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GROWTH PERFORMANCE OF *Hydrangea macrophylla* 'Glowing Embers' ON CULTURE MEDIUM WITH DIFFERENT MACRO-ELEMENT CONCENTRATIONS AND CULTURE CONDITIONS

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ABSTRACT

Hydrangeas are very popular shrubs thanks to their attractive, colorful flowers and foliage. Recently, they have been commercialized for cut-flower production. For mass propagation of this plant species using plant tissue culture, it is necessary to study the effects of culture medium and culture conditions on *in vitro* plant growth. In the first experiment, *in vitro* hydrangea shoots were cultured for 56 days on Murashige and Skoog (MS) media containing one among five different types of macro-elements. The half concentration of NH_4NO_3 on MS macro-elements significantly increased fresh and dry weights of hydrangea shoots in comparison with other treatments. The number of roots of *in vitro* plants in this treatment were the highest, resulting in better absorption of culture nutrient. Cross sections of the shoot base on day 14^{th} showed that reducing only NH_4NO_3 on the MS medium enhanced the formation of adventitious roots. The experiment on culture conditions showed that photoautotrophic condition was suitable for the *in vitro* growth of hydrangea. Plants grown under photoautotrophic (PA) condition had increased fresh weight (341.39 mg/plt), increased dry weight (31.03 mg/plt) with leaf area (7.76 cm^2) significantly greater than those grown under photomixotrophic (PM) condition (259.53 mg/plt, 38.22 mg/plt and 4.73 cm^2 respectively). In addition, the net photosynthetic rate (P_n) of plants under PA condition was statistically higher and increased over the culture period while P_n of plants under PM condition remained constant. This study demonstrated that MS salts with half concentration of NH_4NO_3 and photoautotrophic culture method were appropriate for *in vitro* growth of hydrangea.

Keywords: Hydrangea, macro-elements, photoautotrophic micropropagation, photosynthetic ability.

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INTRODUCTION

Hydrangea macrophylla 'Glowing Embers' plants, in the Hydrangeaceae family, are deciduous shrubs grown as potted plants or garden plants. The inflorescences of *H. macrophylla* form clusters in which all individual flowers belong to a plane, hemisphere, or in some cultivars, a whole sphere. Flower are white in most species, but can be blue, red, pink, light purple, or dark purple in some, due to the availability of aluminum ions depending on the soil pH (Fig. 1). In traditional medicine, hydrangea roots and rhizomes are used for treatment of urinary-tract conditions due to its diuretic properties (Marty, 1999). Hydrangea leaves are also used as an herbal tea in Korea and Japan.

Figure 1. Variation in flower color due to soil pH

Cutting is a popular technique for vegetative propagation of hydrangea species; however, large-scale multiplication of hydrangea plants cannot be achieved in a certain period by conventional propagation methods. Thus, *in vitro* propagation or micropropagation is required to provide genetically uniform, pathogen-free plants in shorter time.

Research literature on *in vitro* culture of hydrangea is limited. Most studies were conducted to investigate the effects of auxin and/or cytokinin on plant growth and shoot multiplication. Gong et al. (2003) found that Murashige and Skoog (MS) medium (Murashige & Skoog, 1962), supplemented with 1.0 mg/L BA and 0.1 mg/L NAA or 0.2 mg/L NAA, was optimal for multiplication of hydrangea shoots cultured *in vitro*. Abou Dahab (2007) successfully developed a protocol for micropropagation for *Hydrangea macrophylla* plants, and indicated that the number of shoots per explant was the highest when explants were cultured on a full strength B5 medium (Gamborg et al., 1968). Ruffoni et al. (2013) also proved that *in vitro* shoots growing on MS medium, supplemented with 0.5 mg/L NAA, showed the best formation of adventitious roots. According to Šiško (2016), shoot proliferation of nodal cuttings was better on the McCown woody plant medium than on the MS medium even though both were supplemented with 2 mg/L BA and 0.005 mg/L NAA. Arafa et al. (2017), when setting up a protocol for large scale production of *Hydrangea macrophylla* by using tissue culture, found that the number of roots was the highest when *in vitro* shoots were cultured on MS media supplemented with 1 mg/L NAA. In Vietnam, Thi et al. (2017) applied the thin cell layer culture method and demonstrated high shoot regeneration rates of hydrangea by culturing leaf blades (88.33%) and transverse nodal layers (91,67%) on modified MS medium supplemented with 3 mg/L BA.

Not only affected by the presence of chemical components, *in vitro* cultures also depend on physical environmental factors, such as light intensity, carbon dioxide concentration, temperature, and relative humidity. During the last 20 years, photoautotrophic (or sugar-free medium) micropropagation has been proved to be beneficial for *in vitro* plant production compared to photomixotrophic (or sugar-containing medium) micropropagation (Nguyen et al., 2016). Therefore, in order to establish an appropriate culture process for

large scale micropropagation of *Hydrangea macrophylla*, the effects of different macro-element concentrations of MS medium and culture conditions (photomixotrophic or photoautotrophic condition) on the growth of hydrangea shoots cultured *in vitro* were investigated.

MATERIALS AND METHODS

Plant materials

Explants for both experiments were derived from *in vitro* shoots of *Hydrangea macrophylla* 'Glowing Embers' previously grown on a proliferation culture medium containing MS macro-and micro-elements, Morel vitamins (Morel & Wetmore, 1951), supplemented with 20 g/L sucrose (Bien Hoa Sugar Factory, Dong Nai, Vietnam), 8 g/L agar (Ha Long Food Co., Hai Phong, Vietnam), under a light intensity (PPFD) of 35 ± 5 $\mu mol.m^{-2}.s^{-1}$ provided by white fluorescent lamps (Dien Quang Co., Ho Chi Minh City, Vietnam) for a 12 h photoperiod at a room temperature of 24 ± 2 ^oC, and relative humidity (RH) of 55 ± 5%. The light intensity (PPFD) was measured by a LI-250A light meter with quantum sensor LI-190R (LI-COR® Inc., Lincoln, USA).

In vitro shoots, each having 2 pairs of unfolded leaves with average fresh weight of 110 mg/shoot, were used as explants for the two following experiments.

Effects of different macro-element concentrations of MS medium on growth of hydrangea shoots *in vitro*

The growth of *in vitro* hydrangea shoots was examined by culturing on 70 mL of 5 modified macro-elements of MS medium: (1) full-strength of macro-elements, (2) half-strength of macro-elements, (3) full-strength of macro-elements with half concentration of NH_4NO_3, (4) full-strength of macro-elements with half concentration of KNO_3, and (5) full-strength of macro-elements with half concentrations of both NH_4NO_3 and KNO_3. The culture medium was supplemented with MS micro-elements, sucrose 20 g/L, Morel vitamins, and agar 8 g/L. The culture vessels were Magenta GA-7 box-type (V = 370 mL) (Sigma, USA). PPFD was set at 35 ± 5 $\mu mol.m^{-2}.s^{-1}$ provided by white fluorescent lamps for a 12 hours photoperiod. Five treatments were conducted in each of 3 replications. Each treatment comprised three vessels, 3 shoots per vessel. Growth parameters were taken using destructive measurements after 56 days of culture.

Effects of culture (photomixotrophic or photoautotrophic) conditions on growth of hydrangea shoots *in vitro*

In the second experiment, two culture conditions were investigated, including (1) photomixotrophic (PM) condition, and (2) photoautotrophic (PA) condition. *In vitro* shoots were cultured on 70 mL of the medium which was found to be optimal in the first experiment and in Magenta GA-7 box-type vessels (V = 370 mL). In PA condition, neither vitamins nor sucrose were added to the culture medium; whereas, two Millipore filter membranes (Nihon Millipore Ltd., Japan) with pore size of 0.45 µm were attached on two holes (φ = 1 cm) of the vessel lid for ventilation. In PM condition, besides macro and micro elements, Morel vitamins and sucrose 20 mg/l were supplemented to the medium, but there were no holes on the vessel lid. PPFD, provided by white fluorescent lamps, was set at 35 $\mu mol\ m^{-2}\ s^{-1}$ from day 0 to day 20, then was gradually raised to 100 $\mu mol\ m^{-2}\ s^{-1}$ on day 35 and kept at this level until the end of experiment (day 49). This experiment included 2 treatments with 3 replicates, each treatment consisting 3 vessels per replicate, with 3 shoots per vessel.

In both experiments, the pH value of the culture media was adjusted to 5.8 before sterilization at 121 ^oC, 1 atm for 20 minutes. The experiments were successively placed in the culture room with room temperature of 24 ± 2 ^oC, RH of 55 ± 5% and a photoperiod of 12 hours per day.

Growth measurement

General growth parameters, such as fresh weight (FW), increased fresh weight (IFW), dry weight (DW), increased dry weight (IDW), dry matter percentage (%DM = Dry

weight/Fresh weight at the end of the experiment); number of shoots (NoS), roots (NoR), and leaves (NoL); shoot length (SL) and root length (RL), were measured at the end of each experiment. All leaves of each *in vitro* plant were detached from petioles, and leaf areas (LA) were measured by the leaf area meter LI-3100C (LI-COR® Inc., Lincoln, U.S.A.). Chlorophyll a and b contents of the third leaf counted from the shoot tip (five samples for each replication per treatment) were measured spectrophotometrically according to the method of Arnon (1949).

In the first experiment, root organogenesis at the basal part of cultured explants was observed. The basal parts of *in vitro* shoot samples were cut into very thin slices then immersed in commercial bleach (Van Phuong Co., Ho Chi Minh City, Vietnam) for 30 minutes. Slices were then washed by water and immersed in acetic acid 10% (v/v) for 15 minutes. Acetic acid was then removed by water washing. Finally, the slices were stained by carmine-iodine solution for 5 minutes. The structure of the shoot tissue section was observed under the optical microscope Nikon Eclipse 80i (Nikon Co. Ltd., Japan).

In the second experiment, the net photosynthetic rate (P_n) of *in vitro* plants was estimated on day 21, 28, 35, and 42 according to the method of Fujiwara et al. (1987) using the following equation:

$$P_n = k \cdot E \cdot V \cdot (C_{out} - C_{in})/n$$

Where: k is the coefficient for converting CO_2 from volume to molecular weight (41.03 $mol.m^{-3}$ at 24 ºC); *E:* The number of air exchanges per hour (h^{-1}) of the culture vessel, estimated by the method of Kozai et al. (1986); *V:* The air volume (ml) of the culture vessel; C_{out} and C_{in}, CO_2 concentrations ($mol.mol^{-1}$) outside and inside of the culture vessel, respectively, under steady state conditions, which were measured by analyzing gas samples with a gas chromatograph (GC-2010, Shimadzu Co., Ltd., Japan); *n*: The number of plants inside of the culture vessel. In this experiment, *E* of culture vessels under PM and PA treatments were estimated to be 0.2 and 3.97 h^{-1}, respectively.

Statistical analysis

The obtained results were subjected to analysis of variance (ANOVA), using MSTATC (Michigan State University, Michigan, USA), version 2.10, to ascertain the significant differences among treatments, one factor per experiment. In all cases, significant differences among means were assessed with LSD-test at $p \leq 0.01$ or $p \leq 0.05$.

RESULTS AND DISCUSSION

Effects of MS macro-elements on growth of Hydrangea shoots *in vitro*

The growth and morphology of hydrangea shoots among five types of macro-elements in MS medium were significantly different (Fig. 2 & Table 1). After 56 days of culture, the greatest fresh weight, dry weight, and increased fresh weight were recorded in MS medium with half concentration of NH_4NO_3 (Table 1).

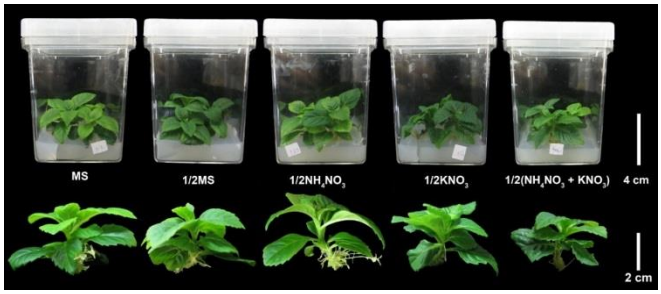

Figure 2. Hydrangea plantlets *in vitro* as affected by different concentrations of macro-elements in MS medium on day 56
Note: For treatment codes, see table 1.

On day 56, treatments with full KNO_3 concentration, MS and 1/2 NH_4NO_3, had slightly higher percentages of dry matter and leaf area than other treatments, but the two parameters were not statistically different among treatments (Table 1). According to Garbin & Dillenburg (2008), KNO_3 contributed to the increase in dry matter percentage of *Araucaria angustifolia* plants. Ivanova & Van Staden (2009) also reported that NO_3^- played a positive role in growth and development of *in vitro Aloe* plants, including increased multiplication rate and decreased plant hyperhydricity. Therefore, reduction of KNO_3 concentration might lead to plant growth retardancy. In this study, KNO_3 concentration in the original MS medium might be adequate for the growth of *in vitro* hydrangea plants.

Table 1. Fresh weight (FW), dry weight (DW), increased fresh weight (IFW), dry matter percentage (%DM), leaf area (LA), and chlorophyll a/b ratio (Chl a/b) of hydrangea plantlets grown *in vitro* as affected by different macro-elements in medium on day 56

Treatment code[z]	FW (mg/plant)	DW (mg/plant)	IFW (mg/plant)	%DM	LA (cm^2)	Chl a/b
MS	597.79 b[x]	92.37 b	490.65 b	15.45	16.73	2.34
1/2 MS	609.01 b	87.81 bc	502.87 b	14.43	16.28	2.29
1/2 NH_4NO_3	709.85 a	109.54 a	602.93 a	15.42	17.08	2.34
1/2 KNO_3	577.59 b	81.98 c	473.89 b	14.20	16.39	2.22
1/2 (NH_4NO_3+KNO_3)	595.13 b	83.84 c	487.65 b	14.09	16.11	2.35
ANOVA[y]	**	**	**	NS	NS	NS
CV (%)	2.04	3.43	2.1	1.62	2.3	3

Notes: [z]MS, 1/2 MS, 1/2 NH_4NO_3, 1/2 KNO_3, 1/2 (NH_4NO_3+KNO_3) represents MS full-strength, MS half-strength, MS with half content of NH_4NO_3, MS with half content of KNO3, MS with half content of both NH_4NO_3 and KNO_3; [y]NS, **: non-significant or significant at $p \leq 0.01$, respectively; [x]Means in the same column followed by the same letters are not significantly different according to LSD-test.

Means of chlorophyll a/b ratio of all treatments, however, were not significantly different, varying from 2 to 3 (Table 1). According to Lichtenthaler (1987), these values indicated that the photosynthetic organ of *in vitro* hydrangea shoots was functioning normally. Overall, the reduction in concentration of some macro-elements in the original MS medium as shown in this experiment did not cause any negative effects on the function of photosynthetic system.

The number of leaves was not significantly different among treatments, though explants cultured on MS medium with half concentration of NH_4NO_3 had more leaf number (12.3 leaves/plt) compared to those of other treatments (Fig. 3). In addition to greater biomass, *in vitro* plants in the treatment with 1/2 NH_4NO_3 had the highest root number (27.8 roots/plt) on day 56 (Fig. 3). Shoot length and root length were also significantly different among treatments. The medium with half concentration of NH_4NO_3 produced the longest root (25.9 mm) and shoot (25.7 mm), while the shortest root (15.5 mm) belonged to the 1/2 MS treatment (Fig. 4).

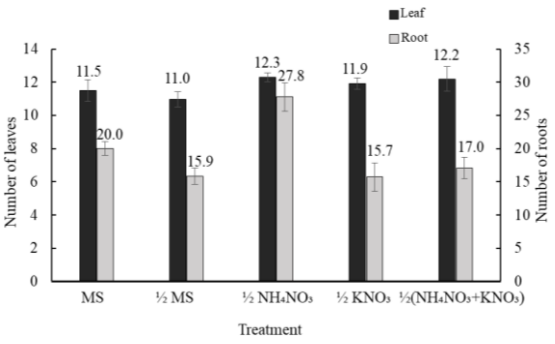

Figure 3. Number of leaves and roots of *in vitro* hydrangea plants affected by different macro-elements in culture medium on day 56

Note: Abbreviations same as table 1.

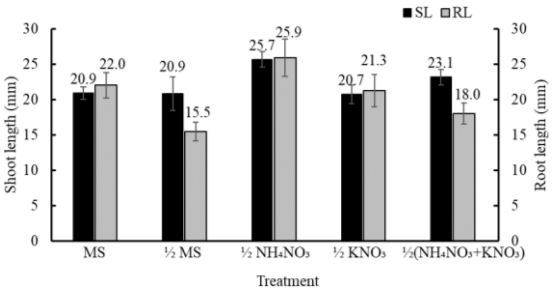

Figure 4. Shoot and root length of *in vitro* hydrangea plants as effected by different macro-elements in culture medium on day 56
Note: Abbreviations same as table 1.

On day 0, cross sections of *in vitro* nodal cuttings showed epidermis cells, cortex cells, and vascular cells to be round-shaped and well organized. This structure remained unchanged in all treatments during the first seven days of culture. On day 14, however, those cells became larger, and changed to hexagonal shape. Cambial cells were greatly divided causing the formation of a dark green ring around the pith (Fig. 5). In the treatment with half concentration of NH_4NO_3, a larger number of root primordia structures were observed (Fig. 5).

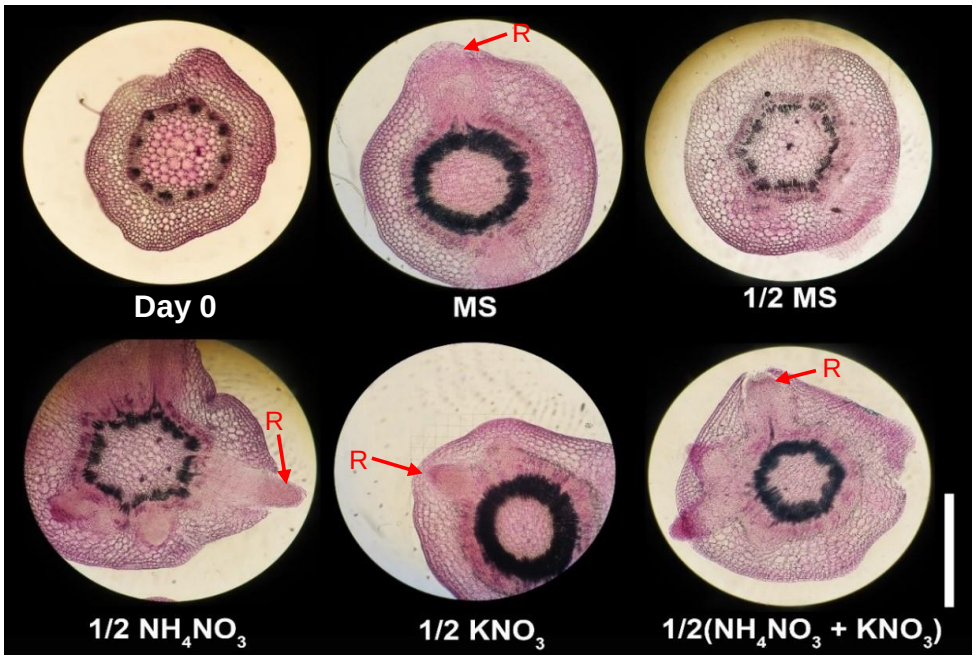

Figure 5. Cross sections of hydrangea shoot base showing the emergence of root primordia on day 14. *Scale Bar = 1 mm (4X). R denotes for root primordium*
Note: Abbreviations same as Table 1.

According to Duong (2003), K^+, NO_3^-, and NH_4^+ ions strongly influenced the growth of *in vitro* plants. George et al. (2008) showed that, in general, plants could absorb nitrogen more efficiently and grew more vigorously if the medium was supplemented with both NO_3^- and NH_4^+ ions. However, high concentration of ammonium (NH_4^+) in culture medium could cause a decrease in root cell activity (George et al., 2008). NH_4^+ was found to have antagonistic properties to K^+, Ca^{2+}, and Mg^{2+} (Bui, 2000). Therefore, a surplus of ammonium ion prevented the absorbance of K^+, Ca^{2+}, and Mg^{2+}, resulting in disturbance of cell permeability, thereby limiting root growth. The better growth of *in vitro* hydrangea shoots in 1/2 NH_4NO_3 treatment was in accordance with the study of Stensvand & Gisler's (1992); which implied that each plant species had a suitable NH_4^+:NO_3^- ratio for its growth. The NH_4^+:NO_3^- ratio in MS medium with half concentration of NH_4NO_3 is the lowest (1:3)

when compared to media including MS, MS half strength, MS with half concentration of KNO_3 and MS with half concentration of both NH_4NO_3 and KNO_3 (1:2, 1:2, 1:1.5, and 1:2 respectively). Le et al. (2015) showed similar results when culturing *Lavandula angustifolia* photoautotrophically on MS medium with half concentration of NH_4NO_3 (the greatest fresh mass and dry mass, and the highest relative growth rate). Park et al. (2007) also reported that the highest biomass of *Wasabia japonica* was obtained from *in vitro* plants cultured in liquid medium with a low NH_4^+:NO_3^- ratio (1:5).

Results of our first experiment suggested that the modified MS medium with a half concentration of NH_4NO_3 was appropriate for a better growth of hydrangea shoots cultured *in vitro*. This medium was then applied for the second experiment and later hydrangea cultures.

Effects of culture conditions on growth of hydrangea shoots *in vitro*

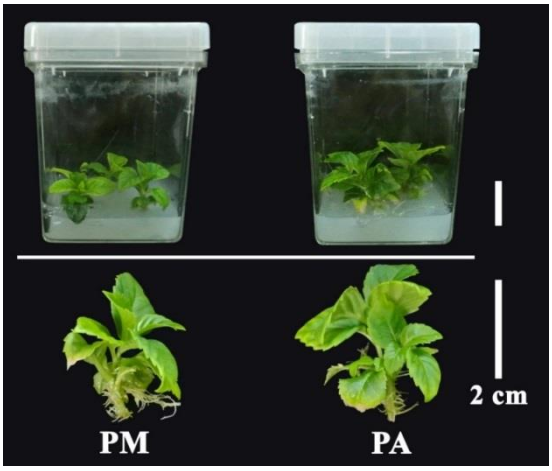

Figure 6. Hydrangea plantlets *in vitro* grown with different culture methods on day 49. PM: photomixotrophic, PA: photoautotrophic

Hydrangea shoots had significantly better growth when cultured under photoautotrophic (PA) condition compared to photomixotrophic (PM) condition (Fig. 6). On day 49, shoots cultured under PA condition (on sugar-free medium and in ventilated vessels) were longer (1.81 cm) and had greater number of roots than those under PM condition (on sugar containing medium and in non-ventilated vessels) (Table 2). These results were consistent with the findings of Nguyen et al. (2012) on thyme plants (*Thymus vulgaris* L.), in which *in vitro* thyme plants cultured under PA condition had shoots longer than those under PM condition. Both conditions, however, did not show any significant effects on the root growth of hydrangea (Table 2).

On day 49, the number of leaves (12.22 leaves/plt) and leaf area (7.76 cm^2) of hydrangea plants cultured under PA condition were statistically greater than those under PM condition (10.07 leaves/plt and 4.73 cm^2, respectively), but the number of shoots showed no difference between treatments (Table 2). Biomass accumulation of hydrangea was also strongly affected by the culture method. Fresh weight (341.39 mg/plt) of plants in PA treatment was significantly higher than that (249.55 mg/plt) in PM treatment.

Although chlorophyll a/b ratio of PM treatment, did not fall below the normal value, the amount of total chlorophyll a + b contents (based on leaf dried weight on day 49) under PM condition was 0.7 times smaller than that in PA condition (Table 2); i.e. the photoautotrophic method in this experiment clearly improved the photosynthetic ability of hydrangea plants *in vitro*. According to Lichtenthaler (1987), inefficient photosynthesis of *in vitro* plants created by PM condition might be due to low contents of both chlorophyll a and b. Short et al. (1987) also demonstrated that cauliflower and chrysanthemum plants cultured on sucrose-free media showed higher photosynthetic rates (measured by O_2 evolution) than those cultured on sucrose-containing ones. Furthermore, Roh & Choi (2004) confirmed that sucrose supplement in the culture medium inhibited the photosynthetic activity of tobacco leaves *in vitro* by decreasing the activity of rubisco, an enzyme involved in the first major step of carbon fixation. In the PA treatment of our study, hydrangea plants cultured in vessels with high number of air

exchanges (3.97 h^{-1}) could absorb more CO_2, an inorganic carbon source from the atmosphere, for their photosynthesis, resulting in greater growth performance (Table 2).

Table 2. Effects of culture methods on growth parameters of hydrangea plants cultured *in vitro* on day 49

Growth parameters	Culture method[z]		ANOVA[y]
	PM	PA	
Shoot length (cm)	1.59	1.81	*
Root length (cm)	1.67	1.53	NS
Number of leaves (leaves/plt)	10.07	12.22	*
Number of roots (roots/plt)	8.28	11.48	**
Number of shoots (shoots/plt)	2.04	1.93	NS
Leaf area (cm^2)	4.73	7.76	*
IFW (mg/plt)	259.56	341.39	**
IDW (mg/plt)	38.22	51.03	**
Chl a + b (mg/g LDW)	1.62	2.31	**
Chl a/b	2.22	2.55	*

Notes: [z]PM: photomixotrophic, PA: photoautotrophic; [y]NS, *, **: non-significant or significant at $p \leq 0.05$ or $p \leq 0.01$, respectively.

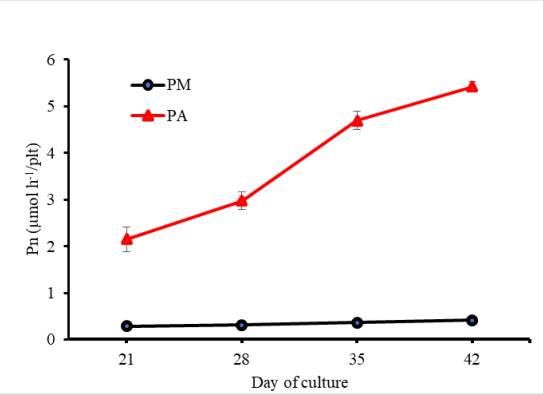

Figure 7. Net photosynthetic rate (P_n) of hydrangea plants *in vitro* under different culture methods. PM: photomixotrophic, PA: photoautotrophic

Regarding the net photosynthetic rate (P_n), P_n of PM treatment was remarkably low and almost constant from day 21 to day 42; whereas, P_n of PA treatment increased dramatically during this culture period (Fig. 7). The low net photosynthetic rate of the plants grown *in vitro* photomixotrophically might be due to low activity of rubisco (Gourt & Price, 1987; Grout, 1988), low light intensity, and inadequate gas exchange (Kozai et al., 1987). In the present study, the low number of air exchange rate (0.2 h^{-1}) of the airtight vessel in the PM condition caused the low CO_2 concentration in the *in vitro* environment; and thus, diminished photosynthesis of *in vitro* hydrangea plants. Kozai et al. (1987) also indicated that low levels of CO_2 in the vessel during the photoperiod in PM condition limited photosynthesis of *in vitro* plants and resulted in a net low or negative balance of CO_2 uptake per day. This explains why *in vitro* plants in PM condition compensates for the CO_2 shortage by using a small amount of sucrose added to the culture medium as a carbon source for their growth when their photosynthetic ability is incompetent (Kozai, 1991). The P_n result of this experiment was also in accordance with that of *Oplopanax elatus* culture, in which the depletion of CO_2 concentration inside the vessel led to lower P_n of *in vitro* plants in photomixotrophic culture compared to photoautotrophic culture (Park et al., 2011).

CONCLUSION

Depending on plant species, an appropriate medium should be used in order to maximize *in vitro* plant growth. In this study, MS medium with half concentration of NH_4NO_3 was shown to be a suitable medium

for obtaining the highest fresh and dry weights and a shorter time for root formation of hydrangea plants cultured *in vitro*. Furthermore, this study verified the positive impact of photoautotrophic culture on the growth performance of hydrangea plants with increases in photosynthetic ability and biomass accumulation.

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