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Establishment of an *In vitro* Rapid Direct Regeneration Protocol For *Holmskioldia sanguinea* Rare Flowering Plant Production

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Abstract: This study was carried out in the Plant Tissue Culture Laboratory, Horticulture Research Institute, Agriculture Research Center, Ministry of Agriculture, during the period from 2011 to 2014. An efficient direct shoot regeneration micropropagation protocol has been established successfully for commercial *in vitro* propagation of *Holmskioldia sanguine*a, a rare plant with ornamental and medicinal values. The optimized sterilization conditions for the single node stem cuttings explants were exposure to 30% Clorox for 20 min (70.83 survival percentage, 4.17 mortality percentage and 25.00 contamination percentage). In establishment stage full strength of Murashige and Skoog medium (MS) supplemented with NAA at 0.5 mg/l was superior in *in vitro* growth of explant in respect of shoot length (2.13 cm) and number of leaves / explant (11.33 leaves). The multiplication stage should be carried out with cutting the shoot apex on MS medium supplemented with 1.0 mg/l BA which recorded 5.33 shoots at the end of the third subculture. For rooting the regenerated shoots, one eighth strength MS medium supplemented with 2.0 mg/l indole-3-butyric acid (IBA) was effective and economic treatment resulted in 100% rooting percentage, 9.67 roots/plantlet, 8.00 cm root length, 8.67 leaves/plantlet and 8.00 cm plantlet length. Growing medium consists of peatmoss and vermiculite at the ratio of 2:1 (v/v) was the best for acclimatization of regenerated plantlets giving 100% survival percentage grew into mature plants recording highest vales after 6 and 12 weeks for plantlet length (10.83 and 15.67 cm, respectively), number of leaves/plantlet (14.67 and 22.33 leaves, respectively), stem diameter (1.80 and 1.97 cm, respectively) and number of shoots/plantlet (2.33 shoots for each). Inter simple sequence repeats (ISSR) analysis detected similarity (13.7%) to the mother tree in the in *vitro* regenerated plants from the nodal explants, that the direct regeneration protocol will be useful for *Holmskioldia sanguinea* production. This plant developmental protocol could be used for large scale regeneration of *Holmskioldia sanguinea.*

Key words: *Holmskioldia sanguinea* · Tissue culture · IBA · NAA · BA · ISSR analysis

INTRODUCTION

Chinese hat plant (*Holmskioldia sanguinea* Retz.), belongs to family Labiatae, formerly Verbenaceae, is a rare beautiful shrub originating from the Himalayas (India, Nepal, Bhutan, Bangladesh and Myanmar) and grows in warm climates. The plant is a spring and summer blooming shrub; it has outstanding ornamental features and is used as cut flowers. It grows well in acidic; slightly alkaline; sand; loam; clay soils. It is usually not affected by pests and tolerates drought moderately. In Egypt, it is an infrequent plant appearing in dense terminal clusters from September to March. It has unique spectacular narrowly trumpet shaped red or orange flowers backed by a broad circular calyx. It has been used for pain relief, an anticancer agent, diuretic and anti-inflammatory. It has anti-inflammatory; antihepatotoxic; antioxidative and antimicrobial activities [1-2]. It is propagated by softwood or hardwood stem cuttings, simple; air and tip layering and also by seeds. Lack of popularity and commercial production may be due to the less symmetrical growth habit and the tendency toward thinning at the bottom of the plant. Besides, vegetative propagation shelters the pathogens, has a low multiplication rate, requires a large number of stock plants and need time to develop plantlets. No available data of literature on *in vitro* micropropagation of *Holmskioldia sanguinea.*

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Selection of the explant and developing a genetically stable regeneration protocol are important in the starting of an *in vitro* study. Direct regenerated shoots from the nodal, axillary and terminal buds explants (meristems) without passing callus phase has fewer somaclonal variation and more genotype-dependency [3] and are true to mother plant type while callus derived plants are variable. Plantlets resulting from meristem culture are free of viruses and parasites due to the absence of vascular system in which viruses travel. Besides, meristematic cells are actively dividing and have high metabolic activity. Shoot apex has high endogenous auxin level. Stem explants is suitable in inducing roots because of the presence of procambial-like tissue in the structure surrounding the vascular tissue. Sterilization aims at making explants contamination free before establishment of cultures. Concentration and duration of exposing to the sterilant are important to keep the explant sterilized. Effectiveness of sodium hypochlorite as an antibacterial is great. For the establishment of a new protocol in tissue culture, a universal basal medium like Murashige and Skoog's MS medium is suitable to supply different requirements for satisfactory growth, besides to its high content of macronutrients, compared to other media formulations. Growth regulators are the most effective variables in plant tissue culture media, especially auxins and cytokinins. The auxins commonly used in plant tissue culture media are indole-3-butyric acid (IBA) and 1-napthaleneacetic acid (NAA). Auxins stimulate growth from shoot apices and shoot stem culture in establishment stage and to induce root formation. Cytokinins commonly used in the culture media in multiplication stage include 6- benzyladenine (BA) and N-(2-furanylmethyl)-1H-puring-6- amine (kinetin).

Tissue culture technique produces homogenized and genetically stabilized plants, having similar banding patterns to that of the mother plant without somaclonal variations. Somaclonal variation occurred at DNA level, clonal stability can be assessed by studying chromosome numbers, isozyme profile and PCR-based molecular markers like random amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSR) and most recently start codon targeted (ScoT) [4-5].

So, this study was carried out as a first attempt with the aim of establishing an efficient *in vitro* regeneration protocol through direct organogenesis for a valuable *Holmskioldia sanguinea* rare flowering medicinal plant using nodal segment; plantlet acclimatization and assessment of genetic stability of the raised plants.

MATERIALS AND METHODS

This study was carried out in the Tissue Culture Laboratory, Horticulture Research Institute, Agriculture Research Center, Ministry of Agriculture, Giza, Egypt, during the period from 2011 to 2014 to determine the suitable protocol for propagating *Holmskioldia sanguinea* plant using tissue culture technique.

Source of Explants: Active growing shoots were excised from 80-years-old tree *Holmskioldia sanguinea* mother plant grown naturally in Zohria Botanical Garden, Cairo, Egypt in March. Single node stem cuttings with its two axillary buds were prepared by dividing the shoots into 1.0-1.5 cm segments. The explants were washed with tap water, followed by a soap solution for 10 min and rinsed under a running tap-water for one hour, then redistilled water for 3 min, before soaking in sterilizers.

This study included five experiments, as follows:

Experiment I: Surface Sterilization of Explants: The aim of this experiment was to evaluate the effect of some sterilization treatments, by using commercial Clorox (containing 5.25% sodium hypochlorite, NaOCl) solution at 20, 30 or 40% for 15, 20, 25 or 30 min, on the contamination, mortality and survival percentages of explants. A few drops (0.1 %) of Tween 20 (polyoxyethylenesorbitan monolaurate) were added to the Clorox solution as a wetting agent per 100 ml of sterilizing solution for each treatment. After sterilization, explants were rinsed in sterilized distilled water (three times) to remove all traces of the disinfectant. After that, the explants were cut further to 0.8 cm and were placed in a 150 ml culture jars containing 20 ml of MS basal medium [6]. This stage contained twelve treatments and each treatment consisted of three replicates and each replicate consisted of three jars and each jar contained three explants. Jars were incubated for 21 days in a growth room at 25 ± 2°C and 16 hrs illumination of 2000 lux (white fluorescent lamps). Contamination, mortality and survival percentages were recorded. All steps of the sterilization experiment have been done under aseptic condition inside the culture cabinet (Laminar air flow).

Experiment II: Establishment Stage: The aim of this experiment was to investigate the suitable MS strength supplemented with various concentrations of NAA for development of sterilized explants during establishment

stage. Uniform sterilized explants produced from the aseptic culture were transferred to the MS medium at full, three quarter, half and quarter salt strength supplemented with NAA at 0.0, 0.5, 1.0, 2.0 or 4.0 mg/l medium. This stage contained twenty treatments and each treatment consisted of three replicates and each replicate consisted of three jars and each jar contained two explants. Jars were incubated as described in experiment I. After four weeks, shoot length (cm) and number of leaves/explant were recorded.

Experiment III: Multiplication Stage The aims of this experiment was increasing shoot formation and determine the most suitable cytokinins concentration, including benzyl adenine (BA) and kinetin, for axillary buds proliferation of *Holmskioldia sanguine*a with or without cutting the shoot apex. In case of cutting the shoot apex, this process was done from the first subculture and applied on the new resulting shoots in the second and third subcultures. Uniform sterilized explants (0.8 cm long shoot) were cultured on full salt strength MS medium supplemented with 0.5 mg/l NAA because it was the best establishment medium as found from the result of experiment II (Table 2). Cultures were incubated under the same conditions previously described in experiment I. for four weeks. The resulted uniform 1.5 cm shoot length with 2 leaves were subcultured on MS medium supplemented with BA or Kin each at 0.1, 0.5, 1.0, 2.0 or 4.0 mg/l in addition to MS medium hormone free as the control. Subculturing the shoots was done onto the same medium every four weeks and three subcultures were done. This experiment consisted of eleven treatments, each treatment consisted of three replicates and each replicate consisted of three jars and each jar contained three explants. After the three subcultures, number of shoots, average shoot length (cm) and number of leaves/shoot were recorded.

Experiment IV: Rooting Stage: The aim of this experiment was to study the effect of MS-salt strength medium and auxins concentration including indole butyric acid (IBA) or naphthalene acetic acid (NAA) on root formation of *Holmskioldia sanguine*a.

According to the results of Experiment III, subculturing the shoots on MS medium supplemented with 1.0 mg/l BA with cutting the shoot apex proved to be the most suitable for the multiplication stage (Table 3). So, uniform shoots, 1.5 cm shoot length with 2 leaves were subcultured (as described in Experiment III) on this medium for producing stock shoots. After that, the resulted uniform shoots with 2.0 cm long with 2 leaves were cultured on MS basal nutrient medium at full, half, quarter and one-eight salt strength supplemented with IBA or NAA each at 1.0, 2.0 or 4.0 mg/l, in addition to a hormone-free MS medium as the control. Activated charcoal (AC) was added to the rooting medium at the rate of 3 g/l in order to improve root formation. This experiment consisted of 28 treatments, each treatment consisted of nine jars and every jar contained three individual shoots. Cultures were incubated for one month under the conditions previously described in experiment I. After one month, at the end of this experiment, rooting percentages, number of roots/ plantlet, average root length, shoot length and number of leaves/plantlet were recorded.

Experiment V: Acclimatization Stage: This experiment was conducted *in vivo* to evaluate the effect of growing media on plantlet growth of *Holmskioldia sanguinea* during acclimatization stage. The shoots (2.0 cm long, with 2 leaves, grown on MS medium supplemented with 1.0 mg/l BA with cutting the shoot apex) were subcultured on 1/8 MS- salt strength medium supplemented with 2.0 mg/l IBA + 3.0 g/l AC (the best rooting medium, as proven by Experiment IV) for 4 weeks, before being used in the acclimatization experiment.

Resulting plantlets of suitable size (3.5 cm long with 4.0 leaves and 7 roots of average 6 cm length) were selected, washed thoroughly with tap water to remove the remains of medium from the root system. The roots of plantlets were rinsed in water containing a fungicide (Benlate, 0.1 %) for 5 min, after that the roots were washed thoroughly with tap water to remove the remains of fungicide from the root system. The plantlets individually transplanted into 5 cm plastic pots filled with one of the following growing media: peatmoss, peatmoss + sand (1:1, 2:1 or 3:1 v/v), peatmoss + perlite (1:1, 2:1 or 3:1 v/v) and peatmoss + vermiculite (1:1, 2:1 or 3:1 v/v). Each treatment consisted of three replicates and each replicate consisted of three pots. The plantlets were held in the greenhouse under 4000-lux light intensity. The relative humidity around the plantlets was kept high by surrounding each plantlet with a white polyethylene bag for two weeks. The polyethylene bags were perforated gradually starting from the third day, with the aim of gradually reducing the relative humidity of the air surrounding the plantlets. The pots were irrigated by diffusion (by placing the pots in water) twice during these weeks and then the bags were removed. After six weeks,

the plantlets were sprayed three times per week for six weeks with a solution containing Kristalon fertilizer (NPK at 19-19-19) at the rate of 0.5 g/l. Also, chelated iron (12% Fe) and chelated magnesium (6% Mg) were added in the solution, each at the rate of 0.1 g/l. The spraying solution contained a wetting agent (Saliant-Film, containing 3% P_2O_5) at the concentration of 3 ml/10 l of water. After 6 and 12 weeks, the survival percentage, plantlet length, number of leaves and number of shoots/plantlet as well as stem diameter were recorded.

In the first four experiments, all culture media contained 30 g/l sucrose and were solidified with 6 g agar/l (pH was adjusted at 5.7 ± 0.1 prior to addition of agar). Media were autoclaved for 20 min at 121°C and 1.1 kg/cm^2, then they were cooled and kept for 7 days before use and contaminated media were discarded. After culturing, the culture jars were directly plugged with polypropylene closure caps.

The first four experiments were factorial with two factors, while the fifth experiment includes one factor. All experiments were conducted using a completely randomized design. All data were averaged and differences among the means of the different treatments were compared using the "Least Significant Difference, L.S.D." test at the 5% level, as described by Little and Hills [7]. In case of survival percentages in the first and fifth experiments and rooting percentage in the fourth experiment, the original data were arcsine-transformed prior to statistical analysis.

Molecular Genetics Identification: The DNA marker system used was inter simple sequence repeats (ISSR).

DNA Extraction: Total genomic DNA was extracted and purified from 0.1 g of freeze dried powered samples as described by Dellaporta *et al*. [8]. DNA present in the supernatant was precipitated according to the described protocol, re-dissolved in sterile, distilled water and quantified.

Data Analysis: The similarity matrices were done using Gel works ID advanced software UVP- England Program. The relationships among genotypes and species as revealed by dendrograms were done by using SPSS windows (Version 10) program. Dice computer package was used to calculate the pairwise difference matrix and plot the phenogram among conifers genotype under investigation. The resultant similarity matrix was employed to construct a dendrogram using Sequential Agglomerative Hierarchical Nesting (SAHN) based Unweighted Pair-Group Method with Arithmetic Means (UPGMA) to infer genetic relationships and phylogeny according to Sensi *et al*. [9].

RESULTS AND DISCUSSION

Experiment I: Surface Sterilization of Explants

Contamination Percentage: Data presented in Table (1) showed that the percentage of contaminated *Holmskioldia sanguine*a explants significantly decreased with increasing Clorox concentration. The significantly lowest percentage of contaminated explants was recorded by using 40% Clorox.

Increasing soaking period from 15 to 25 min significantly decreased percentage of contaminated explants gradually. After that, the percentage of contaminated explants was increased as a result of increasing soaking period to 30 min giving insignificantly higher value than that recorded with 20 min.

Generally, soaking the explants in 40% Clorox for 25 min resulted in the lowest percentage of contaminated explants.

Mortality Percentage: Data presented in Table (1) showed that the mortality percentage of *Holmskioldia sanguine*a explants increased with increasing Clorox concentration. The significantly highest mortality percentage of explants was recorded by using 40% Clorox, followed by that recorded with 30% Clorox, then with 20% Clorox with no significant difference between that soaked in 30% and 20% Clorox.

The highest mortality percentage of *Holmskioldia sanguine*a explants was recorded with soaking explants for 30 min followed by that soaked for 15 min and for 25 min, respectively. There was no significant difference between the percentage of mortality of explants recorded with soaking explants for 30 min and that soaked for 15 min. The lowest percentage of mortality was recorded with explants soaked for 20 min.

Generally, the lowest percentage of mortality explants were resulted from soaking the explants in 20% Clorox for 15 or 20 min as well as 30% Clorox for 20 min.

Survival Percentage: Data presented in Table (1) showed that the highest survival percentage of *Holmskioldia sanguine*a explants was recorded with using Clorox at 30% followed by that recorded with Clorox at 40% and that recorded with Clorox at 20%, respectively with significant differences among them.

Table 1: Effect of different concentrations of Clorox and soaking period on surface sterilization of *Holmskioldia sanguinea* explants

	Clorox% (B)			
Soaking periods (A)	20%	30%	40%	Mean (A)
		Contamination %		
15 min	87.50 a	41.67 d	54.17 c	61.11 a
20 min	83.33 ab	25.00 e	20.83 ef	43.05 b
25 min	54.17 c	20.83 ef	12.50 f	29.17 c
30 min	75.00 b	54.17 c	16.67 ef	48.61 b
Mean (B)	75.00 a	35.42 b	26.04 c	----
		Mortality %		
15 min	4.17 f	33.33 a-c	33.33 a-c	23.61 ab
20 min	4.17 f	4.17 f	37.50 ab	15.28 c
25 min	16.67 d-f	8.33 ef	41.67 a	22.22 bc
30 min	20.83 c-e	25.00 b-d	45.83 a	30.55 a
Mean (B)	11.46 b	17.71 b	39.58 a	----
		Survival %		
15 min	8.33 f	25.00 d	12.50 ef	15.28 c
20 min	12.50 ef	70.83 a	41.67 b	41.67 b
25 min	29.17 cd	70.83 a	45.83 b	48.61 a
30 min	4.17 f	20.83 de	37.50 bc	20.83 c
Mean (B)	13.54 c	46.87 a	34.38 b	----

* For each parameter, within the column for soaking period means, the row for Clorox% means, or the means for combinations of the two factors, means sharing one or more letters are insignificantly different at 5 % level, according the "Least Significant Difference" test.

Table 2: Effect of different concentrations of NAA and MS-salt strength medium on shoot length (cm) and number of leaves/ explant of *Holmskioldia sanguinea* during establishment stage

	Shoot length					Number of leaves/ explant				
	MS – salt strength (B)					MS – salt strength (B)				
NAA (mg/l) (A)	Full	3/4	1/2	1/4	Mean (A)	Full	3/4	1/2	1/4	Mean (A)
0.0 mg/l	1.97	1.70	1.60	1.53	1.70	7.67	6.33	5.00	3.33	5.58
0.5 mg/l	2.13	1.80	1.53	1.40	1.72	11.33	8.00	7.67	5.67	8.17
1.0 mg/l	1.77	1.63	1.47	1.30	1.54	6.33	5.33	4.67	4.00	5.08
2.0 mg/l	1.57	1.43	1.37	1.30	1.42	6.00	5.33	5.00	4.00	5.08
4.0 mg/l	1.50	1.37	1.30	1.20	1.34	3.33	2.67	2.33	2.00	2.58
Mean (B)	1.79	1.54	1.45	1.35	----	6.93	5.53	4.93	3.80	-----
$L.S.D_{0.05}$ NAA (A)			0.09					0.63		
$L.S.D_{0.05}$ MS- salt strength (B)			0.08					0.53		
$L.S.D_{0.05}$ A × B			0.15					1.26		

Increasing soaking periods from 15 min to 20 min and 25 min significantly increased survival percentage gradually, giving the significantly highest value with 25 min soaking period. Increasing soaking periods more than 25 min (to 30 min) significantly decreased survival percentage giving insignificantly higher value than that recorded with soaking period for 15 min.

The highest survival percentage (70.83%) of *Holmskioldia sanguine*a explants was recorded with soaking explants in 30% Clorox for 20 or 25min.

From the above results the best sterilization treatment was soaking explants in 30% Clorox for 20 min (70.83% survival, 4.17% mortality and 25.00% contamination). Antimicrobial action of sodium hypochlorite depends on causing enzymatic inhibition of the bacterial important enzymes. The high pH of sodium hypochlorite interferes in the cytoplasmic membrane integrity with biosynthetic alterations in cellular metabolism and phospholipid degradation [10]. Results are in agreement with prior studies as apical shoots of *Aspidosperma polyneuron* were sterilized by NaOCl at 0.25 % for 10 minutes and resulted in 72.89% survival percentage [11]. Surface sterilization of shoot tips and axillary buds of *Cerbera odollam* explants with 20% Clorox (sodium hypochlorite as commercial bleach) for 20 and 15 min, respectively gave 92.59 and 88.89% survival, 7.41 and 7.41% contamination

in addition to 0.00 and 3.70% mortality of shoot tips and axillary buds, respectively [12]. Dar *et al.* [13] found that the best sterilization conditions for *Hibiscus rosa-sinensis* explants were 40% Clorox- 20 min exposure, 10% Clorox-15 min exposure and 5% Clorox-40 min exposure for the node, internode and shoot tip, respectively. Also, the lowest significant contamination and the highest significant survival percentages of stem nodal explants of cassava plant were recorded with 20% Clorox for 15 min [14].

Experiment II: Establishment Stage

Shoot Length: The significantly tallest shoot was recorded on MS-medium full salt strength. Decreasing the salt strength in MS-medium to 3/4, 1/2 or 1/4 significantly decreased shoot length gradually, giving the significantly shortest shoots with MS-medium 1/4 salt strength (Table 2).

Also, data clearly revealed that supplied MS medium with NAA at 0.5, 1, 2 or 4 mg/l decreased shoot length gradually compared to that cultured on hormone- free MS medium. Shoots established on MS medium supplemented with NAA at 0.5 mg/l were insignificantly different than that established on hormone- free MS medium, while shoots established on MS medium supplemented with NAA at 1, 2 or 4 mg/l were significantly shorter than that established on hormone- free MS medium.

The significantly tallest shoots were recorded on MS-medium full salt strength supplemented with 0.5 mg/l NAA.

Number of Leaves/Explant: The significantly highest number of leaves/explant was formed on explants cultured on MS-medium contains full salt strength, followed by that cultured on 3/4, 1/2 and 1/4 MS – salt strength with significant differences among them.

Concerning the effect of different concentrations of NAA on number of leaves, regardless of the effect of strength of MS medium, data presented in Table (2) clearly revealed that NAA at 0.5 mg/l was the only treatment that significantly increased number of leaves/ explant compared to hormone- free MS medium. Increasing NAA to 1 or 2 mg/l resulted in insignificantly lower number of leaves/explant than that recorded with hormone- free MS medium. Using NAA at 4 mg/l resulted in significantly lower number of leaves/explant than that recorded with hormone -free MS medium.

The significantly highest number of leaves/explant was recorded on MS-medium full salt strength supplemented with 0.5 mg/l NAA.

Fig. 1: Shoot formation of *Holmskioldia sanguine*a explant on MS medium full-salt strength supplemented with 0.5 mg/l NAA during establishment stage

From the above mentioned results the best medium for increasing shoot length and number of leaves was MS-medium full salt strength supplemented with 0.5 mg/l NAA (Fig. 1). These results are in accordance with previous study on Dendrobium orchid, NAA at 0.1 mg/I gave the highest shoot length (2.60 cm) and number of leaves (4.83) [15].

Experiment III: Multiplication Stage

Number of Shoots: Regarding the effect of different cytokinins at various concentrations on number of shoots obtained with or without cutting the shoot apex, data presented in Table (3) and illustrated in Figs. (2 and 3) revealed that the number of shoots was higher with cutting the shoot apex than that recorded without cutting the shoot apex. This result is related to that auxin moves from apical buds down shoots to inhibit axillary bud growth and restricts lateral branching. In most cases, number of shoots was significantly increased as a result of using different concentrations of BA and Kin as compared to the control (hormone-free MS medium). The exceptions to this general trend were recorded with using Kin at 0.1 mg/l which resulted in insignificantly lower values than the control (hormone - free MS medium). Also, MS medium supplemented with BA at 0.1 mg/l, Kin at 0.5 mg/l or 1.0 mg/l resulted in insignificantly higher number of shoots as compared to the control. In most cases, within each cytokinin, increasing cytokinin concentration resulted in an increase in number of shoots. Also, at the same concentration, BA resulted in higher number of shoots as compared with that recorded with Kin. Without cutting the shoot apex,

Table 3: Effect of different concentrations of BA and Kin as well as subcultures number with or without cutting the shoot apex on number of shoots, average shoot length (cm) and number of leaves/shoot during multiplication stage of *Holmskioldia sanguinea* shoots

	Without cutting the shoot apex				With cutting the shoot apex			
	Subcultures (B)				Subcultures (B)			
Cytokinin concentrations (A)	1	2	3	Mean (A)	1	2	3	Mean (A)
				Number of shoots				
Control	1.67	2.33	2.67	2.22	2.33	2.67	3.00	2.67
0.1 mg/l BA	2.00	2.33	3.00	2.44	2.33	3.00	3.33	2.89
0.5 mg/l BA	2.67	2.67	3.00	2.78	2.67	3.00	4.33	3.33
1.0 mg/l BA	2.67	3.33	3.67	3.22	3.00	4.33	5.33	4.22
2.0 mg/l BA	3.33	3.67	3.67	3.56	3.67	4.33	4.67	4.22
4.0 mg/l BA	3.33	3.67	3.67	3.56	4.00	4.00	4.33	4.11
0.1 mg/l Kin	2.00	2.00	2.33	2.11	2.33	2.33	2.67	2.44
0.5 mg/l Kin	2.33	2.67	3.00	2.67	2.67	2.67	3.33	2.89
1.0 mg/l Kin	2.33	2.67	3.00	2.67	2.67	3.00	3.33	3.00
2.0 mg/l Kin	2.67	3.00	3.33	3.00	3.33	3.67	4.33	3.78
4.0 mg/l Kin	2.67	3.00	3.33	3.00	3.33	3.67	3.67	3.56
Mean (B)	2.52	2.85	3.15	----	2.94	3.33	3.85	----
$L.S.D_{0.05}$ cytokinin conc.(A)		0.46				0.51		
$L.S.D_{0.05}$ Subcultures (B)		0.24				0.27		
$L.S.D_{0.05}$ A × B		0.79				0.88		
				Average of shoot length (cm)				
Control	2.23	2.43	2.73	2.46	1.73	1.83	1.90	1.82
0.1 mg/l BA	2.10	1.90	1.77	1.92	1.60	1.53	1.43	1.52
0.5 mg/l BA	1.75	1.76	1.60	1.70	1.47	1.40	1.33	1.40
1.0 mg/l BA	1.77	1.60	1.46	1.61	1.37	1.33	1.23	1.31
2.0 mg/l BA	1.55	1.42	1.44	1.47	1.30	1.27	1.20	1.26
4.0 mg/l BA	1.36	1.38	1.39	1.38	1.14	1.17	1.09	1.13
0.1 mg/l Kin	2.10	2.13	2.07	2.10	1.66	1.68	1.57	1.64
0.5 mg/l Kin	2.03	1.90	1.77	1.90	1.52	1.56	1.37	1.48
1.0 mg/l Kin	1.87	1.70	1.63	1.73	1.43	1.37	1.27	1.36
2.0 mg/l Kin	1.83	1.70	1.55	1.69	1.33	1.30	1.20	1.28
4.0 mg/l Kin	1.70	1.63	1.53	1.62	1.20	1.13	1.15	1.16
Mean (B)	1.84	1.78	1.72	----	1.43	1.42	1.34	
$L.S.D_{0.05}$ cytokinin conc.(A)		0.09				0.06		
$L.S.D_{0.05}$ Subcultures (B)		0.05				0.03		
$L.S.D_{0.05}$ A × B		0.16				0.10		
				Number of leaves /shoot				
Control	4.33	6.67	8.33	6.44	2.33	3.00	3.33	2.89
0.1 mg/l BA	4.33	4.00	3.67	4.00	3.67	3.00	3.00	3.22
0.5 mg/l BA	4.00	3.67	3.00	3.56	3.67	3.00	2.67	3.11
1.0 mg/l BA	3.67	3.00	3.00	3.22	3.33	3.00	2.67	3.00
2.0 mg/l BA	3.33	3.00	2.67	3.00	3.33	2.33	2.33	2.67
4.0 mg/l BA	3.00	2.67	2.33	2.67	3.00	2.00	1.67	2.22
0.1 mg/l Kin	3.33	3.00	2.33	2.89	2.33	2.33	2.00	2.22
0.5 mg/l Kin	3.00	2.67	2.33	2.67	2.33	2.33	2.00	2.22
1.0 mg/l Kin	2.67	2.67	2.33	2.56	2.33	2.00	2.00	2.11
2.0 mg/l Kin	2.33	2.33	2.00	2.22	2.00	1.67	1.67	1.78
4.0 mg/l Kin	2.00	1.67	1.67	1.78	1.67	1.67	1.67	1.67
Mean (B)	3.27	3.21	3.06	----	2.73	2.39	2.27	----
$L.S.D_{0.05}$ cytokinin conc.(A)		0.63				0.55		
$L.S.D_{0.05}$ Subcultures (B)		0.33				0.29		
$L.S.D_{0.05}$ A × B		1.09				0.95		

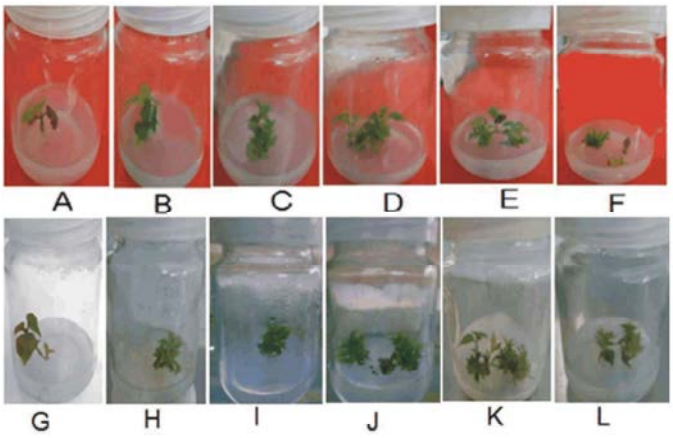

Fig. 2: Effect of BA at 0.0, 0.1, 0.5, 1.0, 2.0 and 4.0 mg/l without cutting the shoot apex, in the third subculture, from left to right in the upper part (A to F), whereas the lower part (G to L) present the effect of the same treatments with cutting the shoot apex in multiplication stage of *Holmskioldia sanguine*a

Fig. 3: Effect of Kin at 0.0, 0.1, 0.5, 1.0, 2.0 and 4.0 mg/l without cutting the shoot apex, in the third subculture, from left to right in the upper part (A to F), whereas the lower part (G to L) present the effect of the same treatments with cutting the shoot apex in multiplication stage of *Holmskioldia sanguine*a.

the highest number of shoots (3.56) was recorded with MS medium supplemented with BA at 2 or 4 mg/l, while with cutting the shoot apex the highest number of shoots (4.22) was recorded with MS medium supplemented with BA at 1 or 2 mg/l.

Data recorded on number of shoots with or without cutting the shoot apex, revealed that increasing subculture number resulted in significant increase in number of resulted shoots. At the same subculture the number of shoots was higher with cutting the shoot apex than that recorded without cutting the shoot apex.

Regarding the interaction effect between the effect of different cytokinins at various concentrations and subculture number, data in Table (3) indicated that MS medium supplemented with 1.0 mg/l BA was the best and economic media for multiplication stage since recorded the highest number of shoots (3.67 shoots without cutting the shoot apex and 5.33 shoots with cutting the shoot apex). Results are in agreement with other studies. The highest number of shoots per explant (14.3±0.9) of *Boscia senegalensis* was achieved on MS medium supplemented with 3 mg/l BA, while the highest shoot length of 3.5±0.4 cm was obtained with 1 mg/l BA [16]. BA was effective on *Pogostemon cablin* shoot regeneration. High number and length of shoots per explant were achieved on Murashige and Skoog (MS) medium containing 0.1–0.2 mg/l IBA [17]. Kinetin was found to be good in stimulating formed shoots of *Labisia pumila* var. alata [18]. Percentage of successful shoots of *Peperomia obtusifolia* regenerated from single node stem segments increased by increasing kinetin concentration then declined at the highest concentration of kinetin (10 mg/ l) [19]. The maximum number of shoots/explants (63.1 ± 1.35) and shoot length (2.8 ± 1.15) within 4 weeks

of *Ophiorrhiza mungos* raised from axillary and terminal buds was observed on MS medium supplemented with 0.25 mg/ l BA and 0.25 mg/ l kinetin, with the highest regeneration frequency (84%) [20].

Cytokinins stimulate cell division; induce shoot formation and axillary shoot proliferation, but retard root formation. Cytokinins promote gene expression in tissue culture that results in the formation of shoots [21]. They are crucial factor in plant meristem activity and morphogenesis. Cytokinins oppose shoots and roots growth when available at high concentrations. At reduced content, they enhance growth through cell cycle regulation; they drive the cell division cycle at normal speed and to obtain the required number of divisions to reach a genetically determined organ size [22]. In this respect Robert and Louis [23] mentioned that percentage of shoot response of *Dipteracanthus prostratus* decreased with increasing the concentration of kinetin.

Average of Shoot Length: Regarding the effect of different cytokinins at various concentrations on average of shoot length recorded with or without cutting the shoot apex, data revealed that the average of shoot length was higher without cutting the shoot apex than that recorded with cutting the shoot apex. Also, in most cases, at the same concentration, Kin resulted in higher average of shoot length as compared with that recorded with BA. The average of shoot length was significantly decreased as a result of using different concentrations of BA and Kin as compared to the control. Also, increasing cytokinin concentration gradually decreased the average of shoot length.

Data recorded on the average of shoot length with or without cutting the shoot apex, revealed that increasing subculture number resulted in significant decrease in average of shoot length, in most cases. The decrease in average of shoot length as a result of increasing subculture number attributed to the increase in number of shoots which can be confirmed by calculating the total length of shoots, by multiplying the average of shoot length by the number of shoots. At the same subculture the average of shoot length recorded without cutting shoot apex was higher than that recorded with cutting the shoot apex.

Regarding the interaction effect between the effect of different cytokinins at various concentrations and subculture number on average of shoot length, data in Table (3) indicated that in the third subculture hormone- free MS medium recorded the highest average of shoot length (2.73 cm without cutting the shoot apex and 1.90 cm with cutting the shoot apex), while the lowest average of shoot length (1.36 cm) was recorded on MS medium supplemented with 4 mg/ l BA in the first subculture without cutting the shoot apex and 1.09 cm on the same medium in the third subculture with cutting the shoot apex.

Number of Leaves /Shoot: Regarding the effect of different cytokinins at various concentrations on number of leaves/shoot obtained with or without cutting the shoot apex, data revealed that the number of leaves/ shoot was higher without cutting the shoot apex than that recorded with cutting the shoot apex, in most cases. Also, at the same concentration, BA resulted in higher number of leaves/ shoot as compared with that recorded with Kin. Number of leaves/ shoot was significantly decreased as a result of using different concentrations of BA and Kin without cutting the shoot apex, as compared to the control. With cutting the shoot apex, most of BA concentrations resulted in insignificantly different number of leaves/ shoot than that recorded on hormone – free MS medium. The only exception to this general trend was recorded with using BA at 4.0 mg/l which resulted in significantly lower value than the control. MS medium supplemented with different concentrations of Kin resulted in significantly lower number of leaves as compared to the control. Without cutting the shoot apex, the highest number of leaves/ shoot (6.44) was recorded with hormone-free MS medium, while with cutting the shoot apex the highest number of leaves/ shoot (3.22) was recorded with MS medium supplemented with BA at 0.1 mg/l.

Data recorded on number of leaves/shoot with or without cutting the shoot apex, revealed that increasing subculture number resulted in insignificant decrease in number of leaves/ shoot in most cases. Similar results were found by Mustafa and Taha [24] on fig as increasing number of subculture increased growth parameter and shoot multiplication increased, but leaf number and shoot length decreased with increasing number of subcultures.

Regarding the interaction effect between the different cytokinins at various concentrations and subculture number, data in Table (3) indicated that hormone- free MS medium resulted in the highest number of leaves/shoot (8.33) in the third subculture without cutting the shoot apex. With cutting the shoot apex, the highest number of leaves/shoot (3.67) was recorded on MS medium supplemented with 0.1 or 0.5 mg/l BA in the first subculture.

Table 4: Effect of different concentrations of IBA and NAA as well as MS-salt strength medium on rooting percentage during rooting stage of *Holmskioldia sanguinea* shoots

	Rooting percentage				
	MS – salt strength (B)				
Auxin concentration (A)	1	1/2	1/4	1/8	Mean (A)
control	60.00 gh	66.67 fg	73.33 ef	96.67 a	74.17 c
1.0 mg/l IBA	66.67 fg	73.33 ef	83.33 cd	100.00 a	80.83 b
2.0 mg/l IBA	76.67 de	86.67 bc	93.33 ab	100.00 a	89.17 a
4.0 mg/l IBA	80.00 c-e	86.67 bc	93.33 ab	100.00 a	90.00 a
1.0 mg/l NAA	53.33 hi	56.67 h	56.67 h	60.00 gh	56.67 d
2.0 mg/l NAA	16.67 l	26.67 k	33.33 jk	46.67 i	30.83 e
4.0 mg/l NAA	6.67 m	13.33 lm	30.00 jk	36.67 j	21.67 f
Mean (B)	51.43 d	58.57 c	66.19 b	77.14 a	---

* Within the column for auxin concentrations means, the row for MS – salt strength means, or the means for combinations of the two factors, means sharing one or more letters are insignificantly different at 5 % level, according the "Least Significant Difference" test.

From the above results, it could be concluded that multiplication stage should be carried out with cutting the shoot apex on MS medium supplemented with 1.0 mg/l BA because it was the best and economic media which recorded the significantly highest number of shoots (5.33) at the end of the third subculture.

Experiment IV: Rooting Stage

Rooting Percentage: Data presented in Table (4) revealed that using IBA at 1.0, 2.0 and 4.0 mg/l resulted in significantly higher rooting percentages as compared to the control. Increasing IBA concentration in the medium significantly increased rooting percentage, in most cases. Using IBA at 4.0 mg/l resulted in the significantly highest rooting percentages followed by using MS medium supplemented with IBA at 2.0 mg/l with no significant difference between them. On contrary using NAA at 1.0, 2.0 and 4.0 mg/l resulted in significantly lower rooting percentages as compared to the control. Increasing NAA concentration significantly decreased rooting percentage. Using NAA at 4.0 mg/l resulted in the significantly lowest rooting percentage. These results are in agreement with the findings of Ling *et al*. [18], on *in vitro* stem explants of *Labisia pumila* var. alata. They reported that 1 mg/l IBA recorded the highest rooting percentage (50.00±7.07). The medium supplemented with 3, 5, or 7 mg/l of IBA achieved rooting of 30.00±7.07%, 10.00±4.71% and 20.00±0.00%, respectively. On the other hand, 40.00±9.43% of stem explants grown on MS medium containing 1 mg/l NAA rooted. Hussein [25] reported that root formation was the best by using IBA at 2.0 mg/l through determining the suitable methodology for propagating three *Aglaonema* species by tissue culture technique. Also, root induction of teak (*Tectona grandis* L.) was recorded by supplementing rooting medium with 2 mg/l NAA [26].

Concerning the effect of MS- salt strength media, regardless of the effect of type and concentration of auxin, using 1/8 MS salt strength medium resulted in the significantly highest rooting percentage (77.14%) followed by 1/4, 1/2 and full salt strength medium (66.19, 58.57, 51.43%, respectively) with significant differences among them.

Using 1/8 MS salt strength medium supplemented with IBA with 1.0, 2.0 or 4.0 mg/l resulted in the significantly highest rooting percentage (100%) followed by 1/8 hormone-free MS salt strength medium (96.67%) and 1/4 MS salt strength medium supplemented with 2.0 or 4.0 mg/l (93.33% for each) with no significant difference among them. Such results are in agreement with the findings of Jirakiattikul *et al*. [27] on *Smilax corbularia*, who reported that the highest rooting percentages occurred on ¼ MS medium supplemented with 2.0 mg/l NAA (65.83%) and ½ MS medium supplemented with 0.5 mg/l NAA (66.67%).

Number of Roots/Plantlet, Root Length, Number of Leaves/Plantlet and Plantlet Length: Data presented in Table (5) revealed that using MS medium supplemented with IBA at 1.0, 2.0 or 4.0 mg/l resulted in significantly higher number of roots/plantlet, root length, number of leaves/plantlet and plantlet length as compared to the hormone-free MS medium. In most cases, using MS medium supplemented with IBA at 4.0 mg/l resulted in the

Table 5: Effect of different concentrations of IBA and NAA as well as MS-salt strength medium on number of roots/plantlet, root length, number of leaves/plantlet and plantlet length during rooting stage of *Holmskioldia sanguinea* explants

	Number of roots/plantlet					Root length (cm)				
	MS – salt strength (B)					MS – salt strength (B)				
Auxin concentration (A)	1	1/2	1/4	1/8	Mean (A)	1	1/2	1/4	1/8	Mean (A)
Control	1.67	2.67	4.33	6.67	3.84	1.00	1.67	2.33	3.33	2.08
1.0 mg/l IBA	2.33	3.67	6.33	6.67	4.75	1.67	2.67	3.00	3.83	2.79
2.0 mg/l IBA	4.00	5.00	7.67	9.67	6.59	2.33	4.33	6.33	8.00	5.25
4.0 mg/l IBA	4.00	5.00	8.33	10.00	6.83	2.33	4.50	6.50	8.17	5.38
1.0 mg/l NAA	1.67	1.67	2.00	2.33	1.92	1.00	1.33	1.33	1.67	1.33
2.0 mg/l NAA	0.67	0.67	1.33	2.00	1.17	0.67	0.67	1.67	2.00	1.25
4.0 mg/l NAA	0.33	0.67	1.33	1.67	1.00	0.33	0.67	1.33	1.67	1.00
Mean (B)	2.10	2.76	4.47	5.57	----	1.33	2.26	3.21	4.10	----
$L.S.D_{0.05}$ Auxin conc. (A)			0.49					0.40		
$L.S.D_{0.05}$ MS- salt strength (B)			0.37					0-.31		
$L.S.D_{0.05}$ A × B			0.98					0.81		
	Number of leaves/ plantlet					Plantlet length (cm)				
Control	2.67	4.67	7.00	11.33	6.42	2.17	2.67	3.17	3.67	2.92
1.0 mg/l IBA	3.33	6.67	7.33	13.00	7.58	2.83	2.83	3.50	4.17	3.33
2.0 mg/l IBA	4.33	7.00	8.67	14.67	8.67	4.33	4.83	5.33	8.00	5.62
4.0 mg/l IBA	4.67	8.00	9.00	14.67	9.09	4.17	4.67	5.17	8.17	5.55
1.0 mg/l NAA	2.67	3.33	6.00	6.33	4.58	2.17	2.33	3.17	3.50	2.79
2.0 mg/l NAA	2.33	3.33	5.67	6.33	4.42	2.00	2.17	3.00	3.33	2.63
4.0 mg/l NAA	2.00	3.00	4.00	6.00	3.75	2.00	2.17	2.83	3.17	2.54
Mean (B)	3.14	5.14	6.81	10.33	----	2.81	3.10	3.74	4.86	----
$L.S.D_{0.05}$ Auxin conc. (A)			0.57					0.29		
$L.S.D_{0.05}$ MS- salt strength (B)			0.43					0.22		
$L.S.D_{0.05}$ A × B			1.14					0.59		

significantly highest values followed by using MS medium supplemented with IBA at 2.0 mg/l with no significant difference between them. The only exception to this general trend was recorded with using MS medium supplemented with IBA at 4.0 mg/l resulted insignificantly shorter plantlet length as compared to using MS medium supplemented with IBA at 2.0 mg/l. Increasing IBA concentration in MS medium gradually increased values of parameters investigated. On contrary, using MS medium supplemented with NAA at 1.0, 2.0 or 4.0 mg/l resulted in significantly lower values for number of roots/plantlet, root length, number of leaves/plantlet and plantlet length as compared to the hormone-free MS medium. Increasing NAA concentration in MS medium gradually decreased values of parameters investigated. Using MS medium supplemented with NAA at 4.0 mg/l resulted in the lowest values of number of roots/plantlet, root length, number of leaves/plantlet and plantlet length. The positive action of IBA over NAA may be due to that polar movement of IBA and its transportation is faster than NAA, Leopold and Lam [28] found that the velocity of NAA was 6 .7 mm/ hr and of IBA was 3.2 mm/ h and this could likely explain the low efficiency in root induction on explants placed in medium supplemented with NAA. Generally, the obtained results are in accordance with another study where IBA and NAA showed a positive rooting response on *Labisia pumila* var. alata stem explants, the longest roots formed from stem explants were 0.35±0.07 cm and (0.05±0.01) cm long at the concentrations of 1 and 3 mg/l NAA, with 1.8±0.4 and 0.5±0.2 roots per explants, respectively [18].

Using 1/8 MS salt strength medium resulted in the significantly highest number of roots/plantlet, root length, number of leaves/plantlet and plantlet length followed by 1/4, 1/2 and full salt strength medium with significant differences among them.

Using 1/8 MS salt strength medium supplemented with IBA at 4.0 mg/l resulted in the significantly highest number of roots/plantlet, root length, number of leaves/plantlet and plantlet length followed by 1/8 MS salt strength medium supplemented with IBA at 2.0 mg/l with no significant difference between them.

Economically, 1/8 MS salt strength medium supplemented with IBA at 2.0 mg/l is the best medium for rooting stage since it resulted in 100% rooting percentage, 9.67 roots/plantlet, 8.00 cm root length, 8.67 leaves/ plantlet and 8.00 cm plantlet length (Fig. 4).

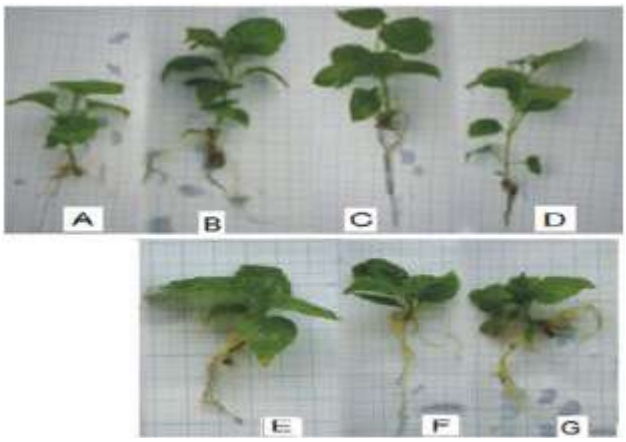

Fig. 4: Effect of IBA and NAA on rooting stage of *Holmskioldia sanguinea.* A: plantlets grown on hormone-free 1/8 MS medium. B- D: Plantlets grown on 1/8 MS medium supplemented with IBA at 1.0, 2.0 and 4.0 mg/l .E –G: Plantlets grown on 1/8 MS medium supplemented with NAA at 1.0, 2.0 and 4.0 mg/l

Table 6: Effect of different growing media on survival percentages during acclimatization stage of *Holmskioldia sanguinea* plantlets after 6 and 12 weeks.

	Survival percentage	
	Weeks	
Growing media	6	12
Peatmoss	83.33 b	66.67 d
Peatmoss + sand (1:1)	83.33 b	73.33 c
Peatmoss + sand (2:1)	100.00 a	100.00 a
Peatmoss + sand (3:1)	83.33 b	80.00 b
Peatmoss + perlite (1:1)	100.00 a	100.00 a
Peatmoss + perlite (2:1)	100.00 a	83.33 b
Peatmoss + perlite (3:1)	83.33 b	73.33 c
Peatmoss + vermiculite (1:1)	83.33 b	83.33 b
Peatmoss + vermiculite (2:1)	100.00 a	100.00 a
Peatmoss + vermiculite (3:1)	83.33 b	63.33 d

* Within each column the means sharing one or more letters are insignificantly different at 5 % level, according the "Least Significant Difference" test.

These values are significantly higher than most of the other treatments and insignificantly different than the highest values recorded with 1/8 MS salt strength medium supplemented with IBA at 4.0 mg/l. These results are in agreement with the findings of Jin *et al.* [17] who reported that growing *Pogostemon cablin* regenerated shoots on half-strength MS medium supplemented with 0.2 mg/l IBA gave maximum number of roots per plantlet and length of roots. Jirakiattikul *et al*. [27] on *Smilax corbularia*, reported that the number of roots per shoot was 9.6 and 8.4 on ¼ MS medium supplemented with 2.0 mg/l NAA and ½ MS medium supplemented with 0.5 mg/l NAA. Also, Abou Dahab *et al.* [29] on *Ruscus hypoglossum* reported that MS medium at ½ strength supplemented with IBA at 2.0 mg/l was the most effective treatment in increasing the number of roots / shootlet.

Experiment V: Acclimatization Stage

Survival Percentage: Data presented in Table (6) revealed that after 6 and 12 weeks, the significantly highest survival percentage (100%) was recorded on growing media containing peatmoss + sand (2:1 v/v), peatmoss + perlite (1:1 v/v) and peatmoss + vermiculite (2:1 v/v), in addition to growing medium containing peatmoss + perlite (2:1 v/v) after 6 weeks only. Acclimatization media provided efficient water supply and water balance in the adaptation of plantlets to *in vivo* conditions as they lacked to complete conductive tissues of roots and their connections, besides to the retardation in leaf thickness, differentiation of leaf mesophyll palisade and spongy parenchyma, stomatal density, cuticle, epicuticular waxes which decreases stomatal restriction of transpiration.

Plantlet Length, Number of Leaves/Plantlet, Stem Diameter and Number of Shoots/Plantlet: Data presented in Table (7) revealed that after 6 and 12 weeks, the significantly tallest plantlets with the significantly highest number of leaves/plantlet and the significantly thickest stem were recorded on the growing medium containing peatmoss + vermiculite (2:1, v/v). There was no significant difference between stem diameter of plantlets grown on peatmoss + sand (2:1, v/v) and that grown on peatmoss + vermiculite (2:1, v/v). After 6 and 12 weeks, there was no significant difference in number of shoots /plantlet recorded on the different growing media investigated.

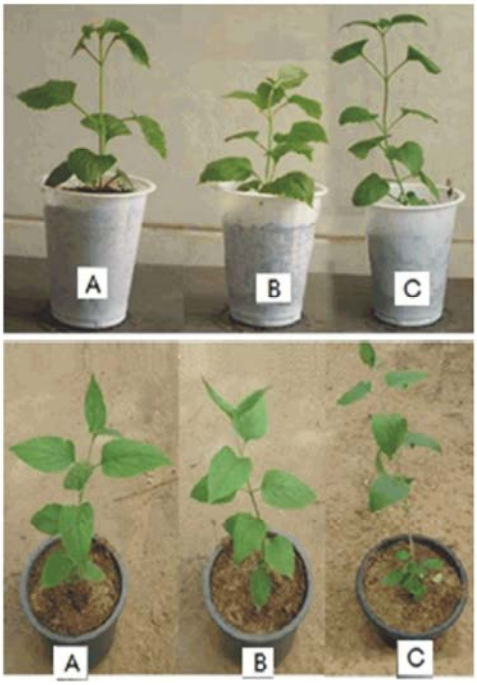

Fig. 5: Effect of growing media on acclimatization stage of *Holmskioldia sanguine*a. In the upper part plantslets after 6 weeks, while in the lower part plantslets after 12 weeks. A – plantlets grown in peatmoss + sand (2: 1, v/v), B- plantlets grown in peatmoss + perlite (1: 1, v/v), C- plantlets grown in peatmoss + vermiculite (2: 1, v/v)

Table 7: Effect of different growing media on plantlet length (cm), number of leaves/plantlet, stem diameter (cm) and number of shoots/plantlet during acclimatization stage of *Holmskioldia sanguinea*

	Plantlet length (cm)		Number of leaves/plantlet		Stem diameter (cm)		Number of shoots/plantlet	
	Weeks		Weeks		Weeks		Weeks	
Growing media	6	12	6	12	6	12	6	12
Peatmoss	4.73	7.00	4.33	8.33	0.97	1.27	1.33	2.33
Peatmoss + sand (1:1)	5.30	8.33	5.67	9.00	1.47	1.73	1.67	2.33
Peatmoss + sand (2:1)	6.10	10.13	8.00	14.33	1.60	1.83	1.67	3.67
Peatmoss + sand (3:1)	7.77	8.10	4.33	13.33	1.23	1.27	1.67	2.00
Peatmoss + perlite (1:1)	6.17	7.17	10.00	12.00	1.20	1.20	2.00	3.33
Peatmoss + perlite (2:1)	4.83	6.13	8.33	10.33	0.97	1.07	1.67	2.67
Peatmoss + perlite (3:1)	3.67	5.17	3.67	8.00	0.93	1.07	1.33	2.00
Peatmoss + vermiculite (1:1)	7.50	9.50	7.67	12.67	1.13	1.23	1.67	2.00
Peatmoss + vermiculite (2:1)	10.83	15.67	14.67	22.33	1.80	1.97	2.33	2.33
Peatmoss + vermiculite (3:1)	9.17	13.07	12.33	18.00	1.43	1.53	1.33	2.00
$L.S.D_{0.05}$	1.14	1.24	1.37	1.47	0.30	0.23	N.S.	N.S.

Table 8: ISSR analysis of DNA extracted from *in vivo* and *in vitro* produced *Holmskioldia sanguine*a

No	Primer	Sequence	Total number of scorable bands	Number of Polymorphic bands	Polymorphism (%)	Size of polymorphic band (bp)
1	HB-08	5GAG AGA GAG AGA GG 3	11	2	18	565.46 -969.67bp
2	HB-09	5GTG TGT GTG TGT GC 3	10	0	0	166.58 - 739.87 bp
3	HB- 10	5 GAG AGA GAG AGA CC CC3’	12	3	25	156.29 - 597.35 bp
4	HB-11	5’ GTG TGT GTG TGT 3’	8	1	12.5	203 - 893.84 bp
5	HB-13	5’ GAG GAG GAG GC 3’	10	1	10	283.10 - 1341.80 bp

The above mentioned result can be explained by that peatmoss holds nutrients and water in the growing media, vermiculite is capable of holding large amounts of air, available water and nutrients needed for plant growth. Whereas perlite is porous, so it improves aeration and drainage and benefits root oxygenation [25, 30].

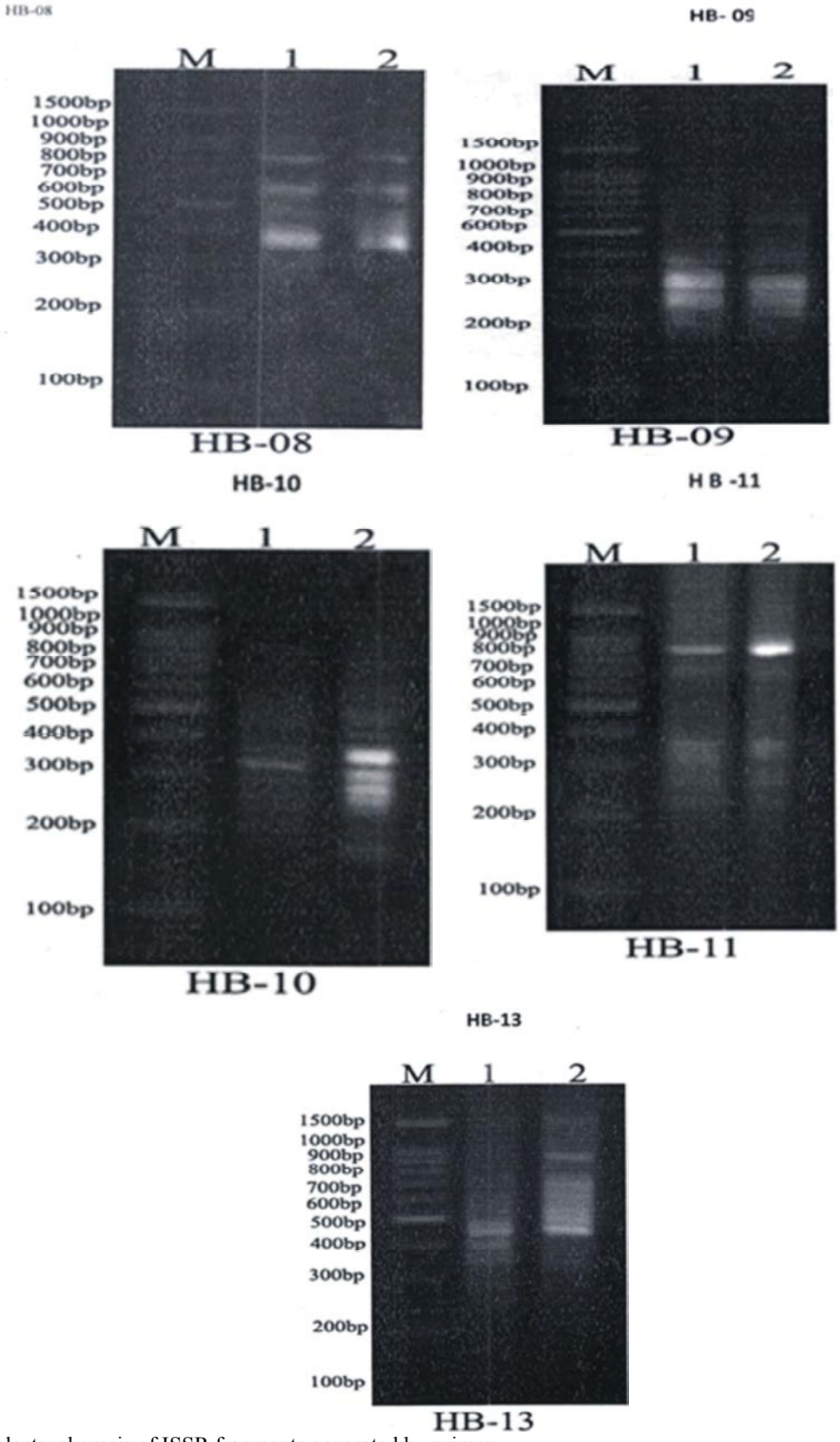

Fig. 6: Gel electrophoresis of ISSR fragments generated by primer

On the other hand growing medium contains peatmoss + perlite (3:1, v/v) as well as that containing peatmoss only proved to be the unsuitable media for acclimatization stage of *Holmskioldia sanguinea* because of resulting in lowest values of plantlet length, number of leaves/plantlet, stem diameter and number of shoots/plantlet. So, mixing or blending several growing media components in various proportions to give the desired mixture characteristics for best plantlet growth is very important and the desired mixture differed according to the genotype investigated [25].

From the above results growing medium consists of peatmoss + vermiculite (2:1, v/v) was the best for acclimatization stage because of giving 100% survival and resulting in the significantly tallest plantlets with the significantly highest number of leaves/plantlet and the significantly thickest stem (Fig. 5).

ISSR Analysis of *In vivo* and *In vitro Holmskioldia sanguinea* Plants: Data in Table (8) demonstrated the results of ISSR analysis of *in vitro* regenerated plants. The ISSR primers applied to investigate the level of polymorphism in *Holmskioldia sanguinea* plant produced clear reproducible bands (sequences presented in Table 8). The 5 primers yielded 51 scorable bands (with an average of 10.2 bands per primer), including seven polymorphic bands (representing 13.7% polymorphism). The total number of bands from each primer varied from eight to 12 bands. The number of polymorphic bands from each primer ranged from zero in HB-09 to three in HB-10. The primer HB-10 produced the highest number of amplified individual fragments (12), recording the highest percentage of polymorphism (25). Primer HB-09 produced the lowest number (zero) of amplicons, followed by HB-11 and HB-13 primers giving only one polymorphic band, producing 12.5 and 10 % polymorphism, respectively. Overall, 4 of the 5 primers tested (HB-08, HB-10, HB-11 and HB-13) produced amplification products. The size of the polymorphic bands produced by these primers ranged from 156.29 bp in HB- 10 to 1341 bp in HB- 13 (Fig. 6).

The present study provides the first information on the molecular basis of polymorphism detected as ISSR markers in *Holmskioldia sanguinea* micropropagated plants. The similarity obtained from the analysis of all the bands recorded showed 13.7% similarity (7 polymorphic fragments out of a total of 51). Prakash *et al.* [31] reported that RAPD analysis of *Curcuma amada* recorded 103 scorable bands from 10 primers, representing 8.7% polymorphism. Obtained results can be explained by that *in vitro* culture results in breakdown of normal cellular controls causing genetic and epigenetic instabilities and therefore alterations in the gene expression and derivation of new phenotypes happens [32 and 33].

Conclusion and Recommendations: The direct shoot regeneration micropropagation protocol for commercial *in vitro* propagation of *Holmskioldia sanguine*a could be summarized as follow: the single node stem cuttings explants should be sterilized by 30% Clorox for 20 min. Full strength MS medium supplemented with NAA at 0.5 mg/l was the best medium for *in vitro* establishment of explants in respect of shoot length and number of leaves / explant. The multiplication stage should be carried out with cutting the shoot apex on MS medium supplemented with 1.0 mg/l BA for three subcultures. For rooting the regenerated shoots, one eighth strength MS medium supplemented with 2.0 mg/l IBA was the effective and economic medium. Growing medium consists of peatmoss and vermiculite at the ratio of 2:1 (v/v) was the best for acclimatization of regenerated plantlets. The inter simple sequence repeats (ISSR) analysis detected similarity (13.7%) to the mother tree in the in *vitro* regenerated plants from the nodal explants.

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