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Genetic diversity and *in vitro* propagation of some *Acacia spp.* trees grown in Taif governorate.

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In current study, genetic relationship between some *Acacia* species tree grown in Taif governorate using inter simple sequence repeat (ISSR) marker and *in vitro* propagation of *Acacia jonwoodii* were investigated. Concerning ISSR analysis, a total of 60 reproducible bands were yielded with an average 7.5 fragments/primer, the bands size ranged from 150 to 1500 bp. The total number of poly-morphic bands 48 ranged from 4 (primer 834-2 and primer 842) to 9 (primer 817) with an average of 6 poly-morphic bands/primer, whereas the total number of mono-morphic bands (NMB) were 12 bands. The results indicated that the percentage of poly-morphic bands (PB %) ranged from 66.7% (primer 818) to 100% (primer 841-2) with an average 80.13 %. This means that ISSR analysis revealed high genetic variation between five *Acacia* species. For *in vitro* propagation of *A. jonwoodii*, the highest average number of shoot/explants 10 was noticed on MS medium augmented with 2.5 mg/l of 6-benzylaminopurine (BAP). The highest average number of shoots produced roots 12 with average number 1.7 (roots/shoot) and average percentage 40 % were resulted in half strength MS medium containing 3 mg/l of Indole-3-butyric acid (IBA). The results indicated that ISSR marker have been successfully applied for studying the genetic relationship between five *Acacia* species and a reliable protocol for *in vitro* propagation of *A. jonwoodii were* established that could be helpful tools for plant breeding programs and for plant preservation.

Keywords: *Acacia* species, genetic variation, ISSR, *Acacia jonwoodii*, *in vitro* propagation.

INTRODUCTION

Multipurpose tree, genus *Acacia* Miller from family Leguminosae, it currently includes about 1300 species (Maslin, 2001). It is widespread in Africa, the Americas, Asia and Australia. It can be grown in arid and moist regions of the world. The tree is highly valued for centuries for gum arabic production, which is used as food additives (Dondain and Phillips, 1999), in pharmaceuticals and other industries (Anderson and Weiping, 1992) and as a source for soil reclamation by fixation of atmospheric nitrogen (Fagg and Allison, 2004). In Saudi Arabia, acacias are distributed according to the variety of climates and soil. In north and central of Saudi Arabia, *A. ehrenbergiana* Hayne and three varieties of *A. tortilis* (Forssk) Hayne are recorded (Aref, 2000). However, in the foot-hill sites, along the red sea coast south Jeddah, *A. albida*, *A. etbaica* (Schweinf) and *A. asak* (Forssk) Willd are grown (Vesey-Fitzgerald, 1955). Despite they have a high reproductive capacity and because of their

narrow genetic diversity and geographical range, acacia population in Saudi Arabia is threatened small population size and low density due to extreme environmental conditions, and indiscriminate cutting of trees (Aref and El Atta, 2013). Studying the genetic diversity within *Acacia* species would be helpful in plant breeding programs and for *ex situ* conservation of plant germplasm (Byrne, 2003). DNA finger printing of all the genetic resources of the timber yielding tree plants is a necessity for generating a molecular database to catalogue as well as to utilize the information in a systematic manner (Gaston and Kunin, 1997). Nowadays, several molecular markers techniques such as inter simple sequence repeat (ISSR) have been widely employed for analysis of genetic diversity between different plant species. ISSR was applied in a lot of molecular genetic studies because it does not need information about the sequence, low cost, simplicity and DNA is required in small amount (Lakshmanan et al, 2007). Genetic diversity has been reported for several *Acacia* species (Byrne et al, 2001). A large scale cultivation of this drought resistant tree on arid lands would help in conserving its gene pool and reclaiming the wastelands. Plant regeneration by tissue culture has been attempted for few species of *Acacia* (Vengadesan et al, 2002). *In vitro* propagation for different *Acacia* species were reported (Rout et al. 2008 and Varshney et al, 2013).

The main objectives for current study to assess the genetic diversity among some *Acacia* species using ISSR DNA based marker and to establish *in vitro* propagation protocol of *A. jonwoodii* grown in Taif governorate. For our knowledge, the present study is the first study on *in vitro* propagation of *A. jonwoodii*.

MATERIALS AND METHODS

Plant material

The seeds of eight species of *Acacia* genus were collected from different sites in Taif governorate (Table 1). Nomenclature was according to Chaudhory (2000). The seeds were pretreated with boiling water for 5 min to facilitate rapid germination according to the Mukherjee and Sharma (1995) and left overnight in half strength (Murashige and Skoog, 1962) MS liquid medium at room temperature (25-30 °C) and cooling temperature at 4°C (Table 2). Seeds were surface sterilized using 70% ethanol for 10 sec, followed by dip in 20% (v/v) commercial bleach sodium hypochlorite (NaOCl) solution for 10 min with continuous shaking, where after they were rinsed three times with sterilized distilled water and then germinated on half strength MS basal salt media.

Genetic diversity analysis

DNA isolation

0.1 g of leaf tissue were collected from 1 month-old seedlings of five *Acacia* species (A_1, A_2, A_3, A_4 and A_5) *A. etabica*, *A. gerrardii ssp. gerrardii*, *A. grrardii ssp. najediensis*, *A. jonwoodii* and *A. ehrenbergiana*, respectively for DNA extraction (Table 2). DNAs were extracted using (Wizard® Genomic DNA Purification Kit, Promega Corporation, USA) according to the manual protocol to isolate genomic DNA from plant tissue; the quantity and quality of DNA were estimated by 1.0% agarose gel. Then the DNA samples were stored at -20 °C.

Inter simple sequence repeat (ISSR)

Twenty ISSR primers, from the University of British Columbia (UBC) series which were synthesized by Macrogen Inc. Biotechnology Company- Seoul- South Korea, were used. Amplification was carried out in 25 µl /reaction containing 12.5 µl Go Taq® Green Master Mix, which contains {*Taq* DNA polymerase, dNTPs, $MgCl_2$ and reaction buffers (Promega Corporation, USA)}, 25 ng genomic DNA (1 µl), 4.0 µl of ISSR primers (10 pmols/µl) and 7.5 µl of double distilled H_2O to make up the volume. PCR amplifications were performed, using thermal cycler (C1000^{TM} BIO Rad), with initial denaturation at 94°C for 4 min followed by 35 cycles of 1 min denaturation at 94 °C, 1 min at the annealing temperature (50 - 53°C) according to the primer, 1 min extension at 72 °C with a final extension at 72 °C for 7 min. The PCR products were separated on 1.5 % agarose gel using 100 bp and 1 kb markers as the band size standard and photographed in a gel documentation system (IN GENIUS, SYNGENE BIO IMAGING).

In vitro Propagation

Culture media Preparation and Culture Conditions

MS medium with 3% (w/v) sucrose, 0.7% (w/v) phytoagar and modifications of cytokinins and auxins concentrations were applied for *in vitro* culture stages. The pH of culture media was adjusted to 5.7-5.8 using 1.0 N potassium hydroxide and 1.0 N hydrochloric acid before adding phytoagar. Culture media were autoclaved

for 20 min at 121°C and 1.5 k/cm^2 pressure. As a control, MS medium without growth regulators was used for all *in vitro* culture treatments. Incubation conditions 25± 2 °C, 16/8 h light/dark and 3,000 Lux light intensity (fluorescent light) were used for *in vitro* culture stages.

In vitro Culture

Shoot tips and axillary buds were excised from one month *in vitro* grown seedlings of *A. jonwoodii* and were cultured for three to four weeks in shoot initiation media containing full strength MS medium supplemented with different concentrations of BAP (0.0, 1.5, 2.0, 2.5 and 3.0 mg/l) and NAA 0.5 mg/l + 3 % Sucrose + 0.7 % (w/v) phytoagar (Table 6). The explants which formed Shoots were subcultured on the same fresh media for three to four weeks. For shoot multiplication and elongation, induced shoots were subcultured on the same media for another three to four weeks. This step was repeated 3-4 times on the same medium for mass production. For rooting stage, elongated shoots (3 to 4 cm length) were transferred in half strength MS basal salts augmented with different concentrations of IBA (0.0,0.5, 1.0, 1.5, 2.0, 2.5, 3.0,3.5,4.0 and 4.5 mg/l) for a further three to four weeks (Table 7). For acclimatization stage, good rooted plantlets were carefully washed with warm H_2O to remove adhered agar and traces of the medium; then they were transplanted to plastic pots diameter (15 cm) containing sterile peat moss under greenhouse conditions. The top of the pots was covered with transparent plastic.

Data Analysis

For genetic variation according to ISSR analysis, the band patterns with high resolution were scored manually as present (1) or absent (0); cluster analysis and dendrogram using average linkage between species were produced by Hierarchical Cluster Analysis using (IBM SPSS STATISTCS Version 20). For *in vitro* propagation, all experiments were carried out in three replicates. The data were collected from different experiments four weeks after culture. A statistical analysis of data, analysis of variance and the comparison between the mean values of treatments were carried out using Duncan Test at a level of 5% of probability, using Statistical Assistance Software (ASSISAT) Version 7.6 beta (2014).

RESULTS

Effect of overnight incubation at RT and 4 °C on seeds germination of some *Acacia* species which pretreated with boiling water for 5 min, was observed after 3-4 weeks from culture on germination medium. As shown in Table 2, the percentages of germinated seeds were varied between different species, which may be a result of seed vitality for each species. The percentage of germinated seeds was increased when the seeds were incubated at RT and 4 °C compared with untreated seeds. These results are in agreement with that reported by Tanabe and Honda (1999) they found that, pre-conditioning by mechanical scarification and incubation in cold water for 24 h was very effective to increase the rate of seeds germination of *A. koa***,** they also reported that hot water treatment increase the seed coat pore size which resulting in an increased germination factors.

Table 1: Some *Acacia* species collected from different regions from Taif governorate.

***Acacia* species**	**Regions**
Acacia asak and *Acacia ehrenbergiana*	AL-seel Alkabeer road
Acacia etabica and *Acacia gerrardii ssp. gerrardii*	Shafa site
Acacia gerrardii ssp. najadiensis	Hada site
Acacia johnwoodii and *Acacia laeta*	Taif-Jeddah (Non Mussilem) road
Acacia tortilis ssp. raddiana	Sediara site

Genetic diversity analysis

Inter simple sequence repeat (ISSR)

The species structure and phylogenetic are the results of interaction between different factors, such as geographical range, evolution of species, mating method, genetic drift, seed dispersal and gene flow (Hogbin and Peakall, 1999).

In the present study, 8 out 20 primers with clear high intensity and relatively high polymorphism bands were selected and used to amplify five *Acacia* species (Table 3). Concerning ISSR analysis, a total of 60 reproducible bands were yielded with an average 7.5 fragments/primer, the band size ranging from 150 to 1500 bp, the total number of poly-morphic bands (NPB) 48 ranged from 4 (primer 834-2 and primer 842) to 9 (primer 817) with an average of 6 PB/primer, where number of mono-morphic bands (NMB) were 12 bands,

Table 2: Effect of pre-conditioning treatment on seed germination of some *Acacia* species.

Species	SN	Cooling (4°C)		RT(25-30°C)	
		NGS	NGS %	NGS	NGS %
Acacia Asak	16	0	0.0	0	0
Acacia gerrardii ssp. gerrardii	25	0	0.0	2	2
Acacia gerrardii ssp. najediensis	26	6	23	2	2
Acacia jonwoodii	23	10	43.5	12	12
Acacia laeta	13	0	0.0	0	0
Acacia tortilis ssp. raddiana	7	0	0.0	0	0
Acacia etabica	15	4	26.7	2	2
Acacia ehrenbergiana	19	14	73.7	19	19

Note: SN: Seeds number, NGS: Number of germinated seeds, RT: Room temperature.

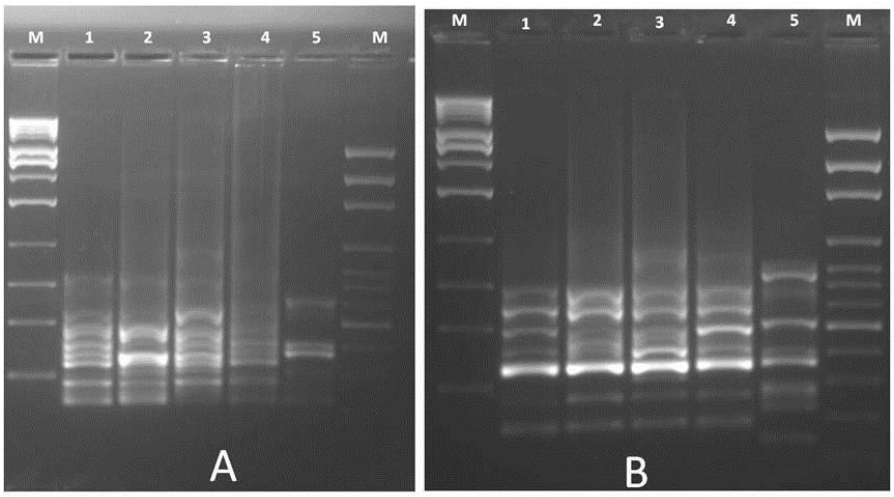


Figure 1: DNA polymorphism based on ISSR analysis of five *Acacia* species. A: ISSR profiles using primer 817, M (Left): DNA marker 100bp, lanes 1-5 indicate to five *Acacia* species and M (Right): DNA marker 1kb. B: ISSR profiles using primer 818, M (Left): DNA marker 100bp, lanes 1-5 indicate to Five A. spp. and M (Right): DNA marker 1kb.

Table 3: ISSR primers and PCR products amplified by ISSR analysis for different genotypes of five *Acacia* species.

Primer	Sequence (5-3)	TB	RBS (bp)	NMB	NPB	PB%
815	$(CT)_8G$	9	1500-300	2	7	77.8
817	$(CA)_8A$	11	1000-200	2	9	81.8
818	$(CA)_8G$	9	750-150	3	6	66.7
834-1	$(AG)_8CT$	7	700-200	2	5	71.4
834-2	$(AG)_8TT$	5	450-150	1	4	80.0
836-1	$(AG)_8CA$	6	1000-200	1	5	83.3
841-2	$(GA)_8TC$	8	650-200	0	8	100
842	$(GA)_8T$	5	500-250	1	4	80.0
Total		60	1500-150	12	48	-
Average		7.5	-	1.5	6.0	80.13

Note: TB: total bands, RBS (bp): Range of band size bp, NMB: number of mono-morphic bands, NPB: number of poly-morphic bands

the representative ISSR profiles using primers 817 and 818 are shown in Figure 1 (A and B). The results indicated that the percentage of poly-morphic bands (PB %) ranged from 66.7 % (primer 818) to 100 % (primer 841-2) with an average 80.13%, indicating high genetic variation between five *Acacia* species (Table 3).

Mehetre et al. (2004) reported that bands which are found in all individuals considered as mono-morphic bands. However, poly-morphic bands are not resulted in all individuals (one or more) and unique bands which are recorded in at least one individual.

As shown in Table 4, the results indicated that, In *A. etabica*, band of molecular size 270 bp (primer 818) was not resulted in this species so that it could be used as negative molecular marker (NMM) for this species. In *A. gerrardii ssp. gerrardii*, bands of molecular size 650 bp (primer 841-2), 400 bp (primer 842) and 250 bp (primer 834-2) which were recorded only in this species could be used as positive molecular marker (PMM) for this species. In *A. grrardii ssp. najediensis*, bands of molecular size 1000 bp (primer 817), 700 bp (primer 834-1) and 550 bp (primer 817) were only found in this species so that it could be used as PMM for *A. grrardii ssp. najediensis*. In *A. jonwoodii* bands of molecular size 200 bp (primer 841-2) was only recorded in this species, a unique band, could be used as PMM for *A. jonwoodii*. In *A. ehrenbergiana*, bands of molecular size 1500 bp (primer 815), 750 bp (primer 818), 600 bp (primer 817 and primer 841-2), 550 bp (primer 815), 400 bp (primer 841-2) and 300 bp (primer 842) which were only resulted in this species, it could be used as PMM for this species. Where, bands of molecular size 650 bp (primer 818), 600 bp (primer 815 and primer 818), 500 bp and 450 bp (primer 815, primer 817 and primer 836-1), 400 bp (primer 834-2), 380 bp (primer 815), 350 bp (primer 817, primer 841-2 and primer 842), 300 bp (primer 817 and primer 834-2) and 200 bp (primer 818) were recorded in all *Acacia* species in present study except *A. ehrenbergiana*. Therefore, these bands could be used as NMM for this species.

As shown in similarity matrix between five *Acacia* species (Table 5) and the dendrogram (Figure 2), five *Acacia* species were divided into two clusters, first cluster consisted of two sub cluster, sub cluster 1 containing A_1 and A_4, sub cluster 2 containing A_2 and A_3. Second cluster containing only A_5, which mean that A_5 species is genetically diverse from other species, this as a result of the geographic distribution of the species, geographically close species have short genetic distance internodes in the dendrogram (Gavlan et al. 2003).

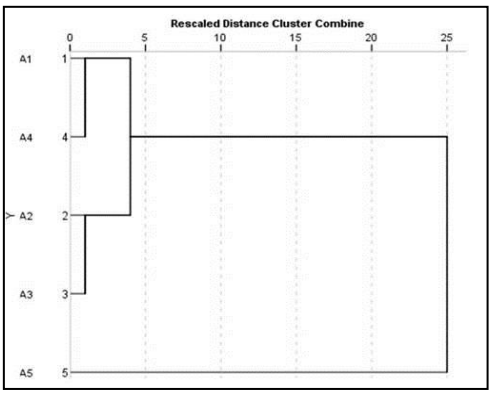


Figure 2: Dendrogram analysis between five *Acacia* species based on ISSR marker.

Our results support the results obtained by Waly and Emad (2012) they reported that, the taxonomical studies of eight *A. species* according to Paracheal and paratracheal parenchyma, investigated species were divided into three groups, group one, the parenchyma is abundant paratracheal and aliform confluent, contained five *A. species* (*A. gerradii, A. raddiana, A. tortilis, A. asak* and *A. hamulosa*). Group two, the parenchyma is predominant a potracheal and rays 1-4 seriate, included only one *A.* species *(A. ehrenbergiana*). The third group, the parenchyma was found in confluent bands alternating with fiber bands, included two *A. species* (*A. nubica* and *A. etabica*). Pometti et al. (2015) reported that morphological analysis between three *Acacia* species (*A. caven, A. farnesiana and A. curvifructa*) showed that they do not harbor consistent morphological discontinuities. On the other hand, molecular analysis for three species using amplified fragment length polymorphism (AFLP) recorded a significant variation between three species, which considered them as different species. The results indicated that ISSR marker could be used as a helpful tool for determining the genetic variation between five *Acacia* species in the present study.

In vitro Propagation

Under *in vitro* conditions, it was so difficult to propagate or regenerate woody plants especially *Acacia* species, but nowadays a lot of success has been reported (Mittal et al. 1989). In the present study different concentrations of BAP with 0.5 mg/l of NAA were applied to study its effect on shoot multiplication of *A. jonwoodii*.

Table 4: ISSR profiles of different genotypes of *Acacia* species.

P-817 (bp)	A_1	A_2	A_3	A_4	A_5
1000	0	0	1	0	0
700	1	1	1	0	0
600	0	0	0	0	1
550	0	0	1	0	0
500	1	1	1	1	0
450	1	1	1	1	0
400	1	1	1	1	1
380	1	1	1	1	1
350	1	1	1	1	0
300	1	1	1	1	0
200	1	1	0	1	0

P- 846-1 (bp)	A_1	A_2	A_3	A_4	A_5
1000	0	1	1	1	0
500	1	1	1	1	0
450	1	1	1	1	0
350	1	1	1	1	1
300	0	1	1	0	0
200	0	1	1	0	0

P-815 (bp)	A_1	A_2	A_3	A_4	A_5
1500	0	0	0	0	1
1000	0	1	1	0	0
600	1	1	1	1	0
550	0	0	0	0	1
500	1	1	1	1	0
450	1	1	1	1	0
380	1	1	1	1	0
350	1	1	1	1	1
300	1	1	1	1	1

P- 841-2 (bp)	A_1	A_2	A_3	A_4	A_5
650	0	1	0	0	0
600	0	0	0	0	1
550	1	1	1	0	0
450	0	0	1	0	1
400	0	0	0	0	1
350	1	1	1	1	0
250	1	0	0	0	1
200	0	0	0	1	0

P-842 (bp)	A_1	A_2	A_3	A_4	A_5
500	0	1	1	0	0
400	0	1	0	0	0
350	1	1	1	1	0
300	0	0	0	0	1
250	1	1	1	1	1

P-818 (bp)	A_1	A_2	A_3	A_4	A_5
750	0	0	0	0	1
650	1	1	1	1	0
600	1	1	1	1	0
500	1	1	1	1	1
400	1	1	1	1	1
350	1	1	1	1	1
270	0	1	1	1	1
200	1	1	1	1	0
150	0	0	0	0	1

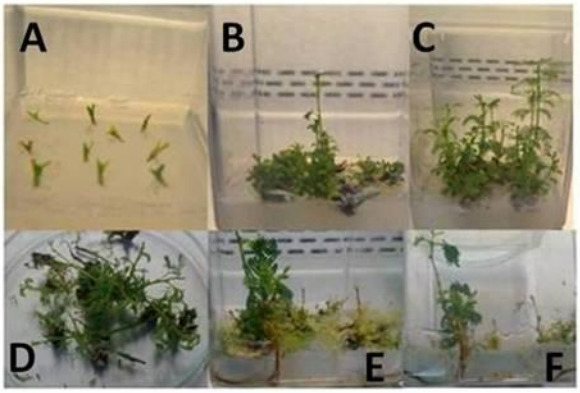
P- 834-1 (bp)	A_1	A_2	A_3	A_4	A_5
700	0	0	1	0	0
500	1	1	1	1	1
400	1	1	1	1	1
350	0	1	1	1	0
300	0	1	1	1	0
250	0	1	1	1	0
200	0	1	1	1	0

P- 834-2 (bp)	A_1	A_2	A_3	A_4	A_5
450	1	1	1	1	1
400	1	1	1	1	0
300	1	1	1	1	0
250	0	1	0	0	0
150	0	1	0	0	1

Table 5: Similarity matrix between five *Acacia* species based on ISSR analysis.

Treatment	A_1	A_2	A_3	A_4	A_5
A_1	0.000	0.817	0.812	0.903	0.500
A_2	0.817	000	0.902	0.880	0.426
A_3	0.812	0.902	0.000	0.877	0.441
A_4	0.903	0.880	0.877	0.000	0.500
A_5	0.500	0.426	0.441	0.500	0.000

Figure 3: *In vitro* propagation of *Acacia johnwoodii* plant.



A: Bud explants on shoot induction medium, B: Shoot multiplication stage, C: Shoot elongation stage, D: Preparation of elongated shoots for sub-culture on rooting medium, E and F: rooting stage

Table 6: Effect of different concentrations of BAP and NAA on shoot multiplication of A. *jonwoodii*.

GR (mg/l)		NE	Shoot tip		NE	Axillary buds	
BAP	NAA		ANS/E	ANS/E %		ANS/E	ANS/E %
0.0	10		0	0	10	6^{c}	60^{c}
1.5	10	10	0	0	10	8^{b}	80^{b}
2.0	10	10	0	0	10	8^{b}	80^{b}
2.5	10	10	0	0	10	10^{a}	100^{a}
3.0	10	10	0	0	10	10^{a}	100^{a}

Note: GR: Growth regulators, NE: Number of explants, ANS/E: Average number of shoots/explants.

Table 7: Effect of half strength basal salt with different concentrations of IBA on root formation of *A. jonwoodii*.

IBA (mg/l)	NS	ANSPR	ANR/S	R %	ANSPC	SPC %
0.0	30	1.0^{e}	1.0^{c}	3.33^{f}	0.00^{i}	0.00^{i}
0.5	30	10^{b}	1.3^{c}	33.3^{b}	9.00^{g}	30.0^{g}
1.0	30	8.0^{c}	1.0^{c}	26.7^{c}	13.0^{e}	43.3^{e}
1.5	30	10^{b}	2.1^{ab}	33.3^{b}	11.0^{f}	36.7^{f}
2.0	30	8.0^{c}	1.3^{c}	26.7^{c}	4.00^{h}	13.3^{h}
2.5	30	4.0^{d}	2.0^{ab}	13.3^{e}	4.00^{h}	13.3^{h}
3.0	30	12^{a}	1.7^{bc}	40.0^{a}	16.0^{d}	53.3^{d}
3.5	30	5.0^{d}	1.6^{bc}	16.7^{d}	18.0^{c}	60.0^{c}
4.0	30	9.0^{b}	1.7^{bc}	30.0^{b}	27.0^{a}	90.0^{a}
4.5	30	4.0^{d}	2.3^{a}	13.3^{e}	24.0^{b}	80.0^{b}

Note: NS: Number of shoot, ANSPR: Average number of shoot produced roots, ANR/S: Average number of roots /shoot, %R: Percentage of roots, ANSPC: Average number of shoots produced callus, %SPC: Percentage of shoots produced callus. The Duncan Test at a level of 5% of probability was applied. The averages followed by the same letter do not differ statistically between themselves.

As shown in Table (6), shoot tip explants did not produce any adventitious shoots when it were cultured on different treatments of MS media with growth regulators to enhance shoot multiplication. However, axillary buds explants produced the highest average number of shoot/explants 10 was noticed on the MS media augmented with 2.5 or 3 mg/l BAP (Figure 3A, B). These results agreed with those of Beck et al. (1998). In *A. koa*, shoots were induced by using BA (Skolmen and Mapes, 1976). A highest average number of multiple shoots 6.3 and 6.7 (shoots/explants) of *in vitro* propagated *A. seyal* Del. were resulted in MS media supplemented with BA and NAA. However, MS with TDZ induced multiple stunted shoots (Al-Wasel, 2000). IAA, IBA and NAA were successfully applied for *in vitro* root induction stage for different *Acacia* species (Gantait et al. 2016).

The Duncan Test at a level of 5% of probability was applied. The averages followed by the same letter do not differ statistically between themselves. The results showed that, after four weeks from subculture of elongated shoots (Figure 3 C, D) on rooting media, the highest average number 12 of shoots produced roots and the highest average percentage 40 % of rooted shoots with average number 1.7 (roots/shoot) were resulted in half strength MS medium containing 3 mg/l IBA (Table 7 and Figure 3 E, F). The previous studies on some *Acacia* species reported that IBA alone or with NAA were used for root formation (Beck and Dunlop, 2001 and Javed et al. 2013). As shown in table 7, callus formation was noticed at the base of the shoots in all treatments that containing IBA and the number of shoots produced callus at the base was increased according to the increase of IBA concentration (Dewan et al. 1992).

CONCLUSION

The results indicated that ISSR markers have been successfully applied for studying and determining the genetic relationship between five *Acacia* species in the present study. ISSR would be a useful tool for genetic variation studies. A reliable protocol for *in vitro* propagation of *A. jonwoodii* was established which could be used for large scale production to transplant this species in its original places. This would be a helpful tool for soil fertility by increasing the amount of nitrogen fixation in the soil. *In vitro* propagation of *Acacia* species is an important tool for improvement of the economical traits of these species and for *ex situ* preservation on the *in vitro* level. Further work will be carry out to survey, classify and determine the genetic relationship using molecular markers techniques between different economical and medicinal plant species grown in Taif provenance.

CONFLICT OF INTEREST

All authors named in the manuscript have no conflict of interest; they are entitled to the authorship and have approved the final version of the submitted manuscript.

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AUTHOR CONTRIBUTIONS

All authors contributed equally in all parts of this study.

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