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RESEARCH ARTICLE

Standardization of an efficient protocol for rapid *in vitro* micropropagation of hydrangea (*Hydrangea macrophylla* Thunb. Ser)

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Abstract

Micropropagation is a valuable tool for producing large numbers of clonal plants via tissue culture through morphogenesis induction. This work aimed to obtain an efficient protocol for *in vitro* regeneration and micropropagation of *Hydrangea macrophylla*. Organogenesis in *H. macrophylla* was achieved on Murashige and Skoog (MS) medium fortified with 2.5 ppm 6-benzylamino purine (BAP), where the minimum number of days for shoot initiation (17.33), maximum number of shoots per explant (2.30), highest number of leaves per explant (8.37) and highest fresh weight (6.26 g) was recorded. Multiple shoot production with combination of MS with 3.5 ppm BAP gave the maximum number of shoots initiated per explant (3.82), maximum number of leaves per explant (16.26) during first sub culturing and during second sub culturing same media combination gave the maximum number of shoots per explant (4.32), maximum number of leaves per explant (21.13) and shoot length (3.48 cm) was recorded. Regenerated shoots rooted best on MS medium fortified with 0.5 ppm naphthaleneacetic acid (NAA), which produced the maximum number of roots (4.54), maximum rooting percent (92.53 %) and early root initiation in (16.24 days). The longest root length (3.91 cm) was recorded on MS medium with 1.0 ppm NAA.

Keywords: hydrangea; *in vitro*; micropropagation; organogenesis; proliferation; tissue culture

Introduction

Hydrangea macrophylla is a widely known perennial summer-flowering ornamental shrub that has a large inflorescence belonging to the family Hydrangeaceae. *Hydrangea* is a genus of ornamental plants cultivated and used traditionally in landscaping and garden work and it is increasingly marketed as potted plants, fresh and dried cut flowers. As a potted plant, *H. macrophylla* inflorescences are spectacular with pink, blue, or white colored hemispherical heads. The flower color of hydrangeas is influenced by the pH level of the soil (1).

Seed-propagated *H. macrophylla* often show reduced quality and vigor in later generations due to genetic segregation. Propagation by stem cuttings is slow, season-dependent, has a low multiplication rate and carries a high risk of disease transmission.

Micropropagation is the *in vitro* culture of plant tissues of the selected plants, which are cultured under aseptic conditions in a medium that comprises macro and micronutrients to yield plants that are disease-free and true to type (2). Consequently, while micropropagation of hydrangeas enables high propagation success rates, it also carries a risk of somaclonal variation, which may affect the stability of quality traits (3).

The present study aimed to develop an efficient protocol for producing quality planting material of *H. macrophylla*. Given its horticultural potential, the species may be suitable for commercial cultivation and greenhouse pot culture under agro-climatic conditions of Bhubaneswar.

Materials and Methods

The experiment to develop an *in vitro* propagation protocol for *H. macrophylla* was conducted (2021-2024) at the Department of Floriculture and Landscaping, Biotechnology-cum Tissue Culture Centre (BTCC), Baramunda, College of Agriculture, Odisha University of Agriculture and Technology (OUAT), Bhubaneswar. The mother plants were grown naturally under shade net house in pots at the Department of Floriculture and Landscaping, College of Agriculture, OUAT, Bhubaneswar and for initial establishment of cultures, various explants, viz., nodes and shoot tips, were utilized from healthy and disease-free plants. Explants were washed under running tap water, treated with 0.1 % Tween -20 for 15 min and then rinsed with double-distilled water. Pretreatment of explants with 0.2 % Bavistin and 0.2 % Ridomil for 30 min, followed by treatment with mercuric chloride ($HgCl_2$)

(0.1 %) for 6 min effectively controlled contamination. Shoot tips and nodal segments were standardized as explants for culture establishment.

Culture establishment and organogenesis

Explants were inoculated on the MS medium supplemented with various proportions of cytokinin, 6-benzylamino purine (BAP), for culture establishment. Based on the result, the best media was selected for further subculturing at an interval of 3 weeks, taking single elongated shoot from their respective cultures. Observations on days to shoot initiation, number of shoots per explant, shoot length, number of leaves per explant and leaf color were recorded after 6 weeks of inoculation.

Shoot proliferation and multiplication

Once the shoots were initiated, the shoot clumps were subdivided into uniform-sized microshoots that were subsequently inoculated on proliferation media supplemented with various combinations of BAP. At each subculture, single elongated shoots were excised and transferred to the second subculture at 3-week intervals. Number of shoots per explant, number of leaves per multiple shoot, shoot length (cm) and leaf color are the observations taken after each subculture.

Rhizogenesis

Rhizogenesis of uniform, healthy micro shoots of about 4 cm height after second subculturing was standardized on rhizogenesis media containing MS (full salt strength) and containing various levels of auxins indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and naphthaleneacetic acid (NAA). Rhizogenesis percentage and roots per plantlet, root length, color and days to root initiation were studied during the course after 30 days for the study of plant growth regulators on root induction.

Media preparation and cultural conditions

The nutrient media, MS used contained 3 % sucrose. The pH of MS media was adjusted to 5.8 by adding hydrochloric acid (HCl) or sodium hydroxide (NaOH) before autoclaving, then 0.8 % (w/v) agar was added and dispensed into flasks and sterilized in an autoclave at 121 °C for 20 min (15 lbs/ inch^{-2}). All experimental explants were subcultured every four weeks and incubated in a growth room at 25 ± 1 °C and 70 % relative humidity with a photoperiod of 16-8 hr (day-night) cycle under fluorescent light of 3000-3200 lux intensity lamps.

Statistical analysis

Data were analyzed using a completely randomized design (CRD) with 3 replications. Twenty explants were cultured per replication. Statistical analysis was performed using OPSTAT software.

Results and Discussion

Initial shoots were raised *in vitro* from nodal segments and shoot tip explants, which were employed for the purpose. Several hormonal combinations were used for organogenesis of *H. macrophylla* comprising cytokinin (BAP at the concentration of 0.5, 1.0, 1.5, 2.0 and 2.5 ppm) (Table 1). Significantly minimum number of days for shoot initiation i.e., 17.33 and highest number of leaves per plant (8.37) was achieved at 2.5 ppm BAP (T_6) and highest fresh weight (6.26 g) was achieved at 2.5 ppm BAP (T_6) which is at par with treatment (T_3) (6.15 g) using 1.0 ppm BAP, lowest shoot length (1.96 cm) in treatment (T_5) but maximum shoot length (3.07) was observed in control (T_1) and maximum number of shoots i.e., 2.30 per explant with 2.5 ppm BAP (T_6) is at par with treatment (T_5) with 2.23 shoots per explant using 2.0 ppm BAP (Fig. 1).

In vitro response of a tissue depends upon the physiological state of the mother plant as well as the explants and also upon the culture environment (4) and the composition of the culture media largely affects the *in vitro* growth and morphogenesis of plant tissue. Hence, exogenous supply of cytokinin is often essential for direct shoot organogenesis.

The 100 % regeneration was achieved from leaf fragments of *H. macrophylla* Hyd1 by adding either 1 or 2 mg L^{-1} 6 -benzylaminopurine (BA), where shoots regenerated directly without forming any intermediate callus (5). MS media was identified as the optimal basal medium for promoting proliferation with plant growth regulators (PGRs) combination of 6-BA 2.0 mg L^{-1}, IBA 0.2 mg L^{-1} and gibberellic acid (GA3) 1.0 mg L^{-1} in *H. bretschneideri* (6). The enhanced regeneration is observed with an optimum level of cytokinin.

Shoot proliferation and multiplication

For standardizing shoot proliferation, sprouted shoots were subcultured on MS medium with different concentrations of BAP (2.0 - 4.0 ppm). The proliferated shoots were subcultured up to 2 subcultures and the prolificacy was adjudged in each medium. The results enumerated showed that multiple shoot production during first sub-culturing, the highest number of shoots per explant (3.82) was recorded in treatment T_5 with MS + BAP (3.5 ppm). The same treatment also produced the maximum number of leaves per explant (16.26).

However, the greatest shoot length (3.84 cm) was observed in the control treatment with MS medium alone (Table 2). The data indicated that multiple shoot production during second subculturing of shoot explants of *H. macrophylla* treatment with combination of MS + BAP (3.5 ppm) gave the maximum number of shoots sprouted per explant (4.32),

Table 1. Effect of plant growth regulator on direct regeneration of shoots from the explant of *H. macrophylla*

Treatments	BAP	Days to shoot initiation	Number of shoots/explant	Number of leaves/explant	Shoot length (cm)	Fresh weight (g)	Leaf color
T_1	MS (Control)	26.33	1.27	4.37	3.07	4.03	Light green
T_2	0.5 ppm	21.67	1.53	5.10	2.63	5.05	Light green
T_3	1.0 ppm	20.33	1.90	6.23	2.18	6.15	Light green
T_4	1.5 ppm	19.33	2.03	6.57	1.32	5.24	Light green
T_5	2.0 ppm	18.67	2.23	7.23	1.26	5.30	Green
T_6	2.5 ppm	17.33	2.30	8.37	1.96	6.26	Green
SE (m) ±		0.43	0.07	0.05	0.04	0.05	
CD (0.05)		1.33	0.22	0.14	0.11	0.15	

SE (m) ± - Standard error of the mean

CD (0.05) - Critical difference at 0.05 significance level

Fig. 1. Direct regeneration of shoots from node explants within 2 weeks of culture with media concentrations (A) T_5-BAP at 2.0 ppm and (B) T_6-BAP at 2.5 ppm.

maximum number of leaves per explant (21.13) in treatment T_5, but maximum shoot length (6.14 cm) was recorded with the control (T_1) (Table 3) (Fig. 2).

Cytokinin at optimal levels stimulates cell division, suppresses apical dominance and promotes shoot proliferation via axillary buds. Hence, to achieve the mass multiplication of any plant species, it is a prerequisite that an optimum level of shoot production is possible at a suitable concentration level of cytokinin employed (7). It was reported that the longest shoot (3.0 cm) was obtained at MS medium containing 0 level of BA in *H. macrophylla* (8) and it was reported that in 'Blaumeise', the longest shoot (2.1 cm) and in 'Vanilla Sky' the longest shoot (1.4 cm) had been obtained at 0 level of BA, without any growth regulators was the best treatment for shoot elongation in both cultivars of *H. macrophylla* (9). It is also found that in *H. quercifolia* 'Snow Queen' BA at any concentration, induced the highest multiplication rate (over 7 shoots per explant at 1 mg L^{-1} BA) and shoot height was not affected by the cytokinin used in each treatment (10).

Shoot induction and multiplication *in vitro* culture were regulated by cytokinin activity and apical dominance (11). It was found to be true that inhibition of apical dominance by cytokinin results in an increased number of multiple shoots in Gerbera (12). The effect of different concentrations of BA on the shoot multiplication of *H. macrophylla* in two cultivars, 'Blaumeise' with BA at 1.0 mg L^{-1} produced highest number of shoots (12.5) and leaves (93.25) and 'Vanilla Sky' with 1.5 mg L^{-1} of BA produced highest numbers of shoots (4.25) and a total of 29 leaves (9).

Table 2. Effect of plant growth regulator on multiple shoot production during first subculturing of shoot explants of *H. macrophylla*

Treatments	BAP	No. of shoots/explant	No. of leaves/multiple shoots	Shoot length (cm)	Leaf color
T_1	MS (Control)	1.24	9.11	3.84	Light green
T_2	2.0 ppm	2.16	12.32	3.16	Green
T_3	2.5 ppm	2.84	14.19	2.83	Green
T_4	3.0 ppm	3.56	15.09	2.18	Green
T_5	3.5 ppm	3.82	16.26	2.53	Green
T_6	4.0 ppm	3.26	13.92	2.90	Green
SE (m) ±		0.05	0.03	0.04	
CD (0.05)		0.16	0.11	0.11	

SE (m) ± - Standard error of the mean

CD (0.05) - Critical difference at 0.05 significance level

Table 3. Effect of plant growth regulator on multiple shoot production during second sub-culturing of shoot explants of *H. macrophylla*

Treatments	BAP	No. of shoots/ explant	No. of leaves/multiple shoots	Shoot length (cm)	Leaf color
T_1	MS (Control)	1.57	12.08	6.14	Green
T_2	2.0 ppm	2.93	15.07	4.05	Green
T_3	2.5 ppm	3.89	18.56	3.84	Green
T_4	3.0 ppm	4.22	19.05	3.15	Green
T_5	3.5 ppm	4.32	21.13	3.48	Green
T_6	4.0 ppm	3.92	18.04	3.88	Green
SE (m) ±		0.03	0.02	0.03	
CD (0.05)		0.08	0.07	0.09	

SE (m) ± - Standard error of the mean

CD (0.05) - Critical difference at 0.05 significance level

Fig. 2. Shoot proliferation and multiplication during the second sub-culturing with media concentration BAP at 3.5 ppm (T_5).

Rooting

The problem of the rooting behaviour of the microshoots of hydrangea was highly influenced by different combinations of IAA, IBA and NAA (Table 4). NAA was found superior to IAA and IBA. with MS media fortified with different plant growth regulators for rooting (T_4) with MS + NAA (0.5 ppm) showed maximum number of roots (4.54), maximum percent rooting (92.53 %) and early root initiation happened in (T_4) within 16.24 days, but maximum root length (3.91 cm) is recorded in (T_5) with MS + NAA (1.0 ppm) followed by T_4 (3.74 cm) (Fig. 3).

Following *in vitro* regeneration, rooting must be induced. Overall, IAA, IBA and NAA behave similarly, though on a qualitative level, certain tissues do exhibit differences. There are plants where fairly large concentrations of auxins will cause the regeneration of roots (13). Higher concentrations of NAA led to callus formation (6). This might be attributed to the fact that auxins are plant growth regulators that serve to speed up the root induction process by increasing the amount of endogenous enzymes present.

It was demonstrated that the concentration of auxins was not the only factor that affected the root initiation and development and the auxin source (IAA/IBA/NAA) also played an important role (14). Among the auxins used, NAA was found to show better results as compared to IBA for inducing good quality and well-developed roots, which contained roots uniformly distributed throughout.

Similar findings were observed in *H. quercifolia* "Snow Queen" (5), that among the auxins tested, the highest *in vitro* rooting success rate (100 %) was observed with 0.5 mg L^{-1} NAA. A study on *H. macrophylla* showed the highest number of roots (5.4) with NAA at 1.0 mg L^{-1} without charcoal (8). Root formation was induced in *H. macrophylla* by culturing them on half-strength MS medium supplemented with 0.25 mg L^{-1} of NAA for a duration of 30 days (15). The highest number of roots were observed in 'Blaumeise' (11 roots) and 'Vanilla Sky' (6.5 roots) when treated with 1.0 mg L^{-1} of IBA in *H. macrophylla* by (9). However, it was concluded that IBA was more effective than NAA for inducing roots in this species of *H. bretschneideri* Dipp (6).

Acclimatization

Rooted plantlets were hardened, using media that must provide good support to the plants, supply water and ensure good drainage and aeration around the plants since they are shallow rooted. Here, the medium selected had soil, sand, farmyard manure (FYM) and cocopeat taken in ratio of 0.5:1:1:2 which satisfies the above requirements.

Conclusion

For developing a propagation protocol for *H. macrophylla,* several sequential steps were followed. Aseptic cultures were established on MS media supplemented with different concentrations of plant growth regulator BAP. The best response for direct organogenesis was obtained with 2.5 ppm BAP from

Table 4. Effect of plant growth regulator on root initiation and rooting of plantlets of *H. macrophylla*

Treatments (mg/L)				Parameters				
Treatments	**IAA**	**NAA**	**IBA**	**Days to root initiation**	**No. of roots/ plantlet**	**Root length (cm)**	**% of culture-producing roots**	**Root color**
T_1	MS media (control)			16.72	2.24	1.88	55.67 (48.25)	Off white
T_2	0.5	-	-	18.49	2.77	2.14	68.33 (55.76)	Off white
T_3	1.0	-	-	19.67	2.90	2.72	71.67 (57.84)	Off white
T_4	-	0.5	-	16.24	4.54	3.74	92.53 (74.13)	Off white
T_5	-	1.0	-	16.92	3.92	3.91	90.35 (71.91)	Off white
T_6	-	-	0.5	16.72	4.03	3.22	80.53 (63.81)	Off white
T_7	-	-	1.0	17.11	3.81	3.15	82.53 (65.29)	Off white
	SE (m) ±			0.02	0.02	0.03	0.23	
	CD (0.05)			0.07	0.07	0.09	0.71	

SE (m) ± - Standard error of the mean

CD (0.05) - Critical difference at 0.05 significance level

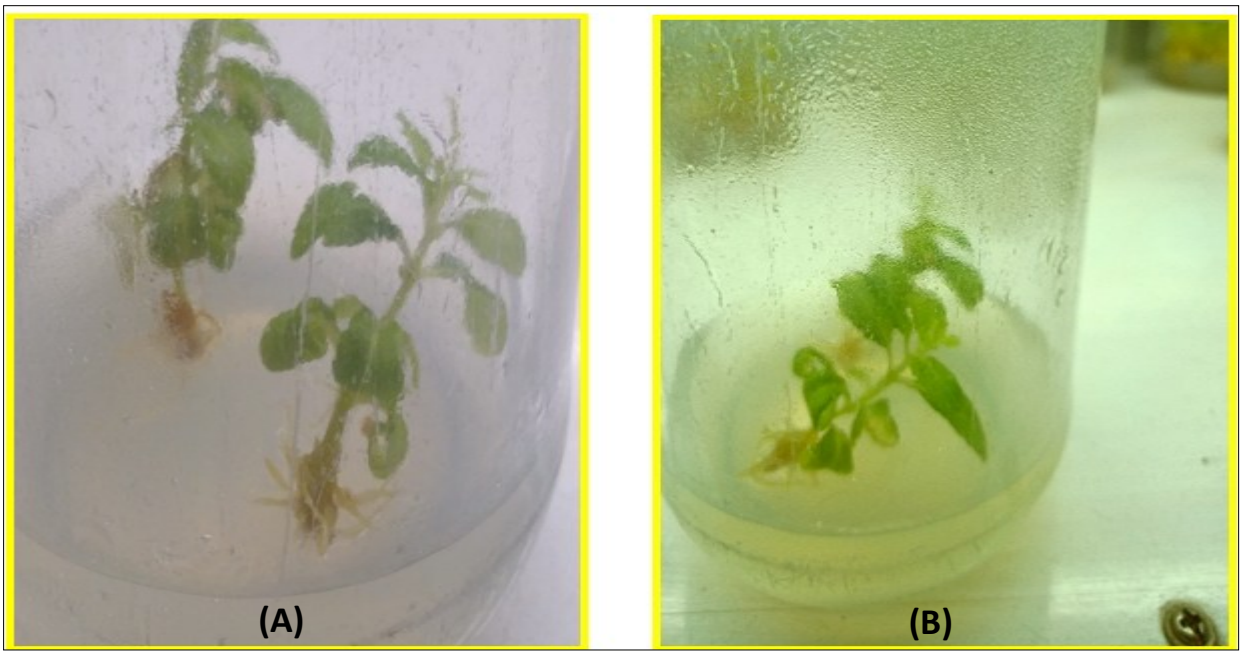

Fig. 3. Root induction during *in vitro* culture of shoot explants with media concentrations (A) MS + NAA at 0.5 ppm (T_4) and (B) MS + NAA at 1.0 ppm (T_5).

node explants. Subsequent shoot proliferation and multiplication were achieved using MS + BAP (3.5 ppm), which produced the maximum number of shoots. Such shoots were then exposed to media containing auxins like IAA, IBA and NAA and the optimal media to induce root formation was MS + 0.5 ppm NAA in best rhizogenesis media. Before hardening them off, the root plantlets had to get acclimated to a potting mix with varied proportions of soil, sand, FYM and cocopeat. Future investigations may explore improved acclimatization techniques by evaluating alternative potting substrates and controlled environmental conditions to enhance plantlet survival. Furthermore, molecular analysis could be employed to assess the genetic fidelity of regenerated *H. macrophylla* across multiple subcultures.

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Authors' contributions

NAP wrote the original draft and conducted the experiment and analysed the data with support from AD. SKP facilitated in conducting the experiment. KM facilitated the acquisition of plant samples and guided the experiment. SB, PNJ and PT conceptualized and contributed by giving essential suggestions. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: The Authors do not have any conflict of interest to declare.

Ethical issues: None

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