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In vitro propagation protocol of *Hibiscus syriacus* L. plants

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Abstract : The *in vitro* experiment was carried to examine the effect of various MS strength culture medium (full or half strength) and growth regulators concentrations of BA at (0.0, 0.1 or 0.2 mg/l) on shootlet multiplication and zeatin, 2,4-D and NAA on callogenesis potentiality) on *Hibiscus syriacus* L. plants. The results showed that using MS (3/4 strength) medium supplemented with BA at 0.2 mg/l resulted in the highest shootlets number/explant (3.33shootlet/explant) and the highest number of leaves (7.67 leaf/shootlet).The *in vitro* plants showed increasing in number of xylem rows, number of vessels and length of vascular bundle as comparison with control (mother plants). For callus induction, zeatin and 2,4-D at 0.5% for each were favored (highest callus percentage/leaf explant (100%) was observed. The response of formed callus to grow as a result of using BA (0.2 mg/) in combination with 2,4-D or NAA (0.25, 0.5 or 1.0 mg/L) for three subcultures was recorded. The high concentration of 2,4-D (1.0mg/l) added to BA (0.2) was favored for callus growth in the third subculture.BA combined with 2,4-D at low concentration (0.25mg/L) had promotion effect on callus dry weight after three subcultures. All shootlet produced from above multiplication treatments were rooted on half strength MS free medium with 3 g/l activated charcoal. The highest survival percentage and longest roots of acclimatized plants were recorded for growth media peat + sand (1: 1) after transplanting (five weeks).
Key words *Hibiscus syriacus*, BA, zeatin, 2,4-D, NAA,leaf anatomy.

Introduction

Hibiscus syriacus L. is a perennial deciduous shrub (family Malvaceae), includes many cultivars with various flower colors (pink, purple or white) and is considered as ornamental[1]. The shrub has economically value for fiber production and medicinal products. It also incorporated in beverage additives in some countries because of its hardiest, it exhibit high resistance to pests. The plant grows in any soil. *Hibiscus syriacus* can be propagated by cutting and seedling methods. Conventional methods of tree improvement and selection offer limited possibilities of meeting the rapidly growing demands of the industry and for reforestation programs. The development and application of molecular tools allowed the better understanding of the genetic structure of several species. While the fundamental techniques to achieve *in vitro* plant morphogenesis have been well established for a number of years[2]*Hibiscus syriacus*is heterozygous and also stock plants can be infected easy by virus diseases. Therefore it is complicated to sustain genetic properties by seedlings. So,*in vitro* plant propagation can solve this problem and we can obtain mass production free virus in short time[3,4 &5]

A few studies on *in vitro* propagation of *Hibiscus spp.*have been reported, this may be attributed to the fact that these species are woody plants[6]. Induced callus from hypocotyl section of *H. syriacus* and isolated

protoplast from callus. The success procedures of consecutive micro-propagation of many woody plants could be influenced by various factors from which plant growth regulators, physical conditions and growing media are the most important ones. The effect of benzyladenine (BA) at different concentrations on shoot multiplication rate was recorded on *Hibiscus rosa-sinensis*[7,8]. The leaf is the key organ for photosynthesis and transpiration. Therefore, leaf morphology and celldistribution may be important in influencing physiological processes[9]The experiment was then conducted to examine the effect of culture medium strength, growth regulators at various concentrations through tissue culture technique on micro-propagation potentiality of shootlets andleaves anatomy as well as callogenesis potentiality of *Hibiscus syriacus.*

Materials and Methods

The work was carried out at tissue culture Laboratory of Ornamental Plants and Woody Trees Department, National Research Center (NRC) and Tissue culture and Germplasm Conservation Research Laboratory, Horti. Res. Institute, Agri. Res. Center (ARC), Egypt during years 2015 and 2016 to establish *in vitro* protocol for shootlet multiplication and callogenesis potentiality of *Hibiscus syriacus* plants

Plant material and culture conditions

Nodal explants with axillary buds of *Hibiscus syriacus* were collected from shrub-grown on Orman Garden- Giza. The excised nodal segments (0.5-1 cm long) were surface sterilized in 70% (v/v) ethanol for 1 min, then in 20% commercial sodium hypochlorite solution and one drop of tween 20(polyoxy ethylene sorbitonmonolaurate) for 10 min after that rinsed tree times with autoclaved distilled water followed with 7-min in 0.1 g/L $HgCl_2$, and rinsed three times with autoclaved distilled water. The sterilized nodal explants were cultured on an induction medium and used as the starting plant material[10](MS) basal medium supplemented with 30 g/L sucrose and 8 g/L agar was adjusted to pH 5.6 ±0.2, and the medium was autoclaved at 121°C under 100 kPa for 20 min. The cultures were incubated at 23 ±2°C, under 16/ 8 h photoperiod with light intensity of 30 umol $m^{-2}s^{-1}$.After three weeks from culture explants on MS free medium the shootlet free contaminated nodal stems as well as leaves were used for micropropagation and callus induction.

Proliferation of auxiliary shoots

Microcutting explants (0.5-1cm length) were excised from *in vitro* shootlet and cultured on MS medium at full or half strengths supplemented with different concentrations (0.0, 0.1 and 0.2mg/l) of 6-Benzyladenine (BA)for axillary shootlet induction. After two subculture, the number of shootlet/explant and the number of leaves/shootlet were recorded after two subculture (each subculture 21 days).

Callus induction from leaf explants

The second or third leaf from the apex of *in vitro*2-month-old shootlet were cut into 0.5 cm by 0.5 cm pieces, then leaf segments were placed on medium supplemented with the growth regulators zeatin (0.5mg/l) or 2,4-D (0.5mg/l) either separetly or in combination of themto test their effects on induction of callus tissues. Callus produced from above treatment were examined on medium supplemented with BA (0.2 mg/l) in combination with 2,4-D or NAA (0.25, 0.5 or 1.0 mg/L). Data of fresh weights (g) of forming callus during three subculture were recorded. After three sub cultures, dry weight (g) was calculated for all treatments

Rooting and acclimatization

All shootlet culture on half strength MS medium supplemented with 3g/L charcoal without growth regulators was rooted. The rooted shoots were transferred to pots containing peat alone or combined with sand in a ratio of 1:1, and then they were covered with polythene bags for 2 weeks before they were transferred to a research greenhouse. The survival percentage, stem length (cm) and root length (cm) were recorded after five weeks from adaptation.

Data analysis:

All experiments used a randomized complete block design with 10 replicates per treatment. Means were compared using L.S.D test at 5% as the method described by[11].

Anatomical study:

At multiplication stage, the samples of leaves section were taken from third one on the stem and were prepared according to the methods described by[12,13] sections were mounted in Canada balsam then examined microscopically and microphotography. The following parameters were recorded:

Number of vascular bundles.- Number of xylem rows- Number of vessels- Length of vascular bundles µm)- Wide of vascular bundles (µm)-Thickness of midvien (µm)-Thickness of lamina (µm)- Epidermis (µm)- Palisade chlorenchyma thickness (µm)- Mesophyll chlorenchyma thickness (µm)

Results and Discussions

Effect of strength of MS medium with different concentrations of BA on multiplication of *Hibiscus syriacus*

The illustrated data in Table (1) indicates that shootlet multiplication of *Hibiscus syriacus* was influenced by strength of medium and BA concentrations interaction used. Using of MS (3/4 strength) medium supplemented with BA at 0.2 mg/L resulted in the highest shootlets number (3.33shootlet/explant) and the highest number of leaves (7.67 leaf/shootlet),followed by half strength of MS supplemented with BA 0.2 mg/l gave 2.67 shootlets as compared to control (1/2 MS free hormones) which decreased this value to the lowest one (1.23 leaf/shootlet).Full or half strength of MS medium without hormones gave lowest number of leaves/shootlet (4.50 and 4.67 leaf/shootlet, respectively). These results could be explained by that cytokinins have important physiological effects, as they have been shown to stimulate cell division as well as cell elongation, to activate RNA synthesis and to stimulate protein synthesis and enzyme activity, as was reviewed by[14]. Also, [15]concluded that optimum shoot proliferation was obtained in full-strength MS salts. Also, it is clear that the addition of BA to the medium increased the shootlets and enhanced theirvigorously. The ability of explants to produce shoots was studied by[7] on *Hibiscus sinensis*. They observed that multiple shoot induction was present in the stem explants that were cultured on the MS medium (3/4 strength) supplemented with BA at the concentration of 2mg/l.

Table (1): Effect of strength of MS medium with different concentrations of BA on multiplication of *Hibiscus syriacus*

Number of leaf /shootlet				Number of shootlets/ explant				Character
Mean B	Half	3/4	Full	Mean B	Half	3/4	Full	Treat mg/l
4.78	4.67	5.17	4.50	1.14	1.23	1.33	1.67	**Cont**
5.72	5.33	6.50	5.33	2.11	2.17	2.33	1.83	**0.1 BA**
6.50	5.50	7.67	6.33	2.83	2.67	3.33	2.50	**0.2 BA**
	5.17	6.44	5.39		2.02	2.33	2.00	**Mean A**
0.839 0.839 1.453				A =0.6507 B =0.6507 AxB = 1.127				**LSD 5%**

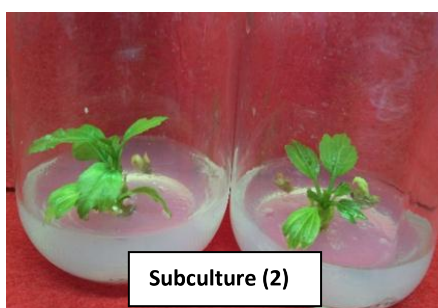


Fig. (1): ***In vitro*** **shoot multiplication 3/4 strength of MS+0.2 mg/L BA) during two subculture of *Hibiscus syriacus*plants.**

Anatomical leaf behavior of *in vitro* propagated plants

Data presented in table (2) show that, The *in vitro*shootlet showed increasing in number of xylem rows, number of vessels and length of vascular bundle as comparison with control (Mother plants). Contrarily, its recorded decreased in thickness of both midvien and lamina and also wide of vascular bundle.The increases in *in vitro* plants surpassed the control by 38.46, 77.77 and 5 % over the control respectively. The decreases undervalue the mother plants by 20, 19.04 and 22.77 % lower thanmother plants respectively.These anatomical differences between the *in vitro* plants and mother plants maybe due to the differences in the environment of the plants. Plantlets *in vitro* facing particular condition, constant temperature and light, contentious source of water and carbohydrates, this condition cause abnormal morphology, physiology and anatomy characteristics. In *vitro* conditions compromise plantlets' leaf morphology and leaf anatomy expansionand these characteristics negatively impact the capacity for ex vitro acclimatization, but the degree to which plants are affected by the *in vitro* environment depends on the plant species[16,17&18]. Growth parameters of *Alpiniapurpurata* were increased mother plants compared to *in vitro* plants[19]. *In vitro* shoots and plantlets are tiny cell wall, sclerenchyma, collenchyma formationAbsent of cuticular wax, limited palisade layer, palisade development is affected by different light levels and reduced as a consequence of relatively low light intensity, xylem vessels were not completed, pericycle and endodermis were darkly[20,21&22].

The spongy layers, palisade layers and epidermis in leaf transaction showed a clear differentiation between palisade and the adjacent spongy layers in both mother plant and *in vitro* plants. This different may due to the components of media *in vitro* and the different atmosphere like different light levels, relatively percentage and light intensity. Many studies on the ecological anatomy of tree species showed that the degree of spongy differentiation is strongly dependent on the degree of exposure to the sun light. The leaves growing in sunshine have more palisade layers compared to leaves in shade and the palisade cells are long, large and closetogether [23,24,21&22]. This may explain why this different between *in vivo* and *in vitro* leaves.

Table (2): Comparison between leaf anatomy behavior of *in vitro* propagation and mother plant of*Hibiscus syriacus*.

Character. / Treat	Thickness of midvein (µm)	Thickness of Lamina (µm)	No. of xylem rows	No. of vessels	Length of vascular bundle (µm)	Wide of vascular bundle (µm)	Epidermis (µm)	Palisade Chlorenchyma thickness (µm)	Mesophyll chlorenchyma thickness Thickness (µm)
Mother plants	75	21	13	45	40	22	1.5	6	13
In vitro plants	60	17	18	80	42	17	2	5.5	11

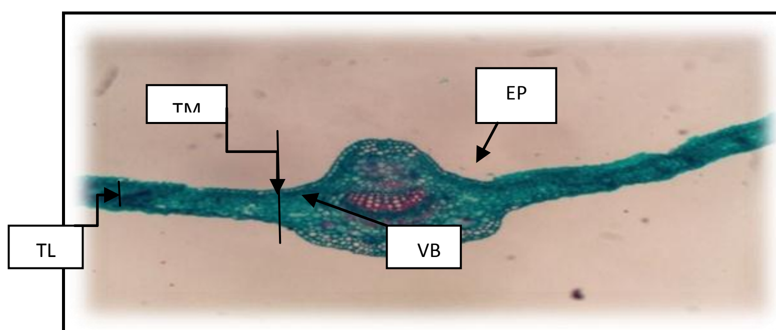


Fig. 2: Mother plants (open field Plant)

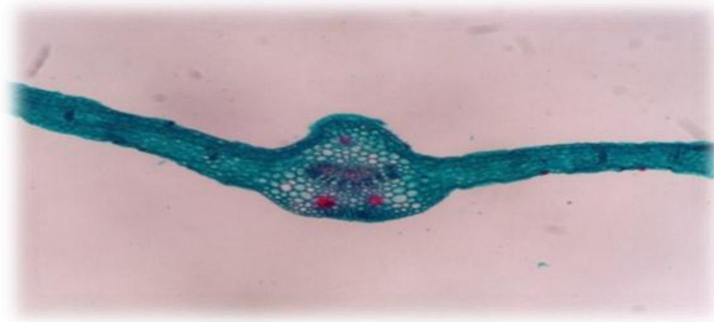


Fig. 3: ***In vitro*** **shootlet**

Fig. 2: Light Microphotograph showing transverse section through the blade of the third leaf developed on the main stem ofMother plantof *Hibiscus syriacus*

L.(x = 10)(Bar=0.05 ml) EP= Epidermis, TM= Thickness of medvein, VR=Vascular bundle and TL=Thickness of lamina

Fig. 3: Light Microphotograph showing transverse section through the blade of the third leaf developed on the main stem of*in vitro* of*Hibiscus syriacus*

L plantlets.(x = 10)(Bar=0.05 ml).

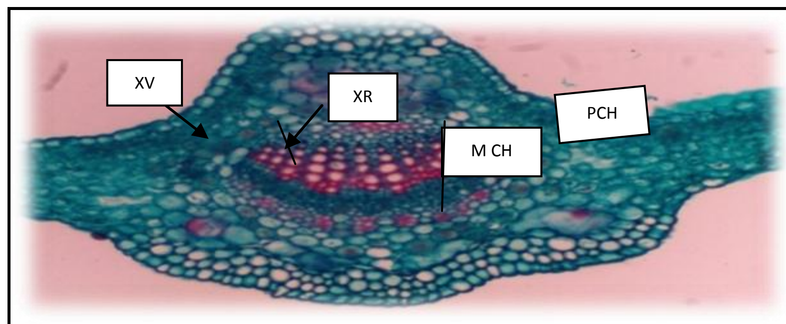


Fig. 4: Mother plant

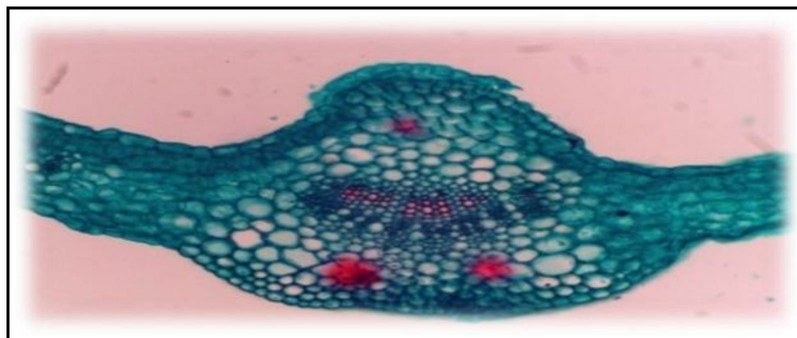


Fig. 5:***In vitro*** **plant**

Fig. 4: Light Microphotograph showing transverse sections through the blade of the third Mother plants leaf developed on the main stem of *Hibiscus syriacus* L. The section shows vascular bundle (number of vessels and number of xylem rows. (x40)(Bar=0.1ml).

XR= Xylem Rows, XV= Xylem vessels, PCH=Palisade Chlorenchyma and MCH=Mesophyll Chlorenchema

Fig. 5: Light Microphotograph showing transverse sections through the blade of the third *in vitro* plant leaf developed on the main stem of *Hibiscus syriacus* L plantlets. The section shows vascular bundle, (number of vessels and number of xylem rows. (x40)(Bar=0.1ml).

Callogenesis potentiality

Callus induction

The effect of growth regulators (zeatin and 2,4-D) on callogenesis potentiality of *Hibiscus syriacus* leaf explants was shown in Fig. (6). The highest callus percentage/leaf explant (100%) was observed when MS culture medium was supplemented with zeatin and 2,4-d at 0.5 mg/L for each followed by zeatin (0.5 mg/L) then 2,4-D (0.5mg/L)[25].

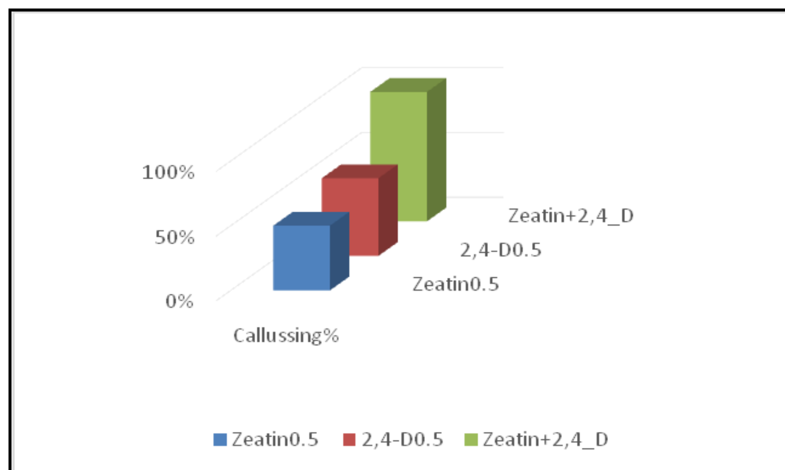


Fig. (6):Effect of growth regulators on callogenesis potentiality of *Hibiscus syriacus* leaf explant.

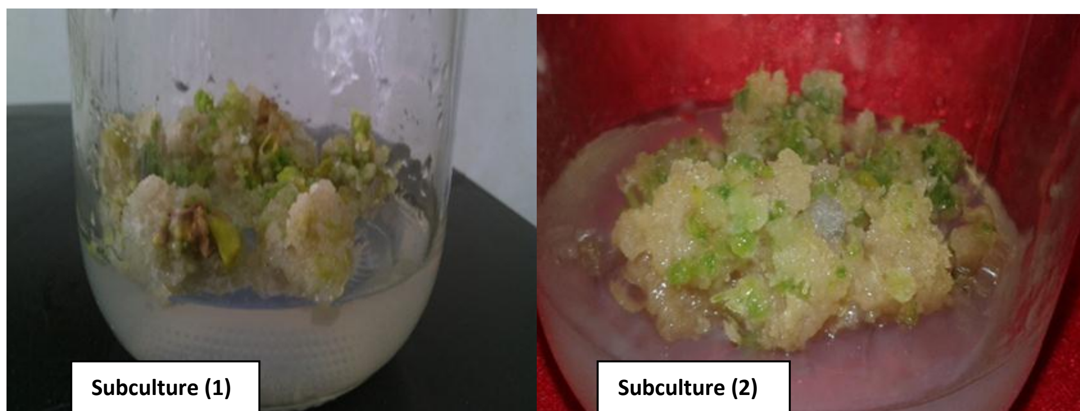


Fig. (7): Callusing of *Hibiscus syriacus* leaf explant during two subculture (Zeatin 0.5 mg/L+ 2,4-D 0.5 mg/L).

Callus growth

The response of callus growth to BA (0.2 mg/L) combined with various concentrations of 2,4-D or NAA (0.25, 0.5 and 1.0 mg/L) was illustrated in Table (3). Callus fresh weight was heaviest (5.27 and 5.61g, respectively) in the second and third subculture, BA combined with 2,4-D at low concentration (0.25mg/L) had promotion effect on callus fresh weight as well as callus dry weight (after three subcultures) (Fig.8) . However, when the effect of repeated subculture interacted with growth regulators, the high concentration of 2,4-D (1.0mg/L) was favored for callus growth in the third subculture. Our results were confirmed by those of[26,27], they found that the callus induction was affected by auxin and cytokinin ratio as well as their actual concentrations. The interaction of cytokinin and auxin can be concentration dependent.

Table (3): Effect of growth regulators and repeated subcultures on callus fresh weights of *Hibiscus syriacus*

Treatment	Sub.1	Sub.2	Sub.3	Mean B
C1	6.70	8.10	9.84	8.21
C2	3.50	3.50	8.80	5.27
C3	2.90	3.38	10.54	5.61
C4	3.14	3.82	4.08	3.68
C5	3.48	3.50	3.90	3.63
C6	3.56	3.66	3.58	3.60
Mean A	0.18	5.27	5.61	
LSD 5%	A: 0.5106	B: 0.722	AxB: 1.251	

C1: BA 0.2 mg/L + 2,4-D 0.25 mg/L, C2: BA 0.2 mg/l + 2,4-D 0.5 mg/LC3: BA 0.2mg/L + 2,4-D 1.0 mg/L

C4: BA 0.2 mg/L + NAA 0.25 mg/LC5: BA 0.2 mg/L + NAA 0.5 mg/LC6: BA 0.2 mg/L + NAA 1.0 mg/L

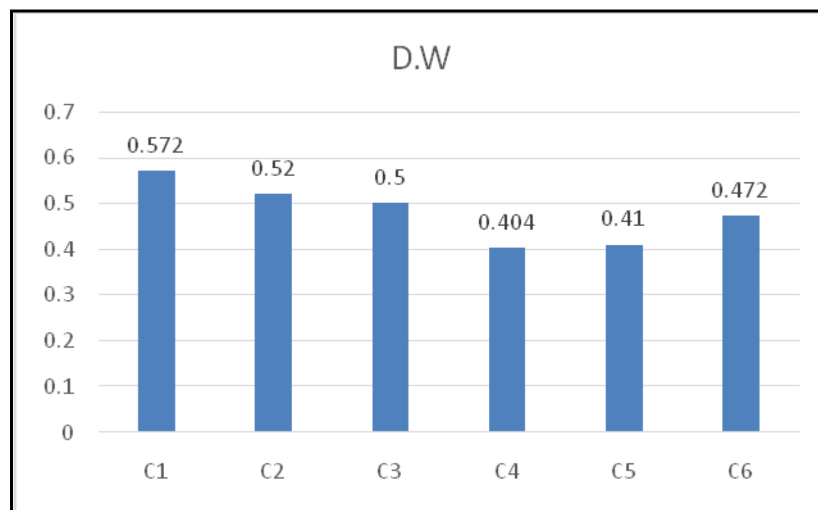
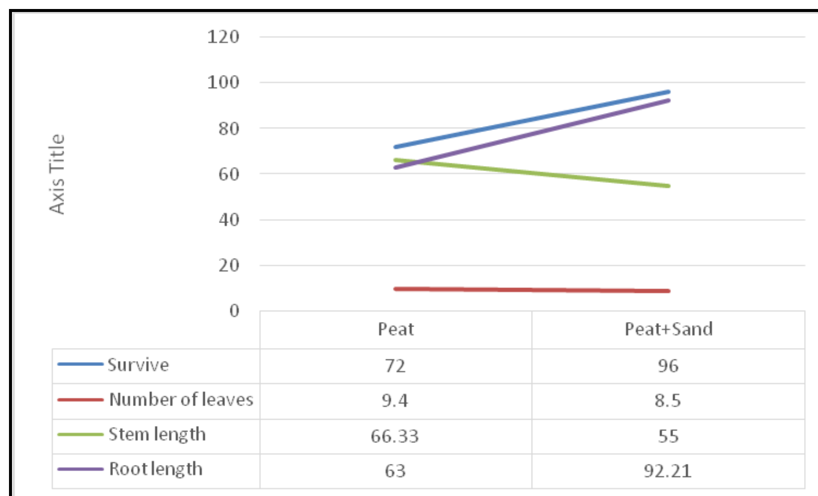


Fig. (8): Effect of growth regulators after third subculture on callus dry weights of *Hibiscus syriacus*.

Rooting and acclimatization

Resulting shoots from nodal stems were transferred to half strength MS medium supplemented with 3g/L charcoal without growth regulators for root initiation and development (Fig 9), the rooted plantlets were removed from rooting media, washed, and then transferred to pots containing peat alone or combined with sand in a ratio of 1:1. Plantlets were covered with polythene bags for 2 weeks before they were transferred to a research greenhouse. Data in Fig (10) mentioned that the highest survival percentage and longest roots of acclimatized plants were recorded for growth media peat + sand (1: 1) after transplanting (five weeks). Using peat alone gave the highest number of leaves/plant and the longest stem. In this regard[28] on *Deutzia scabra* indicated that different growing media had no significant effect on the survival percentage of plant during acclimatization. The tallest plants and greatest number of leaves produced with peat moss growing medium as comparing with peat moss + sand (1:1), peat moss +vermiculite (1:1) or peat moss + sand + sand (1:1:1).[29] on *Paulownia kawakami* showed that the highest percentage of survival (90.00 %) was obtained by using a soil mixture of Peat moss and sand. While, no significant differences between neither stem lengths nor leaves number of the acclimatized rooted plants *ex vitro* were due to different growing media tested. Since, the root length was in highest value (93.33 mm) when plantlets were cultured in mixture of peat moss and sand (1:1).

Fig. (9):*In vitro* rooted Shootlets (MS free + charcoal 3g/L) resulting from nodal stems of *Hibiscus syriacus.*



	Peat	Peat+Sand
Survive	72	96
Number of leaves	9.4	8.5
Stem length	66.33	55
Root length	63	92.21

Fig. (10): Effect of growth media on acclimatization behavior of *Hibiscus syriacus* plantlets

Conclusion:

Using MS (3/4 strength) medium supplemented with BA at 0.2 mg/l resulted in the highest shootlets number/explant (3.33 shootlet/explant) and the highest number of leaves (7.67 leaf/shootlet).The *in vitro* plants showed increasing in number of xylem rows, number of vessels and length of vascular bundle as comparison with control (mother plants). For callus induction, zeatin and 2,4-D at 0.5% for each were favored (highest callus percentage/leaf explant (100%) was observed. The response of formed callus to grow as a result of using BA (0.2 mg/) in combination with 2,4-D or NAA (0.25, 0.5 or 1.0 mg/L) for three subcultures was recorded

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