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Large Scales of *Hydrangea macrophylla* Using Tissue Culture Technique

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A B S T R A C T

Hydrangea (Hortensia) is a highly popular ornamental plant for garden decoration, and now it is commercially produced for cut flower branches. The aim of this study was to set up a protocol for large scale for *in vitro* propagation of *Hydrangea macrophylla*, for this purpose two type of explant (shoot tip and node) were sterilized and the best percentage of survival explants (40%) was obtained when explants immersed for 20 min in 1.0 or 1.5 % NaOCl. For in vitro multiplication, Murashige and Skoog medium supplemented with 1.0 mg/l BA and 2.0 mg/l Kin was the best treatment on shoot number. Using GA3 at 1.0 mg/l was a marked increase in plant length when compared with zero level (control). During the rooting stage, the medium containing 4.0 mg/l IAA with charcoal gave the longest plantlet and the highest number of leaves, while NAA at 1.0 mg/l without charcoal gave the highest number of roots and the longest root was found at 2.0 mg/l IAA without charcoal. The longest plant (6.17 cm) was achieved when peat moss: per lite (2: 1 v/v) acclimatization mixture was used compared with other mixtures.

Introduction

The family Hydrangeaceae includes mainly woody plants and comprises 17 genera and about 170 species. *Hydrangea macrophylla* is one of the most well-known species in the genus and is known by the name of Hortensia (Orozco-Obando, 2005). In most species the flowers are white, in *H. macrophylla* they can be blue, red, pink, light purple, or dark purple, the color depends on the soil pH (Savona *et al*., 2012). *Hydrangea macrophylla* subsp. has been widely cultivated as a garden and potted plant. In addition, cut flower cultivars have been developed Common cultivated species is grown widely in gardens (Schiappacasse *et al*., 2014). In commercial practice, hydrangea is propagated either by seeds or stem cuttings. Each method has its own drawbacks. Seed plants vary and don't allow the propagation of desirable forms, Leaf cuttings occupy considerable space during propagation because of their very large size. Moreover, plants from cuttings are slow to establish and lack good basal branching. Tissue culture methods for vegetative propagation of plants have become increasingly important (Thomas *et al*., 1987).

Tissue culture method is part of biotechnology that is used for massive propagation especially for horticulture crops and ornamental plants. So many factors such as growth regulator, plant and explants type,

environmental condition (temperature, light) influence organogenesis and *in vitro* multiplication. It seems that among these factors, growth regulators have the most effect on plant *in vitro* micropropagation (Jain, 2002). In the last 20 years, few papers were published focusing to Hydrangea *in vitro* propagation; Sebastian *et al.,* (1987) reported the first micropropagation protocol for *Hydrangea quercifolia* Bart. The influence of thidiazuron (TDZ) on *in vitro* shoot proliferation was demonstrated by Preece and Ledbetter (2003) and Ledbetter and Preece (2004), low concentrations of this growth regulator induced a low number of long shoots; high concentrations of TDZ ensured many short adventitious shoots. Abou Dahab (2007) set up a protocol for micropropagation of *Hydrangea macrophylla* for commercial production. To optimize *in vitro* adventitious shoot regeneration in *Hydrangea macrophylla* Thunb. 'Nacthtigall', experiments on salts compositions (MS or B5 - full or half strength), on different cytokinins applied (BAP or TDZ or m-Top) and onto the propagations way (Solid Medium or Temporary Immersion System or Permanent Immersion System) were performed by Doil *et al.,* (2008). In 2012, Sacco and co-authors reported that *Hydrangea quercifolia* 'Snow Queen' showed an efficient in vitro propagation aptitude combined with a good propagation rate when culturing the explants onto agarised MS medium with BA 0.25 mg/L.

The aim of this research was to set up a protocol for large scale micropropagation of *Hydrangea macrophylla*. This purpose was done by studying the effect of sodium hypochlorite (NaOCL) for different times on Sterilization of explants type, effect of cytokinin type (BA or Kin) and explant type on micropropagation, effect of different auxins type (IAA, IBA or NAA) and charcoal addition on rooting and effect of growing media on adaptation of plantlets in greenhouse.

Materials and Methods

Plant materials

The mother plants were grown naturally at the open field condition at Institute of Genetic Engineering and Biotechn ology. University of Sadat City, Egypt, shoot tips and nodes were used as explants

Experimental treatments

Sterilization of explants

Different explants (shoot tip and nodes) of one year old plants of *Hydrangea macrophylla* were washed under a running water for one hour. After washing the explants were dipped in mercuric chloride (HgCl2) at concentration of 0.1 % for 5 minutes then were rinsed in sterilized distilled water.

Chemical disinfectant Clorox (NaOCl 5.25%) were used for shoot tips and nodes surface sterilization with various concentration (0.5,1.0,1,5 2.0 and 2.5 %) of sodium hypochlorite (NaOCL) for different times (10, 15 and 20 minutes). Tween 20 (polyoxythylenesorbitan monolaurate) was used as a wetting agent (one drop / 100 ml). Sterilized explants were rinsed three times with sterilized distilled water to remove all traces of sterilizing substances, and cultured in 25 ml of culture medium. Murashige and Skoog (1962) nutrient medium was used as a basal medium supplemented with 30 g/l sucrose and 7 g/l agar. This experiment consisted of 15 treatments, 3 replicates / treatment, 10 explants / replicate. After 15days of incubation the following characters were estimated: survival percentage, mortality percentage and contamination percentage.

Initial source of explant

Vigorous shoots of *Hydrangea macrophylla* were multiplied *in vitro* onto Murashige and Skoog (MS), 3% sucrose, supplemented with 0.25 mg/L of 6-benzyladenine (BA) and agarized with 8 g/L of technical Agar according to the protocol suggested by Sacco *et al.*, (2012).

Multiplication stage

Effect of different levels of BA and Kin combination on multiplication from shoot tip or nodal segments of *Hydrangea macrophylla* in vitro culture

The explants (shoot tip (0.5 cm) or nodal segment (1 node)) were cultured on full strength MS medium supplemented with the following concentration of BA(0.0, 1.0, 2.0 and 3.0 mg/l) combined with one of the following rates of kin (0.0, 0.5, 1.0 and 2.0 mg/l).This experiment consisted of 16 treatments, 5 replicates (gar / 2 explant). After 45 days of incubation the following parameters were estimated shoot number /explant, leaf number/explant, shoot length (cm), fresh weight (g)

Effect of different GA3 concentration on growth from shoot tip of *Hydrange a macrophylla* in vitro culture

In this experiment, shoot tip as explants were cultured on MS medium supplemented with different concentration of GA3: 0.0, 0.2, 0.4, 0.6 and 1.0 mg/l. This experiment consisted of 5 treatments, 5 replicates / treatment. After 45 days of incubation the following parameters were estimated: shoot length (cm) and leaf number/explant

Rooting stage

Effect of auxins type (IAA, IBA or NAA) and charcoal addition on rooting stage of *Hydrangea macrophylla in vitro* culture

In order to induce an efficient and functional *in vitro* root system, the shoots (2 cm) were cultured on full strength MS medium only and supplemented with indol acetic acid (IAA), indol butyric acid (IBA) or naphthalen acetic acid (NAA) at the same concentrations (0.5, 1.0, 2.0 and 4.0 mg/l) with or without activated charcoal (AC) 1g /l. This experiment consisted of 26 treatment, for each treatment, 15 replect (3 jars with 5 explants each were considered). After 45 days plantlet length (cm), leaf number/ plantlet, plantlet fresh weight (g), root number / plantlet and root length (cm) were recorded

Culture condition

All of this experiments the pH of the medium was adjusted to 5.8 and autoclaved at 121o C for 20 minutes. All the cultures were incubated in growth room at 25 ± 2o C temperature under 16 hours photoperiod using cool-white fluorescent lamp.

Acclimatization stage

Effect of different growing media on adaptation of plantlets *Hydrangea macrophylla* in greenhouse

Plantlet (3 cm, 4 leaves) which produced *in vitro* were washed under tap water to remove agar from the roots which might be a source of contamination, then transplanted to plastic pots (6 cm) containing of different growing media containing a mixture(v/v) of

1- Peat moss only
2- Peat moss + perlite (1:1)
3- Peat moss + perlite (2:1)
4- Peatmoss + perlite (3:1)
5- Peatmoss + vermiculite (1:1)
6- Peatmoss + vermiculite (2:1)
7- Peatmoss +vermiculite (3:1)
8- Peatmoss +sand (1:1)
9- Peatmoss + sand (2:1)
10 - Peatmoss + sand (3:1)

In order to maintain high humidity in culture environment, the pots were covered with a light plastic cover. The plantlet was gradually exposed to normal greenhouse conditions, after 45 days of the following parameters were estimated: Plantlet length (cm), leaf number/ plantlet, root number/ plantlet and root length (cm).

Statistical analysis

All of the experiments were carried out as a factorial experiment at 5 % probability level. Data obtained were statistically analyzed using MSTAT software program (MSTAT Development Team, 1989) for comparing among least significant difference (LSD).

Results and Discussion

Starting stage

Effect of NaOCl concentration and period time on sterilization of tow explants of *Hydrangea macrophylla* cultured *in vitro.*

Shoot tip explants

Results demonstrated in table (1) indicate that, the best concentration of NaOCl was 1.5 % which gave (30 %) survived explants, no mortality (0%) was observed when explants were treated by 0.5, 1.0 and 1.5 %. with increase of NaOCl concentration, the percentage of contaminated explants was decreased. On the other hand, the data indicated that increasing the soaking period of explants increased the survival percentage of explants. Soaking the explants for 20 min was the best time for the highest percentage of survival (30 %), highest percentage of mortality (16 %) and the lowest percentage of contamination (54 %). The data of the interaction between the concentration of NaOCl and the time of soaking indicated that the best percentage of survival explants (40%) was obtained when explants immersed for 20 min in 1.0 or 1.5 % NaOCl and for 15 min in 1.5 % NaOCl. The highest mortality percentage (50 %) was observed when explants were treated by 2.5 % NaOCl for 20 min and the lowest contamination percentage (30 %) was obtained when explants immersed for 10 or 20 min in 2.5 % NaOCl.

Node explants

Data in table (2) showed that the highest survival percentage (26 %) was observed when explants were treated by 1.5 % NaOCl. The highest mortality percentage and the lowest contamination percentage (40 %) and (53.33%) respectively were obtained when explants were treated by 2.5 % NaOCl. Increasing the soaking period of explants increased the survival percentage of explants. Soaking the explants for 20 min was the best time for the highest percentage of survival (22 %), highest percentage of mortality (19.33 %) and the lowest percentage of contamination (58.76 %).

The data of the interaction between the concentration of NaOCl and the time of soaking indicated that the best percentage of survival explants (30%) was obtained when

explants immersed for 20 min in 1.0, 1.5, 2.0 % NaOCl and for 15 min in 1.5 % NaOCl. The highest mortality percentage (50 %) was observed when explants were treated by 2.5 % NaOCl for 20 min and the lowest contamination percentage (40 %) was obtained when explants immersed for 20 min in 2.0 % NaOCl.

This result may be due to the liability of plant tissue of *Hydrangea macrophylla* to excessive surface sterilization with mercuric chloride (MC) which has a lysis effect on microbial cells, as stated by Abou Dahab (2007) reported that the results of explant indicated that the highest percentage of contamination free explants (100%) was obtained by using chlorox at 50% plus mercuric chloride (MC) at the concentration 0.2%.

Multiplication stage

The results shown that the multiplication of *Hydrangea macrophella* was successfully achieved by culture on MS medium supplemented with concentration of BA and Kin.

Effect of BA and Kin concentration on shoot tips of *Hydrangea macrophella*

Table (3) showed that for BA concentrations, culture on MS medium containing 1.0 mg/l BA, the mean higher number of shoots (4.45) and leaf (42.70) giving significant effect as compared with 0.0, 2.0, 3.0 mg/l BA. For Kin concentrations the mean higher number of shoots (3.75) and leaf (36.20) were found on MS medium containing 1.0 mg/l Kin. The interaction between the different concentrations of BA and Kin showed that the best concentration was 1.0 mg/l BA and 2.0 mg/l Kin on shoot number giving (6.60), while the best number of leaves was (56 leaf/explant) at 1.0 mg/l BA with 1.0 mg/l Kin. While data in table (4) showed that the highest values for shoot length (2.95 cm) had been obtained from the control treatment, the largest fresh weight (3.65 g) were found when MS medium containing 3.0 mg/l BA. For shoot length, there were insignificant differences between all the different concentrations of Kin but the longest shoot was (2.80 cm) at 2.0 mg/l Kin, while the best fresh weight was (3.11 g) at 2.0 mg/l Kin. The interaction showed that for shoot length, there were insignificant differences between all the different concentrations but the longest shoot was (3.3 cm) had been obtained from the control treatment. The best concentration for fresh weight (4.40 g) was 3.0 mg/l BA with 2.0 mg/l Kin.

Effect of BA and Kin concentration on nodes explant of *Hydrangea macrophella*

Data in table (5) show that 1.0 mg/l was the best concentration of BA on shoot number, leaf number, they were recorded (8.00) and (79.60) respectively and 0.5 mg/l of Kin was the best concentration for both measurements, the highest number of shoots was (6.75) and biggest leaf number was (69.60).A combination between BA and Kin was positively significant, the highest number of shoots (11) was obtained when MS medium supplemented with 1mg/l BA and 2.0 mg/l Kin, while the best concentration for leaf number (110.0) was 2.0 mg/l BA with 1.0 mg/l Kin.

Results presented in table (6) show that the longest shoot (3.00 cm) was obtained at zero level of BA and the best fresh weight was (5.38 g) at 1.0 mg/l BA.

For shoot length, there were insignificant differences between all the different concentrations of Kin but the longest shoot was (2.90 cm) at 0.5 mg/l Kin and the best fresh weight (4.65 g) had been obtained at the same concentration 0.5 mg/l Kin. For the interaction, results showed that MS medium

without any growth regulators was best treatment for shoot length (3.20 cm), while addition of 1.0 mg/l BA and 0.5 mg/l Kin to MS medium gave the highest fresh weight (7.43 g).

This result agrees with a number of published papers on rooting of *Hydrangea macrophylla* Doil *et al.,* (2008) reported that 6-benzyladenine (BA) rather than TDZ could induce higher regeneration rates for *Hydrangea macrophylla*. Feng Liu (2011) reported that, the highest frequency of leaf explants producing shoots (77%) and the highest mean number of shoots per explant (2.1) were observed on B5 medium supplemented with 2.25 mg/l BA and 0.1 mg/l IBA. Sacco *et al.,* (2012) found that, in the multiplication phase the BA, at any concentration, induced the highest multiplication rate (over 7 shoots/explant) and the highest cluster fresh weight the shoot height was not affected by the cytokinin used in each treatment (data not shown). Very good quality explants were obtained using kinetin but the multiplication rate was not suitable for a commercial production.

Effect of GA3 concentration on plant length and leaf number

Results in table (7) indicate that in general, the addition of GA3 to the medium led to increase in plant length. Increasing the GA3 to1.0 mg/l there was a marked increase in shoot length when compared with zero level (control), it was found (4.12 cm) and there were significant differences between it and different concentrations, while GA3 at 0.6 mg/l gave the highest number of leaves when compared with control.

Rooting stage

Effect of auxins type (IAA, IBA or NAA) and charcoal addition on rooting stage of *Hydrangea macrophylla in vitro.* Results presented in table (8) show that, using IAA at 4.0 mg/l gave the longest plantlet (5.52 cm) and highest number of leaves (12.47), while the best plantlet fresh weight was (1.46 g) at 2.0 mg/l NAA. There was insignificant effect for charcoal addition on plantlet length, while using charcoal gave biggest number of leaves (11.70) and using medium without charcoal produced the best values of plantlet weight (1.05 g). Data of interaction between different concentrations and charcoal addition showed that using 4.0 mg/l IAA with charcoal gave the longest plantlet (5.52 cm) and the highest number of leaves (12.8), 2.0 mg/l NAA with charcoal was the best treatment for plantlet fresh weight (1.63 g).

Table.1 Effect of different NaOCl concentration and times on survival, mortality and contamination percentages of *Hydrangea macrophylla* shoot tips in vitro culture after 2 weeks

NaOCl concentration	Shoot tip											
	Survival % Time (min)			Mean (A)	Mortality % Time (min)			Mean (A)	Contamination % Time (min)			Mean (A)
	10	15	20		10	15	20		10	15	20	
0.5	10.00	30.00	30.00	23.33	0.00	0.00	0.00	0.00	90.00	70.00	70.00	76.67
1.0	10.00	10.00	40.00	20.00	0.00	0.00	0.00	0.00	90.00	90.00	60.00	80.00
1.5	10.00	40.00	40.00	30.00	0.00	0.00	0.00	0.00	90.00	60.00	60.00	70.00
2.0	20.00	30.00	20.00	23.33	10.00	20.00	30.00	20.00	70.00	50.00	50.00	56.67
2.5	30.00	30.00	20.00	26.67	40.00	30.00	50.00	40.00	30.00	40.00	30.00	33.33
Mean (B)	16.00	28.00	30.00		10.00	16.00	74.00		62.00	54.00		
L.S.D at 5% A B AB	**8.32 6.44 14.41**				**5.64 4.37 9.77**				**12.02 9.31 20.81**			

Table.2 Effect of different NaOCl concentration and times on survival, mortality and contamination percentages of *Hydrangea macrophylla* nodes in vitro culture after 15 days

NaOCl concentration	Node											
	Survival %			Mean (A)	Mortality %			Mean (A)	Contamination %			Mean (A)
	Time (min)				Time (min)				Time (min)			
	10	15	20		10	15	20		10	15	20	
0.5	0.00	0.00	20.00	6.66	0.00	0.00	0.00	0.00	100.00	100.00	80.00	93.33
1.0	20.00	20.00	30.00	23.33	0.00	0.00	16.67	5.55	80.00	80.00	53.00	71.11
1.5	20.00	30.00	30.00	26.67	0.00	0.00	0.00	0.00	80.00	70.00	70.00	73.33
2.0	0.00	20.00	30.00	16.66	10.00	20.00	30.00	20.00	90.00	60.00	40.00	63.33
2.5	10.00	10.00	0.00	6.66	40.00	30.00	50.00	40.00	50.00	60.00	50.00	53.33
Mean (B)	10.00	16.00	22.00		10.00	10.00	19.33		80.00	74.00	58.67	
L.S.D at 5% A	7.00				5.19				8.66			
B	5.42				4.02				6.71			
AB	12.12				8.99				15.01			

Table.3 Effect of different BA and Kin concentrations on shoot and leaf number of *Hydrangea macrophylla* shoot tips in vitro culture after 45 days

BA mg/l	Shoot tip									
	Shoot number /explant				Mean (A)	Leaf number/explant				Mean (A)
	Kin mg/l					Kin mg/l				
	0.0	0.5	1.0	2.0		0.0	0.5	1.0	2.0	
0.0	1.00	1.20	1.20	1.80	1.30	15.60	17.60	15.60	18.40	16.80
1.0	2.80	2.80	5.60	6.60	4.45	30.00	34.00	56.00	50.80	42.70
2.0	3.40	2.00	5.00	1.80	3.05	43.60	24.80	47.60	19.60	33.90
3.0	5.00	2.60	3.20	4.00	3.70	50.40	35.20	25.60	42.00	38.30
Mean (B)	3.05	2.15	3.75	3.55		34.90	27.90	36.20	32.70	
L.S.D at 5% A	0.5315					2.685				
B	0.5315					2.685				
AB	1.063					5.370				

Table.4 Effect of different BA and Kin concentrations on shoot length and fresh weight of *Hydrangea macrophylla* shoot tips in vitro culture after 45 days

BA mg/l	Shoot tip									
	Shoot length(cm)				Mean (A)	Fresh weight(g)				Mean (A)
	Kin mg/l					Kin mg/l				
	0.0	0.5	1.0	2.0		0.0	0.5	1.0	2.0	
0.0	3.30	3.00	2.70	2.80	2.95	1.49	1.52	1.71	2.20	1.73
1.0	3.10	2.90	2.80	2.80	2.90	2.26	2.72	4.15	3.73	3.21
2.0	2.40	2.80	2.70	3.00	2.72	4.14	2.66	3.58	2.14	3.13
3.0	2.00	2.40	2.50	2.60	2.37	4.33	3.30	2.55	4.40	3.65
Mean(B)	2.70	2.77	2.67	2.80		3.06	2.55	3.00	3.11	
L.S.D at 5% A	0.5190					0.1108				
B	N.S					0.1108				
AB	1.038					0.2216				

Table.5 Effect of different BA and Kin concentrations on shoot number and leaf number of *Hydrangea macrophylla* nodes in vitro culture after 45 days

BA mg/l	Node									
	Shoot number /explant				Mean (A)	Leaf number/explant				Mean (A)
	Kin mg/l					Kin mg/l				
	0.0	0.5	1.0	2.0		0.0	0.5	1.0	2.0	
0.0	2.00	4.40	3.60	5.80	3.95	23.60	40.80	34.80	50.40	37.40
1.0	7.80	8.40	4.80	11.00	8.00	80.40	90.00	45.60	102.4	79.60
2.0	7.00	9.40	9.60	4.80	7.70	63.60	94.00	110.0	48.00	78.90
3.0	5.80	4.80	5.40	3.40	4.85	57.20	51.60	56.00	44.00	52.20
Mean (B)	5.65	6.75	5.85	6.25		56.20	69.10	61.60	61.20	
L.S.D at 5% A	0.6637					3.479				
B	0.6637					3.479				
AB	1.327					6.958				

Table.6 Effect of BA and Kin concentrations on shoot length and fresh weight of *Hydrangea macrophylla* nodes in vitro culture after 45 days

BA mg/l	Node									
	Shoot length(cm)				Mean (A)	Fresh weight (g)				Mean (A)
	Kin mg/l					Kin mg/l				
	0.0	0.5	1.0	2.0		0.0	0.5	1.0	2.0	
0.0	3.20	2.90	3.10	2.80	3.00	1.65	2.59	2.19	3.06	2.37
1.0	3.10	3.10	2.90	2.80	2.97	5.35	7.43	3.13	5.61	5.38
2.0	2.40	2.80	2.70	2.90	2.70	5.08	5.03	6.20	3.60	4.98
3.0	2.30	2.80	2.7	2.60	2.60	4.31	3.54	3.50	2.87	3.55
Mean (B)	2.75	2.90	2.85	2.77		4.10	4.65	3.75	3.78	
L.S.D at 5% A	0.1920					0.09952				
B	N.S.					0.09952				
AB	0.3839					0.1990				

Table.7 Effect of different GA3 concentration on shoot length and leaf number of *Hydrangea macrophylla* in vitro culture after 45 days

GA3 mg/l	Shoot length(cm)	Leaf number/explant
0.0	**1.780**	**9.760**
0.2	**2.460**	**10.00**
0.4	**2.700**	**10.16**
0.6	**3.280**	**10.72**
1.0	**4.120**	**10.16**
L.S.D at 5%level	**0.2072**	**0.7235**

Table.8 Effect of auxins type (IAA,IBA or NAA) concentrations and charcoal addition on plantlet length (cm), leaf number/ plantlet and /shootlet fresh weight (g)) of *Hydrangea macrophylla*in rooting stage

G.R	Conc.	plantlet **length (cm)**			**Leaf number**/ plantlet			plantlet **fresh weight (g)**		
		Charcoal g/l		**Mean (A)**	**Charcoal g/l**		**Mean (A)**	**Charcoal g/l**		**Mean (A)**
		0.0	**1.0**		**0.0**	**1.0**		**0.0**	**1.0**	
Cont	0.0	4.20	3.60	3.90	12.40	11.07	11.73	0.63	0.68	0.65
IAA mg/l	0.5	3.40	3.73	3.57	12.13	12.40	12.27	0.53	0.60	0.57
	1.0	4.17	2.87	3.52	11.20	10.40	10.80	0.67	0.48	0.57
	2.0	4.27	4.27	4.27	12.13	12.13	12.13	0.98	0.87	0.93
	4.0	5.17	5.87	5.52	12.13	12.80	12.47	1.14	1.08	1.11
IBA mg/l	0.5	3.50	3.70	3.60	10.67	10.67	10.67	0.93	0.78	0.85
	1.0	5.27	4.90	5.08	12.00	12.00	12.00	1.19	0.95	1.07
	2.0	4.77	4.57	4.67	11.73	12.27	12.00	1.27	1.17	1.22
	4.0	3.87	3.93	3.90	10.27	11.07	10.67	1.11	0.79	0.95
NAA mg/l	0.5	4.20	4.07	4.13	12.13	12.40	12.27	1.16	0.88	1.02
	1.0	5.00	5.17	5.08	12.27	12.40	12.33	1.51	1.11	1.31
	2.0	3.60	4.87	4.23	8.53	12.27	10.40	1.28	1.63	1.46
	4.0	3.33	3.37	3.35	7.07	10.27	8.67	1.19	0.63	0.91
Mean (B)		4.21	4.22		11.13	11.70		1.05	0.90	
L.S.D at 5% levelA		0.21			0.61			0.08		
B		0.08			0.24			0.03		
AB		0.29			0.86			0.11		

Table.9 Effect of auxins type (IAA, IBA or NAA) and charcoal addition on root number/shootlet and root length (cm) of shoots(cm, leaf) *Hydrangea macrophylla* in rooting stage

Treatments		**Root number** / plantlet			**Root length (cm)**		
Auxins	**Concentration (mg/l)**	**Charcoal g/l**		**Mean (A)**	**Charcoal g/l**		**Mean (A)**
		0.0	**1.0**		**0.0**	**1.0**	
control	0.0	3.13	3.20	3.17	4.60	3.50	4.05
IAA	0.5	2.53	2.93	2.73	3.40	2.33	2.87
	1.0	3.33	3.47	3.40	3.77	1.36	2.56
	2.0	4.13	3.67	3.90	4.80	4.40	4.60
	4.0	4.20	3.87	4.03	4.67	4.40	4.53
IBA	0.5	4.27	3.07	3.67	2.23	2.70	2.47
	1.0	5.07	4.27	4.67	3.57	4.13	3.85
	2.0	5.27	3.93	4.60	3.93	4.07	4.00
	4.0	2.87	2.87	2.87	2.87	2.27	2.57
NAA	0.5	4.87	3.40	4.13	3.13	2.87	3.00
	1.0	5.40	3.93	4.67	3.47	4.13	3.80
	2.0	3.40	4.87	4.13	2.97	4.67	3.81
	4.0	2.87	3.00	2.93	2.03	2.10	2.07
Mean (B)		3.95	3.57		3.50	3.30	
L.S.D at 5% level A		0.37			0.37		
B		0.15			0.14		
AB		0.52			0.52		

Table.10 Effect of growing media on plant length (cm), leaf number/ plant, root number/plant and root length(cm) of *Hydrangea macrophylla* ex vitro

Mix	Plant length (cm)	Leaf number/ plantlet	Root number/ plantlet	Root length (cm)
Peat moss only	**5.500**	**10.00**	**24.33**	**5.667**
Peat moss +perlite (1:1)	**6.000**	**1.00**	**50.67**	**7.333**
Peat moss +perlite(2:1)	**6.167**	**10.00**	**38.67**	**6.333**
Peat moss +perlite(3:1)	**4.500**	**11.33**	**37.67**	**5.333**
Peat moss +vermiculite(1:1)	**3.667**	**8.667**	**36.33**	**7.000**
Peat moss +vermiculite(2:1)	**4.000**	**10.00**	**37.00**	**7.667**
Peat moss +vermiculite(3:1)	**4.000**	**10.00**	**51.67**	**7.000**
Peat moss + sand(1:1)	**3.500**	**9.333**	**51.33**	**7.667**
Peat moss + sand(2:1)	**3.667**	**8.667**	**51.33**	**5.333**
Peat moss + sand(3:1)	**3.833**	**10.00**	**56.00**	**6.667**
L.S.D at 5%level	**1.151**	**N.S**	**7.422**	**1.150**

Fig.1&2 Sterilization and development of shoot tips and Sterilization and development of node

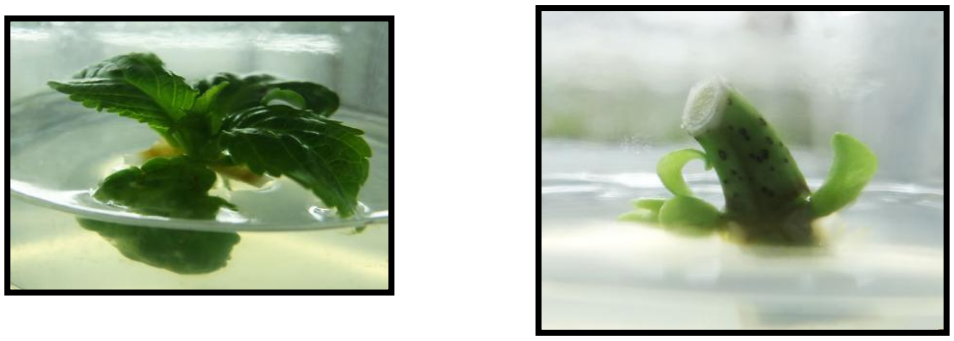

Fig.3 Effect of BA and Kin concentrations on Multiplication stage

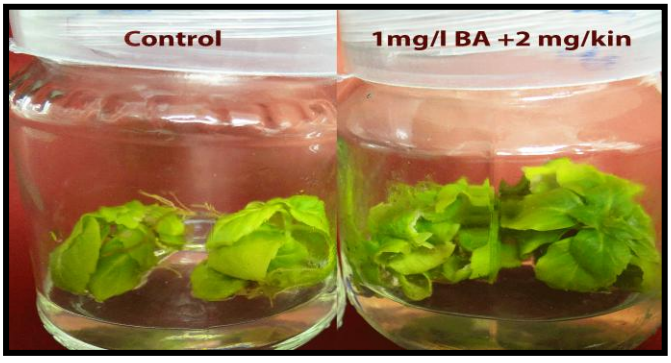

Fig.4 Effect of NAA concentration on growth and development of *Hydrangea macrophylla* in rooting stage

Fig.5 Adaptation of *Hydrangea macrophylla* plant in greenhouse

Fig.6 Flowering plant in greenhouse after 10 months

The data shown in table (9) reveal that 1.0 mg/l IBA or NAA gave the highest number of roots (4.76),while longest roots (4.6 cm) was obtained at 2.0 mg/l IAA. Using medium without charcoal produced the best values of root number and root length, (3.95) and (3.5) respectively. While NAA at 1.0 mg/l without charcoal gave the highest number of roots (5.4) and the longest root (4.8 cm) was found at 2.0 mg/l IAA without charcoal.

Feng *et al.,* (2011) reported that, these roots was exposed to the air, but had insufficient access to nutrients from the medium, resulting in slow growth. Auxins such as NAA or IBA are usually medium supplemented with 0.5 mg/l NAA. When rooted plantlets were transplanted in the greenhouse, they exhibited 100% survival rate. Sacco *et al.,* (2012) reported that, the highest *in vitro* rooting percentage was achieved in the presence of NAA 0.5 mg L-1 (100% rooting).

Acclimatization stage

Effect of different growing media on adaptation of plantlet *Hydrangea macrophylla* in greenhouse

It is clear from the data in table (10) and Fig (4) that the longest plant (6.17 cm) was achieved when peat moss: perlite (2: 1 v/v) mixture was used compared with other mixtures, while there were insignificant different between all mixtures on leaf number. The highest number of roots (56.0) was obtained when used peat moss: sand (3: 1 v/v), on the other hand, the higher mean of root length (7.67 cm) was fond when two different mixture peat moss: vermiculite (2:1 v/v) or peat moss: sand (1:1 v/v) was used. After 10 months of acclimatization, plants flowered and survival rate of each batch of plants was 100% in greenhouse Fig (5). Nguyen *et al.,* (1999) reported that, the higher growth and rooting in perlite substrate is due to the superior aeration supporting root growth. Donna and John (2004) recommended that, there was no significant effect of medium on rooting of microshoots. The mean number of roots was 13.6 and the mean percent rooting was 93%. The microshoots acclimatized and rooted well in every medium tested. Only three shoots died during acclimatization: one each in peat-zite, rockwool, and oasis blocks. Feng *et al.,* (2011) reported that, this rooting protocol could reduce costs in commercial Hydrangea micropropagation due to the lower price of perlite relative to agar.

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