

In vitro tissue culture initiation from potted and garden *Hydrangea macrophylla* explants

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ABSTRACT

The effect of sterilisation treatments on the initiation of the culture of three *Hydrangea macrophylla* genotypes *in vitro* was studied. The results indicated difference among different disinfection treatment; the consequence of treatments with dichloroisocyanuric acid, in combination with silver nitrate, was a significantly higher survival rate comparing to treatments based on NaOCl. Success of sterilisation was significantly influenced by studied genotypes. Two potted hydrangea genotypes (G2 and G3) showed higher survival rate (45.00 %, 55.00 %, respectively) comparing to the genotype 1 (originated from a local garden), which exhibited the highest percentage of contamination rate (58.75 %). Culturing the explants on a McCown woody plant medium supplemented with BAP at 2 mg/L and NAA at 0.005 mg/L produced the highest number of shoots with multiplication rate of 1.31 in 5 weeks. The highest rooting percentage (100.00 %) was obtained with the genotype 2 on a McCown woody plant medium supplemented with 0.5 mg/L IBA.

Key words: micropropagation, tissue culture, hydrangea, *Hydrangea macrophylla*, plant disinfection

INTRODUCTION

Hydrangeas (*Hydrangea macrophylla* (Thunb.) Ser., Hydrangeaceae) are potted florists plants or garden plants growing as deciduous shrubs, and are up to 2 m tall and approximately 2.5 m wide. The term 'macrophylla' means large or long leaves (the leaves can grow to 15 cm in length) (Harrison 2012). The inflorescence of *H. macrophylla* is a corymb with all individual flowers placed in a plane or hemisphere, or even a whole sphere in some cultivated forms. Blooms can be blue, red, pink, light purple, or dark purple. The colour is affected by soil pH. An acid soil (pH below 7) will usually induce blue floral colour, whereas in alkaline soil (pH above 7) flowers will be more pinkish. The colour change is associated with the presence of aluminium ions.

There are a few reports on the micropropagation of hydrangeas. Sebastian and Heuser (1987) established *in vitro* propagation of *H. quercifolia* through differentiation of leaf callus into plantlets and stimulation of axillary bud released from the dormant buds. The plantlets obtained from leaf callus were suitable for commercial propagation. Abou Dahab (2006) successfully conducted experiments

aimed at developing an *in vitro* propagation protocol for *H. macrophylla* plants. Ruffoni et al. (2013) placed *Hydrangea* spp. explants on MS medium supplemented with benzyl adenine (BA) (0.25 mg/L) and sucrose (30 g/L). The rooting of proliferated shoots was 100% on a medium containing naphthalene acetic acid (NAA) (0.5 mg/L). The main aim of this experiment was to study the effect of some disinfection treatments on the survival rate of *H. macrophylla* explants cultured *in vitro*.

MATERIALS AND METHODS

Plant material and establishment of *in vitro* culture

The plant material was obtained during April from vigorous one year old shoots of *H. macrophylla* plant growing in a local garden (G1), and from potted *H. macrophylla* plants (G2 and G3). The plant material was cut into 1 cm single node explants.

Four sterilisation treatments were evaluated for the disinfection of the plant material: (a) the explants were dipped

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in 70 % ethanol for 2 min and then 2 min immersion, with agitation, in 4.6% NaOCl containing two drops of Tween 20 (T1); (b) the explants were dipped in 70 % ethanol for 2 min and then 10 min immersion, with agitation, in 4.6% NaOCl containing two drops of Tween 20 (T2); (c) the explants were dipped in 70% ethanol for 2 min and then in solution of 16 % dichloroisocyanuric acid in combination with 8 % silver nitrate for 2 min, with agitation, containing two drops of Tween 20 (T3); (d) the explants were dipped in 70% ethanol for 2 min and then in solution of 16 % dichloroisocyanuric acid in combination with 8 % silver nitrate for 10 min, with agitation, containing two drops of Tween 20 (T4). After the application of all four sterilisation methods, the explants were rinsed into sterilised distilled water (3 times) in order to remove all traces of the sterilising substances. All manipulation of plant material was done under aseptic condition.

After sterilisation, the explants were cultured on induction media (Table 1) in petri dishes. Plants were incubated in growth chamber at 21 °C with a 15 hours photoperiod (15.000 Lux) and investigated for contamination every 3 to 4 days after incubation. Explants receiving different treatments were incubated for one month. For each treatment, the contamination rate (Cb) was calculated as a percentage of contaminated explants, survival rate (Sb) as a percentage of non-contaminated and vital explants, and deceased rate (Db) as a percentage of non-vital explants.

The experiment included 4 treatments in four replicates, with each replicate consisting of 5 explants for 3 different genotypes. The obtained results were subjected to analysis of variance (ANOVA) using Statgraphics Centurion XV (2005)

to ascertain significant differences between treatments ($P < 0.05$). In order to improve the normality of distribution all the data were arcsin-transformed. Differences between means were revealed by Duncan's multiple range test ($\alpha = 0.05$).

Proliferation

One month after induction, all vital and not contaminated explants were transferred on five propagation media (P1, P2, P3, P4, and P5), based on two different basal media (MS and McCown woody plant medium) supplemented with different plant growth regulators (Table 1). On media P1, P2, P3, and P4, 24 explants were cultured on each media in jars. Each jar contained one explant. After five weeks, there were no new shoots developed on four media used in the experiment. Because of bad regeneration on these media, all obtained explants (95) were transferred on the medium P5. After five weeks, the number of shoots per explant was recorded, and multiplication rate was calculated for each media.

Rooting *in vitro*

This experiment was carried out to study the effect of two different plant growth regulators on root formation. Two media were prepared (Table 1) one with 0.5 mg/L IBA and the other with 0.5 mg/L NAA. Shoots of the three studied genotypes, in length from 1.5 cm to 3 cm, were placed on each medium: 13 shoots of the garden *H. macrophylla* genotype 1, 8 shoots of potted *H. macrophylla* genotype 2, and 15 shoots of potted *H.*

Table 1: Composition of the media used for micropropagation of *H. macrophylla*.

Component	Induction medium	Propagation media					Rooting media	
		P1	P2	P3	P4	P5	R1	R2
MS including vitamins (g/L)	4.3	4.3	4.3	4.3	4.3	-	-	-
McCown woody plant medium	-	-	-	-	-	2.46	2.46	2.46
Sucrose (g/L)	30.0	30.0	30.0	30.0	30.0	20.0	20.0	20.0
6-benzylaminopurine (BAP) (mg/L)	-	5	2	5	4	2	-	-
2iP (mg/L)	-	2	2	2	-	-	-	-
Naphthoxyacetic acid (NOA) (mg/L)	-	-	-	0.1	-	-	-	-
Indole-3-butyric acid (IBA) (mg/L)	-	-	-	-	-	-	0.5	-
Naphthalene acetic acid (NAA) (mg/L)	-	-	-	-	-	0.005	-	0.5
L-prolin (mg/L)	200.0	200.0	200.0	200.0	200.0	-	-	-
Myo-inositol (mg/L)	400.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Nicotinic acid (mg/L)	-	-	-	-	-	1.0	1.0	1.0
Tiamin (mg/L)	-	-	-	-	-	2.0	2.0	2.0
Pyridoxine hydrochloride (mg/L)	-	-	-	-	-	1.0	1.0	1.0
Agar (g/L)	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
pH	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8

macrophylla genotype 3. The number of rooted plants was counted every 14 days.

RESULTS AND DISCUSSION

Effect of disinfection treatments on successful sterilisation and survival rate of explants

The difference among different disinfection treatment was established. The T3 and T4 (both included dichloroisocyanuric acid in combination with silver nitrate) gave significantly higher survival rate (63.33 % and 55.00, % respectively) comparing to T1 and T2 (based on NaOCl) with lower survival rate (10.00 % and 26.67 %, respectively).

The data presented in Table 2 indicate that there were no significant differences among different disinfection treatments in the genotype 1. For genotype 2, the treatment 3 resulted in significantly higher mean percentages of contamination-free explants (90.00 %). In genotype 3 there were also significant differences among different treatment methods. The highest survival rate was obtained with T3, T4, and T2 (80.00 %, 75.00 % and 55.00 %, respectively).

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In Table 3, the impacts of genotype and disinfection method on the survival rate are shown. Interaction between genotype and disinfection treatment was not statistically significant. The differences in survival rate among three genotypes were statistically significant at $P < 0.001$. The genotypes 2 and 3 had higher survival rate comparing to the genotype 1 of which the highest percentage of contamination rate was obtained (58.75 %). Those results were expected, due to the reason that G1 was growing outside, in a garden, where plants could be more contaminated comparing to potted plants (G2, G3) which were grown under controlled condition in a greenhouse. If an explant is isolated from a plant grown outside, there is a much higher chance of infection taking place (Pierik 1997).

The influence of medium composition on proliferation

Vital explants of three different genotypes were transferred on five different shoot proliferation media (Table 1). Media P1-P4 included MS (Murashige and Skoog 1962) as basal

Table 2: The effects of four different disinfection methods (T1, T2, T3, and T4) for three *H. macrophylla* genotypes (G1, G2, and G3) on the survival, contamination, and deceasing rate of explants.

	Survival rate (%)			Contamination rate (%)			Deceasing rate (%)		
	G1 ^{ns}	G2*	G3*	G1**	G2*	G3*	G1 ^{ns}	G2 ^{ns}	G3 ^{ns}
T1	5.0	15.0 b	10.0 b	90.0 a	70.0 a	70.0 a	5.0	15.0	20.0
T2	0.0	25.0 b	55.0 ab	90.0 a	60.0 a	35.0 ab	10.0	15.0	10.0
T3	20.0	90.0 a	80.0 a	35.0 b	5.0 b	15.0 b	45.0	5.0	5.0
T4	40.0	50.0 ab	75.0 a	20.0 b	50.0 a	20.0 b	40.0	0.0	5.0

***, **, * Significant at $P < 0.001$, $P < 0.01$, $P < 0.05$, respectively; ns = not significant.

Table 3: The effects of hydrangea genotype (G) and disinfection treatment (T) on the buds survival rate (Sb), contamination rate (Cb), and rate of deceased buds (Db).

Source of variation	Sb (arcsin)	Cb (arcsin)	Db (arcsin)	
Genotype (G)	***	*	ns	
Disinfection treatment (T)	***	***	ns	
G×T	ns	ns	ns	
	Mean ± SEM			
Genotype				
	G1	16.25 ± 8.21b	58.75 ± 11.18a	25.00 ± 8.66
	G2	45.00 ± 12.04a	46.25 ± 11.93ab	8.75 ± 4.46
	G3	55.00 ± 11.90a	35.00 ± 10.57b	10.00 ± 4.47
Disinfection treatment				
	T1	10.00 ± 5.77b	76.67 ± 10.96a	13.33 ± 5.69
	T2	26.67 ± 12.87b	61.67 ± 12.18a	11.67 ± 6.26
	T3	63.33 ± 12.99a	18.33 ± 9.36b	18.33 ± 9.03
	T4	55.00 ± 14.38a	30.00 ± 12.91b	15.00 ± 8.57

***, **, * Significant at $P < 0.001$, $P < 0.01$, $P < 0.05$, respectively; ns = not significant.

^{a-b} Means (± SEM) followed by different letters within particular factor are significantly different (Duncan, $\alpha = 0.05$)

medium, medium P5 was based on McCown woody plant medium (Lloyd and McCown 1980). On media with MS, no multiplication was obtained. On P5 medium, 126 shoots were obtained from 95 explants after 5 weeks, which gave multiplication rate of 1.31. For the media P1, P2, P3, and P4, the multiplication rate was 1.0. Among the three studied genotypes, no significant differences in multiplication rate were observed.

Table 4: Multiplication rate for *H. macrophylla* on five different media (n=24).

Medium	Number of explants	Number of shoots after 5 weeks	Multiplication factor
P1	24	24	1
P2	24	24	1
P3	24	24	1
P4	23	23	1
P5	95	126	1.33

Effect of different media on rooting

Shoots in size of 1.5 to 3 cm were placed on two rooting media with McCown woody plant medium supplemented with IBA at 0.5 mg/L (R1) and McCown woody plant medium supplemented with NAA at 0.5 mg/L (R2) (Table 1). In the case of genotype 1, more shoots developed roots on the medium R2 (62.5 %) comparing to the medium R1 (50.00 %). The best genotype for rooting was genotype 2, where the percentage of rooted plants was the highest on R1 medium (100.00 %). In genotype 3, 66.67 % of rooted shoots were obtained on medium R1.

CONCLUSIONS

In the present study the method to initiate *in vitro* culture of potted and garden *H. macrophylla* and its multiplication and *in vitro* rooting was studied. The presented results indicate that the effective *in vitro* regeneration of *H. macrophylla* was established. Based on the results shown above, it is possible to conclude that there was a significant impact of the studied genotypes on successfully obtained sterile culture of *H. macrophylla*. When using potted plants, the number of successfully disinfected explants was much higher (up to 55 %) comparing to those grown in a garden (with 16.25 % disinfected and survived explants). Disinfection treatments with dichloroisocyanuric acid in combination with silver nitrate gave a significantly higher survival rate comparing to treatments based on NaOCl. The successful shoot multiplication was achieved on McCown woody plant medium, supplemented with BAP at 2 mg/L and NAA at 0.005 mg/L. The best rooting was obtained with genotype 2 on McCown woody plant medium, supplemented with 0.5 mg/L IBA (100.00 % of rooted plants).

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Iniciacija rastlinske tkivne kulture lončne in vrtno hortenzije (*Hydrangea macrophylla*) v in vitro razmerah

IZVLEČEK

Proučevali smo vpliv različnih načinov sterilizacije na uspešno vzpostavitev tkivne kulture treh genotipov hortenzije (*Hydrangea macrophylla*). Ugotovili smo, da obstajajo razlike med različnimi načini sterilizacije. Sterilizacija z dikloroizocianurno kislino v kombinaciji s srebrovim nitratom je bila uspešnejša od sterilizacije z uporabo NaOCl. Na uspešnost sterilizacije je vplival tudi genotip. Lončne hortenzije (genotipa G2 in G3) so kazale večjo sposobnost sterilizacije (pri genotipu G2 smo tako dobili 45.00 %, pri G3 pa 55.00 % uspešno steriliziranih poganjkov) v primerjavi z vrtno hortenzijo, kjer je bilo največ okuženih poganjkov (58.75 %). Za namnoževanje poganjkov se je kot najboljše izkazalo gojišče McCown z dodatkom 2 mg/L BAP in 0.005 NAA, na katerem je bil faktor namnoževanja 1,31 v petih tednih. Najbolje je koreninil genotip 2 na McCown osnovnem gojišču z dodano 0,5 mg/L IBA in sicer je na tem gojišču koreninilo 100 % poganjkov.

Ključne besede: mikropropagacija, tkivna kultura, hortenzija, *Hydrangea macrophylla*, sterilizacija rastlin