

# Effect of media composition and growth regulators on mass propagation of Juniper (*Juniperus Procera*) (#107112)

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# Effect of media composition and growth regulators on mass propagation of Juniper (*Juniperus Procera*)

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**Abstract :** The medicinal *Juniperus procera* trees found in Saudi Arabia mostly, in the Enemas region. Die-back disease and problems with seed reproduction, including as dormancy, malformed embryos, and a germination rate of less than 40%, pose dangers to it. Therefore, alternate techniques for reproducing *Juniperus procera* are essential for their conservation and large-scale production for use in pharmaceuticals. We described the successful in vitro shoot multiplication of *J. procera*. Explants were cultivated in MS media with varying combinations of plant growth regulators (PGRs) added at varying amounts. The concentration of BAP at 1.0 mg/l showed the highest survival rate (70%) followed by 0.5mg/l then the control treatment. Similarly, concentration of BAP 1.0 mg/l produced higher number of responded explants (2.66) and shoot number (2.67) compared with the other treatments. In multiplication media BAP at 2.0 mg/l without NAA produced higher percent of responded shoots; the lower concentrations of BAP gave lower response. The highest shoot number was observed into multiplication medium supplemented with BAP at 2.0 mg/l then by BAP at 2.0 mg/l +0.2 mg/l NAA. Meanwhile, shoot length showed a different trend in this experiment, as the highest shoot length was occurred at the control treatment (0.0 BAP +0.0 NAA) followed by all BAP treatments, while, addition of NAA to BAP into multiplication medium gave lower shoots length. Juniper shoots are hardly to root as, most of the treatments were inefficient. OM medium was responsible for rooting only when addition of IBA was implemented. The maximum percentage of rooted shoots was obtained with olive medium supplemented with IBA at 1.0 mg/l.

# Effect of Media Composition and Growth Regulators on Mass propagation of Juniper (*Juniperus Procera*)

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**Abstract:** The medicinal *Juniperus procera* trees found in Saudi Arabia mostly, in the Enemas region. Die-back disease and problems with seed reproduction, including as dormancy, malformed embryos, and a germination rate of less than 40%, pose dangers to it. Therefore, alternate techniques for reproducing *Juniperus procera* are essential for their conservation and large-scale production for use in pharmaceuticals. We described the successful in vitro shoot multiplication of *J. procera*. Explants were cultivated in MS media with varying combinations of plant growth regulators (PGRs) added at varying amounts. The concentration of BAP at 1.0 mg/l showed the highest survival rate (70%) followed by 0.5mg/l then the control treatment. Similarly, concentration of BAP 1.0 mg/l produced higher number of responded explants (2.66) and shoot number (2.67) compared with the other treatments. In multiplication media BAP at 2.0 mg/l without NAA produced higher percent of responded shoots; the lower concentrations of BAP gave lower response. The highest shoot number was observed in multiplication medium supplemented with BAP at 2.0 mg/l then by BAP at 2.0 mg/l +0.2 mg/l NAA. Meanwhile, shoot length showed a different trend in this experiment, as the highest shoot length was occurred at the control treatment (0.0 BAP +0.0 NAA) followed by all BAP treatments, while, addition of NAA to BAP into multiplication medium gave lower shoots length. Juniper shoots are hardly to root as, most of the treatments were inefficient. OM medium was responsible for rooting only when addition of IBA was implemented. The maximum percentage of rooted shoots was obtained with olive medium supplemented with IBA at 1.0 mg/l.

**Keywords:** Micropropagation; *Juniperus procera*; Plant Growth Regulators; Medicinal Endanger plant.

## 1. Introduction

The second most common family of conifers on Earth is the genus *Juniperus*, which belongs to the Cupressaceae family. Numerous juniper species can be found in the Northern Hemisphere, Africa, Central America, and Europe and Asia. Certain juniper species can withstand arid regions

and adjust to challenging environmental circumstances *Juniperus procera*, also known as "Arar" in Arabic, is a plant that grows in the Enemas region in south Saudi Arabia (Seca *et al.*, 2015; Hazubska-Przybył, 2019; Loureiro *et al.*, 2007). Coniferous evergreen shrub or tree is called *Juniperus procera*; *Juniperus* comprises more than 75 species (Loureiro *et al.*, 2007). *Juniperus procera* leaves, fruits and seeds are rich with bioactive compounds, with high anticancer, antimicrobial, and antioxidant activities (Nuñez *et al.*, 2008; Tumen *et al.*, 2013; Abdel Ghany *et al.*, 2014; Bitew, 2015). According to reports, *J. procera* leaves are a source of novel flavonoids (Mujwah, 2010). In addition, fruits can be used medicinally to treat skin conditions and headaches. Its resin was used as a stimulant and medication to treat liver disorders and ulcers when combined with honey (Jansen, 1981; Ahani *et al.*, 2013; Tounekti *et al.*, 2019). Nevertheless, juniper forests are disappearing from many of today's woods because of both human activities and natural causes. The species has been steadily diminishing in many parts of the world; the urgency for conservation of *J. navicularis* is mostly due to drought, soil erosion, and increased runoff (Sriskandarajahet *et al.*, 1994; Ortiz *et al.*, 1998; Aref *et al.*, 2016). Additionally, the distribution of populations of many juniper species may have been negatively impacted by climate change. Because of this, certain juniper species are currently considered rare or endangered and need to be protected right away (Romme *et al.*, 2009; Abrha *et al.*, 2018; Miller *et al.*, 2019). The development of efficient ex situ conservation techniques, reproduction for upcoming reintroduction and restoration projects is therefore critically needed (Mestanza-Ramón *et al.*, 2020). Traditional forestry reintroduction procedures as seed propagation, rooted cuttings, and grafting have been employed with conservation strategies (Varshney, and Anis, 2014). With the ability to conserve and mass clonally propagate many coniferous tree species, in vitro culture technology is becoming more and more popular (Lynch, 1999). Certain species of *Juniperus* have either produced extremely few viable or anatomically underdeveloped seeds, moreover high percentage of empty seeds (Ortiz *et al.*, 1998; Mohammadi *et al.*, 2018), in addition to mechanical dormancy and presence of germination inhibitors (Juan *et al.* 2006). As a result, in vitro culture technique is gaining more and more attention as a potential substitute for mass clonal propagation and conservation of many coniferous tree species (Hazubska-Przybył, 2019). In vitro propagation techniques are useful for mass production, conservation and restoration of forestry trees and overcome the problem of deforestation (Zaidi *et al.*, 2012). On the other hand, plant in vitro propagation can solve the issues with conventional conservation

methods because it is affected by the environment conditions, ageing of the plants, diseases and pests (Panis *et al.*, 2020). As a result, it has proven to be a trustworthy technique for plant multiplication, particularly when it comes to producing uncommon and endangered species (Francis *et al.*, 2007; Joshi and Dhawan, 2007; Offord and Tyler, 2009; Gonçalves *et al.*, 2010). The first research on the *in vitro* propagation of juniper associates carried out by Javeed *et al.* (1980). On the topic of juniper *in vitro* propagation, a few investigations have been conducted and published (Zaidi *et al.*, 2012; Gomez and Segura, 1994; Gomez and Segura, 1995; Negussie, 1997; Khater and Benbouza, 2019). Because micropropagation may be the sole other means of plant reproduction for this group, *in vitro* propagation of juniper species should be overstated (Hazubska-Przybył, 2019). Prioritizing it would be beneficial for both mass propagation for pharmaceutical applications and the possibility of conservation (Harry *et al.*, 1995). Plant tissue culture is an effective way to increase the production of secondary metabolites, claims (Hussain *et al.*, 2012). Because *J. promethea* regeneration through seeds is difficult, bulk propagation material for pharmaceutical purposes can be produced using the micropropagation technology, which will preserve natural regeneration through seeds in the wild. Consequently, the main goal of the current experiment was to optimize the medium and growth regulator concentrations for *in vitro* proliferation of *J. procera* shoots and roots to overcome the issues with vegetative reproduction and seed regeneration.

## 2. Materials and Methods

### 2.1. Plant material and explant sterilization

Shoots of selected Juniper trees (*Juniperus procera*), were collected from Baljurashi, Al-Baha region (41°35'4"E, 19°50'53.7"N) on the western south of Kingdom of Saudi Arabia(Figure, 1) and immediately transferred to the tissue culture lab., shoots were divided into segments each included 2-3 nodes

Shoot segments were washed under running water for half an hour then rinsed with alcohol at 70% (Taha, 2022). Four sterilization treatments were processed using Clorox (commercial bleach containing 5.5% of NaOCl) and mercuric chlorate (MC) as the following

1. Clorox at 50% for 15 min. followed by MC at 0.2 g/l.
2. Clorox at 50% for 30 min. followed by MC at 0.1 g/l.
3. Clorox at 50%for 30 min followed by Clorox at 30% for 15 min
4. Clorox at 50%for 30 min followed by Clorox at 30% for 30 min

Explants were rinsed three times with distilled sterilized water and cultured on autoclaved MS media (Murashige and Skoog, 1962), supplemented with benzyl amino purine (BAP) at 0.5 mg/l, sucrose at 30 g/l and agar at 8.0 g/l, cultured explants were incubated at growth chamber ( $25 \pm 2$  °C and 16 h photoperiod) for three weeks. Survival rate and contamination percentage were conducted.

## 2.2. Initiation stage

Juniper sterilized explants were cultured in MS media, supplemented with sucrose at 30 g/l and agar at 8.0 g/l and BAP at 0.0, 0.5 and 1.0 mg/l for initiation. Cultured explants were incubated under growth chamber conditions (16 hours light, 8 hours dark a day with 1500 flux and  $23 \pm 2$  °C). Nine jars each contained one explant were used for each treatment. Four weeks later, survival rate, number of sprouted buds and number of shoots per explant were recorded.

## 2.3. Multiplication stage

The sprouted Juniper explants from initiation media were and sub-cultured into multiplication medium. MS medium supplemented with BAP at 0.5, 1.0 and 2.0 mg/l with NAA at 0.0, 0.2 and 0.4 mg/l in addition to the control treatment (MS free growth regulators) were used for multiplication rate assessment. Nine jars each contained two shoots were used for this treatment. Sprouted shoots percentage, average shoot number per explant, average leaf number and shoot length were estimated.

## 2.4. Elongation stage

Every shoot from the earlier therapies was moved to  $\frac{3}{4}$  MS + 0.5 mg/l kinetin + 1.0 mg/l IAA as described by (Taha *et al.*, 2021). Two subcultures exploited this medium (4-weeks interval). Cultures were incubated at  $23 \pm 2$  °C and 2000 lux of light intensity is required for the transfer to rooting media.

## 2.5. Rooting stage

In vitro growing Juniper shoots with length of 2-3 cm from elongation medium were cultured in half strength MS or full strength olive medium (Rugini, 1984), supplemented with IBA at 0.0, 0.5, 1.0 and 1.5 mg/l. Ten shoots were cultured for each treatment and incubated at growth chamber with 16 hours light of 3000 flux. Plantlet length, rooting percentage, root number and root length were determined.

## 2.6. Statistical design:



The treatments of the current research were arranged in complete randomized design, with three replicate (three jars with three explants in each jar) for each treatment, and each replicate involved. Data were data was subjected to analysis of variance (ANOVA) and means were compared by LSD test (Snedecor and Cochran 1967).

### 3. Results

#### 3.1. Effect of type of sterilization agent, concentration and duration on *Juniperus procera* explants

Data in Figure(2) revealed that the highest survival rate (80%) for juniper explants was recorded when Clorox was used at 50% then 30% each for 30 min. followed by the same concentrations but for 30 min and 15 min, respectively. The lowest survival rate was recorded when Clorox was used at 50% for 15 min then explants were immersed in mercuric chloride at 0.2 g/l. This later treatment also showed a dramatically high contamination level. MC showed a toxic effect on juniper explants and did not decrease the contamination levels to a satisfied level. Fortunately, treating explants with Clorox at 50% then 30%, each for 30 min, lowered the contamination to the lowest level (40%). Moreover, sterilize shoot tip and nodal bud using sodium hypochlorite.

#### 3.2. Effect of BAP concentration on initiation stage of *Juniperus procera* explants

Data in Figure (3) indicated that BAP is crucial for sprouting of juniper explants. The concentration of 1.0 mg/l showed the highest sprouting rate (70%) followed by 0.5 mg/l compared with the control treatment. Similarly, the concentration of 1.0 mg/l obtained that the highest number of sprouted buds per explants. Average number of shoots per explants also took the same previous trend as the concentration of 1.0 mg/l showed the highest shoot number (Figure, 4).

#### 3.3. Effect of BAP and NAA concentrations on multiplication stage of *Juniperus procera* shoots

Data in Figure (5-8) indicated that growth regulator (BAP) had a significant effect on multiplication rate of juniper shoot. The highest percentage of responded shoots was occurred with 2.0 mg/l BAP without NAA while; the lower concentrations of BAP gave lower response Figure (5). NAA combination with BAP, lower the percentage of responded shoots. The best results with addition of NAA were noticed when combined with BAP at 2.0mg/l. With respect to shoot number parameter, data in Figure (6) showed that the highest shoot number was observed into medium supplemented with BAP at 2.0 mg/l followed with BAP at 1.0mg/land 2.0 mg/l

BA+0.2 mg/l NAA (Figure 9B and C). The higher concentration of NAA negatively affected shoot number. Meanwhile, shoot length showed a different trend as the highest shoot length was occurred at the control treatment (0.0 BA +0.0 NAA) followed by all BAP treatments without NAA while, addition of NAA to BAP into multiplication medium recorded lower shoot length (Figure 7). Leaf number had the same trend with shoot length as the control treatment gave the highest leaf number of juniper shoots. The higher concentration of BAP was added the lower leaf number was obtained. NAA had a negative effect on leaf number of juniper shoots (Figure 6).

The profiled shoots on the different multiplication media were transferred to  $\frac{3}{4}$  MS supplemented with 0.5 mg/l kinetin and 1.0 mg/l IAA. After two subcultures the cultured shoots showed enhanced shoot length and become suitable to sub-culture on the rooting media (Figure 9, D and E).

Shoots (2-3cm in length) produced from elongation medium were selected to use in rooting experiment (Figure 8, D and E). Data assured that juniper shoots are hard to root; most of the examined media and IBA concentration were inefficient to induce root formation on *Juniperus* micro-shoots. All juniper shoots cultured on  $\frac{1}{2}$  MS media with all IBA concentration failed to root, OM medium was responsible for rooting only when addition of IBA was implemented; Olive medium supplemented with IBA at 1.0 mg/l gave the highest percentage of rooted shoots (Figure, 10 and 13).

With respect to shoot length, the highest value was occurred at  $\frac{1}{2}$  MS+1.5 IBA and OM+1.0 IBA followed by  $\frac{1}{2}$  MS+0.5 IBA and OM+0.5 IBA or 1.5 IBA. With respect to leaf number, the highest value was occurred at  $\frac{1}{2}$  MS+1.5 IBA and OM+1.0 IBA followed by  $\frac{1}{2}$  MS+0.5 IBA then OM+0.5 IBA (Figure, 11 and 12).

#### 4. Discussion

*Juniperus procera* is a medical plant that grows in the Saudi Arabia (Seca *et al.*, 2015), Juniper leaves, fruits and seeds are rich with bioactive compounds and antioxidant (Nuñez *et al.*, 2008; Tumen *et al.*, 2013; Abdel Ghany *et al.*, 2014; Bitew, 2015). Nevertheless, juniper forests are steadily diminishing and many juniper species are considered rare (Romme *et al.*, 2009; Abrha *et al.*, 2018; Miller *et al.*, 2019). *In vitro* culture technique has potential substitute for mass clonal propagation and conservation of many tree species (Hazubska-Przybył, 2019). Contamination with different microorganism is a serious problem, of micro-propagation; eliminate microbial

contamination is a basic requirements for establishment of cultured plant tissues. Our results indicated that Clorox (5% sodium hypochlorite) is an important sterilization agent for juniper and its duplicated treatment (50% then 30%) gave the highest survival rate and the lowest contamination percentage. The sterilization efficiency of sodium hypochlorite was reported previously; *Juniprus navicularis* micro-cuttings was sterilized using 70% ethanol, 3% commercial bleach, and in 1% Castro *et al* 2011. Also, *J. excels* shoot tip explants showed higher sterilizing when treated with 2.5% sodium hypochlorite (Kashani et al., 2018). A high sterilized degree was obtained by using ethanol, followed by immersion in commercial bleach, and 1% Benlate solution Snedecor and Cochran 1967. Immersing of explant in a fungicide solution, followed by immersion in NaOCl solution, a very high level of sterilization was obtained (Khater and Benbouza, 2019). Sodium hypochlorite recorded higher survival percentage for pomegranate explants (Sanjy et al., 2010). Using a combination of NaOCl successfully sterilize axially bud and segments in pomegranate and Jack fruit with good survival rate (Damisno and Padro, 2008 and Faisal et al., 2010). Shoot multiplication is highly affected by plant genotype, growth medium, and cytokinin; our results indicated that BAP was essential for proliferation and multiplication of juniper explant. Similarly, Qarachoboogh et al. (2022) found that the optimal culture medium for shoot growth of was supplemented with BAP at 1.0 mg/l and IAA at 0.1 mg/l *J. foetidissima*, while Salih et al. (2021) assured that the highest shoot multiplication was obtained with 0.5  $\mu$ M BAP combined with 0.5  $\mu$ M IAA or 0.5  $\mu$ M IBA. Higher shoots number was produced in medium containing 0.5 mg/l BAP as *J. excelsa* produced 6 shoots, *J. horizontalis* 8 shoots while *J. chinensis* produced 9 shoots per explant. However, presence of auxin maybe inefficient or retard shoots growth as we noticed in the current experiment; NAA had a negative effect on shoot number and shoot length. Kuritskaya et al. (2016) assured that when the IBA concentration was raised in the medium of *J. chinensis* var. *sargentii* the number of buds and the length of shoots reduced. In addition, 0.5mg/L of BAP resulted in a greater shoot proliferation rate (5.37 shoots per explant) of a dwarfing cherry rootstock (Mahdavian et al., 2011). In addition, sweet cherry cultivar "Lapins" demonstrated high frequency shoot proliferation when grown on the basic MS medium with reduced BAP concentrations added. Conversely, a greater concentration of BAP also produced good shoot elongation (Ruzic and Vujovic 2008). The highest shoots number of Jack fruit explant was obtained on MS medium with BAP (Damisno and Padro, 2008). Many researchers tried to produce juniper rooted plantlets

but it seems that juniper is hard to root. Ioannidis et al. (2023) studied *Juniperus drupacea* micropropagation; they found that using IBA, NAA, or IAA in various concentrations was proven to be ineffective for its rooting. According to Qarachoboogh et al. (2022) juniper is rarely propagation by cuttings due to the poor rooting of stem cuttings; which may be overcome by in vitro rooting. Root induction depends on the composition of culture media and phytohormones; moreover, auxin concentration and method of treatment are important factors affecting root induction (Amiri et al., 2019). IBA is the commonly applied at low concentrations for root induction (Beyramizadeh et al., 2020), further increase in auxin concentration inhibited rooting growth (Negussie, 1997). Farzan et al. (2023) reported that root formation of juniper shoots was not observed until six months, but about 10% of these regenerated shoots produced roots eight months after shoot proliferation. They claimed that various factors such as genotype, polyploidy or hybrid formation, slow plant growth (which is common in conifers), or secondary compounds are the reasons for the low rate of regeneration, especially rooting. Also, the duration of cultivation can be effective in rooting. Nevertheless, rooting occurred in three species of juniperus using shoots with length of 4-7cm. The best roots produced in WPM with IBA at 1.0mg/l but these roots not enough to support plantlet (Zaidi et al., 2012). Similarly, Castro et al. (2011) reported that more rooting was obtained in juniper micro-shoots cultured on Olive Medium (OM) supplemented with IBA at 12.3µM. However, Negussie (1997) indicated that spontaneous rooting at a low percentage (10.0%) could be observed on WPM media with 0.1 mg/l IBA after a long period of cultures, further increase in IBA concentration inhibited root growth. In addition, Kuritskaya et al. (2016) assured that adventitious roots developed in *M. decussate* after three months of cultivation on MS medium with 0.1 mg/l IBA. Nevertheless, Kocer et al. (2011) said that adventitious root-like structures were formed in multiple experimental trials using 0.005, 0.03, and 0.05 mg/l of indole-3-butyric acid; however, none of these structures went on to grow into a real root system. Rugini medium has enriched composition compared to MS and contains folic acid which was found to be useful in root induction (Mustafa et al., 2018). OM supplemented with 3mg/L IBA achieved rooting percentage of 85% (Peixe et al., 2007). Also, MS medium containing 2.0 mg/L IBA has been found essential for obtaining good rooting during in vitro rooting (Sanjay et al., 2010).

## 5. Conclusions

Juniper is valuable medicinal tree growing in Saudi Arabia, it challenges with seed dormancy, underdeveloped embryo, and low germination rate. In vitro propagation considered as an alternate technique for large-scale production. The current study aimed to optimizing in vitro propagation of Juniper trees. However Juniper is a recalcitrant species to in vitro conditions; the micropropagation of Juniper is highly depends on the nutrient media and growth regulators. The highest proliferation rate, shoot number was recorded for BAP at 2.0 mg L<sup>-1</sup>; addition of NAA negatively affected multiplication rate and shoots growth. Juniper shoots demonstrated a low rooting potential, as, most of the examined treatments were inefficient; addition of 1.0 mg l<sup>-1</sup> IBA to Olive media exhibited better results than the other treatments. Future studies are required to improve the current micropropagation protocol.

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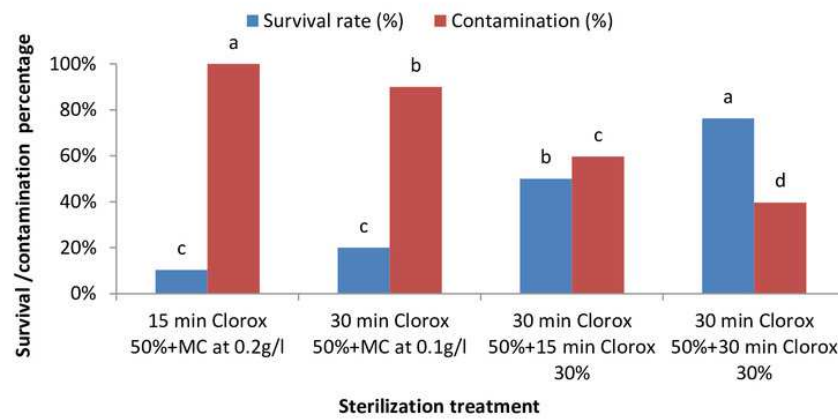
# Figure 1

Map of the sample collection with its ordination, Baljurashi, Al-Baha, Kingdom of Saudi Arabia.



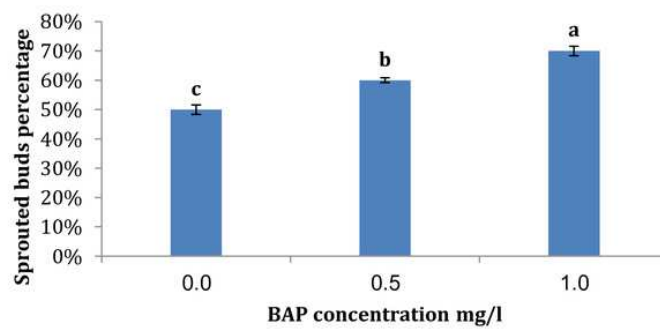
# Figure 2

Effect of sterilization agent, concentration and duration on *Juniperus procera* explants; different letters indicate statistical differences between treatments at 1% according LSD Test. Error bars represent the standard deviation.



# Figure 3

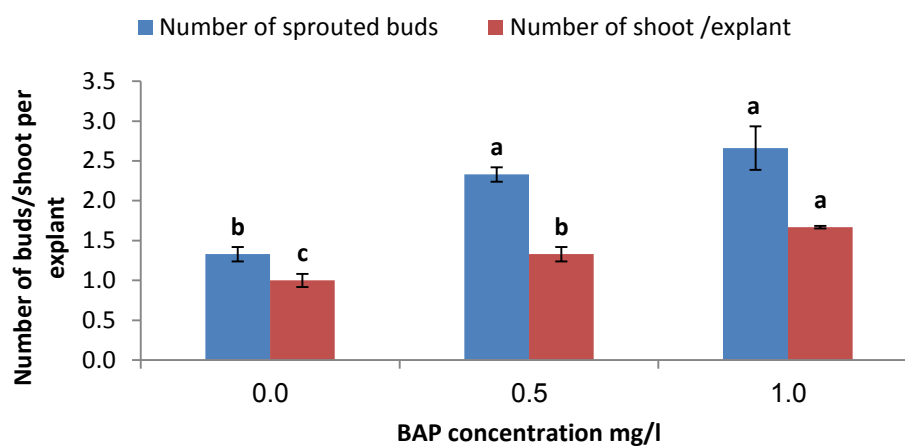
Effect of BAP concentration on sprouting percentage of *Juniperus procera* explants; different letters indicate statistical differences between treatments at 1% according LSD Test. Error bars represent the standard deviation



# **Figure 4**(on next page)

Effect of BAP concentration number of sprouted buds and shoot number per explant of *Juniperus procera*; different letters indicate statistical differences between treatments at 1% according LSD Test. Error bars represent the standard deviation.

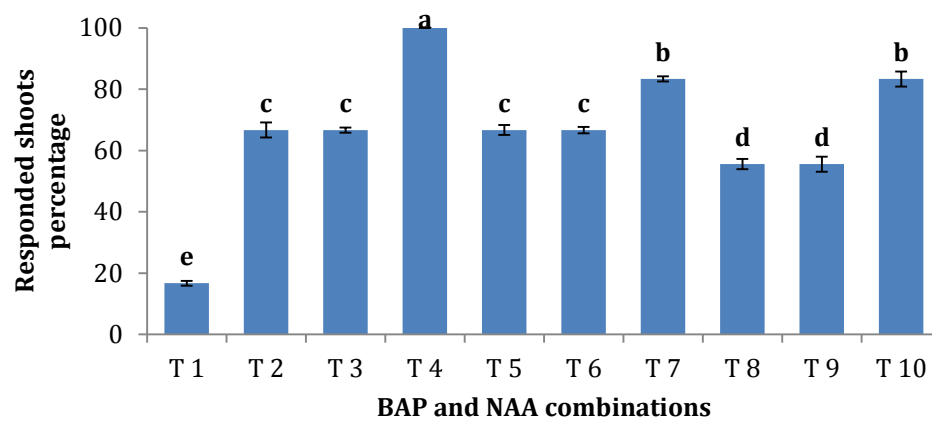






# **Figure 5**(on next page)

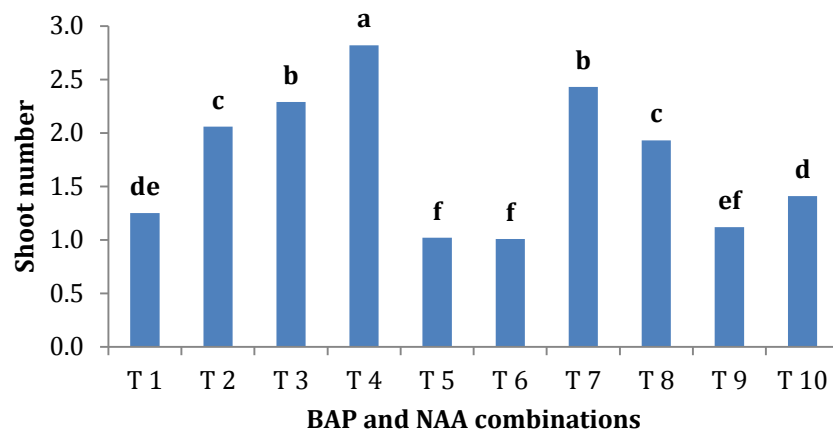
Effect of BAP and NAA combination on responded shoots percentage of Juniperus procera; different letters indicate statistical differences between treatments at 1% according LSD Test. Error bars represent the standard deviation.





# **Figure 6**(on next page)

Effect of BAP and NAA combination on shoot number of *Juniperus procera*; different letters indicate statistical differences between treatments at 1% according LSD Test. Error bars represent the standard deviation

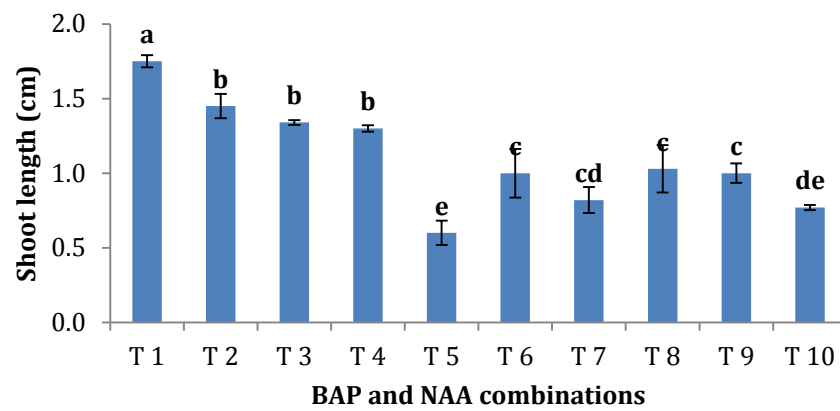






# **Figure 7**(on next page)

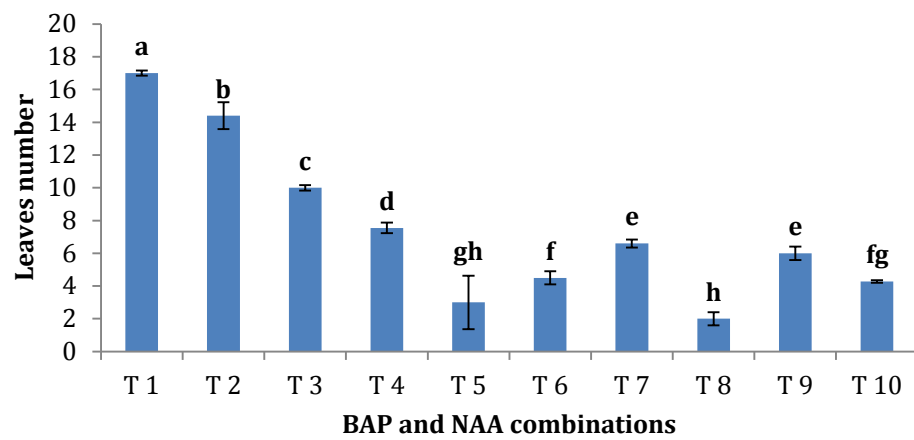
Effect of BAP and NAA combination on shoot length of *Juniperus procera*; different letters indicate statistical differences between treatments at 1% according LSD Test. Error bars represent the standard deviation.





# **Figure 8**(on next page)

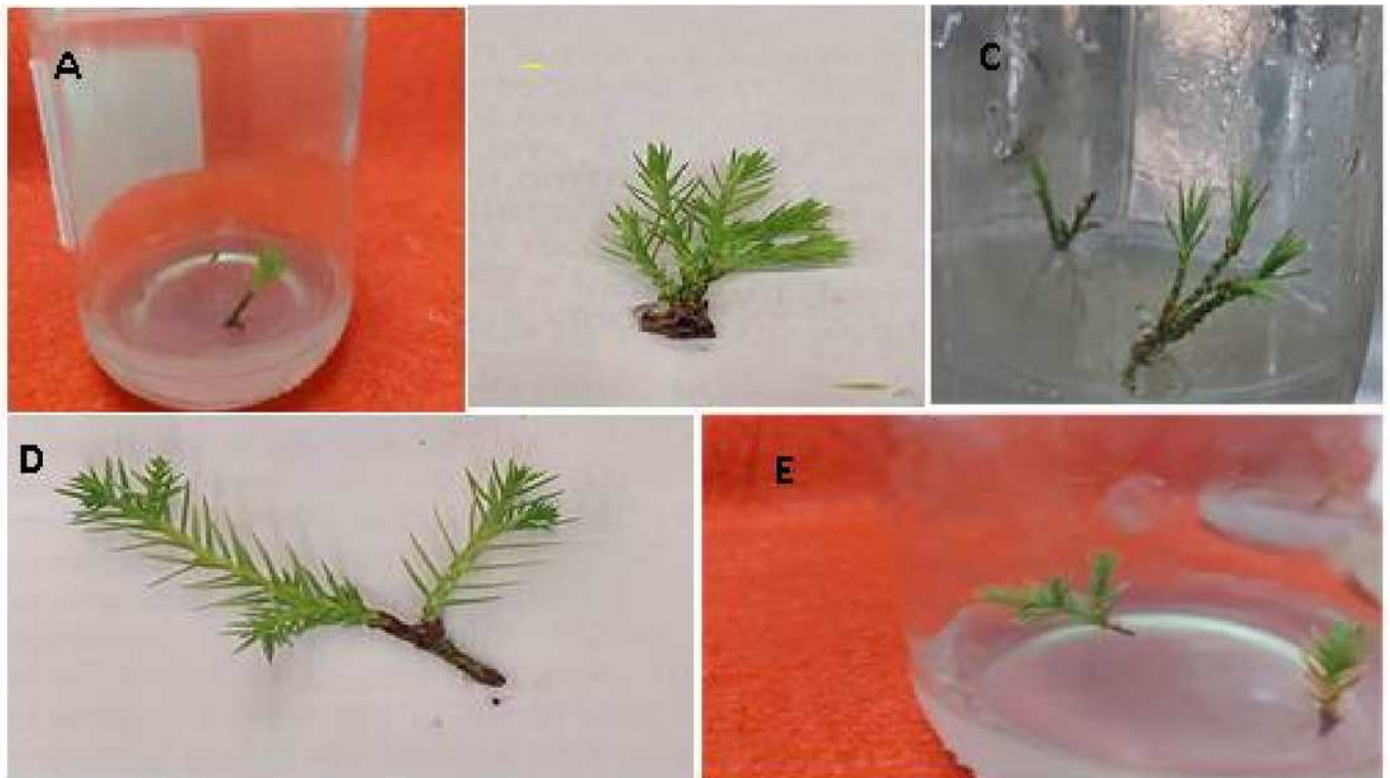
Effect of BAP and NAA combination on shoot length of *Juniperus procera*; different letters indicate statistical differences between treatments at 1% according LSD Test. Error bars represent the standard deviation





# Figure 9

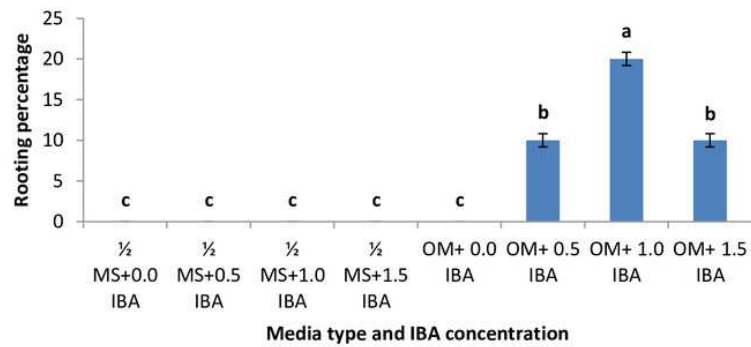
Sprouted juniper shoots (A); multiple shoots produced on MS medium supplemented with 2.0mg/l BAP (B and C); elongated shoots cultured on  $\frac{3}{4}$ MS medium with 0.5 mg/l kinetin + 1.0 mg/l IAA (D and E).



# Figure 10

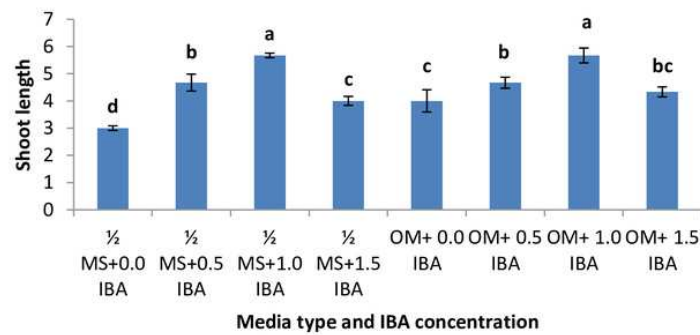
Effect of type of media and IBA concentrations on rooting percentage of *Juniperus procera* shoots; different letters indicate statistical differences between treatments at 1% according LSD Test. Error bars represent the standard deviation.





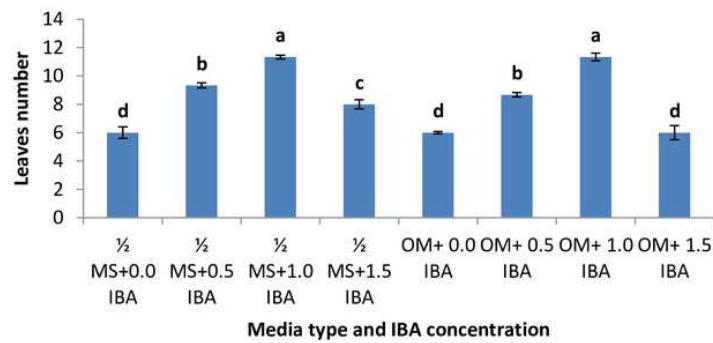
# Figure 11

Effect of type of media and IBA concentrations on shoot length of *Juniperus procera* shoots; different letters indicate statistical differences between treatments at 1% according LSD Test. Error bars represent the standard deviation.



# Figure 12

Effect of type of media and IBA concentrations on leaves number of *Juniperus procera* shoots; different letters indicate statistical differences between treatments at 1% according LSD Test. Error bars represent the standard deviation



# Figure 13

Rooted *Juniperus procera* shoots growing on OM supplemented with IBA

