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Effect of different types of the medium on micropropagation of *Ixora coccinea* L.

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ABSTRACT

***Ixora coccinea* is one of the important ornamental plants, commercially propagated by stem cuttings. This study aims to determine the effects of different media on micropropagation and rooting and to investigate the genetic fidelity of regenerated plants using the Inter Simple Sequence Repeat (ISSR) marker. For this purpose, Murashige and Skoog (MS), and Woody Plant Medium (WPM) media were used for micropropagation and rooting experiments. All media were supplemented by 5.5g /l gelling agent (Agar) for micropropagation, MS media with different level of sucrose (15,30 ,45 and 60 g/l), 2.0 mg/l BAP with different concentrations of IBA (0.0, 0.1, 0.25 and 0.5 mg/l), for rooting 10mg /l NAA used. *In vitro* rooted plantlets were transferred to plastic pots containing; peat moss with vermiculite (1:1; 2:1, and 1:2 v/ v) for acclimatization to *ex vitro* conditions. The ISSR markers were used for the assessment of genetic fidelity between the mother plant and acclimatized plantlets ISSR marker used. Total DNA was extracted from leaves of the acclimated plant by the cetyltrimethyl ammonium bromide method (CTAB), and the data obtained were analyzed and used to generate a similarity matrix using Jaccard's similarity coefficient. The best result in terms of regeneration of shoots was obtained from 30 g/ l sucrose on a full MS medium. MS media during the incubation period gave the best results in terms of vegetative growth parameters compared to WPM. The estimation of the genetic similarity coefficient based on the ISSR band indicated that acclimatized plantlets derived from the 6th subculture were 100% similar to the mother plant.** **Keywords:** growth regulator; acclimatization; *in vitro*; genetic fidelity; DNA

INTRODUCTION

*Ixora coccine*a is belong to the Rubiaceae family, in Sudan it is called Ixora (NISIR,2002, El Amin,1990). There are around 562 species according to WCSP (2017). Ixora is native to the tropical area but today it can be growing in the tropical and subtropical climates of the world (Thakur and Kumar, 2014, Idowu *et al*.,2010). Traditionally Ixora is propagated from stem cuttings. Plant tissue culture techniques have allowed strong and continued growth within the micropropagation industry (Onsa *et al*. 2018a, b). The MS media salts whether in the form of full or half strengths are considered to be the most common basal medium. Some reports also suggested there is the usage of other media such as the medium of the woody plant (WPM) (Lloyd and Mc Cown, 1980). Onsa *et al*. (2018 a, b) obtained the highest

regeneration rate at *I. coccinea* on full MS medium supplemented with 2.0 mg/L BAP, 0.5 mg/l IBA, and 30 g/l sucrose, using shoot tips as explants. Li *et al*. (2019) and Sakr *et al.* (2010) obtained a high survival rate (80%) from *I. chinensis* and the best vegetative growth in *I. undulata* when they used full MS cultured media, respectively. In contrast, Lakshmanan *et al.* (1997) used full WPM media for the best result. The highest percentage of plant survival rate (60%) occurred when Sakr *et al*. (2010) used peat moss: sand (1:1v/v) to *I. undulata* in the acclimatization stage. Random amplified polymorphic DNA (RAPD) and ISSR markers are molecular-typing approaches that have been used to detect, identify and determine relationships between species and cultivars among plants (Raina *et al*. 2001; Williams *et al.*1986; Zeitkiewicz *et al*. 1994). Rajaseger *et al.* (1997) recorded the first report of the use of a DNA-based polymorphism assay to assess the level of variability in 22 Ixora cultivars depending on the RAPD marker. This study aims to develop an efficient micropropagation protocol of *Ixora coccinea* by using a different type of medium and then assess the genetic fidelity of micropropagation plantlets by using the ISSR marker.

MATERIALS AND METHODS

In vitro multiplication experiments

The biological material used to initiate the *in vitro* culture of *Ixora coccinea* ('pink') was sampled from a mature healthy shrub. The plant material was washed under running tap water then transferred to a laminar airflow cabinet, rinsed with 70% ethanol for 10 seconds, washed with distilled water followed by immersing in 20% sodium hypochlorite solution (v/v) and 2-3drops of Tween 20 with continuous shaking to 15 minutes. The explant was carefully washed with sterile distilled water. For the initiation of *in vitro* culture, the following basal culture media were used Murashige and Skoog (MS) and Woody Plant Medium (WPM). These media were supplemented with 2.0 mg/l BAP, 0.5 mg/l IBA, and 5.5 g/l agar. MS media supplemented with different levels of sucrose (15, 30, 45, and 60 g/l). The culture media were sterilized by autoclaving at 121°C for 15 minutes. The pH of media was adjusted to 5.8 ± 0.02. The cultures were transferred to the growth room where controlled conditions of temperature (25±2^{0}C), with 16 hours of light and 8 hours of darkness, and light intensity (1000 lx, using white fluorescent lamps) were ensured. Four variants of the MS medium were used (quarter, half, full and double). The sterile shoot tip explants of *I. coccinea* were cultured and vegetative growth parameters such as; the number of leaves, the number of shoots, the length of shoots, and the number of nodes were measured weekly until the 8th week. The regenerated shoots were cultured on MS and WPM medium with 2.0 mg/l BAP and different concentrations of IBA (0.0, 0.1, 0.25, and 0.5 mg/l) to evaluate the effect of MS versus WPM on shoot morphogenesis of Ixora shoot tip explants. *In vitro* roots of Ixora plantlets with an average of 3.0 roots/ plant and 6.0 leaves /plant were taken out from the test tubes and washed thoroughly under running tap water to remove the medium from roots. The plantlets were transferred into plastic pots (5×10 cm) containing vermiculite, completely covered with plastic bags for acclimatization, and kept in an incubation room. By the end of the 3^{ed} week, the plantlets were transferred to a 50% shade house for adaptation to *ex vitro* conditions. Plantlets were transplanted to plastic pots containing nutrient substrate peat moss with vermiculite by the different ratios (1:1, 2:1 and 1:2 v/v). The experiments were performed using a completely randomized design of three treatments. Growth parameters such as; the number of leaves, number of shoots, and length of shoots were measured then the data were recorded. The recorded data were analyzed by the Statistic Analysis System software program (SAS) using analysis of variance (ANOVA). Comparisons of means data were performed using Duncan Multiple Range Test (DMRT) at a probability of 5%

Genetic stability assessment:

To the assessment of genetic fidelity between the mother plant and acclimatized plantlets, Inter Simple Sequence Repeat (ISSR) marker was used. The source of plant material used in this test was derived from plantlets after six subcultures, after the acclimatization of plants, the total DNA was extracted from the leaves of the five acclimatized plants individually by cetyltrimethylammonium bromide method (CTAB) (Doyle and Doyle 1987). The concentration and purity of isolated DNA were determined using the NanoDrop 2000 (Thermo Fisher Scientific Wilmington, USA). In this experiment, four ISSR primers were screened by Integrated DNA Technologies company and used for the analysis of genetic stability among five tissue culture acclimatized plantlets (AP) and their mother plant (MP). Polymerase chain reaction (PCR) amplification was carried out for four ISSR primers in a 25 µl reaction volume. The PCR reaction contained 70 mg/µL genomic DNA, 2 X Dream Taq TM Green PCR Master Mix (Fermentas, International Inc.), and 0.4µM forward and reverse primers. Amplification was performed in a thermal cycler (Bio-Rad Laboratories, Inc.) for a total of 40 cycles after an initial denaturation of the template DNA at 94°C for 3 min. This was followed by 10 cycles of 94°C for the 40s. This was followed by 30 cycles of 95°C for the 40s, a final annealing temperature for 30s and 72°C for 1 minute, and a final extension of 72°C for 10 min. The amplification products were analyzed on 2% gel with a 50 bp DNA ladder (Ready to use). The gel was stained with Midori green, and visualized under ultraviolet light by a gel documentation system (Bio-Rad Company). Clear ISSR bands were scored '1' if they were present and '0 if it absents within the gel was considered as a single dominant ISSR marker locus. The binary data obtained were analyzed and used to generate a similarity matrix using Jaccard's similarity coefficient (Jaccard, 1908).

RESULTS AND DISCUSSIONS

Referred to table 1 MS media with different concentrations of sucrose (15, 30, 45, and 60 g/l), 2.0 mg / l BAP, and 0.5 mg /l IBA after 8 weeks from culture, showed a highly significant difference between 30 g/ l and other concentrations. No growth parameters were recorded on media with 15 g/ l and 60 g/ l of sugar. This comes in the same line with Gamborg *et.al* (1976) who mentioned that carbon and energy source at 2-4% is preferred by most plant cells. George (1993) and Karhu (1997) stated that plant cell, tissue, or organ culture normally requires the incorporation of a carbon source into the culture medium. The result agreed with Onsa *et al*. (2018, b) and Amine *et al*. (2002). Although, Ixora is a woody plant that required large energy in culture media to grow well it didn't respond to the higher concentration of sugar (60 g/l).

Table 1. Effect of MS media supplemented with different concentrations of sucrose, 2.0 mg/l BAP and 0.5 mg/l IBA on shoot multiplication of *Ixora coccinea* after 8 weeks of culture

Sucrose concentrations (g/l)	Vegetative Parameters				
	Mean± SE				
	Number of leaves	Number of shoots	Length of shoots(cm)	Length of explants (cm)	Number of nodes
15	0.0^{d}	0.0^{c}	0.0^{c}	0.0^{b}	0.0^{b}
30	15.2±0.18^{a}	2.4±0.25^{a}	3.0±0.06^{a}	1.5±0.09^{a}	7.3±0.43^{a}
45	4.0±0.20^{b}	1.3±0.20^{b}	0.5±0.04^{b}	0.3±0.03^{b}	0.0^{b}
60	2.0±0.51^{c}	0.0^{c}	0.0^{c}	0.0^{b}	0.0^{b}

**Means with the same letters in the same column are not significantly different at 5% using Duncan Multiple Range Test. SE=Standard Error*

The media strength had a significant effect on the vegetative parameters of the shoot tip of the *Ixora* explant (Table 2). The highest value of all parameters measured was obtained on full MS without significant difference from ½ MS except for the length of a shoot. 2MS didn't show any growth, this might be due to the toxic effect of a higher concentration of salts, in the same way, ¼ MS gave a poor result, thus, the two media could be excluded from *in-vitro* micropropagation of *I. coccinea*. It appeared from this study that *I. coccinea* shooting performs well under full MS salts mixture. This finding confirmed the previous one of Thakur and Kumar (2014) who used full MS to *Ixora parviflora* and Sakr *et al.* (2010) to *Ixora andulata*. Amin *et al.* (2002) reported that the response of *Ixora fulgens* cultured on full MS was significantly better than on ½ or ¼ MS. Figure 1 showed the strength medium effect on shoot tips after 8 weeks from culture.

Table 2. Effect of MS medium strengths supplemented with 2.0 mg/l BAP and 0.5 mg/l IBA on shoot regeneration of *Ixora coccinea* after 8 weeks from culture

Media	Vegetative Parameters				
	Mean ± SE				
	Number of leaves	Number of shoots	Length of shoots(cm)	Length of explants(cm)	Number of nodes
¼ MS	2.6±0.32^{b}	0.3±0.30^{b}	0.07±0.00^{c}	1.3±0.33^{b}	0.0^{b}
½ MS	10.3±0.41^{a}	2.1±0.11^{a}	1.2±0.11^{b}	1.7±0.11^{a}	6.8±0.19^{a}
1MS	15.7±0.50^{a}	2.3±0.36^{a}	3.1±0.24^{a}	1.7±0.10^{a}	7.0±0.28^{a}
2MS	0.0^{c}	0.0^{b}	0. 0^{c}	0.0^{b}	0.0^{b}

**Means with same letters in the same column are not significantly different at 5% using Duncan Multiple Range Test. SE=Standard Error*

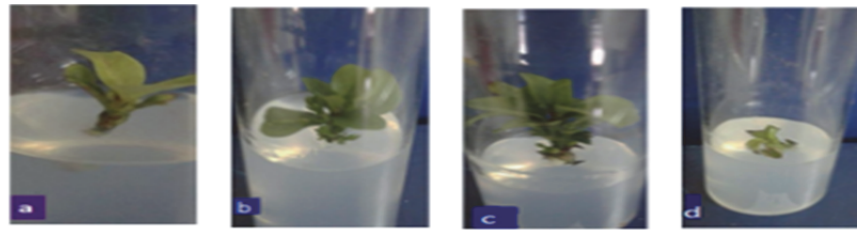

Figure 1. Effect of MS medium strengths on shoot tip initiation of *Ixora coccinea* explant after 8 weeks from culture (a) ¼ MS (b) ½ MS (c) Full MS (d) 2MS

The result of adding 2.0 mg / l BAP with different concentrations of IBA (0.1, 0.25,0.5 mg/l) to MS and WPM to enhance the growth and development of *I. coccinea* is shown in table 3. The combination of 2.0 mg /l BAP with 0.5 mg /l IBA on MS media recorded a significantly higher number of leaves, length of shoot, and explant length than other treatments used, while, WPM with 2.0 mg/l BAP + 0.1IBA recorded significantly higher growth parameters for Ixora except for the length of explant. This result agrees with Amin *et al.* (2002) who found that axillary buds of *lxora fulgens* showed sprouting within two weeks of incubation on MS medium fortified with 0.5 mg/l BAP and 0.1 mg /l NAA. In contrast, Lakshmanan *et al.*1997 found the number of regenerated axillary shoots of *l. coccinea* cultured in WPM medium increased with increasing the concentration of BA from 0.5 to 2.5 mg/l. Generally, all the results provided by MS media were far better than that obtained from WPM (Figure 2), so it could be recommended that the MS media is a suitable one for Ixora micropropagation.

After three days of acclimatization under room temperature, most of the plantlets showed some wilting symptoms like slight stem bending and yellowing of leaves. After one week, the plantlets recovered slowly and started growing well again with a 70 % survival rate. After

three weeks, the plantlets were transferred to a 50% shade house for adaptation to *ex-vitro* situations. In this stage, new leaves unfolded and the plantlets recovered very well.

Table 3. MS versus WPM supplemented with 2.0 mg/l BAP and different concentrations of IBA on *Ixora coccinea* regeneration after 8 weeks from culture

Medium	Different Combinations of BAP and IBA (mg/l)	Number of leaves	Number of shoots	Length of shoots (cm)	Length of explants (cm)
MS	0.0BAP+0.00BA	0.0^{e}	0.0^{d}	0.0c	0.0c
WPM		0.0^{e}	0.0^{d}	0.0^{c}	0.0^{c}
MS	2.0BAP+0.1IBA	15.7±0.27^{a}	2.3±0.30^{a}	1.3±0.14^{b}	0.6±0.16b^{c}
WPM		11.2±0.42^{b}	2.2±0.15^{a}	1.7±0.04^{b}	1.5±0.09a^{b}
MS	2.0BAP+0.25IBA	9.6±0.33^{c}	1.3±0.30^{b}	1.3±0.17^{b}	1.0±0.20ab^{c}
WPM		4.2±0.48^{d}	0.2±0.06^{c}	0.1±0.03^{c}	1.5±0.13a^{b}
MS	2.0BAP+0.5IBA	16.3±0.37^{a}	2.3±0.11^{a}	2.3±0.21^{a}	1.9±0.23^{a}
WPM		7.4±0.30^{c}	0.2±0.06^{c}	0.1±0.03^{c}	1.8±0.16^{a}

**Means with same letters in the same column are not significantly different at 5% using Duncan Multiple Range Test. SE=Standard Error*

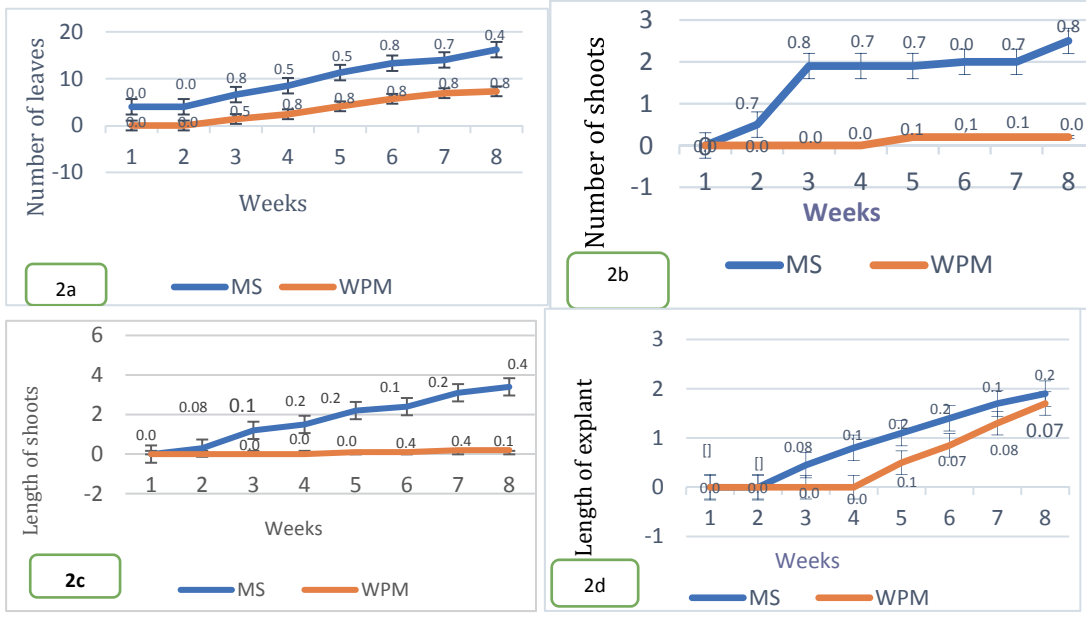

Figure 2 (2a ,2b,2 c, 2d) Comparison between the number of leaves, number of shoots, length of shoots and length of explant of *Ixora coccinea* shoot tip explant during incubation period cultured on MS and WPM supplemented with 2.0mg/l BAP+0.5mg/l IBA. Vertical bars are indicating the standard deviation (±SD)

Figure 3 showed the survival rate among the three potting media used during acclimatization. The highest survival rate (60%) was obtained in a medium consisting of peat moss with vermiculite (2:1v/v) followed by 40% in (1:1v/v), and the lowest rate 20% was obtained in (1:2v/v). A similar observation was reported by Sakr *et al.* (2010) who got a 60% survival rate and 90% by Amin *et al.*(2002) when coco-peat was used.

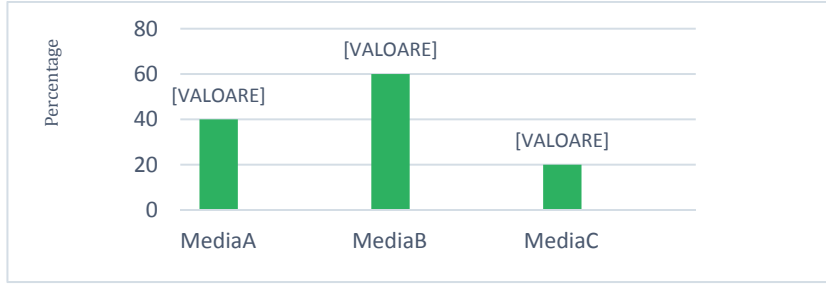

Figure 3. Survival rate of *Ixora coccinea* plant on different soil media after seven weeks. (A) peat moss + vermiculite (1:1v/v) (B) peat moss + vermiculite (2:1v/v) (C) peat moss + vermiculite (1:2v/v).

Table 4 revealed the significant differences in vegetative parameters. The media containing peat moss with vermiculite (2:1v/v) produced the highest number of leaves (8.0 leaves/ plant), shoot number (1.0/plant), and plant height (7.5 cm) This result agreed with Hartmann *et al*. (2007) who stated that when vermiculite combined with peat moss, cation exchange properties could possess which can hold and make ammonium, potassium, calcium, and magnesium available to the growing plants and promote quick anchorage to young roots. Kim (2011) emphasis that a mixture of more than 60% peat moss creates an optimum condition for plant growth. Nevertheless, in our study, the mixture of peat moss with vermiculite (2:1) resulted in an increased percentage of plant survival and plant height of Ixora so it could be a recommended medium for the acclimatization period.

Table 4. Effect of different soil media on acclimatization of *Ixora coccinea* plant after 8 weeks of planting

Type of media / Vegetative parameters	Media A	Media B	Media C
	Mean± SE		
Number of leaves	6.0±0.40^{b}	8.0±0.50^{a}	4.0 ± 0.26^{b}
Number of shoots/plants	0.0^{b}	1.0±0.30^{a}	0.0^{b}
Length of a plant (cm)	5.1±0.16^{b}	7.5±0.29^{a}	4.1± 0.59^{b}

**(A) peat moss+ vermiculite (1:1v/v); (B) peat moss + vermiculite (2:1v/v); (C) peat moss +vermiculite (1:2v/v); *Means with the same letters in the same row are not significantly different at 5%using Duncan Multiple Range Test. SE=Standard Error*

To Assessment of genetic fidelity DNA samples extracted from acclimated plantlets that derived from the 6th subculture were analyzed using Nano Drop Spectrophotometer at 260nm/280 nm, the reading on all DNA samples is shown in Table (5). The high purity ratio of DNA samples was within 1.80 - 1.88 and the high concentration of DNA ranged from 997.9 to 1200 ng/µl in plants 1 and 5 respectively.
The extracted *I. coccinea* genomic DNA showed good purified extracted DNA obtained by running the DNA samples on 0.8% agarose gel.

Table 5. Purity of extracted DNA analyzed using Nano Drop SpectrophotometerA260nm/A280nm.

Sample	Purity Ratio(A260/A280)	DNA Concentration (ng/µl)
Mother plant	1.85	1001.2
Plant 1	1.84	997.9
Plant 2	1.88	1100.5
Plant 3	1.83	998.1
Plant 4	1.80	1004.7
Plant 5	1.84	1200.1

The screening with selected four ISSR primers resulted in 78 scorable bands, the number of bands for each primer varied from 18 in primer 827,873 and 880 to 24in primer 855 with an average of 19.5 bands per ISSR primers (Table 6).

Table 6. Total number and amplified fragments size range and number of polymorphic fragments amplified by four ISSR primers in micropropagated shoots of *Ixora coccinea*

Primer	Primer Sequence	Tm	Total bands scored	No. of polymorphic band	No. of monomorphic bands	Similarity Index (SI)
UBC 827	5′-ACA CAC ACA CAC ACA CG-3′	53.0	18	0.00	18	1.00
UBC 855	5′-ACA CAC ACA CAC ACA CYT-3′	53.1	24	0.00	24	1.00
UBC 873	5′-GAC AGA CAG ACA CACA -3′	47.4	18	0.00	18	1.00
UBC 880	5′-GGA GAG GAG AGG AGA-3′	47.9	18	0.00	18	1.00
Total	4		78	0.00	78	
Average			*19.5*	*0.00*	*17*	

In this study, the estimation of the genetic similarity coefficient based on the ISSR band indicated that acclimatized plantlets regenerated by *in vitro* culture were 100% similar to the mother plant where the similarity index equaled 1.0. This result emphasized that the amplified products exhibited monomorphisms among all the *in vitro* plants that were similar to their mother plant which proved no polymorphisms or changes in the amplified DNAs within micro propagated plants. During *in vitro* culture, the propagation methods, genotype, nature of the tissue, type and concentration of growth regulators, and duration of subcultures are important factors that determine the frequency of variation (Pierik, 1997, Bairu *et al*.,2006) this result same to Smikal *et al*.,(2007) who found pea maintained over an elongated period (24 years) remained genetically secure and was similar to the original genotype and different from Rodrigues *et al.* (1998) who found 3.8% somaclonal variants in Nanicao appeared after 11 subcultures, that emphasize the extended time in culture increase the number of somaclonal variants as that noticed in wheat regenerate and banana (Hartmann *et al*., 1989, Bairu *et al*.2006)

CONCLUSION

The conclusion that could be drawn from this study is that full MS media supplemented with 30 g/l sugar was the best media for Ixora micropropagation and MS media was far better than WPM because it is much richer in macro and micronutrients than WPM. The estimation of the genetic similarity coefficient based on the ISSR band indicated that acclimatized plantlets derived from the 6th subculture were 100% similar to the mother plant.

REFERENCES

1. Amin M.N., Ahmed S., Alam M.R and Azad M.A. (2002). *In vitro* rapid clonal propagation of an ornamental plant *Ixora fulgens*. Journal of biological sciences, vol. 2:485-488.
2. Bairu M.W., Fennell C.W. and Staden J. (2006). The effect of plant growth regulators on somaclonal variation in *Cavendish Banana* (Musa AAA Cv.'Zelig'). Scientia Horticulturae, vol. 108: 347-351.
3. Doyle J.J. and Doyle J.L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull.vol. 19: 11-15.

4. El Amin H. M. (1990). Ixora- in Trees and shrubs of Sudan. Food and Agricultural Organization (FAO) press. 491pp.
5. Gamborg O. L, Murashige T. Thorpe T.A. and Vasil I.K. (1976). Plant tissue culture media. *In vitro*, vol.12:473-78.
6. George E.F. (1993). Plant propagation by tissue culture. Part1-The technology. Edington: Exegetics limited, pp. 337-356.
7. Hartmann C., Henry Y., Buyser J., Aubry C. and Rode A. (1989). Identification of New Mitochondrial Genome Organizations in Wheat Plants Regenerated from Somatic Tissue Cultures. Theoretical and Applied Genetics, vol.77: 169- 175.
8. Hartmann H.T., Kester D.E., Davies F.T. and Geneve R.L. (2007). Plant propagation: principles and practices. 6thEdition. Chapter 18. Techniques of *in vitro* culture for micropropagation and biotechnology. Prentice-Hall, Upper Saddle River. p. 590-624.
9. Idowu T.O, Ogundaini A.O., Salau A.O., Obuotor E.M., Bezabih M. and Abegaz B.M. (2010). Doubly linked, a-type proanthocyanidin trimer and other constituents of *Ixora coccinea* leaves and their antioxidant and antibacterial properties. Phytochemistry; 71:2092-2098
10. Jaccard P. (1908). New researches on the floral distribution. Bull Soc Vaudoise Sci Natl 44: 223–270
11. Karhu S. T. (1997). Auxalary shoot proliferation of blue Honeysuckle. Plant cell, Tissue and organ culture.vol.48:195-201.
12. Kim H.S. (2011). Physical properties of the horticultural substrate according to mixing ratio of peat moss, perlite and vermiculite. Korean Journal of Soil Science and Fertilizer 44(3) 321-330.
13. Lakshmanan P., Lee C.L. and Goh C.J. (1997). An efficient *in vitro* method for mass propagation of a woody ornamental *Ixora coccinea*. Plant Cell Rep.vol.16: 572-577.
14. Li T., Cai H., Wang T., Fu Y., Yang W., Zhao A., Cui Z. and Wang, J. (2019). Plant regeneration in *Ixora chinensis* from young leaves. Plant Cell, Tissue and Organ Culture, 139(3), 605-608.
15. Lloyd G., and McCown B. (1980). Commercially Feasible Micropropagation of *Mountain laurel ,Kalmia latifolia* by Use of Shoot Tip Culture. The International Plant Propagators society vol 30,421-427
16. Murashige T. and F. Skoog. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant, vol.15: 473–97.
17. NISIR, National Institute of Science Communication and Information Resources. (2002). The wealth of India, dictionary of Indian raw materials and industrial products—raw materials. New Delhi.
18. Onsa R.A.H., Abdellatif I.A., Osman, M.G. and Abdullah T.L. (2018). Comparison between uses of growth regulators and natural adjuvant on *in vitro* micropropagation of *Ixora coccinea* L. International journal of science, environment and technology .7(5),1794-1801.
19. Onsa R.A.H., Abdellatif I.A., Osman M.G. and Abdullah T.L. (2018). Effect of Growth Regulators in in vitro micropropagation of *Ixora coccinea* L. International Journal of Scientific and Research Publications. 8(11), 144-149
20. Pierik R.LM. (1997). *In vitro* Cultures of higher plants. Dordrecht, the Netherlands: Kluwer academic Publishers, p 21-125.
21. Raina S.N, Rani V, Kojima T, Ogihara Y, Singh K.P. and Devarumath R.M. (2001) RAPD and ISSR fingerprints as useful genetic markers foranalysis of genetic diversity, varietals identification, and phylogeneticrelationships in peanut (*Arachis hypogaea*) cultivars and wild species. Genome 44:763–772
22. Rodrigues P., Tulmann A., Cassieri P. and Mendes B. (1998). Influence of the Number of Subcultures on Somaclonal Variation in Micropropagated Nanicao (*Musa Spp*.) Acta Horticulturae. vol.490: 469-473.
23. Rajaseger G. H. T. W., Tan H. T. W., Turner I. M. and Kumar, P. P. (1997). Analysis of Genetic Diversity among *Ixora* Cultivars (Rubiaceae) using Random Amplified Polymorphic DNA. Annals of Botany, 80(3), 355
24. Ska S.S., Habib A.M., El shamy M.A. and Soliman H.B. (2010). Micropropagation of *Ixora andulata* by tissue culture. Journal of horticulture science and ornamental plant, vol.2:111-117.
25. Smikal P., Valledor L., Rodriguez R. and Griga M. (2007). Assessment of genetic and epigenetic stability in long-term *in vitro* shoot culture of Pea (*Pisum Sativum* L.). Plant Cell Reports. 26: 1985-1998.
26. Thakur P.C. and Kumar H. (2014). *In vitro* morphogenic response of *Ixora parviflora.*
IJ EK. vol. 1 :4-7.
27. WCSP (2017). World Checklist of Selected Plant Families. facilitated by the royal botanical garden, kew http: //wcsp.scince.kew.org/Retrieved.
28. Williams E. G. and Maheswaran G. (1986). Somatic embryogenesis:Factors influencing coordinated behavior of cells as an embryogenic group. Annals of Botany vol.57(4): 443-462.
29. Zeitkiewicz E, Rafalski A, and Labuda D (1994) Genome finger printing by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomic 20:176–183