Effect of Light and Darkness on Callus Induction and Plant Regeneration of Roselle Plant Leaves (*Hibiscus sabdariffa* L.) Cultured on Right and Reverse Orientation

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Abstract

This study aimed to obtain seedlings of the Roselle plant (*Hibiscus sabdariffa* L.), take leaf discs from these seedlings, and induce callus, and also test the possibility of regenerating the plant from the callus induction, root formation and finally acclimating the plants resulting from regeneration to different media (peatmoss, sand, and peatmoss + sand 1:1). A high percentage of germination rate and callus induction (100%) were obtained. The length of the hypocotyl was (10.8 cm), the number of roots (12 roots/ explant), and the length of the roots (10.0 cm) at a concentration of (1.5 $mg.l^{-1}$) BA, in addition to obtaining high percentages of fresh weight and dry weight of callus (36.537 g and 10.100 g), respectively, when the leaves discs were cultured right and in the dark for a period of time (15 days) in the culture medium supplemented with (1.5 $mg.l^{-1}$) of benzyl adenine, (4 $mg.l^{-1}$) of 2,4-D, and (0.3 $mg.l^{-1}$) of NAA, with the addition of (0.5 $mg.l^{-1}$) of gibberellin acid. The resulting callus was yellowy-white in color and its consistency was compact and had a very intensive as compared with the other concentrations. For plant regeneration test, the concentrations of BA (2 $mg.l^{-1}$) and NAA (0.2 $mg.l^{-1}$) gave the highest mean length of shoots (8.9 cm), number shoots (5 shoots/explant), number of leaves (13 leaves/ explant) length of roots (15 cm) and number of roots (17 roots/ explant) as compared with other concentrations. At the end it has been shown that the acclimatization process on the different cultural media, petmoss gave the best results compared to other media, and the success rate was 100%.

Keywords: Tissue culture, growth regulators, Callus, Regeneration, (*Hibiscus sabdariffa* L.).

Introduction

Hibiscus sabdariffa L. (Malvaceae), commonly known as Roselle (English) and Oseille de Guinée (French) is an erect annual herb and cultivated for its seeds, petals and leaves. It is used for preparation of local non-alcoholic cold beverage and as an infusion. Roselle is known for its delicacy and medicinal properties. It is a plant which is widely grown in Central and West Africa and South East Asia. This plant is utilized by people in Africa and particularly in Côte d'Ivoire in direct or indirect ways in the treatment of several diseases. The red color of Roselle petals, essentially the anthocyanins, is an attractive source of natural food colorants (1).

The Roselle plant is propagated by sexual propagation using of seeds is the preferred method, therefore they are the only traditional ways to propagate Roselle plant nowadays, micropropagation is considered the most important technique for propagation of most

plants, and thus micropropagation may be a better success method to overcome all problems (2). Generally, micropropagation by plant tissue culture offers promising possibilities. Moreover, in recent years, the application of plant tissue culture as a micropropagation technique has become an important biotechnological tool in the multiplication of various plants that have a great economic importance (3). Likewise, micropropagation techniques offer additional advantages such as the rapid propagation rate, lack of seasonal restrictions, provision of disease free plants, maintenance of self-incompatible inbred lines**.** Different pathways of regeneration can be adopted in micropropagation. These include direct methods such as axillary bud proliferation and direct organogenesis and indirect techniques involving an intermediate callus phase. Direct methods of plant regeneration usually ensure genetic stability whereas when plant tissues are cultured via callus phase, the plants that are regenerated may exhibit variation (4). Commonly callus is induced either in absence or presence of light. So far as we know, till now there are not enough reports on the effect of white light and dark to callus induction and plant regeneration in respect of physiological and morphological development in Roselle plant.

Usually, plant growth regulators must be supplied in the medium for the growth and development of the explants. Plant growth regulators exert dramatic effects at low concentrations. They regulate the initiation and development of shoots and roots on explants and embryos on semisolid or in liquid medium cultures. They stimulate cell division and expansion. The most important classes of the plant growth regulators used in tissue culture are the auxins and cytokinins. The relative effects of auxin and cytokinin ratio on morphogenesis of cultured tissues were demonstrated by Skoog and Miller (1957) and still serve as the basis for plant tissue culture manipulations today.

The purpose of this research was therefore to establish optimal conditions for *in vitro* seed germination and seedling development based on the responses to various seed cultures and growth media compositions. This is also required to provide seedling material that can serve as an aseptic explant source for callus induction, plant regeneration, root formation and acclimatization for all year round *ex vitro* establishment of plants. Also the present study was undertaken to observe the effect of light and dark on callus induction, plant regeneration, related morphology of calli and its growth and development.

Materials and Methods

The study was carried out during growing season 2023 in lab plant tissue culture of Horticulture Department, College of Agricultural Engineering Sciences, University of Duhok, Kurdistan Region /Iraq. The seeds of the Roselle plant were obtained from Egypt country. The seeds choose were uniform in shape and size. The study include:

Seed Sterilization:

The explants were surface disinfestation by immersion in 1.5, 2.0 and 2.5% sodium hypochlorite (NaOCl) solution for fifteen minutes with addition 1-2 drops of Tween 20 and then were be washed three times with sterilized distilled water. Another sterilant material was used for explants disinfestation represented by mercuric chloride ($HgCl_2$) at 0.1% w/v for 15 minutes. After sterilization, the explants were rinsed in sterilized distilled water three times for five minutes each. All steps of sterilization were carried out under aseptic conditions (laminar Ari flow hood).

Seed Germination: According to the results of sterilization, the best treatment chosen ensured that the explants were free of contaminations for

obtaining growth. The seeds were cultured on MS media (Murashige and Skoog, 1962). As known, MS medium contains all mineral nutrients necessary for plant growth and development including both macro- and micronutrients.This medium was purchased from Caisson Company, USA as a pre-mixed medium. After adding 30 gl^{-1} sucrose as an energy source and the pH was adjusted to 5.7± 0.1 with 1N NaOH or HCl and 7 gl^{-1} agar as a gelling was added. The media were supplied with different concentrations of BA and Kinetin were tested (0.0, 1.5 and 2) mgl^{-1}, (0 and 0.5 mg^{-1}) IBA and 0.3 mg^{-1} GA_3.

The seeds were cultured in vessel and ten replications were used for each treatment under conditions of (16) light hours and (8) darkness hours. After four weeks of incubation, germination rate %, mean length of hypocotyl, number of roots per seed, and length of roots were recorded.

Callus induction:

At callus induction, leaves discs (1*1 cm) of Roselle plants were excised from seedlings grown *in vitro* were used as explants source and culture on right orientation and reverse orientation in MS medium enriched with different combinations between BA (0.5,1 and 1.5 $mg.l^{-1}$), 2,4-D (2, 3 and 4 $mg.l^{-1}$), NAA (0.3 $mg.l^{-1}$), and GA_3 (0.5 $mg.l^{-1}$). After adding 30 gl^{-1} sucrose as an energy source and the pH was adjusted to 5.7± 0.1 with 1N NaOH or HCl and 7 gl^{-1} agar as a gelling was added.

Cultures were divided into groups, the first were put cultures under (16) hours light and (8) hours dark, photoperiod (light provided by white fluorescent tubes), the second were incubated the cultures in complete darkness for (15) days and covered with aluminum foil (continuous dark), and they were shifted to 16 hours light and 8 hours darkness daily to (1000 lux) illumination.

The leaves discs were cultured in vessels with 10 replications. A record of the response percentage% of callus induction were made after six weeks of incubation in addition to the fresh weight and dry weight of callus, color of callus, consistency of callus, and density of callus.

Shoot Regeneration:

At plant regeneration on callus initiated, the callus which were produced from leave explants were cultured on MS medium. After adding 30 gl^{-1} sucrose as an energy source and the pH was adjusted to 5.7± 0.1 and 7 gl^{-1} agar was added. And supplemented with different concentrations of BA (2, 4, and 6 mg^{-1}) and NAA (0.2 mg^{-1}). Also were cultured in MS medium enriched with different combinations between Kin (2, 4, and 6 mg^{-1}) and NAA (0.2 mg^{-1}) for shoot development. All treatments were established in a vessels containing 25ml solidified MS medium and 10 replications were will be used for each treatment. After six weeks the mean length of shoots, number of shoots per explant, number of leaves per explant, length of root, and number of roots per explant were recorded. The cultures will be maintained at 25± 1 ºC, and 50-60 % relative humidity in a culture room under a (16) hours photoperiod provided by white fluorescents tubes and 8 hours darkness. The photoperiod was maintained by an automatic timer system.

Root Formation:

At root formation stage, were transferred the shoots produced from plant regeneration to a rooting media MS medium. After adding 30 gl^{-1} sucrose as an energy source and the pH was adjusted to 5.7± 0.1 and 7 gl^{-1} agar was added. And supplemented with two kinds of auxins were tested including IBA and NAA at concentrations (0.0, 0.2, 0.4 and 0.6) $mg.l^{-1}$. After six weeks in culture, the number of roots

per explant, mean length of roots and rooting percentage were recorded as rooting parameters.

Acclimatization:

For acclimatization stage, the well rooted plantlets were carefully removed from the culture vessels and were washed in tap water to remove agar from the roots which might be a source of contamination. It is important to avoid cutting any part of the roots during washing. The plantlets are then put in Benlate fungicide solution (0.1%) for (1-2) minutes before being planted in plastic pots, to avoid fungus infections and then planted in plastic pots filled with a steam-sterilized soil mix for *in vitro* shoot culture in different substrate. (peatmoss 100%, sand 100%, peatmoss + sand 1:1) v:v In order to maintain high humidity in culture environment, the pots were covered with light beakers which permits light passing. The potted plants were placed in incubation room for (14) days. The plantlets were irrigated with quarter salt strength of MS salt according to their need, and gradually the beakers were opened from time to time and after four weeks the beakers were removed then the plantlets were transplanted and grown under a regular greenhouse. After six weeks in culture, the survival rate (%), mean length of shoots (cm), number of shoots/ explant, number of leaves/ explant, mean length of roots (cm), number of roots/ explant, chlorophyll content (SPAD).

Statistical Analysis:

The whole experiments were arranged according to Completely Randomized Design (CRD) and the comparison among means was done according to Duncan multiple range test at 0.05% and the whole data were statistically analyzed by SAS computerized program (5).

Results and Discussion

Generally, the results of this experiment were very successful in improving a reliable and effective micropropagation protocol for Roselle plant. After testing several disinfestation treatments including sodium hypochlorite (NaOCl) with different concentrations and durations, but unfortunately, one hundred percent of contamination was occurred. That's why, mercuric chloride was finally used at 0.1% for 15 minutes which gave ideal results with producing healthy and aseptic cultures from the inoculation of seeds on MS medium (Fig. 1).

At initiation stage, Table (1) and Table (2) shows the effect of BA and Kinetin concentrations and their interactions with IBA levels on seed germination of Roselle plant. It can be noticed that germination rate were 100% in all treatments. The tables indicates clearly that when BA and Kinetin were added to the medium, the highest length of hypocotyls (10.80 cm) and (10.5 cm) respectively, the highest number of root per seeding (12.0) and (11.0) respectively, and the longest roots (10.5 cm) and (10.3 cm) respectively were recorded in MS medium Supplemented with 1.5mg.l^{-1} BA and kinetin (Fig.1).

Table 1. Effect of BA and IBA on initiation seed germination of Roselle (*Hibiscus sabdariffa* L.) grown on MS medium enriched with 0.3 mg.l^{-1} GA_3 after four weeks.

BA+ IBA (mg.l^{-1})	Germination Rate (%)	Mean length of hypocotyl (cm)	Number of roots/ seed	Length of root (cm)
0 + 0	100 a	8.9 b	11.0 b	9.9 a
0 + 0.5	100 a	7.6 c	8.0 c	8.0 b

1.5 + 0	100 a	10.8 a	12.0 a	10.5 a
1.5 + 0.5	100 a	9.0 b	8.0 c	8.8 b
2 + 0	100 a	7.1 c	8.0 c	8.0 b
2 + 0.5	100 a	6.0 d	7.0 c	7.0 c

Table 2. Effect of Kinetin and IBA on initiation stage of seed germination of Roselle (*Hibiscus sabdariffa* L.) plantlets grown on MS medium enriched with 0.3 mg.l^{-1} GA_3 after four weeks in culture.

Kinetin+ IBA (mg.l^{-1})	Germination Rate (%)	Mean length of hypocotyl (cm)	Number of roots/ seed	Length of root (cm)
0 + 0	100 a	8.6 b	10.0 b	8.9 b
0 + 0.5	100 a	7.5 c	7.0 c	7.2 c
1.5 + 0	100 a	10.5 a	11.0 a	10.3 a
1.5 + 0.5	100 a	8.8 b	7.0 c	7.4 c
2 + 0	100 a	6.4 d	6.0 d	7.0 c
2 + 0.5	100 a	8.1 b	7.0 c	7.1 c

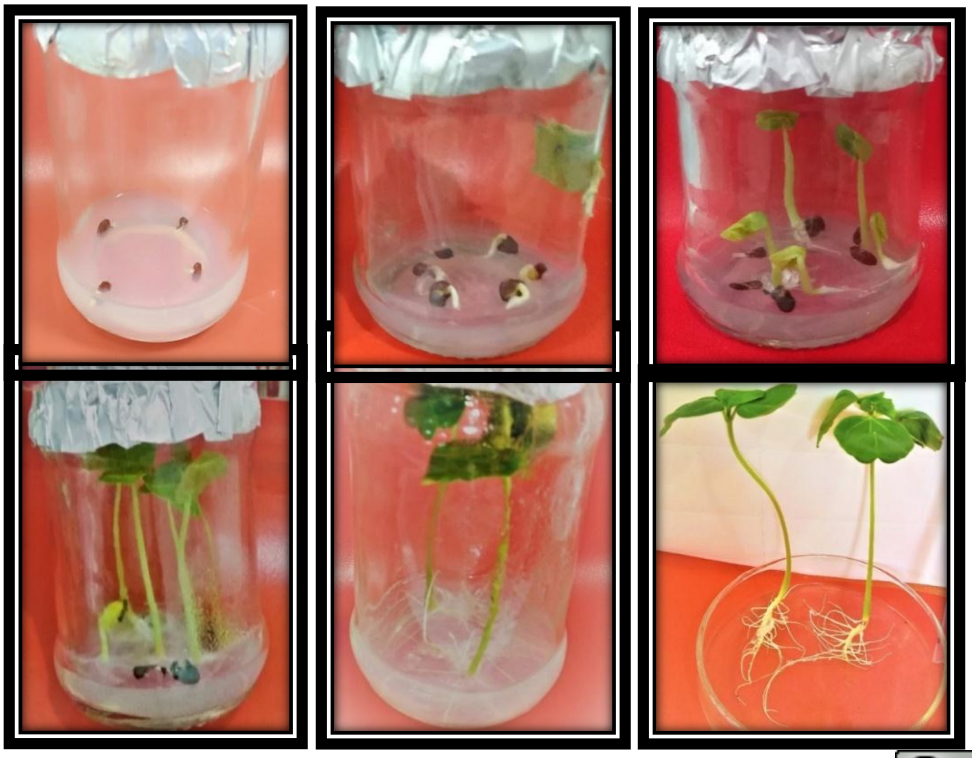

Figure 1. Steps of germination of Roselle (*Hibiscus sabdariffa* L.) plantlets produced from *in vitro* seed culture.

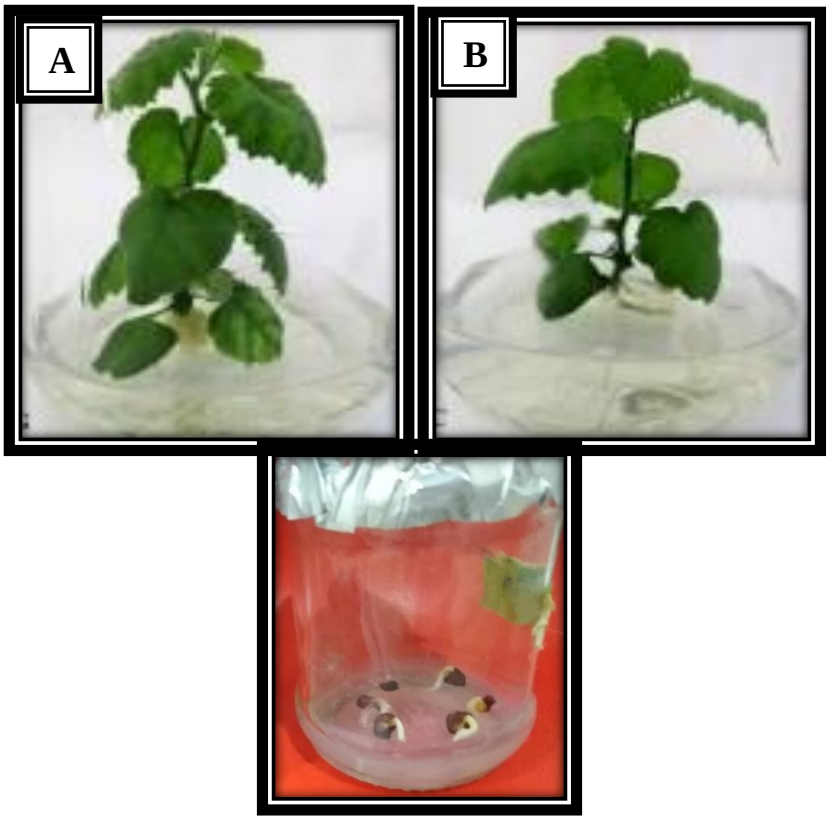

Figure 2. Initiation stage of seed germination of Roselle (*Hibiscus sabdariffa* L.) plantlets cultures by adding BA and Kinetin with IBA after four weeks in culture on MS medium.

A. (1.5 mg.l^{-1} BA + 0.0 mg.l^{-1} IBA + 0.3 mg.l^{-1} GA_3)

B. (1.5 mg.l^{-1} Kin + 0.0 mg.l^{-1} IBA + 0.3 mg.l^{-1} GA_3)

The initiation stage was a very important stage in plant propagation through *in vitro* culture techniques in which a large number of sterilization seedlings was produced when seeds were cultured in nutrient media supplemented with different PGRs.

The ratio of auxins and cytokinins is important with respect to morphogenesis in the culture system. Equally important is the concentration of these two groups of growth regulators (6). However, the *in vivo* seed cultures required constant care and maintenance of adequate moisture to ensure good germination performance. On the other hand, germination of seeds in the closed environment of *in vitro*

cultures can provide constant moisture levels, resulting in fast germination and more uniform seedlings (7). Growth and morphogenesis *in vitro* were regulated by the interaction and balance between the growth regulators supplied in the medium, and the growth substances produced endogenously (8).

Seedlings derived from *in vitro* germinated seeds can be used as an aseptic explant source for *in vitro* multiplication of valuable plants as

reported for *H. cannabinus* (9), *H. sabdariffa* (10). An overall conclusion from these results can be made that BA has significantly stronger activity than Kinetin and this explains why explants cultured on media supplemented with BA produced higher number of shoots as compared with those cultured in media supplemented with Kinetin, since BA is composed of three double bonds in its side change, whereas Kinetin is composed of two double bonds (11). Kinetin is exclusively activates the flavonoid synthesis gene, benzyl adenine affected more significantly the synthesis of proteins, and enhance photosynthesis and plant tolerance-related genes (12).

At callus induction, Table (3) displays callus initiation on Leaf- discs of Roselle plantlets taken from *in vitro* grown plantlets after six weeks cultured on right orientation in MS medium enriched with different combinations between BA (0.5, 1 and 1.5 $mg.l^{-1}$), 2, 4-D (2, 3 and 4 $mg.l^{-1}$) NAA (0.3 $mg.l^{-1}$) and GA_3 (0.5 $mg.l^{-1}$). Results clearly that the high concentrations of BA (1.5 $mg.l^{-1}$), 2, 4-D (4 $mg.l^{-1}$), NAA (0.3 $mg.l^{-1}$) and GA_3 (0.5 $mg.l^{-1}$) and also dark periods efficient effect on Callus induction. The optimum Production of callus occurred in Continuous dark for 15 days. This treatment gave the highest response percentage (100%), fresh weight of callus (36.537g) and dry weight of callus (10.100g) also this table shows the color of callus was yellowy white, consistency of callus is compact and density of callus is very intensive as compared with the other treatments. (Fig 3)

Table 3. Callus initiation on leaf-discs of Roselle (*Hibiscus sabdariffa* L.) plantlets taken from *in vitro* grown plantlets after six weeks cultured on right orientation in MS medium enriched with different combinations between BA, 2,4-D, NAA and GA_3 .

Explant culture orientation	Light Condition	Treatment BA + 2,4-D + NAA+ GA_3 ($mg.l^{-1}$)	Response percentage (%)	Fresh Weight of Callus (g)	Dry weight of callus (g)	Color of callus	Consistency of callus	Density of callus
	Light	0.5+2+0.3+0.5	85	16.993 e	5.257 e	Yellow	Friable	++
		0.5+3+0.3+0.5	100	27.792 c	7.104 c	Whitley yellow	Friable	+++
		0.5+4+0.3+0.5	0	0 g	0 g	0	0	0
		1+2+0.3+0.5	60	8.256 f	3.101 f	Creamy	Nodular	+
		1+3+0.3+0.5	0	0 g	0 g	0	0	0
		1+4+0.3+0.5	100	20.861 d	7.541 c	Whitley yellow	Nodular	+++
		1.5+2+0.3+0.5	85	18.673 d	6.503 d	Yellowy white	Compact	++

Right Orient		1.5+3+0.3+0.5	85	14.567 e	4.115 f	Whitley yellow	Compact	++
		1.5+4+0.3+0.5	100	26.537 c	9.05 a	Whitley Green	Compact	+++
	Dark 15 day	0.5+2+0.3+0.5	85	18.953 d	8.256 b	White	Friable	++
		0.5+3+0.3+0.5	100	32.899 b	9.909 a	White	Friable	+++
		0.5+4+0.3+0.5	0	0 g	0 g	0	0	0
		1+2+0.3+0.5	60	9.555 f	4.010 f	Whitley yellow	Friable	+
		1+3+0.3+0.5	0	0 g	0 g	0	0	0
		1+4+0.3+0.5	85	17.777 e	6.707 d	Creamy	Friable	++
		1.5+2+0.3+0.5	100	20.789 d	7.703 c	Yellow	Nodular	+++
		1.5+3+0.3+0.5	85	17.111 e	6.250 d	Yellowy white	Compact	++
		1.5+4+0.3+0.5	100	36.537 a	10.100 a	Yellowy white	Compact	+++

*** (+ "low intensity", ++"Moderately intensity", +++ "high intensity" and - for "0") to describe the density status of the callus.**

Right orient

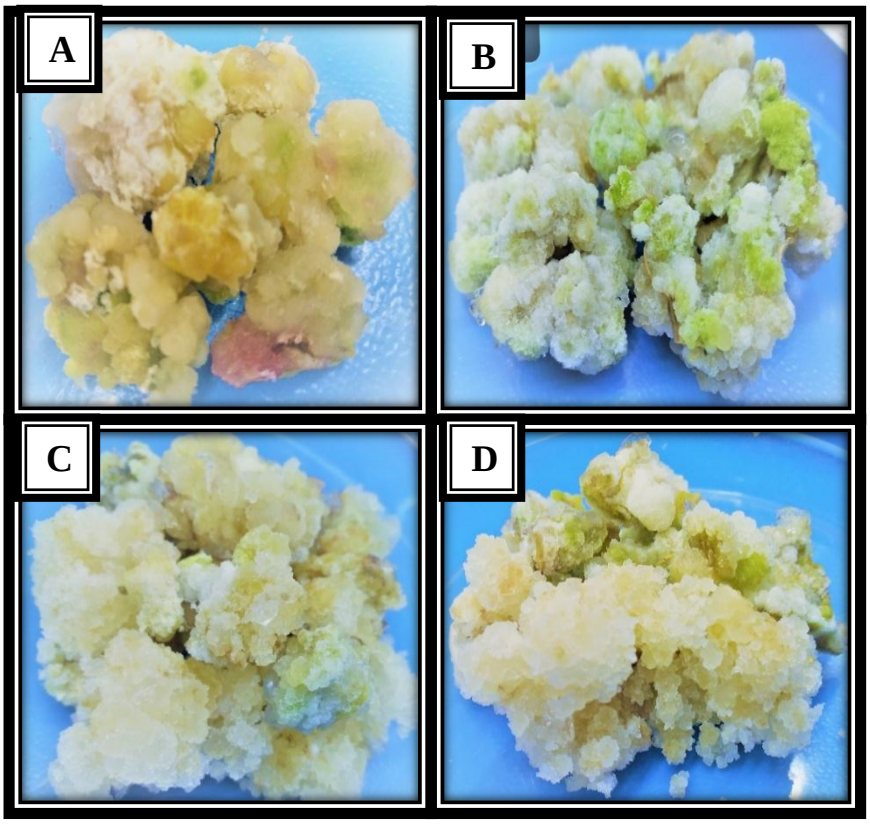

Figure 3. Callus initiation in light and darkness on leaf-discs of Roselle (*Hibiscus sabdariffa* L.) plantlets taken from *in vitro* grown leaves discs after six weeks in MS medium enriched with different combinations between BA, 2,4-D , NAA and GA_3.

A-B light C-D dark

A. (0.5 mg.l^{-1} BA + 3 mg.l^{-1} 2, 4-D + 0.3 mg.l^{-1} NAA + 0.5 mg.l^{-1} GA_3)

B. (1.5 mg.l^{-1} BA + 4 mg.l^{-1} 2, 4-D + 0.3 mg.l^{-1} NAA + 0.5 mg.l^{-1} GA_3)

C. (1.5 mg.l^{-1} BA + 4 mg.l^{-1} 2, 4-D + 0.3 mg.l^{-1} NAA + 0.5 mg.l^{-1} GA_3)

D. (0.5 mg.l^{-1} BA + 3 mg.l^{-1} 2, 4-D + 0.3 mg.l^{-1} NAA + 0.5 mg.l^{-1} GA_3)

On the other hand, Table (4) illustrate callus initiation on leaf-discs of Roselle plantlets taken from *in vitro* grown plantlets after six weeks cultured on reverse orientation in MS medium enriched with different combinations between BA (0.5, 1 and 1.5 mg.l^{-1}), 2,4 -D (2, 3 and 4 mg.l^{-1}), NAA (0.3 mg.l^{-1}) and GA_3 (0.5 mg.l^{-1}), It was clear in this table the high response percentage of callus (100%) were obtained at concentration (1.5 mg.l^{-1}) BA, (4 mg.l^{-1}) 2, 4 -D, (0.3 mg.l^{-1}) NAA and (0.5 mg.l^{-1}) GA_3 in the dark for 15 days, it can be noticed also at the same concentration gave the highest fresh weight and dry weight of callus (30.542g and 9.215g) respectively. The color of callus was creamy, consistency of callus is compact and density of callus was very intensive as compared with the other treatment. (Fig.4).

Table 4. Callus initiation on leaf-discs of Roselle (*Hibiscus sabdariffa* L.) plantlets taken from *in vitro* grown plantlets after six weeks cultured on reverse orientation in MS medium enriched with different combinations between BA, 2,4-D, NAA and GA_3.

Explant culture orientation	Light Condition	Treatment BA + 2,4-D + NAA+ GA_3 (mg.l^{-1})	Response percentage (%)	Fresh Weight of Callus (g)	Dry weight of callus (g)	Color of callus	Consistency of callus	Density of callus
	Light	0.5+2+0.3+0.5	85	16.910 cd	5.244 e	Yellow	Nodular	++
		0.5+3+0.3+0.5	100	20.129 b	5.501 e	Yellow	Nodular	+++
		0.5+4+0.3+0.5	0	0 f	0 g	0	0	0
		1+2+0.3+0.5	60	6.440 e	2.201 f	Yellowy white	Compact	+
		1+3+0.3+0.5	0	0 f	0 g	0	0	0
		1+4+0.3+0.5	85	19.081 b	5.431 e	Yellow	Compact	++
		1.5+2+0.3+0.5	85	16.673 c	4.401 f	Creamy	Friable	++

		1.5+3+0.3+0.5	85	15.571 cd	5.102 e	Creamy	Friable	++
		1.5+4+0.3+0.5	100	20.610 b	5.722 e	Yellowy white	Friable	+++
Reverse Orient	Dark 15 day	0.5+2+0.3+0.5	85	15.986 cd	8.010 b	Yellow	Compact	++
		0.5+3+0.3+0.5	100	22.933 b	8.015 b	Yellow	Compact	+++
		0.5+4+0.3+0.5	0	0 f	0 g	0	0	0
		1+2+0.3+0.5	60	8.444 e	3.010 ef	White	Nodular	+
		1+3+0.3+0.5	0	0 f	0 g	0	0	0
		1+4+0.3+0.5	100	20.542 b	7.012 c	Creamy	Friable	+++
		1.5+2+0.3+0.5	85	17.505 c	6.111 d	Yellowy white	Friable	++
		1.5+3+0.3+0.5	85	13.051 d	4.220 f	White	Nodular	++
		1.5+4+0.3+0.5	100	30.542 a	9.215 a	Creamy	Compact	+++

*** (+ "low intensity", ++"Moderately intensity", +++ "high intensity" and - for "0") to describe the density status of the callus.**

Reverse orient

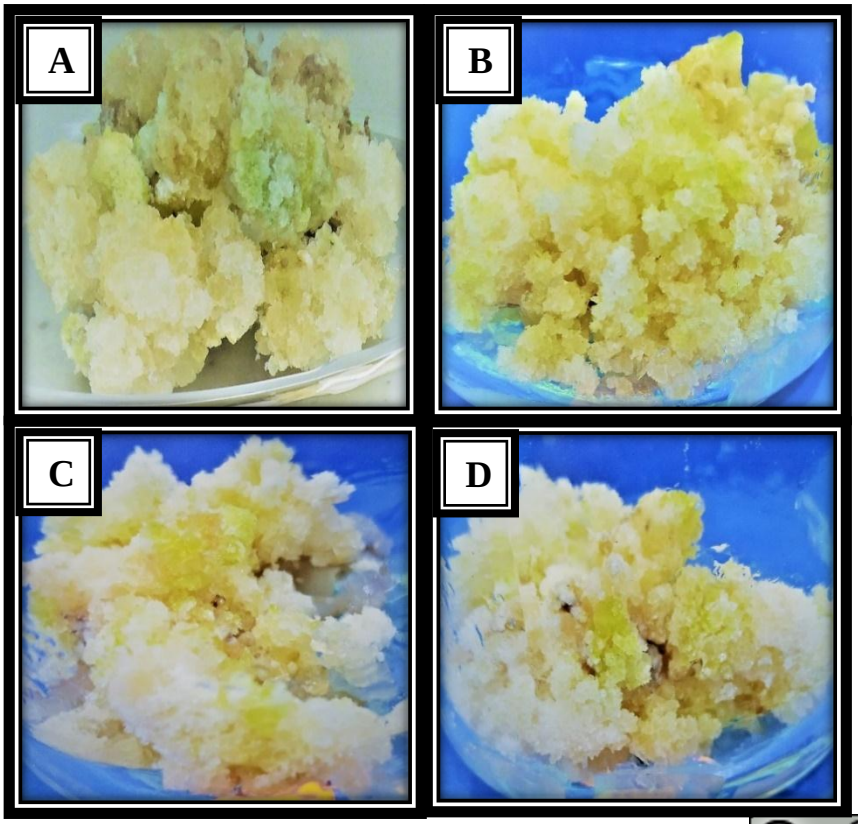

Figure 4. Callus initiation in light and darkness on leaf-discs of Roselle (*Hibiscus sabdariffa* L.) plantlets taken from *in vitro* grown leaves discs after six weeks in MS medium enriched with different combinations between BA, 2,4-D , NAA and GA_3.

A-B light C-D dark

A. (1.5 mg.l^{-1} BA + 4 mg.l^{-1} 2, 4-D + 0.3 mg.l^{-1} NAA + 0.5 mg.l^{-1} GA_3)

B. (0.5 mg.l^{-1} BA + 3 mg.l^{-1} 2, 4-D + 0.3 mg.l^{-1} NAA + 0.5 mg.l^{-1} GA_3)

C. (1.5 mg.l^{-1} BA + 4 mg.l^{-1} 2, 4-D + 0.3 mg.l^{-1} NAA + 0.5 mg.l^{-1} GA_3)

D. (0.5 mg.l^{-1} BA + 3 mg.l^{-1} 2, 4-D + 0.3 mg.l^{-1} NAA + 0.5 mg.l^{-1} GA_3)

Generally, it was clear from the above results that the use of cytokinins and auxins were often used in tissue culture to stimulate growth and development; they usually promote cell division especially if they added together. The auxins promote, mainly in combination with cytokinins, the growth of calli, cell suspensions and organs, and also regulate the direction of morphogenesis (13). The amount of callus produced in the dark conditions was more than the amount of callus produced in light, according to (14) the peroxidase (POD) activity in tea callus cultured in the dark was 1.6 fold higher than that in callus cultured with a 16 h photoperiod. Thus culture in the dark efficiently increased peroxidase (POD) activity of callus conventionally sub cultured and growing in the light. The leaves which were incubated for one and three weeks in the dark conditions produced best amount of callus. This agreed with the results found by (15) who incubated leaflets of rose in the dark for one week for callus induction. (16) Found that a 100% response was obtained from callus induction on fig (*Ficus carica* L.) plant on MS medium enriched with 0.2 mgl^{-1} kinetin and 2.0 mgl^{-1} 2, 4-D. This confirm the fact of adding higher concentrations from auxins with relatively lower concentrations of cytokinins for callus initiation on any explants (17).

At plant regeneration on callus initiated, Table (5) shows the plant regeneration on callus initiated on leaf- discs of Roselle plantlets taken from in vitro grown plantlets after six weeks in MS medias enriched with different combinations between BA (2,4 and 6 mg.l^{-1}) and NAA (0.2 mg.l^{-1}) also between kinetin (2, 4 and 6 mg.l^{-1}) and NAA (0.2 mg.l^{-1}). The results reveals that the low concentrations of BA (2 mg.l^{-1}) and (0.2 mg.l^{-1}) NAA gave the highest mean length of sheets, number of shoots, number of leaves, length of roots and number of roots (8.9cm, 2.8 shoots/explant, 13.0 leaves/explant, 15.0 cm and 17.0 roots/explant) respectively.

Concerning the interaction between Kinetin and NAA it can be noticed the high concentrations of kinetin (6 mg.l^{-1}) and NAA (0.2 mg.l^{-1}) gave the highest mean length (5.4 cm), number of shoots (1.8 shoots/explant), number of leaves (6.5 leaves/explant), length of roots (9.0cm) and number of roots (13.0 roots/explant) as compared with the other treatment.(Fig.5)

Table 5. Plant regeneration on callus initiated on leaf-discs of Roselle (*Hibiscus sabdariffa* L.) plantlets taken from *in vitro* grown plantlets after six weeks in MS medium Enriched with different combinations between BA + NAA and Kin + NAA.

Treatment ($mg.l^{-1}$)	Mean length of shoots (cm)	Number of shoots/ explant	Number of leaves/ explant	Length of roots (cm)	Number of Roots/ explant
BA+NAA					
2 + 0.2	**8.9 a**	**5 a**	**13.0 a**	**15.0 a**	**17.0 a**
4 + 0.2	**7.3 b**	**4 b**	**11.0 b**	**12.0 b**	**15.0 b**
6 + 0.2	**4.1 d**	**3 c**	**8.0 c**	**9.0 c**	**11. cd**
Kin + NAA					
2 + 0.2	**4.8 d**	**2 c**	**6.0 c**	**8.0 c**	**12.0 c**
4 + 0.2	**3.9 d**	**1 d**	**3.0 d**	**7.0 c**	**9.0 e**
6 + 0.2	**5.4 c**	**3 c**	**7.0 c**	**9.0 c**	**13.0 c**

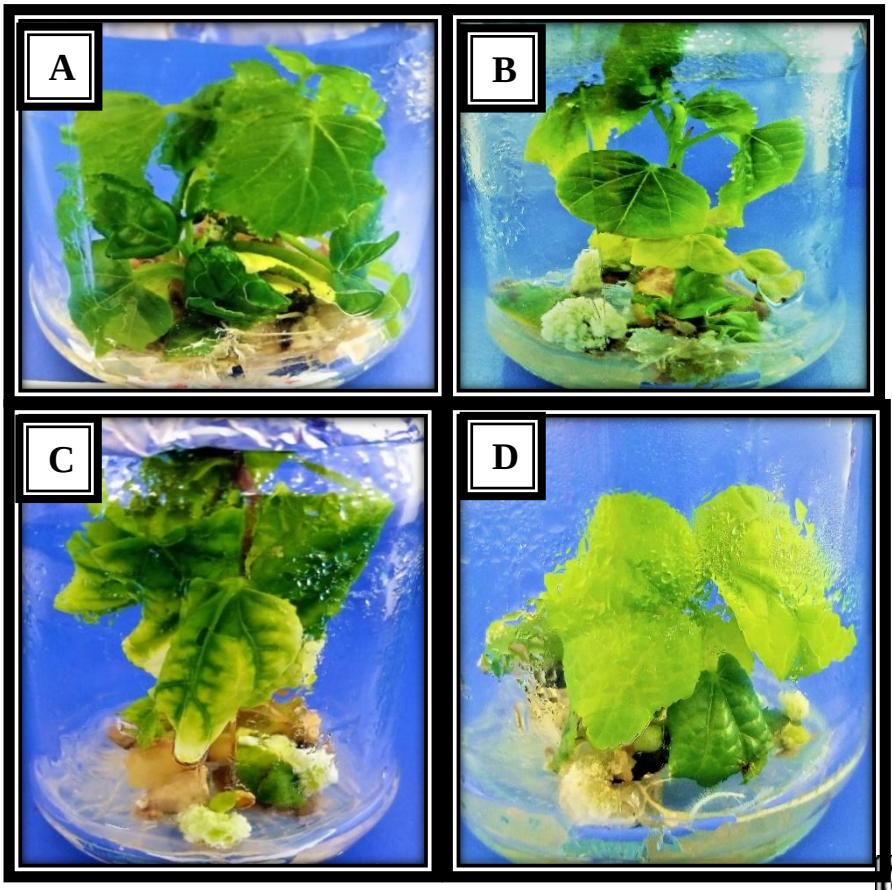

Figure 5. Plant regeneration on callus initiated on leaf-discs of Roselle (*Hibiscus sabdariffa* L.) plantlets taken from *in vitro* grown plantlets after six weeks in MS medium Enriched with different combinations between BA, NAA and GA_3.

A. (2 mg.l^{-1} BA + 0.2 mg.l^{-1} NAA)
B. (4 mg.l^{-1} BA + 0.2 mg.l^{-1} NAA)
C. (6 mg .l^{-1} KIN + 0.2 mg.l^{-1} NAA)
D. (4 mg.l^{-1} KIN + 0.2 mg.l^{-1} NAA)

The organogenesis stage is very important in plant propagation via tissue culture techniques by which a large number of shoots is achieved when different explants are cultured in nutrient medium supplemented with high concentrations of cytokinins. Cytokinins are often used to stimulate growth and development. Kinetin is the most common cytokinins being used. It usually promotes cell division if added together with auxin, in higher concentrations, it can induce adventitious shoot formation by promoting auxiliary bud growth via decreasing apical dominance and generation attraction region in buds and promoted fast transferred of nutrients, other growth substances, vitamins and mineral elements which leading to promote the initiation and buds growth (18).

In other studies, many researchers have found that cytokinins, especially BA, stimulates auxiliary bud development, but at high concentration, shoot elongation is suppressed (19). An overall conclusion from these results can be made that Kinetin was less effective than BA at the same concentrations. The reasons behind BA superiority might be due to its internal molecular structure and the numbers of double bonds on its side chain of benzyl ring, in which these bonds increased the activity of this plant growth regulator than other cytokinins (20). In addition, BA is the most effective cytokinin in cell division as compared to other cytokinins (21). At rooting stage, the results showed that plant tissue culture is an effective means to be used instead of the conventional propagation methods in Roselle. Table (6) shows the effects of tow auxins (IBA and NAA) concentrations on Roselle microshoots grown on MS medium. It is clear that the addition of 0.2 mg.l^{-1} NAA was significantly the best treatment by recording the best root formation parameters. Since, it produced 28.80 roots/ explant, 10.40 cm as longest roots and 100% of rooting (Fig. 6).

Table 6. Effect of IBA and NAA on root formation stage of Roselle (*Hibiscus sabdariffa* L.) plantlets grown on MS medium after six weeks in culture.

Auxins (mg.l^{-1})	Number of roots/ explant	Mean length of roots (cm)	Rooting Percentage (%)
Control 0.0	6.20 h	3.60 e	65 e
IBA 0.2	23.40 b	9.80 b	95 b
IBA 0.4	22.60 b	8.20 c	90 c
IBA 0.6	10.40 f	7.78 c	85 d
NAA 0.2	28.80 a	10.40 a	100 a
NAA 0.4	23.20 b	9.08 b	95 b

NAA 0.6	**12.20 e**	**8.08 c**	**90 c**

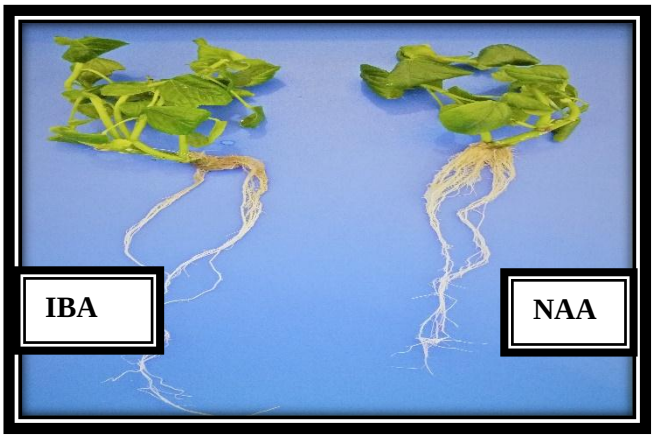

Figure 6. Effects of IBA and NAA on root formation stage of Roselle (*Hibiscus sabdariffa* L.) plantlets grown on full strength salts of MS medium enriched with 0.2 mg.l^{-1} IBA and NAA after six weeks in culture.

The current results assured auxins roles in enhancing rooting process by promoting adventitious roots initiation in the bases of cultured shoots (22). The differences in the For Acclimatization stage, It is clear from this tables (7) that the highest response percentage (100%) was obtained on culture media supplemented with peatmoss (100%), also highest mean length of shoots (67 cm) number of shoots (7 shoots/explant), number of leaves (45 leaves/explant), mean length of roots (18.6 cm), number of roots (69 roots/explant) and chlorophyll content (77.80 SPAD) were recorded at peatmoss cultures media (100%).(Fig. 7)

potency of IBA and NAA in promoting rooting might be due to the structure of the different auxins under testing, the endogenous hormone concentration, also the genetic makeup of species under consideration (23).

These results approve that peatmoss is always the best choice for acclimatizing plantlets produced through tissue culture technique. Peatmoss has many advantageous characteristics like high water absorbing capacity, preventing soil compaction, holding soil nutrients, free of bacteria, fungi and weed seeds, being perfect for acid-loving plants, and availability and cheapness (24).

Table 7. Acclimatization response of Roselle (*Hibiscus sabdariffa* L.) plantlets produced from regeneration plant grown in different culture media mixture.

Culture Media Mixture	Survival rate (%)	Mean length of shoots (cm)	Number of shoots/ explant	Number of leaves/ explant	Mean length of Roots (cm)	Number of Roots/ explant	Chlorophyll Content (SPAD)
Peatmoss 100%	**100 a**	**67 a**	**7 a**	**45 a**	**18.6 a**	**69 a**	**77.80 a**

Sand 100%	**95 b**	**50 c**	**3 c**	**29 c**	**15.5 c**	**58 b**	**61.42 c**
Peatmoss+ Sand 1:1	**100 a**	**64 b**	**5 b**	**37 b**	**17.3 b**	**63 a**	**67.80 b**

Figure 7. Acclimatized Roselle (*Hibiscus sabdariffa* L.) plantlets produced from *in vitro* regeneration plants

Conclusion

This study has shown that *H. sabdariffa* can be propagated by vegetative methods *in vitro*. The present findings indicate also the possibility of using micropropagation methods for the *in vitro* regeneration of *H. sabdariffa*. The use of direct and indirect organogenesis as

suggested in this work proved to be a quick and efficient method for in vitro regeneration of *H. sabdariffa.* In addition, by making use of leaves explants. The fact that no or low levels of cytokinins induced higher sprouting efficiency also reduces the risks of somaclonal variation as a result of the use of plant growth regulators in tissue culture.

Conflict of interest

The authors declare no conflict of interest.

References

1. Lin, T**. L., Lin H. H., Chen CC., Lin M.C., Chou M.C. and C.J. (2007).** Wang. *Hibiscus sabdariffa* extract reduces cholesterol in men and women. Nutr. Res., 2007, 27:140-145.
2. **Ahuja, M. R. (1991).** Woody Plant Biotechnology, p. 373. Plenum Press, New York.
3. **Hartman, B.H., D.E. Kester and F.T. Davies. (1994).** Plant Propagation: Principles and Techniques. Prentice Hall, New Jersey.
4. **Collin, H.A. and S. Edwards. (1998).** Plant Cell Culture. Bios Scientific Eds., Guilford.
5. **SAS. (2010).** SAS/ STAT User's Guide for Personal Computers. Release 6.12. SAS Institute Inc. Cary, NC, USA.
6. **Razdan, M. K. (2003).** Introduction to Plant Tissue Culture. 2nd Edition. Science Publishers, INC. USA.
7. **RONI, Z.K.; ISLAM, S.; SHIMASAKI, K. (2018).** *In vitro* seed germination and tracking the seedling growth of eustoma. New Zealand Journal of Crop and Horticultural Science, v.46, n.3, p.224-242, 2018. DOI: https://doi.org/10.1080/01140671.2017.1391300.
8. **George, E. F. (1996).** Plant Propagation by Tissue Culture: In Practice. Part II. 2nd ed. Exegetics Limited Press, London.
9. **Sultana. R.; Quader, A.K.M.L.; Haque, M.; Mazumder, S.; Paul, S.K. (2016).** *In vitro* studies on shoot proliferation of kenaf (*Hibiscus cannabinus* L.). World Journal of Agricultural Sciences, v.12, n.1, p.25-30, 2016. DOI: http://www.idosi.org/wjas/wjas12(1)16/.
10. **Kumar, S.S.; Manoj, P.; Giridhar, P. (2016).** Micropropagation for mass multiplication and enriched production of ascorbic acid in tissue culture foliage of roselle (*Hibiscus sabdariffa* L.) *In Vitro* Cellular & Developmental Biology, v.52, p.427-436, 2016. DOI: https://doi.org/10.1007/s11627-016-9785-2.
11. **Pilate. B. G. Scott, B. Maldiney, R. Jacques, L. M. Sossooutzov and E. Miginiae. (1989).** Abscisic Acid, IAA and cytokinins changes in bud pseudotsuga. Physiol. Plant. 76:100-106.
12. **Maheshwaran, M., R. Gnanam and R. Seerangasamy. (2000).** Plant Tissue Culture, Principle and Practices. CPMB.
13. **George, E. F., A. H. Michael and D. K. Greek-Jan. (2008).** Plant Propagation by Tissue Culture (3rd Edition). Vol. 1. The Background. Springer.
14. **Aoshima, Y. and M. Takemoto. (2006).** Increase in peroxidase activity in tea callus in response to darkness, 2, 4-dichlorophenoxyacetic acid and carbohydrates. Plant Biotech. 23: 405 – 408.

15. **Ibrahim, R. and P. C. Debergh. (2001).** Factors controlling high efficiency of adventitious bud formation and plant regeneration from in vitro leaf explants of roses (Rosa hybrida). Sci. Hort., 88: 41-57. In: Acta Physiologiae Plantarum. 16 (1): 53-60.
16. **Danial, G. H., D. A. Ibrahim, S. A. Brkat and B. M. Khalil (2014).** Multiple Shoots Production from Shoot Tips of Fig Tree (*Ficus carica* L.) and Callus Induction from Leaf Segments.Int. J. Pure Appl. Sci. Technol., 20(1): 117-124.Available online at www.ijopaasat.in.
17. **Skoog, F. and C. O. Miller. (1957).** Chemical regulation of growth and organ formation in plant tissue culture *in vitro*. Symp. Soc. Exp. Biol. 11: 118-148.
18. **Pierik, R. L. M. (1997).** *In vitro* Culture of Higher Plants. Martinus Nijhoff publisher. Dordrecht, the Netherlands. P: 209-218.
19. **De Silva, A. L.; M. Rogalski and M. P. Guerra. (2003).** Effect of different cytokinins on *in vitro* multiplication of Prunus 'Capdeboscq' rootstocks. Crop Breeding and Applied Biotechnology. 3 (2): 149-156.
20. **Hopkins, W. G. and N. P. A. Hiiner. (2004).** Introduction to Plant Physiology, 3rd ed. John Wiley and Sons, Inc.
21. **Al-Ansary, A. M. F.; A. A. Rizkalla and A. M. Badr-Elden. (2007).** Micropopagation and biochemical genetic markers detection for drought and salt tolerance of pear rootstock. Australian J. Basic and App. Sci., 1(4): 625-636.
22. **Sharma, T., M. Modgil and M. Thakur (2007).** Factors affecting induction and development of *in vitro* rooting in apple rootstocks. Indian journal of experimental biology, 45: 824-829.
23. **Toma, R. S. (2019).** Cost Effective Culture Medium for Micropropagation of Paulownia (*Paulownia tomentosa* Steud.) and Catalpa (*Catalpa bignonioides* Walt.). Arab Journal for Agricultural Sciences, 2(4): 33-46. Doi: 10.21608/ASAJS.2019.52886.
24. **Arodin, J. (2022).** What are the pros and cons of peatmoss? Available online at: https://lawnlove.com/blog/peat-moss-pros-and-cons.