Medium pH between 5.5 and 7.5 has Minimal Effects on Tissue Culture of Apple

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Abstract. Medium pH is generally adjusted to 5.8 to 6.0 for plant tissue culture. Our research indicated that pH generally falls between 5.5 and 7.5 in an ordinarily made medium which can be directly used for apple tissue culture without adjusting pH. Repeated adjustment of pH by adding NaOH and HCl leads to the increase in Na⁺ and Cl[−] concentration and decrease in Mg²⁺ and Ca²⁺ concentration in the medium due to precipitation. To determine the pros, cons, and necessity of pH levels while making medium in plant tissue culture, subculture proliferation, adventitious root induction, and organ regeneration, the apple cultivars Fuji, Golden Delicious, Jonagold, and Gala were used and hardness of the medium and the ion content of Na⁺, Cl[−], Mg²⁺, and Ca²⁺ in the medium under different pH were measured. In the lower pH range of 5.0–5.5, plantlets could be subcultured and grew normally; however, the medium did not solidify or solidified poorly resulting in problems associated with handling. No significant difference was found among the treatments when pH ranged 6.0–8.0 in terms of proliferation, adventitious root induction, and adventitious bud regeneration from leaves, except a slight decrease in shoot number proliferation in ‘Jonagold’ and in adventitious bud regeneration from leaves in ‘Fuji’ and ‘Golden Delicious’ at pH above 7.5. The hardness of the medium increased with the increased pH. The superfluous Cl[−] and Na⁺ generated during the process of overadjusting pH to 7.0 by adding NaOH and then readjusting to 6.0 by adding HCl significantly affected the proliferation, rooting, and organ regeneration of apple plantlets. A relative broad range of medium pH (5.5–7.5) is suitable for apple tissue culture. We suggest that it is not necessary to always adjust medium pH to 5.8–6.0 in apple tissue culture; especially the repeated adjustment should be avoided.

Plant tissue culture technique has been improved remarkably in the last few decades. The technique is widely used in plant propagation (Pattanaik and Chand, 1997), virus elimination (Ramírez-Malagón et al., 2006; Subba Reddy and Sreenivasulu, 2011), genetic transformation (Falco et al., 2000; Taskin et al., 2003; Xue et al., 1999), germplasm preservation (Forsline et al., 1998; Gagliardi et al., 2002; Orlikowska, 1992; Reed et al., 1998; Zacchini and Agazio, 2004), and secondary metabolite production (Bourgaud et al., 2001; Cheng et al., 2008). The most commonly used culture medium is the Murashige and

Skoog (MS) medium (Murashige and Skoog, 1962) or the variant of it. The pH of the medium is generally suggested as 5.5–6.0 and not much research on it has been reported (Beyl, 2011).

Generally, the medium is adjusted to a certain pH depending on the plant species used and the purpose. For example, tetraploid black locust showed the highest proliferation rate at pH 5.6–6.0, whereas the Erqiao black locust preferred pH 6.4 (Huang and Liu, 2003). Growth and nutrient uptake of teak (*Tectona grandis*) seedlings in vitro was optimum on a medium at pH of 6.0 (Hong and Yin, 2010; Zhou et al., 2009). The pH of the medium is also responsible for the occurrence of vitrification (a phenomenon of plantlets appearing turgid, glaucous, and watery), which has been shown on *Prunus salicina* within pH 5.4 to 5.8 (Ding et al., 2008). In the case of apple, the medium pH regulated root formation of the microcuttings of the cultivars Gala and Triple Red Delicious by affecting

indole-3-butyric acid (IBA) uptake (Harbage and Stimart, 1996; Harbage et al., 1998), and for cultivar Jork9, better rooting occurred at pH 5.3 (Klerk et al., 2008).

However, not all plant species are that sensitive to pH variation of the medium within a certain range. For instance, the proliferation and growth of *Oncidium* plantlets did not show significant difference with variable pH from 5.2 to 6.5 (Cui et al., 2004). Likewise, plantlets of *Plantago almogravensis* and *Plantago algarbiensis* performed equally with variation of medium pH from 4.50 to 5.75 (Martins et al., 2011). Although optimal medium pH varied from plant species to species, in most cases, it is adjusted to the range of 5.0–6.0 (Li, 2002).

Adjusting medium pH is normally done by adding diluted HCl or NaOH solution. In many cases, repeated adjustment by adding HCl and NaOH in turn is needed to get an exact pH value. It is quite understandable that adding these chemicals changes the ion concentration which might have adverse effects on plant growth. The objectives for this study were 1) to analyze the changes of the ion concentration in medium after adjustment of pH, 2) to assess the effect of medium pH range on apple subculture proliferation, rooting, and adventitious bud regeneration from leaves, and 3) to determine the necessity of pH adjustment while preparing medium for apple tissue culture.

Materials and Methods

Plant materials. The plantlets of *Malus domestica* ‘Fuji’, ‘Golden Delicious’, ‘Jonagold’, and ‘Gala’, and one apple rootstock M₂₆ were studied. These were subcultured in vitro for years using the MS medium containing benzyladenine (BA) 1.0 mg·L^{−1}, naphthaleneacetic acid (NAA) 0.05 mg·L^{−1}, sucrose 35.0 g·L^{−1}, and agar (from Haiyan Agar Limited, Qingdao, China) 6.0 g·L^{−1}, in a culture room under the condition of 2000 lx light intensity with 14-h-photoperiod at 25 ± 3 °C. The medium pH was adjusted to 5.8–6.0 before autoclaving at 121 °C for 20 min.

Analysis of ion content in different pH medium after adjustment. The MS medium containing BA 1.0 mg·L^{−1}, NAA 0.05 mg·L^{−1}, and sucrose 35.0 g·L^{−1} was prepared and divided into four subsets. The pH of two of them were adjusted to 6.0 and 7.0 by adding 1 mol·L^{−1} NaOH and/or 1 mol·L^{−1} HCl depending on the initial medium pH. The third one was adjusted to 7.0 first, and then readjusted to 6.0, resulting in three treatments, and the fourth one was taken as control without adjusting pH. The medium pH was measured using a pH meter (Sartorius PB-10; Germany). The amount of 1 mol·L^{−1} NaOH or 1 mol·L^{−1} HCl added was recorded. Volumetric differences among treatments were equalized by deionized and distilled water. The medium was autoclaved at 121 °C for 20 min before use.

The content of Na⁺, Mg²⁺, and Ca²⁺ ions in the medium was determined by using inductively coupled plasma atomic emission spectrometry (VISTA-MPX, Varian Inc., CA), and

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that of Cl^- ion was determined by using ion chromatography (Metrohm 882 Compact IC plus, Herisau, Switzerland).

Effect of medium pH on proliferation of apple plantlets in vitro. Subculture medium, basic MS containing BA $1.0 \text{ mg}\cdot\text{L}^{-1}$, NAA $0.05 \text{ mg}\cdot\text{L}^{-1}$, sucrose $35.0 \text{ g}\cdot\text{L}^{-1}$, and agar $6.0 \text{ g}\cdot\text{L}^{-1}$ was prepared. Six different media with pH adjusted to 5.5, 6.0, 6.5, 7.0, and 7.5, and an adjusted 7.0 medium readjusted to 6.0 were used. The subcultured apple plantlets with similar growth status were subcultured onto the medium. Each treatment consisted of 54 plantlets equally distributed in nine 100-mL conical flasks. The number of total new shoots and the effective new shoots that were longer than 1.5 cm (able to be used for rooting) were recorded at 35 d after subculture.

Effect of medium pH on adventitious root induction. New shoots longer than 1.5 cm were inoculated onto the rooting medium containing $\frac{1}{2}$ -strength MS, indoleacetic acid $1.0 \text{ mg}\cdot\text{L}^{-1}$, IBA $0.4 \text{ mg}\cdot\text{L}^{-1}$, sucrose $25.0 \text{ g}\cdot\text{L}^{-1}$, and agar $6.0 \text{ g}\cdot\text{L}^{-1}$. The pH of the medium was adjusted as of the subculture experiment. Nine shoots were inoculated in each conical flask. The materials were cultured in a cabinet with a 14-h-photoperiod at $25 \pm 3^\circ\text{C}$. Rooting rate, number of roots per plant, and length of roots were recorded 35 d after inoculation. Rooting rate was calculated as the percentage of the shoots that produced roots.

Effect of medium pH on adventitious bud induction from leaves in vitro. The top second to fourth leaves (expanding or fully expanded) were collected from the plantlets that subcultured for about 30 d. Each leaf was crosscut twice without breaking and inoculated on the MS medium containing BA $1.0 \text{ mg}\cdot\text{L}^{-1}$, thidiazuron $0.5 \text{ mg}\cdot\text{L}^{-1}$, NAA $\text{mg}\cdot\text{L}^{-1}$, sucrose $30.0 \text{ g}\cdot\text{L}^{-1}$, and agar $6.0 \text{ g}\cdot\text{L}^{-1}$ with the pH adjustment as of the above sections. Eight leaves were inoculated in each conical flask. The materials were dark-cultured for 20 d before light culture with a 16-h-photoperiod at $25 \pm 3^\circ\text{C}$. Adventitious bud regeneration rate and number of adventitious buds per leaf were evaluated 30 d after light culture.

Data and statistical analysis. This research was repeated in years 2009, 2011, and 2012. The result of different years showed very reproducible data. Thus, the data from the year 2012 have been analyzed and interpreted to prepare this manuscript. The experimental design used in this research was completely randomized with three replications. Data were analyzed using analysis of variance of Data Processing System (DPS®) software (Tang and Feng, 2007; Tang and Zhang, 2013). The percentage data were converted to a decimal value and transformed as the inverse sine of the square root to normalize the data. Treatment differences were tested at the level of $\alpha = 0.05$ using Least Significant Ranges means.

Results

Analysis of ion content in different pH medium. The initial pH of the MS medium prepared in this experiment was read as 5.1.

Since the initial pH value was lower than expected, NaOH was added to the medium to increase the pH value. As expected, the content of Na^+ ion was increased due to the addition of NaOH (Table 1). Likewise, the addition of HCl to readjust the pH resulted in the increase of Cl^- ion content. However, the content of Ca^{2+} ion in the solution decreased significantly with the addition of NaOH. The content of Mg^{2+} ion also changed slightly during this process (Table 1).

Precipitation had been observed as of adding NaOH while adjusting the medium pH, which is hardly noticed when agar is added during the conventional process of medium preparation. To determine what caused this precipitation, the salts involved in the MS medium formula were used individually in making solution in the same concentration as in the MS medium. Adding NaOH resulted in precipitation in the solutions made from $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ and $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, which confirmed the above chemical analysis results.

Effect of pH on hardness of the medium. The medium solidity is dependent on pH value. When the pH value was 5.0, it hardly became solid and the plantlets had to be grown in this medium using sponge as an extra supporting material. Although the plantlets grew normally, this medium did not appear convenient due to the difficulty in handling. It was observed that the medium started to solidify when its pH reached 5.5, although it was soft and could crack while the conical flask was tilted. The medium solidity was optimal when pH value ranged from 6.0 to 6.5. It was noted that when the pH was adjusted up to 7.0 or higher, the medium became too hard for the tender plantlets to be inoculated which probably affected the nutrition uptake as evidenced by the yellowish leaves. This hard medium also showed a tendency of cracking itself.

Effect of medium pH on proliferation of subculture. The proliferation efficiency of subculture in four apple varieties and M_{26} was unaffected by the medium pH within the range from 5.5 to 7.5. The number of shoots and the number of effective shoots were not influenced by the tested pH, except for 'Fuji' apple at pH 7.5 (Table 2). It appeared that the apple plantlets were compatible with a wide pH range of medium during the subculture stage. On the other hand, the readjustment of pH from 7.0 back to 6.0 significantly lowered the number of shoots and the number of effective shoots in 'Fuji' and 'Jonagold' apple. The readjustment by adding HCl resulted in the increase of Cl^- ion (Table 1). Even though the medium pH was relatively

optimal, the side effect of the ion concentration was prominent.

Effect of medium pH on adventitious root induction. Root induction was not significantly influenced by medium pH, except for 'Fuji' apple at pH 7.5, which had significant low rooting (Table 3). In the case where pH was readjusted, lower rooting percentage and number of roots have been recorded in all varieties and M_{26} , although root length was not significantly affected.

Effect of medium pH on adventitious bud regeneration from leaves. The leaves cut from subcultured plantlets of 'Fuji', 'Golden Delicious', 'Jonagold', 'Gala', and M_{26} were inoculated onto the medium with different pH to induce the adventitious buds. The data showed that the adventitious bud regeneration rate and number of buds regenerated per leaf (Table 4) are not significantly influenced by the medium pH. However, the medium of which pH had been readjusted showed significantly decreased adventitious bud regeneration of 'Fuji', 'Jonagold', and M_{26} , but those of 'Golden Delicious' and 'Gala' were not significantly affected by the readjustment.

Discussion

Apple is an important fruit species worldwide which can be readily propagated by tissue culture. It has been documented in different plant species that the medium pH is an important factor for the growth and development of plantlets in vitro. Repeated use of NaOH and HCl to adjust the medium pH results in imbalanced ion concentration in the medium leading to the problem of nutrient uptake by the young plantlets. Thus, the present study investigated the influence of medium pH on tissue culture performance of different apple cultivars and the consequence of pH readjustment on growth of apple plantlets.

Our major conclusions are that pH falls between 5.5 and 7.5 in an ordinarily made medium which can be directly used for apple tissue culture without adjusting pH. Repeated adjustment of pH by adding NaOH and HCl leads to the increase in Na^+ and Cl^- concentration and decrease in Mg^{2+} and Ca^{2+} availability in the medium due to precipitation. In the lower pH range of 5.0–5.5, plantlets could be subcultured and grew normally; however, the medium did not solidify or solidified poorly resulting in problems associated with handling. No significant difference was found among the treatments when pH ranged from 6.0 to 8.0 in terms of proliferation, adventitious root induction, and adventitious bud regeneration from leaves, except a slight decrease in shoot number proliferation in 'Jonagold' and in adventitious bud regeneration from leaves

Table 1. Effect of pH adjustment on ion concentration in medium solution.

Medium pH	Na^+ ($\text{mg}\cdot\text{L}^{-1}$)	Cl^- ($\text{mg}\cdot\text{L}^{-1}$)	Ca^{2+} ($\text{mg}\cdot\text{L}^{-1}$)	Mg^{2+} ($\text{mg}\cdot\text{L}^{-1}$)
5.1 (Initial)	11.6 c ^z	320.0 b	188.0 a	39.4 a
Adjusted to 6.0	49.4 b	323.1 b	160.4 b	38.5 a
Adjusted to 7.0	106.0 a	321.6 b	122.0 c	35.0 b
Adjusted to 7.0, readjusted to 6.0	104.8 a	363.0 a	117.2 c	34.2 b

^zDifferent letters following the data within each column represent the significance at the level of $\alpha = 0.05$.

Table 2. Influence of medium pH on proliferation efficiency of apple cultivars in vitro.

Medium pH	Fuji		Golden Delicious		Jonagold		Gala		M ₂₆	
	No. of shoots	No. of effective shoots	No. of shoots	No. of effective shoots	No. of shoots	No. of effective shoots	No. of shoots	No. of effective shoots	No. of shoots	No. of effective shoots
5.5	5.24 a ²	2.37 a	6.36 a	2.69 a	4.90 a	3.47 a	7.54 a	3.55 a	7.80 a	2.60 a
6.0	4.73 ab	2.03 a	6.55 a	3.20 a	4.23 ab	3.13 ab	7.16 a	2.80 a	9.33 a	2.73 a
6.5	4.60 ab	2.30 a	6.87 a	2.80 a	4.13 ab	3.08 ab	7.08 a	2.51 a	7.93 a	2.97 a
7.0	5.10 a	1.80 ab	7.08 a	2.20 a	3.89 ab	2.82 ab	7.72 a	2.42 a	8.00 a	2.55 a
7.5	4.42 ab	1.50 b	6.51 a	2.51 a	4.28 ab	3.24 ab	7.27 a	2.31 a	8.25 a	3.42 a
6.0 (Readjusted)	3.93 b	1.31 b	6.82 a	2.42 a	3.40 b	2.30 b	7.03 a	2.29 a	8.47 a	2.64 a

²Different letters following the data within each column represent the significance at the level of $\alpha = 0.05$.

Table 3. Effect of medium pH on rooting ability of apple shoots in vitro.

Medium pH	Fuji			Jonagold			Gala			M ₂₆		
	Rooting (%)	No. of roots per plant	Root length (cm)	Rooting (%)	No. of roots per plant	Root length (cm)	Rooting (%)	No. of roots per plant	Root length (cm)	Rooting (%)	No. of roots per plant	Root length (cm)
5.5	69.56 a ²	3.69 a	3.82 a	98.90 a	4.63 a	4.21 a	100.00 a	6.63 a	4.57 a	100.00 a	7.54 a	5.92 a
6.0	78.21 a	3.76 a	3.25 a	100.00 a	4.48 a	4.05 a	94.23 a	6.12 a	4.26 a	100.00 a	7.30 a	5.00 a
6.5	70.23 a	3.65 a	2.89 a	98.02 a	3.83 a	4.02 a	100.00 a	5.16 a	4.20 a	97.63 a	5.40 a	4.72 a
7.0	73.94 a	3.09 ab	3.63 a	89.56 ab	4.34 a	4.72 a	93.30 a	5.02 a	3.28 a	97.63 a	5.40 a	4.26 a
7.5	52.31 b	2.89 b	2.56 a	88.30 ab	3.38 a	4.62 a	98.30 a	6.32 a	4.74 a	89.56 ab	6.33 a	3.97 a
6.0 (Readjusted)	40.23 b	1.83 c	2.23 a	66.67 b	3.17 a	3.83 a	74.26 b	5.78 a	4.79 a	68.96 b	5.00 a	5.09 a

²Different letters following the data within each column represent the significance at the level of $\alpha = 0.05$.

Table 4. Effect of medium pH on adventitious bud regeneration from leaf explants of apple in vitro.

Medium pH	Fuji		Golden Delicious		Jonagold		Gala		M ₂₆	
	Regeneration (%)	No. of buds	Regeneration (%)	No. of buds	Regeneration (%)	No. of buds	Regeneration (%)	No. of buds	Regeneration (%)	No. of buds
5.5	46.60 a ²	2.33 a	96.91 a	16.57 a	100.00 a	16.72 a	100.00 a	35.12 a	100.00 a	25.71 a
6.0	50.00 a	2.78 a	95.34 a	19.07 a	97.63 ab	16.93 a	99.48 a	29.07 a	100.00 a	21.13 a
6.5	39.34 a	2.25 a	93.39 a	17.78 a	83.05 bc	16.90 a	99.51 a	29.13 a	98.34 a	19.03 a
7.0	44.35 a	3.50 a	89.50 a	14.81 a	95.77 abc	15.33 ab	98.05 a	29.63 a	99.30 a	21.07 a
7.5	41.54 a	2.33 a	89.07 a	16.44 a	89.23 abc	15.81 ab	100.00 a	24.34 a	92.11 a	17.27 ab
6.0 (Readjusted)	16.06 b	0.92 b	93.07 a	18.84 a	75.05 c	9.29 b	100.00 a	33.77 a	74.79 b	14.83 b

²Different letters following the data within each column represent the significance at the level of $\alpha = 0.05$.

in 'Fuji' and 'Golden Delicious' at pH above 7.5. The hardness of the medium increased with increased pH. The superfluous Cl^- and Na^+ generated during the process of overadjusting pH to 7.0 by adding NaOH and then readjusting to 6.0 by adding HCl significantly affected the proliferation, rooting, and organ regeneration of apple plantlets.

In most cases, the medium pH ranging from 5.8 to 6.0 was considered as optimal for tissue culture. The initial pH of the medium could be varied depending on the medium components (Bennett et al., 2003; Woodward et al., 2006), the water used (Beyl, 2011), and the ways whether acid or alkaline solution was used for the plant growth regulator to dissolve. In this research, a wider pH range (5.5 to 7.5) was tested to get a better understanding. In our experiment, the initial medium pH was 5.1 in the ready-made MS medium. BA was one of the most common plant growth regulators used in plant tissue culture and was dissolved in $1 \text{ mol} \cdot \text{L}^{-1}$ HCl normally as a stock solution. For MS medium preparation, the pH could be lowered by adding BA that was prepared by dissolving in HCl, resulting in poor solidity of the medium and more NaOH was needed for the pH adjustment. Therefore, NaOH solution was added to increase the pH value and the concentration of $1 \text{ mol} \cdot \text{L}^{-1}$ NaOH is recommended in this process. However, the

results of this experiment clearly indicate that adding NaOH changed the ion concentration of medium solution. And this is particularly true in the case where addition of NaOH and HCl was implemented to readjust the medium pH. This ion concentration change showed adverse influence on plant nutrient uptake. Moreover, precipitation was found in the solution during the process of pH adjustment, and the reaction of Mg^{2+} and Ca^{2+} with OH^- contributed to this phenomenon, which led to changes in some ion concentrations in the medium. In the real operating process, this phenomenon was hardly noticed due to the presence of agar.

The medium pH has considerable influence on the physical strength or hardness of the medium which has also been reported in several works on different species. The medium was too soft and had a poor solidity at the pH lower than 5.5, which posed a handling problem. Thus, it is not recommended to use such a low pH unless specifically needed for some species. In this work, the plantlets of four apple cultivars and a rootstock M₂₆ showed broad medium pH adaptability in the aspects of subculture proliferation efficiency, rooting, and adventitious bud regeneration from leaves. But the pH readjustment resulted in significant limitation in plantlet growth and regeneration, especially in rooting. The changes in some ion concentrations,

especially the increase of Cl^- ion, could be one of the most important reasons. Thus, the most important conclusion from this research is that for apple tissue culture it is not recommended to adjust medium pH when it ranges from 5.5 to 7.5. Particularly, the readjustment using HCl and NaOH in turn should be avoided as this changes the concentration or availability of some ions.

It was also found that the influence of the pH adjustment is variable among apple cultivars. 'Gala' appeared as the most sensitive to medium pH, whereas 'Golden Delicious' and M₂₆ showed almost no effect by the medium pH difference in the culture. 'Fuji' was relatively difficult to root and regenerate, and showed sensitivity to the changes of culture conditions. However, the mechanism of this effect was still unclear. Some scientists have reported that the medium pH could influence the membrane permeability, the respiratory metabolism, polyamine metabolism, protein synthesis, and the activity of hormones, and indirectly affected the growth and development of the culture materials (Cao and Han, 2003; Ding et al., 2008; Zhang, 2006). Sometimes proliferation or rooting problems were encountered even though the same routine protocol was used. The precipitation and change of ion concentration due to the pH adjustment reported in this research might provide a solution to these problems.

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