more conducive, while clonal growth has withstood dry spells. Given the severe conditions of the central Asian drylands, this combination of infrequent sexual repeat and many generations of just vegetative reproduction may be a common strategy. Additionally, RAPD was employed in a set of random primers with typical lengths of 10–15 bases, according to Kuang et al. (2018). A PCR amplicon was created following the annealing of these primers. The size and quantity of the amplicons were then assessed using gel electrophoresis, which revealed several bands. Various amplicons of varying sizes are produced by sequence changes in distinct genomes, which results in a varied banding pattern. The primary objectives of this research were to propagate J. phoenicea using tissue culture by identifying a promising protocol for using kinetin to break the secondary dormancy of seeds. In addition, RAPD fingerprinting is used for molecular analysis to determine the total genomic DNA of the mother plant compared to the laboratory plant obtained.

2 Materials and Methods

Healthy mature *J. phoenicea* seeds and leaves were produced by the parent plants, which were bushes in the Al-Jabal Al-Akhdhar region of Libya. This experiment tested the optimal medium for in vitro seedling germination using three different half-strengths of basal MS medium, ROM, and WPM supplemented with 0.5 mg/l-1 Kin, 15 g/l^{-1} sucrose, and 8 g/l^{-1} agars. Before adding the solidifying agent, the pH media was brought to 5.7. They were then autoclaved for 20 minutes at 121ºC. Each autoclaved media was separately poured

into 250 ml glass jars (50 medium/ jar), and then all sterile wear glass culture (250 ml jars) sealed tightly with parafilm and stored at room temperature 25 ±2 ºC at least three days under complete darkness to examine contamination (Mansur et al., 2021).

2.1. Surface sterilization of *J. phoenicea* seeds

Seeds of J. phoenicea were soaked in Toptapsin fungicide (5g /l-1) for 24 hrs., 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. On 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 and 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 step, the seed's surfaces were sterilized by dipping in 70% (v/v) ethanol for 2 min, and then they were immediately rinsed with a sterile double distilled water to remove ethanol traces. In a complete aseptic condition all sterilized seeds were grown on the germination media. An equal number (three seeds) from total sterilized seeds were cultivated in each 50 ml medium of 250ml jars, the replicates of each media type were 40 jars and then, all were incubated in a growth room 25 ±2 ºC with a daily 16 hrs photoperiod under standard cool white fluorescent tubes, until germination seeds (Mansur et al.,2021).

2.2. Molecular analysis

Using a modified version of Cassells and Doyle (2005) Cetyl Trimethyl Ammonium Bromide (CTAB) method, total DNA was extracted from the leaves of three in vitro J. phoenicea plants that germinated and the leaves of the in vivo J. phoenicea parents as a control. A list of the primers (5-mer primers, Operon Technologies Inc., Alameda, California) their names and sequences is shown in Table 1.

Table 1. List of nucleotide sequence of the PCR primers.

Primers name	Nucleotide Sequence (5' to 3')	Molecular weight
BA-K1	5'-GGG ACC CAA CTCTGG TCA AT -3'	6102.02
BA-K2	5'- ACT CGG TGC ACTCGT ATG AT -3'	6108.03
BA-K3	5'- AGG GAC TTC AAGAAC CAC GA -3	6144.07
BA-K4	5'- CAC GCT CTT TGACTC CCT CT -3'	5954.90
BA-K5	5'- GTT GGT GCA GGTGAA GGT C -3'	5939.92

2.3. Statistical analysis

Differences between treatment means and interactions were examined using the test of least significance (LSD) at a significance level of 0.05%. The MSTAT-C software program was used to statistically analyze the data using the Gomez and Gomez (1984) approach.

3 Results

Seed germination

The findings demonstrated that the most significant increase in germination percentage (60%) was obtained using a half-strength WPM medium supplemented with 0.5 g/l-1 kin: Fig. 1 and Table 2. Within 14 days of culture, seeds germinated on the half strength of the WPM medium. However, the lowest germination rate (8%) was obtained with 1/2 MS (Table 2). Seed germination on 1/2 MS and 1/2 ROM material demonstrated limited growth and development even after a long incubation period. Based on the initial seed germination experiment, WPM outperformed MS and ROM as the optimum germination medium. While some societies showed a predisposition for a particular medium for the establishment and spread of cultures, others responded similarly across all media types.

Table (2). Seed germination of *J. phoenicea* after 20 days of cultivation on three different half-salt strength media.

Germination Percentage (%)			
Treatment (PGR)	**Medium Type**		
Kin	½ MS	½ ROM	½ WPM
	8^{d}	40^{b}	60^{a}

Fig. 1. *In vitro J. phoenicea* seed germination after 20 days of cultivation on half-salt strength of WPM medium.

RAPD-PCR analysis

The RAPD-PCR analysis indicated that only two random primers produced amplifiable PCR products, with a variable number of bands from five tested due to insufficient genomic variability or primer inefficiency. The failure of some primers in PCR can result in numerous nonspecific DNA products of varying sizes that appear as ladder-like or distorted bands on agarose gels. Sometimes, no products are formed at all. Another potential problem occurs when mutations are inadvertently introduced into the amplicons, resulting in a heterogeneous population of PCR products.

Out of the 19 fragments that were produced by the two selected RAPD primers, 37.22% were polymorphisms, according to the results. Of the two RAPD primers used, the BA-K5 primer produced the highest percentage of polymorphism (44.44%), revealing four polymorphic bands and one unique (1500) band from nine amplified fragments (Table 3). However, the BA-K2 primer recorded the lowest percentage (30%) by revealing three polymorphic (without unique) bands in 10 amplified fragments as shown in Table 3. Other primes BA-K1, BA-K3, and BA-K4 failed to respond to any RAPD-PCR reaction. Also as shown in Table 4 the generated RAPD-PCR products using primer BA-K2 showed the same DNA amplification (monomorphic bands) with plantlets grown *in vitro* and parents plant as control (C). The fragments of DNA products ranged in molecular weight size from 300,500,700,1000, 1500, 2000, 3000, 4000, 5000, and 20000 bp. In addition to primer BA-K2 was characterized by the absence of the band with molecular weight size 300 and 1000 bp from in vitro germinated plants was grown on MS (M1) and ROM (M2) media as shown in Table 5. The primer BA-K5 presented a total of seven bands in the parent plant as a control. and all treatment samples plant growing on MS (M1), plant growing on ROM (M2), plant growing on WPM (M3). Also, the BA-K5 primer amplified nine bands, four of them were polymorphic by percentage 44.44% polymorphism and five of them were DNA monomorphic bands as observed in Table 5. The smallest molecular weight size of the amplified products was 1000 bp and the largest size of the amplified product was 20000 bp (Table 5). Parents plant (C) and germinated plant on MPW (M3) were characterized by the absence of the band with molecular weights of 3000 bp and 10000 bp. Also, germinated plants cultured on MS (M1) and ROM (M2) media were characterized by the absence of the band with sizes 1000 bp and 1500 bp. The results obtained showed a high percentage of polymorphism resulting from using BA-K5 as a primer compared to BA-K2. In addition, using BA-K5 primer distinguished more genetic stability among different half basal media.

Table (3). Total bands produced by BA-K2 and BA-K5 primers in in vitro germination plantlets cultured on different basal mediums.

Primer	Band. No	Monomorphic	Polymorphic bands		Polymorphism (%)
			Non Unique	Unique	
BA-K2	10	7	3	-	30
BA-K5	9	5	4	1	44.44
Total Bands	19	12	7	1	37.22

Table (4). Data matrix illustrating the presence (1) or absence (0) of RAPD bands produced by primer BA-K2 of *in vivo J. phoenicea* parent plant leaves as control and *in vitro* germination plantlets cultured on different basal medium invested with 0.5 mg l^{-1} of kin.

Band number	M.W. (bp)	C	M_1	M_2	M_3
1	20000	**1**	**1**	**1**	**1**
2	5000	**1**	**1**	**1**	**1**
3	4000	**1**	**1**	**1**	**1**
4	3000	**1**	**1**	**1**	**1**
5	2000	**1**	**1**	**1**	**1**
6	1500	**1**	**1**	**1**	**1**
7	1000	**1**	**0**	**0**	**1**
8	700	**1**	**0**	**0**	**1**
9	500	**1**	**1**	**1**	**1**
10	300	**1**	**0**	**0**	**1**
Total bands		**10**	**7**	**8**	**10**

Table (5). Data matrix illustrating the presence (1) or (0) absence of RAPD bands produced by primer BA-K5of *in vivo J. phoenicea* parents plant as control and *in vitro* germination plantlets cultured on different basal medium invested with 0.5 mg l^{-1} of kin.

Band number	M.W. (bp)	C	M_1	M_2	M_3
1	20000	1	**1**	1	1
2	10000	0	**1**	1	0
3	7000	1	**1**	1	1
4	6000	1	**1**	1	1
5	4000	1	**1**	1	1
6	3000	0	**1**	1	0
7	2000	1	**0**	1	1
8	1500	1	**0**	0	0
9	1000	1	**1**	0	1
Total bands		7	**7**	7	7

Where: - C= parents plant as control M_1= seedling plant growing on MS M_2= seedling plant growing on ROM

4 Discussion

The development of woody tissue distinguishes a group of woody plants. Compared to other plant species, woody plants are more challenging to propagate. Because the embryos are not viable for the majority of the duration, the seeds are unable to germinate. In this instance, effective germination depends on the right season. Also, slow growth is a disadvantage since the apical and axillary buds become latent during specified periods. Woody plants therefore require a great deal of respect to understand when seeds should germinate and when buds should form. According to this research, reduced seed output in juniper trees can be attributed to several factors, including poor pollen viability, lack of pollination, and/or malformed or disturbed embryos. To remove the inhibiting influence among these materials, separated embryos or seeds are incubated in vitro on culture media under handled conditions to promote the germination procedure. Researchers applied this strategy to improve the germination rate of individual juniper species. Several woody plant seeds were cultivated in vitro under aseptic control. Kin, a growth regulator inducer, is useful for both shoot elongation and seed germination. Three distinct medium types—WPM, MS, and ROM—were employed in our study to facilitate *J. Phoenicea* seed germination under ideal circumstances. 60% germination was the largest significant increase in WPM medium with 0.5 g l^{-1} kin.

The results gained were consistent with those of certain researchers (Máchová et al., 2013; Kaviani and Negahdar 2017) who found that using kin as a plant growth regulator in them *in vitro* culture conditions produced positive outcomes. Furthermore, Ghimire et al. (2017) reported that Kin and GA_3 were significantly beneficial for shoot elongation among the several growth regulators tested in Melastoma malabatricum. Nevertheless, GA_3 had a detrimental effect on the quantity and length of shoots in Pyrus boissieriana (Aasim et al., 2013).

Plants grown from isolated embryos also demonstrated a high degree of soil acclimatization. The results from this study indicate that there is a positive correlation between genomic template stability when using Kin as a growth regulator. RAPD technology and generated DNA changes in *J. phoenicea* were revealed in vitro culture, and it was demonstrated that RAPD can be used successfully as a tool for botany investigation.

Mutations and DNA damage are distinct types of DNA errors. DNA damage is a defect or change in the chemical structure of DNA, such as a break in a piece of DNA, the loss of a nitrogenous base, or a change in the chemical structure of the base itself. Mutation is a change in the order of standard base pairs. DNA damage alters the structure of the genetic material and prevents the replication mechanism from functioning properly.

The inefficiency of some primers in our study may stem from base sequence mismatches or from seed germination in chemically defined artificial media. Additionally, the hormone kinetin may have significantly contributed to these differences (Atienzar et al., 2002). Our obtained results are in agreement with Abd-El-Haleem et al. (2009) reported that RAPD analysis indicated high levels of genetic variability, even with the use of a limited batch of primers. This high level of polymorphism among individuals implies that RAPD techniques can be useful for *Juniperus* for the maintenance of germplasm banks and the efficient selection of parents for breeding.

In addition, Asili et al. (2010) reported molecular markers developed by analysis of randomly amplified polymorphism DNA (RAPD) has shown excellent prospect to assist choice of quantitative traits, and Abd-El-Haleem et al. (2009) investigated the detection of DNA alterations using RAPD technique was established on the changes in amplified band intensities, disappearance of bands and appearance of new bands in the RAPD profiles yielded from *in vitro* micropropagation explants comparison to the control mother plant. Using non-conventional approaches such as molecular markers as one of strategy to obtain plants with higher implementation by recognizing the genes and banding patterns that took place when the plant grew under septic *in vitro* conditions accelerated the improvement of breeding programs. Finally, utilizing BA-K5 primer in this study was illuminating for detecting in vitro-induced specific DNA alterations. The primer BA-K5 heightened total bands of seven polymorphic (44% polymorphism), five monomorphic DNA bands in seedlings, and one unique band. However, many constraints in applying the RAPD procedure involving amplification and/or electrophoretic separations like contaminated DNA, amplification competition, and homology of band depend on Abd-El-Haleem et al. (2009). This results of RAPD-PCR indicated the existence of differences in RAPD fragments and the quantitative polymorphism occurred because of the modifications of some nucleotide sequences aligned area by arbitrary primers. These were brought on by the effects of kinase or by the steroidal hormones' activation of Tag polymerase activity and/or promotion of sequence recognition, which improved annealing between primers and DNA templates.

Conclusions

Germination in zygotic embryos using an in vitro culture technique may be a viable way to overcome the challenges of poor juniper germination rates and boost seed multiplication. Additionally, we believe that the capacity to in vitro germinate zygotic embryos offers the potential to utilize them as a source of sterile explants for the induction of J. phoenicea cultures by the application of in vitro propagation procedures. These methods include organogenesis and somatic embryogenesis.

Conflict of interest:

The authors declare that there are no conflicts of interest

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