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RESEARCH NOTE

Shoot organogenesis in leaf explants of *Hydrangea macrophylla* 'Hyd1' and assessing genetic stability of regenerants using ISSR markers

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Abstract For the first time, an in vitro regeneration protocol of *Hydrangea macrophylla* 'Hyd1' was developed. Effects of different plant growth regulators (PGRs) on shoot regeneration were investigated jointly with selecting optimal basal media and cefotaxime concentrations. The highest frequency of shoot organogenesis (100%) and mean number of shoots per explant (2.7) were found on Gamborg B5 basal medium supplemented with 2.25 mg/l 6-benzyladenine (BA), 0.1 mg/l Indole-3-butyric acid (IBA), 100 mg/l cefotaxime and 30 g/l sucrose solidified by 7 g/l agar. Regenerated shoots were rooted by culturing on perlite plus half strength liquid B5 basal medium with 0.5 mg/l NAA. Rooted plantlets were transplanted to the greenhouse with 100% survival rate. Genetic stability of 32 plantlets (one mother plant and 31 regenerants) was assessed by 44 ISSR markers. Out of 44 ISSR markers, ten markers produced clear, reproducible bands with a mean of 5.9 bands per marker. The in vitro regeneration protocol is potentially useful for the genetic transformation of *Hydrangea macrophylla* 'Hyd1'.

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Keywords *Hydrangea macrophylla* 'Hyd1' · In vitro regeneration · Somaclonal variation · ISSR

Abbreviations

B5	Gamborg B5 medium
BA	6-benzyladenine
IAA	Indoleacetic acid
IBA	Indole-3-butyric acid
ISSR	Inter simple sequence repeat
MS	Murashige and Skoog medium
NAA	α-Naphthalene acetic acid
PCR	Polymerase chain reaction
RAPD	Random amplified polymorphic DNA
WPM	Woody plant medium

Introduction

Hydrangea species originate from China and Japan (Kesumawati et al. 2006), and are well known ornamental plants in Europe, North America and Asia. They are grown commercially as potted plants and as cut flowers with large, round flowerheads of various colours ranging from white to purple, pink and red. Production schedules to fulfill the demands for holiday markets at Christmas, Valentine's Day, Easter and Mother's Day are well established by mimicking the natural phenology, subjecting plants to specific temperature and humidity regimes (Bailey 1989). Low temperature (4–7°C) and high humidity are indispensable for flower bud breaking (Guo et al. 1995). During this cold period, *Hydrangea* is vulnerable to the fungus *Botrytis*. As the cold period is unavoidable, breeders are turning to

genetic engineering to increase resistance to *Botrytis*. For *Hydrangea*, a transformation system for in vivo analysis of gene functions is not yet available. Therefore, the development of an efficient regeneration system is a prerequisite for genetic modification of this important ornamental plant.

As with many woody species, the establishment of an efficient regeneration system in *Hydrangea* has not yet been successful. Very little has been reported on the micropropagation and regeneration of *Hydrangea* in vitro. Sebastian and Heurser (1987) reported that if unfolded new leaves from *Hydrangea quercifolia* were placed on MS medium (Murashige and Skoog 1962) with 10 μM zeatin combined with gibberellic acid (GA_3) and indoleacetic acid (IAA), leaves would differentiate into callus and shoots would proliferate. Those experiments showed that adventitious shoots could form from leaf callus, but the detailed data on adventitious shoot formation are lacking. Ledbetter and Preece (2004) reported effects of Thidiazuron (TDZ) on *H. quercifolia* adventitious shoot production. Combining MS medium with 1 μM IBA, and a low concentration of TDZ (0.05 μM) stimulated the formation of fewer, but more elongated shoots, whereas the highest concentration of TDZ (5 μM) resulted in leaf explants heavily callused and with many tiny short shoots. However, for practical applications in *Hydrangea* propagation this phytohormone is currently too expensive. Doil et al. (2008) reported that 6-benzyladenine (BA) rather than TDZ could induce higher regeneration rates for *Hydrangea macrophylla* 'Nachtigall'. Boccon-Gibod et al. (2000) found that sugar was an important factor determining regeneraton of the *Hydrangea* cultivars 'Blaumeise', 'Messalina' and 'Red Barons'. They reported that maltose and sucrose out of five sugars gave the best regeneration rate of approximately 80%.

Somaclonal variation can occur during in vitro culture and regeneration. Molecular marker techniques are at present powerful and reliable tools to analyze genetic stability of in vitro regenerants. Randomly Amplified Polymorphic DNA (RAPD), Simple Sequence Repeat (SSR), flow cytometric analysis and morphology analysis (Jin et al. 2008) can be used to determine stability of regenerants. The Inter Simple Sequence Repeats (ISSR) technique has been used most frequently, because of its reproducibility, simplicity and cost-effectiveness. Applications of ISSR have been well documented by Joshi and Dhawan (2007) and Lakshmanan et al. (2007).

Cefotaxime is an antibiotic used to control *Agrobacterium tumefaciens* after infection to induce plant genetic transformation. Cefotaxime is known to generally reduce or even inhibit plant regeneration, except in wheat where it promotes regeneration (Yu and Wei 2007). It may be important, therefore, to test effects of different concentrations on regeneration of different plant species prior to using it in transformation experiments.

The aim of the study presented here was to develop a reproducible and efficient regeneration system for *H. macrophylla* 'Hyd1', which is one of the elite cultivars of the *Hydrangea* Breeders Association B.V. in the Netherlands. The ability of the regeneration protocol of 'Hyd1' to deliver genetically identical and stable regenerants was assessed by ISSR markers. The study provides the first necessary step for the establishment of a genetic transformation system for *Hydrangea* by *Agrobacterium tumefaciens*.

Materials and methods

Plant materials and establishment of stock cultures

Shoot buds were obtained from elite plants of *H. macrophylla* 'Hyd1' growing in the greenhouses of Agriom B.V. in the Netherlands. They were washed with running tap water for 60 min, soaked in 70% (v/v) ethanol for 1 min, containing 1% (v/v) sodium hypochlorite and 5–10 drops/l of Tween-20 as wetting agent for 20 min and, finally, they were rinsed six times with sterile distilled water. Under laminar flow, meristem tips were isolated from the shoot buds using a stereomicroscope. Tips, approximately 1 mm in length, were cultivated axenically on half strength Gamborg B5 basal medium (Gamborg et al. 1968) without plant growth regulator (PGR) and supplemented with 10 g/l sucrose and 7 g/l agar. After 60 days of culture, shoot buds and stems obtained were excised and re-cultivated in the same basal medium solidified with 7 g/l agar for shoot propagation. The stock culture was sub-cultured at 30 days intervals. The pH of media was adjusted to approximately 6.0 before autoclaving at 121°C for 20 min.

Induction of adventitious shoots on 'Hyd1' leaf explants

Two pairs of leaves unfolding from the apical meristem were excised from stock cultures in vitro (Fig. 1a). Each leaf explant with the petiole and leaf tip removed was given a transverse cut twice across the middle vein (Fig. 1a) and placed with their abaxial sides on the medium.

In this culturing stage, we evaluated plant growth regulators PGRs types and concentrations, basal medium composition and cefatoxime effects on shoot regeneration.

Effects of PGRs-Incubating leaf explants on a B5 basal medium supplemented with 30 g/l sucrose and 7 g/l agar against various combinations of PGRs: 0, 1.0, 2.25, and 3.5 mg/l BA in combination with 0, 0.05, 0.1, and 0.2 mg/l Indole-3-butyric acid (IBA), or with 0, 0.05, 0.1, and 0.2 mg/lα-Naphthalene acetic acid (NAA) were investigated (Table 1).

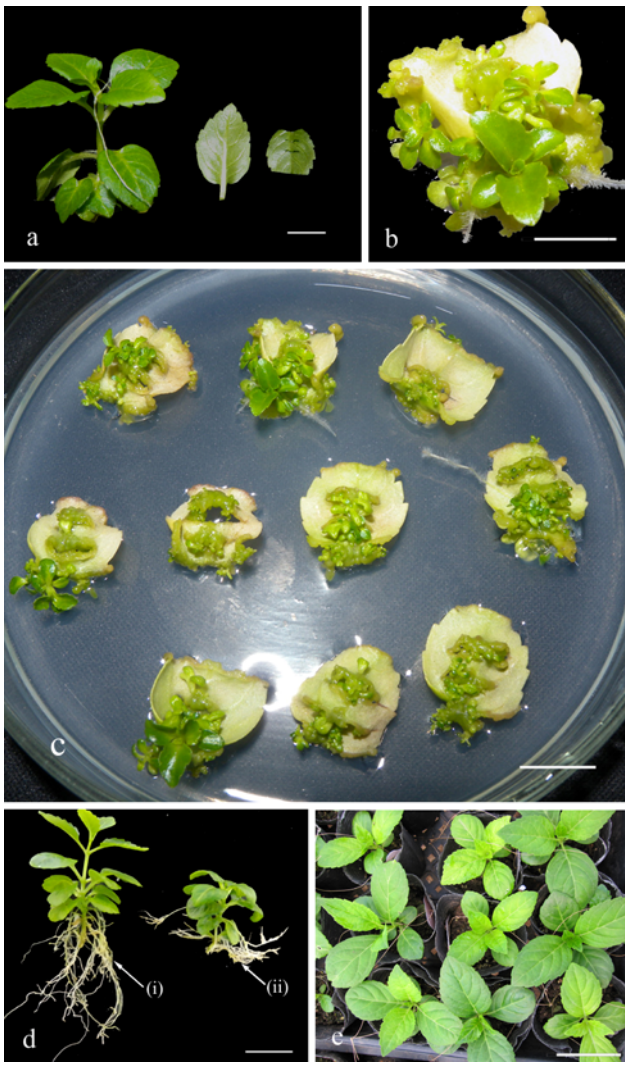

Fig. 1 In propagation of *Hydrangea macrophylla* 'Hyd1' via direct organogenesis from the leaf explants. **a** Shoot propagated from a meristem of 'Hyd1' stock culture, and two leaf explants derived from the *top two pairs* of leaves of the apical shoot. The leaf on the *right* shows the way the explant was cut before cultivation on the media (Bar: 1 cm). **b** Adventitious shoots regenerated from the cut edge of leaf explant of 'Hyd1' (Bar: 1 cm). **c** Induction of adventitious shoots via direct organogenesis on B5 medium supplemented with 2.25 mg/l BA and 0.1 mg/l IBA 50 days after culture (Bar: 1 cm). **d** Comparison of rooted plantlets (*i*) cultured on perlite: liquid half strength B5 basal medium = 2:1 (v/v) plus 0.5 mg/l NAA and (*ii*) cultured on half strength B5 basal medium containing 0.5 mg/l NAA, 30 g/l sucrose and solidified with 7 g/l agar (Bar: 1 cm). **e** Acclimatized plantlets growing ex vitro conditons (Bar: 5 cm)

Table 1 The effect of PGRs on shoot formation from leaf explants of *H. macrophylla* 'Hyd1'

IBA (mg/l)	NAA (mg/l)	BA (mg/l)	Organogenes frequency (%)	Mean no. of shoots per explant
–	–	–	0 ± 0k	0 ± 0f
–	–	1.0	17 ± 2.9gh	1.0cd
–	–	2.25	17 ± 2.9gh	1.0cd
–	–	3.5	8.3 ± 5.0ij	1.0cd
0.05	–	–	0 ± 0k	0 ± 0f
0.05	–	1.0	3.3 ± 2.9jk	0.7 ± 0.6de
0.05	–	2.25	33 ± 2.9d	1.4 ± 0.2bc
0.05	–	3.5	50 ± 2.9c	1.1 ± 0.2cd
0.1	–	–	0 ± 0k	0 ± 0f
0.1	–	1.0	13 ± 2.9hi	1.1 ± 0.2cd
0.1	–	2.25	79 ± 2.3a	2.2 ± 0.3a
0.1	–	3.5	23 ± 2.9ef	1.2 ± 0.2c
0.2	–	–	0 ± 0k	0 ± 0f
0.2	–	1.0	12 ± 5.8hi	1.0cd
0.2	–	2.25	12 ± 2.9hi	1.0cd
0.2	–	3.5	26 ± 2.1ef	1.1 ± 0.1cd
–	0.05	–	0 ± 0k	0 ± 0f
–	0.05	1.0	27 ± 7.6e	1.0cd
–	0.05	2.25	67 ± 5.8b	1.7 ± 0.1b
–	0.05	3.5	45 ± 5.0c	1.6 ± 0.2b
–	0.1	–	0 ± 0k	0 ± 0f
–	0.1	1.0	20 ± 5.0fg	1.0cd
–	0.1	2.25	33 ± 2.9d	1.4 ± 0.2bc
–	0.1	3.5	5.0 ± 5.0jk	0.7 ± 0.6de
–	0.2	–	0 ± 0k	0 ± 0f
–	0.2	1.0	0 ± 0k	0 ± 0f
–	0.2	2.25	5 ± 5jk	0.7 ± 0.6de
–	0.2	3.5	1.7 ± 2.9k	0.3 ± 0.6ef

Means within the same column with different letters are significantly different at 0.05 level of probability based on Duncan's multiple range test

Effects of basal media-Basal media B5 (Gamborg et al. 1968), MS (Murashige and Skoog 1962) or WPM (Lloyd and McCown 1980) supplemented with 30 g/l sucrose, 7 g/l agar, and 2.25 mg/l BA and 0.1 mg/l IBA were tested (Table 2).

Effects of cefotaxime sodium salt (BBI, Canada)-Concentrations of 0, 100, 150, 200 and 250 mg/l in B5 medium supplemented with 2.25 mg/l BA, 0.1 mg/l IBA, 30 g/l sucrose and 7 g/l agar were investigated (Table 3).

The pH of the medium was adjusted to 6.0 before autoclaving at 121°C for 20 min. Cefotaxime sodium salt was sterilized by filtration and added to the autoclaved medium when it temperature was about 50°C.

Culture conditions

Stock culture and leaf explants were initially incubated in darkness in a culture chamber at 25 ± 2°C. Subsequently, explants were incubated under a 14/10-h (light/dark) photoperiod with light supplied by cool-white fluorescent lighting at an intensity of 60 μmol m^{-2} s^{-1}.

Table 2 Effect of basal medium on shoot obtained from the leaf explants of *H. macrophylla* 'Hyd1'

Basal medium	Organogenes frequency (%)	Mean no. of shoots per explant
B5	77 ± 5.0a	2.1 ± 0.2a
WPM	32 ± 2.9b	1.4 ± 0.1b
MS	5.0 ± 1.0c	1.0 ± 0c

Means within the same column with different letters are significantly different at 0.05 level of probability based on Duncan's multiple range test

Table 3 Effect of cefotaxime on shoot formation from leaf explants of *H. macrophylla* 'Hyd1'

Cefotaxime (mg/l)	Organogenes frequency (%)	Mean no. of shoots per explant
0	74 ± 4.0b	1.9 ± 0.1b
100	100a	2.7 ± 0.1a
150	80 ± 8.7b	2.8 ± 0.5a
200	83 ± 7.6b	2.8 ± 0.1a
250	87 ± 10b	2.4 ± 0.2a

Means within the same column with different letters are significantly different at 0.05 level of probability based on Duncan's multiple range test

Rooting and acclimatization

Regenerated shoots were excised from individual leaf explants when they were 3–5 mm long, and transferred to vessels containing 30 ml half strength B5 basal medium supplemented with 10 g/l sucrose and solidified with 7% agar for shoot growth. Shoots of 1 cm were used to optimize in vitro rooting of 'Hyd1'. Eight treatments were tested: Liquid half strength B5 basal medium was added to perlite in a ratio of 1:2 (v/v) with 4 different concentrations of NAA (0.2, 0.5, 1.0 and 2.0 mg/l), and in a second series half strength B5 basal medium with 4 different concentrations of NAA (0.2, 0.5, 1.0 and 2.0 mg/l) was supplemented with 3% sucrose, and solidified with 7% agar. Rooted plantlets of 3 cm were transplanted to plastic pots containing autoclaved vermiculite-soil (1:1 v/v). The pots were covered with plastic film for 2 weeks, and then moved to a greenhouse with watering at 2–3 days intervals.

DNA extraction and PCR amplification conditions

Total DNA was extracted from leaf material of stock cultures that were used as leaf explants for the regeneration experiments, and from 31 regenerants growing in vitro according to Aljanabi and Martinez (1997). Quality and quantity of DNA was monitored by spectrophotometry and gel inspection. Each sample was diluted in 0.5 × TE-buffer (Fermentas, Vilnius, Lithuania) at concentrations ranging from 300 to 900 mg/l and stored at 4°C. Ten primers were selected from a total of 44 ISSR primers (Sangon Biotech Inc, Shanghai, China) for genetic analysis (Table 5). ISSR amplifications were performed in a volume of 20 µl containing 20–30 ng template DNA, 1.0 µM primer, 1×reaction buffer (Fermentas), 2.0 mM $MgCl_2$, 0.3 mM dNTPs and 1U *Taq* DNA polymerase (Fermentas). The amplification reaction consisted of an initial denaturation step at 94°C for 3 min, followed by 38 cycles of denaturation at 94°C for 30 s, annealing at 50–57°C (Table 5) for 1 min, extension at 72°C for 90 s and a final extension at 72°C for 10 min. Amplifications were performed in a PTC-100 thermocycler (Bio-RAD, Singapore). DNA amplification products were separated in a 2% agarose gel using 1×TAE buffer and stained with ethidium bromide (EB).

Experimental design and data analysis

Each regeneration experiment was replicated three times with at least 20 leaf explants per treatment. The frequency of shoot organogenesis and mean number of shoots per explant were determined 50 days after inoculating leaf explants on different media. For the rooting experiment, 16 shoots were selected per treatment. The experiment was done in two replications. All data were analyzed by SAS v. 8.0. Analysis of variance (ANOVA) was used to test the statistical significance, and the significance differences of means was calculated using Duncan's multiple range test at $P \leq 0.05$ level. For genetic analysis, only clear and reproducible bands were scored. Data were scored as 1 for the presence and 0 for the absence of a DNA band in each sample. The similarity coefficients were determined using the NTSYSpc 2.10e software package (Rohlf 2000).

Results and discussion

Effects of different PGR combinations on adventitious shoot induction

To generate in vitro materials explants were sterilized from elite lines growing in the greenhouse. Contaminated shoot tips were observed and removed within 1–3 weeks. Clean healthy shoots of 'Hyd1' were used for micro-propagation (Fig. 1a). Most cultured nodal segments produced 1–3 shoots from axillary buds within approximately 1 week. One month later, leaves from these shoots were used as explants for plant regeneration (Fig. 1a).

Leaf explants from proliferating shoots were inoculated on different adventitious shoot induction media. After

30 days in culture, adventitious shoot buds emerged directly from the cut edges of leaf explants (Fig. 1b, c). Among all 28 treatments (Table 1), the combination of 2.25 mg/l BA and 0.1 mg/l IBA resulted in the highest regeneration efficiency (78.7% of leaf explants to form adventitious buds) and the highest mean number of shoots per explant (2.2 shoots per explant) (Table 1). The second highest regeneration efficiency (67%) was achieved by a treatment of 2.25 mg/l BA and 0.05 mg/l NAA (Table 1). Prolific roots were observed on explants on medium with NAA combinations. Leaf explants which spontaneously produced roots lost the capacity of shoot organogenesis during subculturing (data not shown). ANOVA analysis demonstrated significant differences for both frequency of shoot organogenesis and mean number of shoots per explant among combination of 2.25 mg/l BA and 0.1 mg/l IBA, the rest combinations of BA and IBA and combinations of BA and NAA.

Early research by Skoog and Miller (1957) demonstrated that the developmental fate of regenerating tobacco pith cells in tissue culture could be directed by the balance of cytokinin and auxin added to the growth medium. In relative terms, higher cytokinin/auxin ratios promoted shoot formation, and lower ratios favored the formation of roots. In the present study, the regeneration frequencies increased when the ratios of BA/IBA increased from 1.0/0.05 to 3.5/0.05 (Table 1). However, organogenesis frequencies displayed a parabolic shape when combinations of BA/IBA varied among 2.25/0, 2.25/0.05, 2.25/0.1 and 2.25/0.2 (Table 1). This indicated that specific BA/IBA ratios were more optimal for shoot formation than lower or higher ones. The strong influence of increasing BA concentrations on shoot formation in treatments relative to fixed IBA or NAA concentrations was described in a regeneration study of pistachio too (Tilkat and Onay 2009), and similarly for somatic embryogenesis and organogenesis in callus cultures of *Albizzia richardiana* King (Tomar and Gupta 1988). IBA was more effective than NAA on *Euphorbia esula* shoot induction (Xu et al. 2008). Our results similarly demonstrate that also for *H. macrophylla* 'Hyd1', IBA has the strongest positive influence on shoot induction compared to NAA. Ledbetter and Preece (2004) reported that TDZ had a highly significant effect on production of adventitious shoots and dry weight accumulation when *Hydrangea quercifolia* leaf explants were used. However, pre-experiments showed that the regeneration rate could not be improved further by replacing TDZ with Kinetin when our explants were used (data not shown).

Effect of basal media on adventitious shoot induction

The composition of the basal media B5, WPM and MS significantly influenced the efficiency of adventitious shoot formation and mean number of shoots per explant. 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 (Table 2). These results were coincided with Doil et al. (2008) who reported that half strength B5 medium was more effective than MS medium on the shoot induction of *Hydrangea macrophylla* 'Nachtigall'. B5 medium might led to a higher frequency of shoot formation of 'Hyd1' leaf explants due to the lower concentration ammonium salts compared to MS and WPM media. WPM was reported to be an appropriate medium for woody plants regeneration (Gu and Zhang 2005), but apparently not for *H. macrophylla* 'Hyd1' which is also considered a woody plant species.

Effect of cefotaxime on adventitious shoot induction

Results of regeneration experiments using the bacterial antibiotic cefotaxime showed that shoot formation was most efficient (100%) on medium supplemented with 100 mg/l cefotaxime (Table 3). Higher cefotaxime concentrations (150, 200, 250 mg/l) reduced organogenesis frequencies to 80–87% (Table 3). These results interestingly demonstrated that cefotaxime did not inhibit 'Hyd1' shoot induction, but could even promote the regeneration. Similar results were obtained for wheat mature embryo regeneration (Yu and Wei 2007). In this report, a possible explanation was made that cefotaxime was converted by cell motebolism to an unknown compound with phytohormone activity. But no convincing evidence was presented. Agrobacterium *tumefaciens* kill curves indicated that 200 mg/l cefotaxime inhibits bacterial growth satisfactorily (data were not shown) and should be used in future transformation experiments.

Rooting and acclimatization

For any micropropagation protocol, successful rooting of in vitro microshoots is a pre-requisite to facilitate establishment in soil. Comparing perlite and agar as medium for rooting and subsequent transplanting, shoots of 'Hyd1' embedded in perlite performed much better than in agar (Fig. 1d). This result was similar to a report about rooting of in vitro shoots of *Simmondsia chinensis* (Mills et al. 2009). The higher growth and rooting in perlite substrate is due to the superior aeration supporting root growth (Nguyen et al. 1999). Shoots cultivated in agar only developed thin short roots on the stem section outside the medium rather than on the stem section inside the medium (Fig. 1d). 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

Table 4 Effect of different media and levels of NAA on roots induction from shoots of *H. macrophylla* 'Hyd1'

Media	NAA (mg/l)	Root number (≥0.2 cm)	Root length (≥0.2 cm)
P: L1/2B5[a]	0.2	6.6 ± 1.7a	1.5 ± 0.3a
	0.5	7.3 ± 0.9a	1.1 ± 0.2b
	1.0	5.8 ± 0.9a	0.9 ± 0b
	2.0	4.1 ± 1.1b	0.5 ± 0.1c
S 1/2B5[b]	0.2	3.4 ± 0.7bc	0.9 ± 0.1b
	0.5	2.2 ± 0.4 cd	0.5 ± 0.1c
	1.0	1.7 ± 1.5d	0.4 ± 0.2c
	2.0	1.0 ± 0.8d	0.3 ± 0.2c

Means within the same column with different letters are significantly different at 0.05 level of probability based on Duncan's multiple range test

[a] Perlite: liquid half strength Gamborg's B5 medium (v/v)=2:1

[b] Half strength Gamborg's B5 medium plus 3% sucrose and solidified by 7% agar

Table 5 List of ISSR primers used to score genetic variation in 'Hyd1'

Primers	5′-3′ motif	Annealing temperature (°C)	Number of scorable bands per primer	Size range (bp)
P836	$(AG)_8YA$	55	6	300–1,100
P840	$(GA)_8YT$	57	5	300–1,000
P846	$(CA)_8RT$	53	5	200–1,000
P848	$(CA)_8RG$	57	4	250–1,200
P856	$(AC)_8YA$	55	8	400–1,100
P859	$(TG)_8RC$	57	5	250–1,000
P864	$(ATG)_6$	56	8	250–1,700
P866	$(CTC)_6$	56	6	300–1,400
P873	$(GACA)_4$	54	4	300–1,200
P876	$(GATA)_4$	50	8	250–1,400

used as root induction PGRs. Higher NAA concentrations reduced root number and root length in both agar and perlite. The highest root number (7.3) was found in perlite medium supplemented with 0.5 mg/l NAA (Table 4). When rooted plantlets (Fig. 1e) were transplanted in the greenhouse, they exhibited 100% survival rate. This rooting protocol could reduce costs in commercial *Hydrangea* micropropagation due to the lower price of perlite relative to agar.

Somaclonal variation analysis

Forty-four ISSR primers were used to screen for the regeneration plantlets. Only 10 primers gave more than four clear and scorable bands, and were used for ISSR-PCR. Fifty-nine scorable bands were selected, ranging in size from 250 bp to 1.7 kb (Table 5). The number of bands for each primer varied from 4 to 8, with an average of 5.9 bands per ISSR primer. A total of 1,857 bands were generated by ISSR techniques, giving rise to monomorphic patterns across 32 plantlets analyzed. Similarity coefficients among 32 samples ranged from 0.980 to 0.983 with a mean of 0.982. Thirty-two regenerants did not deviate at all from the parental genotype. Figure 2 is an example of monomorphic bands obtained with ISSR primers.

Many studies have used PCR-based techniques such as SSR, RAPD and AFLP to determine somaclonal variation of regenerants. The variations can be due to gene amplification, chromosomal irregularities, point mutation and alteration in DNA methylation during in vitro culture (Saker et al. 2000). Genetic stability was reported for plants such as gerbera micropropagated from capitulum, leaf and shoot tips (Bhatia et al. 2009), a monopodial orchid hybrid with multiple shoots induced from seedlings (Kishor and Devi 2009), micropropagation in banana (Lakshmanan et al. 2007), *Cymbopogon flexuosus* regenerated from somatic embryogenesis (Bhattacharya et al. 2008) and almond plantlets propagated from axillary branches (Martins et al. 2004). In the case of *Hydrangea* 'Hyd1', ISSR markers were used to evaluate the genetic stability of plantlets regenerated through direct organogenesis. The results obtained suggested that direct organogenesis on 'Hyd1' leaf explants induced by BA and IBA carry a low risk of generating somaclonal variants. A further study of phenotypical

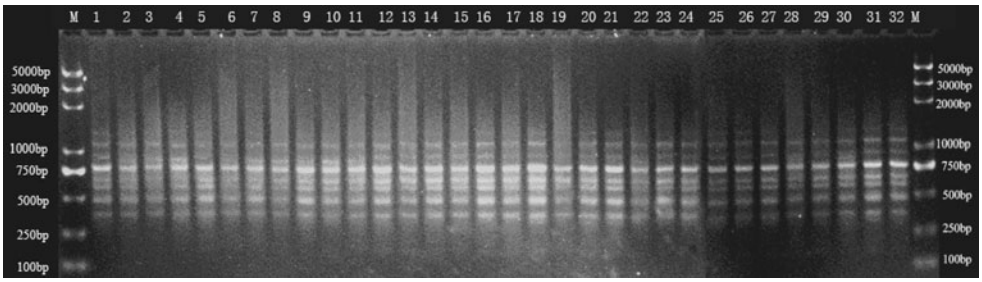

Fig. 2 Inter simple sequence repeats (ISSR) amplification pattern obtained for a mother plant (1), and regenerants (2–32) of in vitro *Hydrangea macrophylla* 'Hyd1' using primer P856. M: DL2000

differences in plant habit and flowering of 'Hyd1' regenerants will be done as additional indexes of true-to-type micropropagation.

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