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Impact of Diatom on Improvement of *in vitro* Rooting, Acclimatization and Molecular Characterization of *Taxodium distichum* Tree

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Abstract: In Egypt and many countries, it is important to raise mass production of various woody trees to cultivate in reclaimed and semi-arid regions. *Taxodium* could be one of the promising woody trees for cultivation in these regions. Development of an *in vitro* rapid mass propagation system is a unique solution to face the shortage of trees needed. *In vitro* rooting and acclimatization are considered the most important stages of commercial production of trees through tissue culture. In this study, the influence of diatom on *in vitro* rooting and acclimatization of *Taxodium distichum* was studied. Diatom was added with different concentrations (0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 g/l) to MS medium supplemented with 0.5 mg/l IBA and 1g/l activated charcoal. Supplementation of MS medium with 0.6 g/l diatom achieved of rootlets shoots formation compared with other concentrations. Also, roots formation in the media contained the different concentrations of diatom was earlier formation than control treatment. Furthermore, diatom improved acclimatization the greenhouse as results of irrigation with 0.5 g/l diatom. The results of molecular analysis of RAPD and ISSR were obviously revealed that there was no variation between the *in vitro* derived plants treated with diatom and the mother plant. To our knowledge, this is the first report to study the effect of diatom on *in vitro* rooting and acclimatization of woody tree.

Key words: *Taxodium distichum* · Diatom · *In vitro* rooting · Acclimatization · RAPD · ISSR

INTRODUCTION

Forests are important renewable natural resources, because they provide us with several important products, including fuel, timber, lumber, paper and fodder. They are the main wildlife habitat and also serve other purposes such as recreation and as air and water sheds. Forests regulate the level of rainfall necessary for the existence of vegetation on earth. They also help in recycling moisture. There are many tree species grown in various areas of the world, but cultivation of woody trees (including *Taxodium*) in Egypt has been faced with several problems, like failure to provide the necessary provision for plantation and lack of interest by farmers and crop growers. Further, there are only four *Taxodium distichum* trees in all of Egypt; *Taxodium* could be one of the promising trees to be cultivated in Egypt [1]. *Taxodium* wood has a multitude of uses and is well known for its ability to resist decay. Oil extracted from the wood is believed to give baldcypress high decay resistance. For this reason, cypress wood has long been favored in building construction, fences, planking in boats, river pilings, furniture, interior trim, cabinetry, sills, rafters, siding, flooring and shingles, garden boxes, greenhouses and many other uses [2]. A number of reports concerning isolation and characterization of bioactive compounds from various parts of *Taxodium distichum* have appeared in the literature. Such compounds include cytotoxic diterpenoid quinine methides, 2-furaldehyde, tannins, flavone and its derivatives, sterols and fatty acids and proanthocyanidin [3]. In this respect, Hirasawa *et al.* [4] isolated two new abietane-type diterpenes, taxodistines A and B, from the fruits of *Taxodium distichum* by the guidance of their inhibitory effect on tubulin polymerization and the structures were elucidated by using 2D NMR data. Taxodistine B showed inhibition of tubulin polymerization.

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Development of a protocol for *in vitro* propagation of *Taxodium* is a unique solution to face the shortage of needed plants for cultivation. However, the slow growth of the survival tissue culture derived plantlets during rooting and acclimatization stages are critical problems facing the success of micropropagation as a commercial technique of taxodium propagation. Moreover, to exploit genetic gains in forestry species, efficient vegetative propagation techniques are needed. However, as with many woody species, propagation from trees more than 6 to 8 years old is difficult and necessitates rejuvenation [5]. Soluble silicon is the plant available portion of the silica that makes up over 40% of soil chemistry. It is generally present in plant tissues in amounts similar to that of macronutrients (N, P, K, Ca, Mg and S) and in somegrasses and grass family crops, often at higher levels than other macronutrients. In addition, silicon's have many impacts on plant health: increases cell wall strength, increased shoot and root density, regulates uptake of toxic elements (silicon's activity in the soil matrix has been proven to improve micronutrient uptake (boron, copper, iron, manganese, zinc) and reduce toxic metal uptake (aluminum) as well as sodium), increases heat tolerance and slows transpiration [6]. Silicon is considered an essential nutrient for a group of algae known as diatoms [7-11]. Diatom is microscopically small fossilized skeletons of single-celled plants called Diatoms which lived in fresh and sea water many years ago. Diatoms are delicate unicellular organisms that have a yellow-brown chloroplast that enables them to photosynthesize, their cell walls are made of silica called the frustule. The frustule is composed almost purely of silica, made from silicic acid and is coated with a layer of organic substance [9, 12].

Genetic markers have become indispensable tools for understanding, managing and improving natural and planted forest tree populations [13]. The many marker systems and their uses, as well as the choice of optimal system for various research goals, are well reviewed [14-16]. The RAPD technique provides genetic markers which have been used extensively in many different applications and in different plant species because of its simplicity [17-22]. The ISSR technique is a powerful, rapid, simple, reproducible and inexpensive way to assess genetic diversity or to identify closely related cultivars in many species [23]. ISSR technique permits the detection of polymorphisms in microsatellites and intermicrosatellites loci without previous knowledge of the DNA sequence [24].

This study was conducted with the aim of use diatom to improve the *in vitro* rooting, acclimatization and molecular identification of *Taxodium distichum* plantlets.

MATERIALS AND METHODS

Plant Material: Nodal explants (starting plant material) were taken from young branches of *Taxodium distichum* trees growing in Orman Garden, Giza, Egypt. The explants were dipped in soapy water using septol soap, agitated in a Savlon disinfectant solution (3%) for 20 min, then washed with running tap water for one hour. The explants were soaked for 1 min in 70% ethanol under aseptic conditions in a laminar air-flow cabinet. The explants were then immersed for 5 min in 20% Clorox followed by 0.2% Mercuric chloride (MC) for 5 min. The explants were rinsed three times with sterile distilled water and then cultured on half salt strength of a basal MS medium [25]. The culture medium was solidified using 0.7 % agar prior to autoclaving at 1.2 kg/cm^2 for 15 min and pH of the culture medium was adjusted to 5.8. Culturing was done in 300 ml glass jars containing 50 ml of the medium. All cultures were incubated for 4 weeks in a growth chamber at 24±2°C. The photoperiod was 16 hours light/8 hours darkness and Illumination intensity was 3000 lux from cool white fluorescent lamps.

Shootlets Stage: The survival explants were subcultured on woody plant (WPM) medium [26] supplemented with 0.4 mg/l Benzyl adenine (BA) as described by Abou Dahab *et al.* [1], which gave the longest shootlets with high multiplication rate. This step aimed to provide a stock of *Taxodium* shoot cultures which will be subjected in the followed stages.

Rooting Stage: This stage was conducted to investigate the effects of different concentrations (0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 g/l) of diatom (Agri Silica SA Company, South Africa) on *in vitro* rooting. Shootlets produced after the third subculture from the shootlets stage (3 to 5 cm) were individually separated and cultured on half salt strength MS or WPM media supplemented with 1.0 g/l activated charcoal (AC) and 0.5 mg/l indole butyric acid (IBA). Cultures were incubated for 8 weeks under the conditions previously described. Rooting as response to the different treatments was recorded, in terms of rooting percentage (%), number of roots and root length (cm).

Acclimatization Stage: This stage was divided to two steps: 1) Pre-acclimatization (under control condition), 2) Acclimatization (in greenhouse). *In vitro* rooted plantlets were washed with antifungal (Topsin 0.2%, w/v) then transplanted into 0.2 liter capacity pots filled with sand + peat (1:1, v/v). The pots were covered with semitransparent plastic covers and incubated under control condition (16/8 h light/dark and 25±2°C) for8 weeks. During these eight weeks the pots were irrigated by 0.5 or 1.0 g/l diatom. At the end of the first step of acclimatization (pre-acclimatization) data were recorded as survival percentage and the survived pots were transferred to the greenhouse for acclimatization. In greenhouse the pots were irrigated regularly with water containing 0.5 or 1.0 g/l diatom. After 12 weeks in the greenhouse data were recorded as survival percentage, plant height and branches number/plant.

Molecular Analysis: In the present study, two PCR-based techniques, RAPD and ISSR, for the identification of the variation between the mother plant and acclimatized plants which irrigated with diatom for their simplicity and cost-effectiveness were used. The use of the two types of markers, which amplify different regions of the genome, allows better chances for identification of genetic variation in the plantlets [27].

Total Genomic DNA Extraction: Total genomic DNA was extracted from leaflets of mother plant and the derived *in vitro* new branches, which taken after acclimatization, irrigated plants with 0.5 or 1.0 g/l diatom and grinded into a fine powder in liquid nitrogen using a pestle and mortar following the steps of CTAB (hexadecyl trimethyl ammonium bromide) protocol [28].

RAPD-PCR Analysis: A set of four random primers was used in the detection of polymorphism among mother plant and *in vitro* derived plants which irrigated with 0.5 or 1.0 g/l diatom. RAPD-PCR was carried out according to the procedure given by Williams *et al.* [29] with minor modifications. The amplification reaction was carried out in 25 µl reaction volume containing 1X PCR buffer, 1.5 mM $MgCl_2$, 2 mM dNTPs, 1 U *Taq* DNA polymerase and 25 ng templates DNA andl µM primer from each of random primers (Operon) (Table 1). PCR amplification was performed in a Biometera Uno thermal cycles programmed to fulfil 40 cycles after an initial denaturation cycle for 5 min at 94°C. Each cycle consisted of a denaturation step at 94°C for 1 min, an annealing step at 36°C for 1 min and an elongation step at 72°C for 1.5 min. The primer extension segment was extended to 7 min at 72°C in the final cycle. The amplification products were resolved by electrophoresis in a 1.5% agarosegel containing ethidium bromide (0.5ug/ml) in 1X TBE buffer at 90 volts. Gel was photographed under UV light with Tracktel GDS-2 gel documentation system. The size of the amplification products was estimated from 100 bp DNA ladder.

Table 1: Ssequences of RAPD primers

	RAPD primers	
No.	Name	Sequences5'--------- 3'
3	AM6	TCTGCGGCTGTAGTTCCAGT
4	AM7	CTTCGGCAGCATCTCTTCAT
5	AM8	CAGTGTGGAAGCCGATTATG
6	AM9	ATGTGTTGTCTGGCTTGGTA

Table 2: Sequences of ISSR primers

No.	Primers name	Primers sequences 5´---------- 3´
1	AM 1	GCTATGCAATGGCAG
2	AM 2	GGATGGAATAGTCTC
3	AM 3	GCATGGCAAGCTGCA
4	AM 4	GAGAGAGAGAGAGAGAC

ISSR-PCR Analysis: ISSR scorable primers were designed and screened for PCR amplification (Table 2). PCR was performed in 25 µl reaction volume containing 1X PCR buffer, 1.75 mM $MgCl_2$, 5 mM of each dNTPs, 40 µM oligonucleotide primer from each of the ISSR primers (Operon) (Table2), 25ng genomic DNA and 1 U of Taq DNA polymerase. PCR amplification was performed in a Biometera Uno thermal cycles programmed to fulfill 35 cycles after an initial denaturation step for 5 min at 94°C followed by ten touch down cycles (94°C/30 sec, 65-55°C/45 sec, 72°C/1 min). The PCR products were separated by electrophoresis in a 2.0% agarose gel containing ethidium bromide (0.5ug/ml) in 1X TBE buffer at 90 volts. Gel was photographed under UV light with Tracktel GDS-2 gel documentation system. The size of the amplification products was estimated from 100 bp DNA ladder.

Statistical Analysis: All analyses were performed in triplicate and data reported as mean ± standard deviation (SD). Data were subjected to analysis of variance (ANOVA) ($P<0.05$). Results were processed by Excel (Microsoft Office 2010) and SPSS Version 17.0 (SPSS Inc., Chicago, IL, USA).For both RAPD and ISSR profiles, the well resolved and consistently reproducible fragments ranging form100 bp to 1.5 kb were scored as present or absent. For detecting any genetic change, all the RAPD and ISSR results were compared with each other for all the DNA samples.

Table 3: Effect of diatom at different concentrations and type of culture medium on *in vitro* rootlets shoot formation of *T. distichum* shootlets after 8 weeks of culturing

Treatments	Rooting (%)	Roots number	Root length (cm)
½ MS + 0.5 mg/l IBA + 1.0 g/l AC (Control)	20	1.00±0.00	1.17±0.41
1/2 MS + 0.5 mg/l IBA + 1.0 g/l AC + 0.2 g/l Diatom	25	1.33±0.52	1.33±0.52
1/2 MS + 0.5 mg/l IBA + 1.0 g/l AC + 0.4 g/l Diatom	35	1.33±0.52	2.50±0.55
1/2 MS + 0.5 mg/l IBA + 1.0 g/l AC + 0.6 g/l Diatom	70	3.33±0.52	5.50±0.55
1/2 MS + 0.5 mg/l IBA + 1.0 g/l AC + 0.8 g/l Diatom	65	2.83±0.98	4.50±0.55
1/2 MS + 0.5 mg/l IBA + 1.0 g/l AC + 1.0 g/l Diatom	60	3.00±0.89	5.83±0.98
1/2 WPM + 0.5 mg/l IBA + 1.0 g/l AC (Control)	15	1.00±0.00	1.00±0.00
1/2 WPM + 0.5 mg/l IBA + 1.0 g/l AC + 0.2 g/l Diatom	20	1.33±0.52	1.33±0.52
1/2 WPM + 0.5 mg/l IBA + 1.0 g/l AC + 0.4 g/l Diatom	35	1.50±0.55	4.00±0.63
1/2 WPM + 0.5 mg/l IBA + 1.0 g/l AC + 0.6 g/l Diatom	50	2.50±0.55	5.50±1.05
1/2 WPM + 0.5 mg/l IBA + 1.0 g/l AC + 0.8 g/l Diatom	50	2.50±0.55	4.33±1.51
1/2 WPM + 0.5 mg/l IBA + 1.0 g/l AC + 1.0 g/l Diatom	50	2.67±0.82	4.00±0.89

Values are expressed as mean ± SD; n=12

RESULTS AND DISCUSSION

Rooting Stage Experiment: This experimental was conducted to improve the rate of *in vitro* rootlets/shootlets formation of *Taxodium*. Data in Table 3 presents the effect of the fine diatom concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 g/l) and the two types of culture media (MS and WPM) on roots formation and their characteristics. The percentage of roots formation was obviously elevated by the different concentrations of diatom tested. Such result was observed with the two types of culture media. Rooting percentage was gradually increased by increasing diatom concentration. However, it was found that adding diatom to MS medium gave higher rooting percentage compared with those added to WPM. The highest percentage of roots formation (70%) was recorded as a result of culturing the shootlets on half-strength MS medium with 1.0 g AC/l + 0.5 mg IBA/l + 0.6 g/l diatom, while the lowest percentage of roots formation (15) was observed with those shootlets grown on WPM free diatom. Regarding the roots number/shootlet, data showed pronounced increment as a result of adding diatom compared with control treatment. The highest roots number/shootlet (3.33) was recorded in the MS medium containing 0.6 g/l diatom. While, the cultures medium (MS or WPM) free diatom were recorded the lowest (1.00) roots number/shootlet. Regarding root length data revealed that the highest mean root lengths (5.83 cm) was obtained with MS culture medium containing 1.0 g/l diatom. As same obtained results of roots number/shootlets, the lowest root length was recorded with culture medium free diatom. Literature in hand suggested that diatom substance might have a role in root development [6]. Rooting stage is considering a critical stage in *in vitro* propagation of several plants especially woody trees. Therefore diatom was subjected in rooting stage aiming to enhance root formation of *Taxodium*. Data obtained from this study revealed that rooting percentage was gradually increased by increasing diatom concentrations. Supplementation of half-strength MS medium by 0.6 g/diatom gave higher percentage of root formation compared with those added to WPM. These results support the suggestion of the role of diatom in root development. It is worth mentioning here that the essentiality of silicon for plant growth has long been a question of interest to plant nutrition researchers. Uptake of silicon varies by species and by plant group [30-32]. Recently, Cornelis *et al.* [33] suggested that silicon uptake is passive in forest trees. The results obtained in this stage support to those obtained by Manisha *et al.* [34] on *Alnus nepalensis* and Gad *et al.* [35] on *Khaya ivorensis*. On the other hand, Abou-Dahab *et al.* [1] found that the half salt strength WPM medium, supplemented with different concentrations of IBA, was more effective for promoting root elongation in both *T. distichum* and *T. distichum* var, 'distichum', compared to the MS medium. In this connection it might be mentioned that activated charcoal stimulates rooting of microcuttings in some woody species [36]. Normally, AC is included in the root development medium after root-inducing auxin treatment [37-39].

Acclimatization Stage: Regarding the first stage of acclimatization (pre-acclimatization), transplants growing in pots were irrigated by two concentrations of diatom (0.5 or 1.0 g/l) results were recorded at the end of pre-acclimatization on step (after 8 weeks). Results obtained revealed that there were no obvious differences

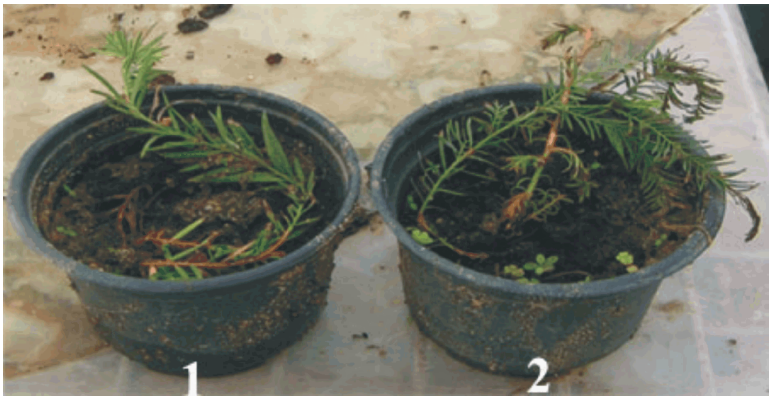

Fig. 1: *T. distichum* plants after 8 weeks of pre-acclimatization stage with irrigation by 1) 0.5 g/l diatom and 2) 1.0 g/l diatom.

Fig. 2: *T. distichum* plants after 12 weeks of acclimatization in greenhouse with 1.0 g/l diatom.

Table 4: Effect of irrigation by diatom on survival percentage after 8 weeks of pre-acclimatization of *T. distichum* transplants

Treatments	Survival (%)
Control	70 ± 0.58
Irrigated with 0.5 g/l diatom	70 ± 0.60
Irrigated with 1.0 g/l diatom	75 ± 0.60

Values are expressed as mean ± SD; n=20

between the treatments (Table 4). Although, irrigation by water containing 1.0 g/l diatom gave higher survival percentage (75%) of plants compared with control treatment or irrigated plants with 0.5 g/l diatom which gave 70% of survival plants (Fig. 1).

Regarding, the second stage of acclimatization, which carried out in the greenhouse for 12 weeks with irrigation by two concentrations of diatom (0.5 or 1.0 g/l). Data presented in Table 5, showed that the highest survival percentage (100%) was recorded with plants irrigated with diatomcompared with the control treatment (Fig. 2). Concerning plant characterization, data showed that there was no significant difference between the two treatments of irrigation with 0.5 or 1.0 g/l diatom on plants length (19.67 and 21.00 cm, respectively). While, the lowest plant length was recorded with the control treatment (7.67 cm). Data of number of branches /plant showed the same trend like plant length, in which there were no significant differences between the two treatments of irrigation with 0.5 or 1.0 g/l diatom (18.67 and 23.33, respectively) and the lowest number of branches/plant (10.67) was recorded with the control treatment.

One of the major obstacles to the practical application of plant tissue culture to mass propagation has been the difficulty of successful transfer of plantlets from *in vitro* conditions to a soil medium [40]. Acclimatization stage is considering the critical stage in *in vitro* propagation of plants especially woody trees. The environments in *in vivo* are quite different when compared to *in vitro* conditions, in terms of relative humidity (RH), constant temperature, air ventilation, nutrient levels, etc. [41, 42, 43]. *In vitro* acclimatization, or hardening, is one of the main processes in the production of healthy plantlets before their transplantation to *in vivo* [44, 45]. The results obtained in this study reported that irrigation with diatom increased the survival percentages, plant length and number of branches/plant of taxodium. In this respect, the results obtained are in harmony with those reported by Bocharnikova [46]. Also, Matichenkov [47], reported that optimization of silicon nutrition results in increased mass and volume of roots, giving increased total and adsorbing

Table 5: Effect of irrigation by diatom on acclimatization of *T. distichum* plants after 12 weeks of culturing

Treatments	Survival (%)	plant length (cm)	Number of branches /plant
Control	75±0.43	7.67±0.58	10.67±3.06
Irrigated with 0.5 g/l diatom	100	19.67±3.06**	18.67±3.06*
Irrigated with 1.0 g/l diatom	100	21.00±2.00**	23.33±1.15*

Values are expressed as mean ± SD; n=12, significance of differences is indicated (* p=0.05; ** p=0.01).

Table 6: Total number of amplicons, monomorphic amplicons, polymorphic amplicons and percentage of polymorphism by RAPD markers as results of diatom irrigation (at the end of acclimatization stage) and the mother plant of *T. distichum*

Primers	Total # amplicons	Monomorphic amplicons	Polymorphic amplicons	Polymorphism %
AM6	7	7	0	0
AM7	8	8	0	0
AM8	6	4	2	50
AM9	5	3	2	40
Total	26	22	4	--
Average	6.5	5.5	1.0	22.5

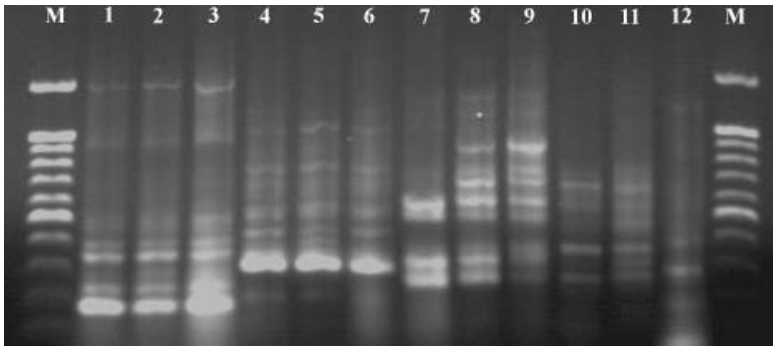

Fig. 3: RAPD DNA amplification pattern obtained for mother plant and acclimatized plants which irrigated with 0.5 g/l diatom and 1.0 g/l diatom, respectively, generated by primers: AM6 (Lan 1, 2 and 3), AM7: (Lan 4, 5 and 6), AM8: (Lan 7, 8 and 9) and AM9: (Lan 10, 11 and 12) M: 100 – 1500 pb DNA Ladder.

surfaces.As a result of application of silicon fertilizer, the dry weight of barley increased by 21 and 54% over 20 and 30 days of growth, respectively, relative to plants receiving no supplemental silicon [48].

Molecular Analysis: True-to-type clonal fidelity is one of the most important prerequisites in the micropropagation of any crop species. A major problem encountered with the *in vitro* culture is the presence of somaclonal variation amongst sub-clones of one parental line, arising as a direct consequence of *in vitro*culture of plant cells, tissues or organs. A better analysis of genetic stability of plantlets can be made by using a combination of two types of markers which amplify different regions of the genome [49]. In this respect, it should be important to note that Palombi and Damiano [27] suggested the use of more than one DNA amplification technique as advantageous in evaluating somaclonal variation while working on micropropagated plants of kiwi fruit. Hence, in the present study, two PCR based techniques, RAPD and ISSR were adopted for evaluation the variation between the mother plant and acclimatized plants which irrigated with diatom.

RAPD-PCR Analysis: Regarding the first molecular analysis (RAPD-PCR analysis), four random primers (AM6, AM7, AM8 and AM9) were screened in RAPD analysis for their ability to produce sufficient amplification products.In thepresent study, the total number of fragments produced by the four primers was 26 with an average of 6.5 fragments/primer (Table 6 and Fig. 3). While, the number of polymorphic fragments ranged from 0 to 2. A maximum number of amplicons (8) were amplified with primer AM7, while the minimum number of fragments (5) was amplified with primer AM9. The highest number of polymorphic bands (2) was obtained with primers AM8 and AM9. Primer AM8 exhibited the highest percentage (50%) of polymorphism. Data presented in Table 6 also revealed that the total number of polymorphic amplicons obtained by the four studied primers was 4.

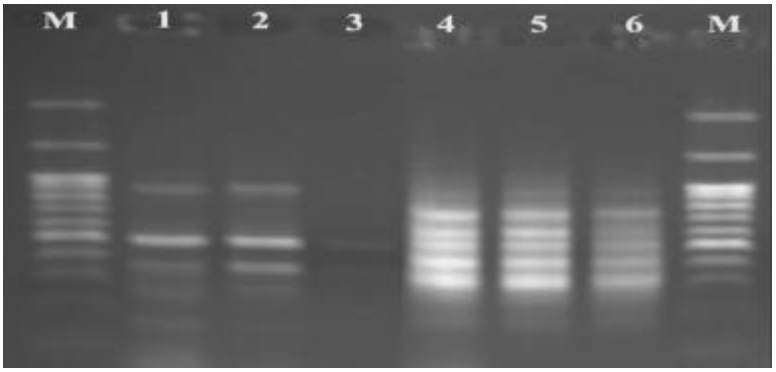

Fig. 4: ISSR profiles of the *T. distichum* plants using the primers: AM1: Lane 1) 0.5 g/l diatom, Lane 2) 1.0g/l diatom and Lane 3) Mother plant. AM2: Lane 4) 0.5 g/l diatom, Lane 5) 1.0g/l diatom and Lane 6) Mother plant. Lane M) 1.5 Kb ladder DNA markers.

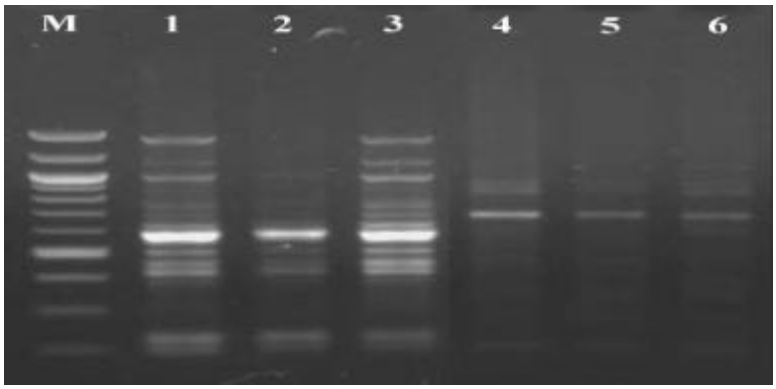

Fig. 5: ISSR profiles of the *T. distichum* plants using the primers: AM3: Lane 1) 0.5 g/l diatom, Lane 2) 1.0g/l diatom and Lane 3) Mother plant. AM4: Lane 4) 0.5 g/l diatom, Lane 5) 1.0g/l diatom and Lane 6) Mother plant. Lane M) 1.5 Kb ladder DNA markers.

This corresponds to a level of polymorphism of 15% and an average number of polymorphic fragments/primer of 0.6. In this respect, RAPDs have been used for many purposes, ranging from studies at the individual levels (e.g. genetic identity) to studies involving closely related species. Due to their high genomic abundance, RAPDs have also been applied in gene mapping studies. In this respect, RAPD markers have been able to assess the genetic stability of micropropagated plants of *Deutzia scabra* and the effect of slow growth preservation of the same plant almonds [22, 49], turmeric [50] and yams [51]. As RAPD markers amplify different regions of the genome, their simultaneous analyses give a better interpretation of the genetic stability of the *in vitro* regenerates [49].

ISSR-PCR Analysis: Regarding the second molecular analysis (ISSR-PCR analysis), four ISSR primers were used in this study, the four ISSR primers produced good reproducible and scorable patterns and the amplification profiles were screened for the presence of polymorphisms among and within the three *T. distichum* treatments (Fig. 4 and 5). The total number of fragments produced by the four ISSR primers was 26with an average of 6.5 fragments/primer (Table 7). While, the number of polymorphic fragments ranged from 0 to 4. A maximum number of amplicons (10) was amplified with primer AM3, while the minimum number of fragments (5) was amplified with primers AM1 and AM4 (Figs. 4 and 5). The highest number of polymorphic bands (4) was obtained with primers AM3. Data also revealed that the total number of polymorphic amplicons obtained by the four studied primers was 6. This corresponds to a level of polymorphism of 20% and an average number of polymorphic fragments/primer of 1.5. Because ISSR amplify different parts of the genome, the data generated from this analysis was obtaining more balanced values for genetic variation among genotypes targeted and representation of their relationships. This allowed us to explore the DNA polymorphism in the

Table 7: Total number of amplicons, monomorphic amplicons, polymorphic amplicons and percentage of polymorphism by ISSR markers as results of diatom irrigation (at the end of acclimatization step) and the mother plant of *T. distichum*

Primers	Total # amplicons	Monomorphic amplicons	Polymorphic amplicons	% polymorphism
AM1	5	3	2	40
AM2	6	6	0	0
AM3	10	6	4	40
AM4	5	5	0	0
Total	26	20	6	--
Average	6.5	5	1.5	20

collection of genotypes analyzed and generate many polymorphic markers ensuring a good coverage of the genome [52, 53].

CONCLUSION

From the above results it can be concluded that adding 0.6 g/l diatom tothe *in vitro* rooting medium gave the best results of roots formation. Also, diatom improved acclimatizationof the transplants in the greenhouse as results of irrigation water including 0.5 g/l diatom. Analysis of both RAPD and ISSR obviously revealed that there was no variation between the *in vitro* derived plants as a result of diatom treatments and the mother plant.

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