In vitro Mineral Nutrients and Sucrose Affecting Growth and Development of Micropropagated Red Raspberry Shoots

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Abstract

Red raspberry (*Rubus idaeus* Linn.) has been grown for fruit production in the north of Thailand by the Royal Project foundation. Mass production of red raspberry plant stock is needed for commercial fruit production. This alternative crop might be interesting for increased incomes of upland farmers. The aim of this study was to investigate the effects of mineral nutrients and sucrose on plant growth and development of micropropagated red raspberry 'Amity'. Previous studies showed that minerals in Murashige and Skoog (MS) medium were not suitable for this plant. Therefore, in the current study, the effects of the mineral components in three groups; nitrogen, mesos and micronutrients were tested as the relative concentrations compared to MS (0.5x, 1.0x, 1.5x and 2.0x MS) and then sucrose concentrations were optimized. Plants were evaluated for shoot quality, leaf color, multiplication and shoot length. The results showed that reduced mesos negatively affected plant quality, leaf color and shoot number. The modified MS suitable for this cultivar was half the MS nitrogen concentration and twice the MS mesos concentration. The leaf color measured by SPAD meter for greenness correlated with the chlorophyll content of the leaf tissues. The best sucrose concentration for improving growth of micropropagated red raspberry shoots was 1.5 %.

Keywords: chlorophyll content; mineral nutrition; red raspberry; SPAD reading; sucrose

1. Introduction

Plant tissue culture medium consists of 13 minerals, growth regulators, carbon source, vitamins and gelling substances. Mineral nutrition from those 13 minerals plays an important role in plant growth and development (Murashige and Skoog, 1962; Anderson, 1980). Although the Murashige and Skoog (1980) formulation is commonly used for *in vitro* culture of various plants, it was designed to grow tobacco callus, and is not suitable for all plants. Several studies indicate that some plants cannot

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grow or be propagated well on MS (Murashige and Skoog). Shoot or organ cultures showing non-optimal growth on MS were observed in many plant species including red raspberries (*Rubus idaeus* L.) (Ruzic *et al*., 2000; Dantas *et al*., 2001; Greenway *et al*., 2012; Reed *et al*., 2013; Poothong and Reed, 2014). A recent study of micropropagation in red raspberry showed that MS mineral nutrients were not suitable for growth, multiplication and development of the five red raspberry cultivars tested (Poothong and Reed, 2014). Investigating the effects of *in vitro* mineral nutrients is very challenging because these minerals are very important components for *in vitro* plant growth and plant species or cultivars may have diverse requirements for nutrition (Niedz and Evens, 2007; Reed *et al*., 2013). Sucrose is a common carbon source for providing energy for *in vitro* culture systems and the optimal concentration of sucrose required varies with the plant species. In red raspberry, there are few studies focusing on the effect of sucrose on plant growth (Deng and Donnelly, 1993).

Growing red raspberry in tropical countries such as Thailand is challenging because it is a temperate small fruit crop and was only recently tested to grow in the northern highland areas. Micropropagation is useful for producing healthy temperate small fruits for growing in the highlands. Shoot cultures of red raspberry cultivars grown in Thailand had stunted growth, poor shoot quality, and symptoms of mineral deficiencies or toxicities, which indicate that MS medium might not be suitable for growing these red raspberry cultivars (Poothong and Reed, 2014; Poothong and Reed, 2015). Many studies about the effects of *in vitro* mineral salts showed that optimizing nutrients could improve growth and development including greenness of leaves or chlorophyll content (García-Jiménez *et al*., 2006; Poothong and Reed, 2015; Poothong and Reed, 2016; Kovalchuk *et al*., 2017; Kovalchuk *et al*., 2018). Therefore, in this study the objective was to investigate the effect of MS mineral salts and sucrose on growth and development of red raspberries for improving effective micropropagation and promoting better commercial production.

2. Materials and Methods

2.1 Plant material and culture conditions

For establishing shoot cultures, plant materials of the red raspberry 'Amity' commonly grown in Northern of Thailand were used for this study. The shoot tips were used as explants. After surface sterilization with 10 % Clorox® with tween 20 for 10 mins and rinsing with sterilized water three times for 5 mins, meristems about 0.5-1.0 mm were cut under stereo microscope for plant stock culture. All shoots were grown on MS (Murashige and Skoog, 1962) medium with 1.0 mg/L N6-benzylaminopurine (BAP), 0.01 mg/L indole-3-butyric acid (IBA), 30 g/L sucrose, 8 g/L agar (UnionSciences Lot: 2P6000430, Chiang Mai) at pH 5.7. Shoots were grown in glass bottles (8 oz.) with 30 ml of medium per container. These shoots were cultured at 25±1 ^{o}C with a 16 hr photoperiod with cool white fluorescent bulbs (PPFD = 35 µmol m^{-2} s^{-1}).

Table 1 The three factors used to construct 10 treatments, their component MS salts, and concentration range expressed as x MS levels. Treatment 4 is the MS control.

Treatment (Trt.)	Relative concentration compared to MS (x)		
	Nitrogen	Mesos	Micronutrient
Trt. 1	0.5	1.0	1.0
Trt. 2	1.0	0.5	1.0
Trt. 3	1.0	1.0	0.5
Trt. 4	1.0	1.0	1.0
Trt. 5	1.5	1.0	1.0
Trt. 6	1.0	1.5	1.0
Trt. 7	1.0	1.0	1.5
Trt. 8	2.0	1.0	1.0
Trt. 9	1.0	2.0	1.0
Trt. 10	1.0	1.0	2.0

2. 2 The effects of *in vitro* mineral nutrients

Experimental treatments were designed using three factors of MS mineral salts, which the treatments were varied using a completely randomized design (CRD). The three factors were nitrogen (KNO_3 and NH_4NO_3) , mesos ($CaCl_2.2H_20$, $MgSO_4.7H_2O$ and KH_2PO_4) and micronutrients (Zn-Mn-Cu-Co-Mo-B-I-EDTA-chelated iron). Each factor was varied as relative concentration compared to MS medium (0. 5x, 1.0x, 1.5x and 2.0x). There were 10 treatments (Table 1) and for each treatment, there were 5 replications with four shoots per bottle. Treatment 4 was the standard MS control. Shoots, approximately 0.5-1.0 cm, were planted and transferred to fresh treatment medium at four- week intervals. They were harvested after 12 weeks. For data collection, shoot growth response data were taken from shoots grown on each treatment (Table 1) . Response data (described below) were used for statistical analysis. Overall quality ratings were assigned to each shoot on a scale of 1 (poor quality) , 2 (moderate quality) and 3 (good quality) . Leaf color was measured with a portable Soil Plant Analysis Development (SPAD) - 502 meter (Konica Minolta) (relative chlorophyll content of leaves) . The numbers of shoots were counted and shoot length of the longest shoot was measured in mm (from base to shoot tip) . For statistical analysis, the data were analyzed and performed with the software of IBM SPSS Statistics 20. 0. Data were subjected to an analysis of variance (ANOVA) followed by separating for mean differences using the Duncan's multiple range tests (DMRT) at $p < 0.05$.

2. 3 The correlation between leaf greenness (SPAD reading) and chlorophyll content

Leaves were randomly collected from shoots grown on various conditions for providing leaf greenness reading. Each sample was measured using SPAD reading protocol and analyzed by spectrophotometer for chlorophyll content. Twelve samples were used for data analysis.

2. 4 Experimental designs for optimi-zation

Optimized culture medium was conducted using the results from previous study (the effects of *in vitro* mineral nutrients), which had half of the nitrogen concentration and twice the mesos concentration, to test the effects of modified MS (defined as mMS). This mMS medium was used to grow red raspberry shoots compared to MS basal medium for 4 weeks. Shoot growth responses; total phenolic and the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity for antioxidant activity of shoots were investigated. Plant leaves were prepared by grinding 10 mg of fresh sample in 70% ethanol. The amount of total phenolic content and antioxidant activity were determined using Folin-Ciocalteu assay with modification and measured using DPPH radical scavenging assay with modification as described in Katsube *et al*. (2004).

2.5 Experimental design for studying the effect of sucrose

Four sucrose concentrations (0, 1, 2 and 3 %) were used with two mineral formulations (MS and mMS). This experiment was carried out using a completely randomized design (CRD). Shoot were grown on 8 treatments and cultured for 4 weeks. For data collection, the criteria explained in 2.2 (effects of *in vitro* mineral nutrients) were applied.

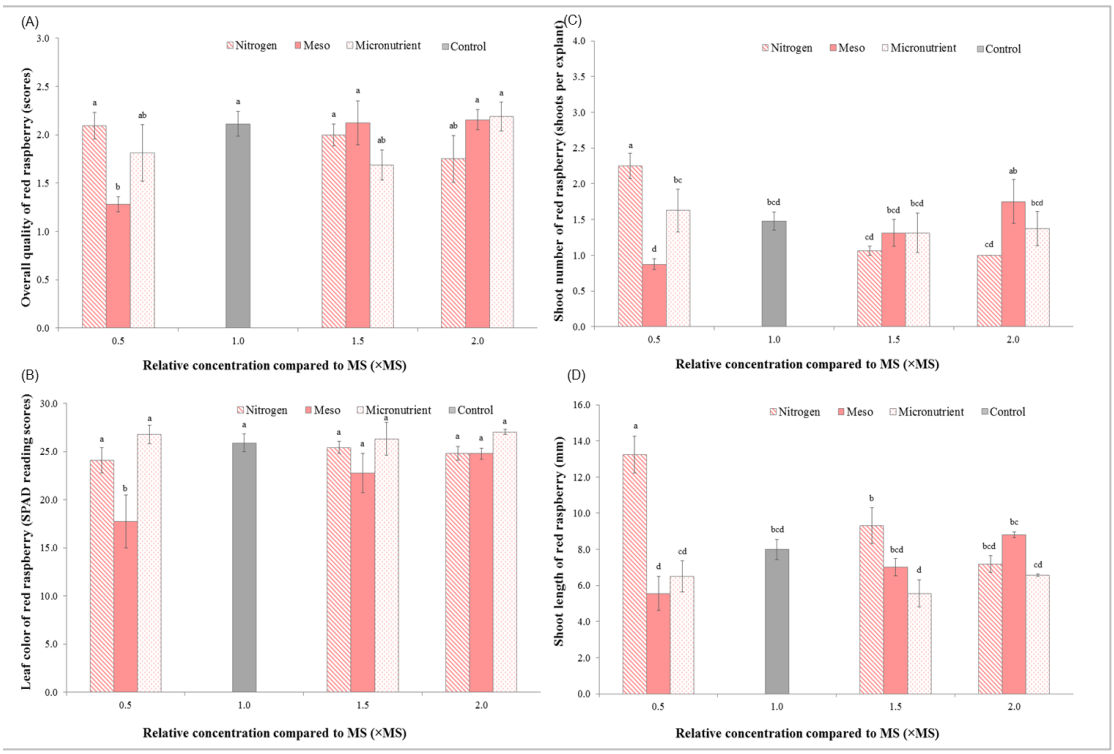

Figure 1 The effect of *in vitro* mineral nutrients on (A) overall quality, (B) leaf color, (C) shoot number and (D) shoot length of 'Amity' red raspberry after cultured for 12 weeks with a four-week intervals.

3. Results and Discussion

3. 1 The effects of *in vitro* mineral nutrients on plant growth

There were no significant differences in the quality of shoots grown on the four concentrations of nitrogen or minor nutrients. However, the overall quality of shoots of 'Amity' grown on 0. 5x mesos concentration had significantly lower quality compared to shoots grown the other treatments (p- value = 0. 006) (Figure 1A). The changes of leaf color were similar to the overall quality response (Figure 1B). These results indicate that the mesos component was critical for both quality and leaf color, as reduced mesos produced decreased quality and leaf color. For shoot number, shoots grown on high mesos (2x) had high multiplication and produced significantly more shoots than the other treatments (Figure 1C). Reduced amounts of nitrogen (0. 5x) produced significantly more shoots the MS control or the other nitrogen treatments (Figure 1C). Finally, nitrogen also seemed to be the interesting factor affecting shoot length because shoots grown on reduced nitrogen had greater shoot length than any other treatments or the MS control (Figure 1D). Mesos and micronutrients had significant effects on shoot number or shoot length only at the highest level 2x (Figure 1C and 1D). Plant growth appearance of each treatment was different and shoots grown on low mesos (0. 5x) showed obvious stunted growth (Figure 2). Therefore, MS medium for this cultivar was modified by reducing nitrogen to 0. 5x and increasing mesos to 2x MS concentration. The plant response to these treatments can be seen in Figure 2.

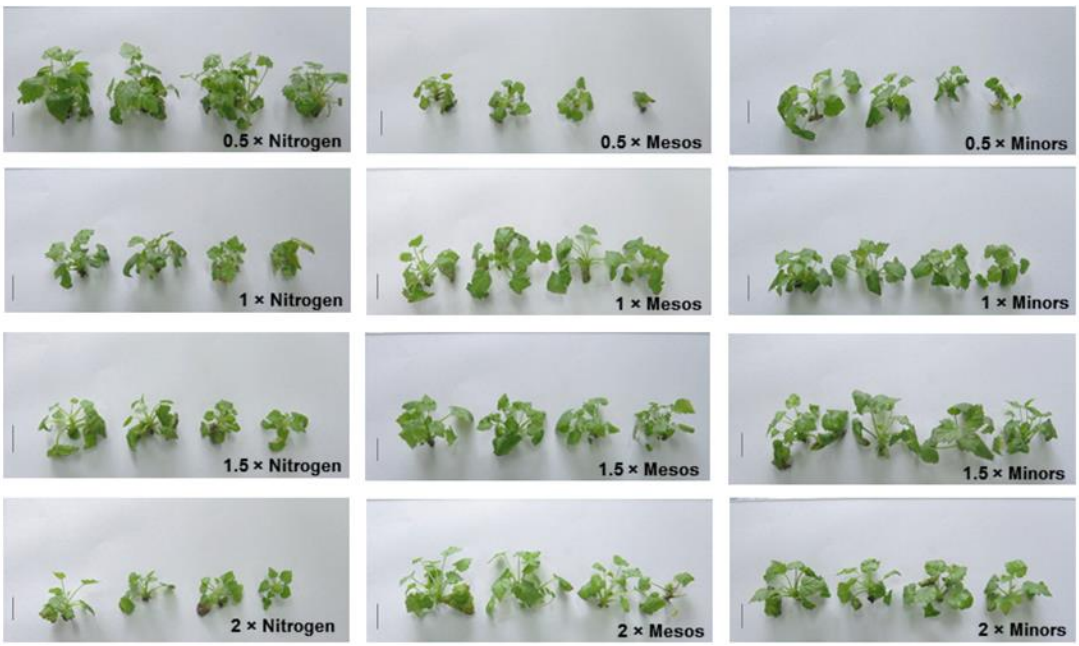

Figure 2 Plant growth appearance of shoot grown on nitrogen, mesos and micronutrient treatments (bar = 1 cm).

3. 2 The correlation between leaf greenness (SPAD reading) and chlorophyll content

Leaf samples were evaluated for leaf color based on SPAD meter readings for quantifying the relative chlorophyll content. Samples were analyzed for chlorophyll content using a spectrophotometer. Results showed that the SPAD reading scores had strong correlation to chlorophyll a, b and total chlorophyll (Figure 3). When leaves were measured having high scores of SPAD reading, they also had high chlorophyll content (Fig. 3).

3.3 The effects of optimized MS mineral nutrients on plant growth, the phenolic compounds and antioxidant activity

Shoots grown on optimized medium with 0.5x nitrogen and 2x mesos (mMS) had good growth and shoot length (Figure 4A and 4B). However, for leaf color (SPAD reading) and shoot number, there were no significant effects of the optimized medium on these responses (Figure 4A and 4B). Phenolic compounds and antioxidant activity were not significantly different for the two medium formulations (Figure 4C and 4D).

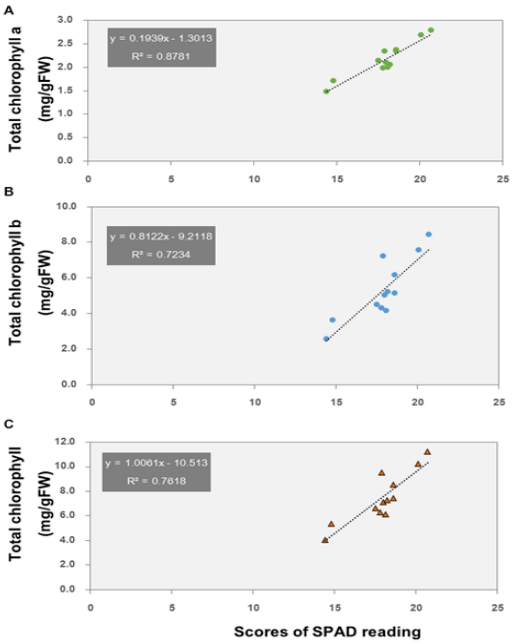

Figure 3 The correlation between leaf greenness (SPAD reading) and (A) chlorophyll a content (B) chlorophyll b content and (C) total chlorophyll content.

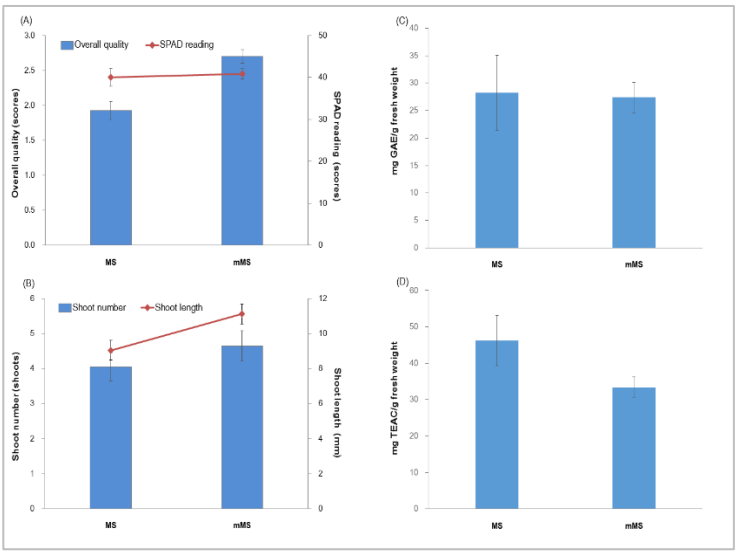

Figure 4 The effect of modified MS (mMS) and MS on (A) overall quality and SPAD reading, (B) shoot number and shoot length, (C) phenolic compounds and (D) antioxidant activity after culture for four weeks.

3. 4 The effect of sucrose on plant growth

According to the ANOVA, sucrose concentrations did not affect overall quality or leaf color when used with MS medium (Figure 5A and 5B). Quality of shoots in mMS medium were not significantly different from those grown on MS (Figure 5A), but shoots grown on mMS had significantly lower SPAD readings (Figure 5B). Using mMS medium with reduced (1.5 %) sucrose or the typical concentration (3 %), shoots had higher multiplication than MS medium or lower sucrose concentrations (Figure 5C). However, for shoot length, shoots grown on MS medium without sucrose had the greatest length and other treatments were not significantly different (Figure 5D). Only shoots grown on the 1.5 % sucrose concentration were not significantly different in length when compared to MS, all others were shorter (Figure 5D).

Although increasing nitrogen, mesos and minors did not significantly improve growth and development of 'Amity' red raspberry, reducing either nitrogen or mesos produced poor growth and leaf color. When the three mineral components were modified, shoots had slightly better quality compared to the control (MS) (Figure 1). Poothong and Reed (2014) reported that optimization of mesos components in culture

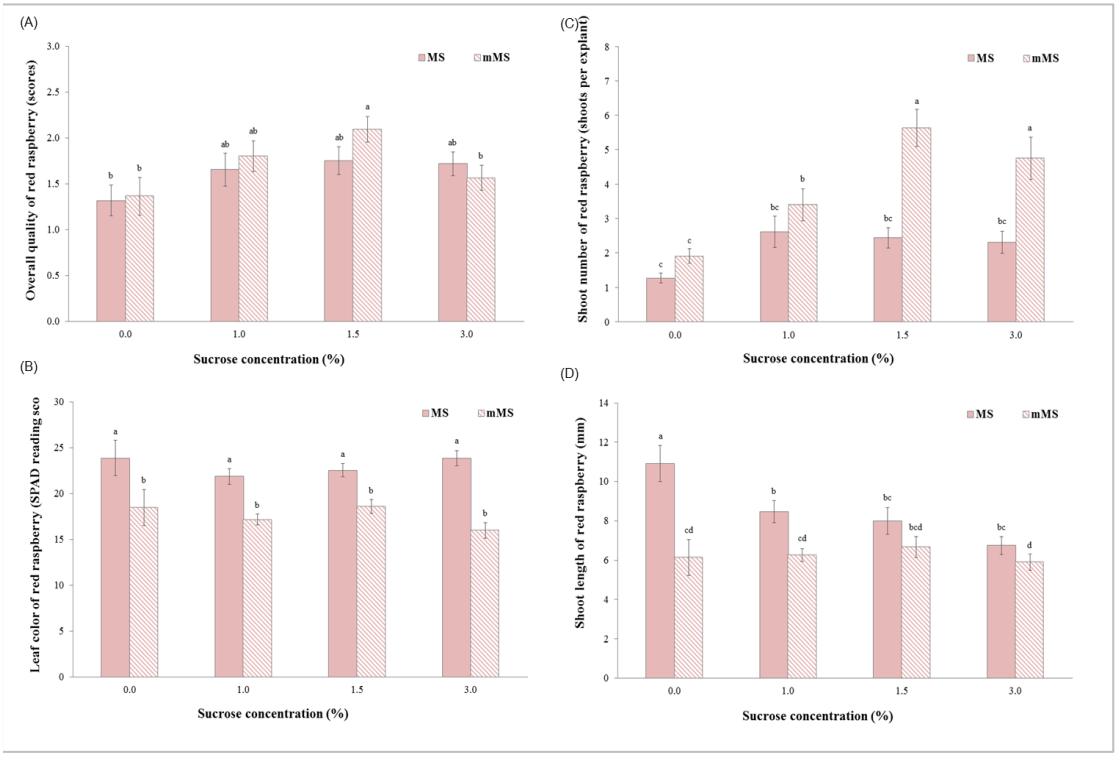

Figure 5 The effect of modified *in vitro* mineral nutrients and sucrose on (A) overall quality, (B) leaf color, (C) shoot number and (D) shoot length of 'Amity' red raspberry after culture for four weeks.

medium was useful for investigating the effects of each component on plant growth of five red raspberry cultivars. In that study, increased mesos were the most significant factor affecting plant quality, shoot length and multiplication of 'Canby', 'Nootka', 'Indian Summer', 'Willamette' and 'Trailblazer' (Poothong and Reed, 2014). The results showed that all three mesos components needed to be increased to 2x MS concentrations for improving shoot quality (Poothong and Reed, 2014). These results demonstrate that the mesos components in MS medium are insufficient for many red raspberry cultivars (Poothong and Reed, 2015). In micropropagated red raspberry, mesos was significant for leaf color as the effect of magnesium and calcium (Niedz and Evens, 2007; Poothong and Reed, 2014). Wu *et al*. (2009) found that the strength reduction of MS basal medium as one-third, with 0.49 µM indole-3-butyric acid and 0.05 % activated charcoal, and culturing shoots under reduced light intensity, could alleviate chlorosis and improve plant quality of micropropagated *Rubus* plantlets (Wu *et al*., 2009). Sufficient mineral nutrients in the culture medium could provide optimal growth of *in vitro* plants, resulting in successful micropropagation without increasing plant growth regulators (Preece, 1995). In apricot, the yellow leaves with dark green veins, a typical symptom for magnesium deficiency, was observed in low KH_2PO_4 and high $CaCl_2$ medium. Potassium, calcium and magnesium ions compete with each other for uptake (Kovalchuk *et al*., 2017). In the current study, increased nitrogen reduced shoot multiplication in 'Amity'. This was similar to the study of red raspberry study with five cultivars, where decreased KNO_3 with higher mesos and less iron increased shoot multiplication (Poothong and Reed, 2014). In the five factors study of red raspberry, most cultivars except 'Willamette', shoots grown on reduced NH_4NO_3 were stunted and had little elongation (Poothong and Reed, 2014). In the current study optimized MS mineral nutrients with 0.5x nitrogen and 2x mesos concentrations provided better quality for 'Amity'. Although there were no significant differences in the total phenolic compounds and DPPH between MS and mMS, in other responses shoots grown on optimized mMS had high quality and shoot length.

Although there is less photosynthesis in *in vitro* cultured plants, leaf greenness is very important for evaluating the effects of mineral nutrients on plant growth (García-Jiménez *et al*., 2006; Poothong and Reed, 2015; Poothong and Reed, 2016; Kovalchuk *et al*., 2018). Measurement of the greenness of leaves was subjective using scores for leaf colors. This scoring system is not practical for interpreting how the color scores relate to the photosynthetic capacity of leaves. SPAD reading is used to read the greenness based on the light reflection and most researchers use readings to estimate the chlorophyll content of leaves (Rolando *et al*., 2015). However, higher SPAD readings sometimes do not correlate well with actual chlorophyll content (Uddling *et al*., 2007). For this study, the correlation of between greenness

(SPAD reading) and chlorophyll content was determined. The strong correlation of greenness and chlorophyll a, b and total chlorophyll content showed that this SPAD reading was a reliable method for estimating chlorophyll content of *in vitro* shoot cultures. The destructive methodology for chlorophyll extraction using organic solvents is very common in photosynthetic studies. However, the portable chlorophyll SPAD-502 meter can be used to analyze the photosynthetic pigments as well. Netto *et al.* (2005) found that there was a correlation between photosynthetic pigments in *Coffea canephora* Pierre leaves and the SPAD-502 readings (Netto *et al*., 2005).

Optimizing sucrose concentration for improving growth and development has been studied in many plant species (Nhut *et al*., 2001; Faria *et al*., 2004). In the current study, the result showed that 1.5 % of sucrose, half the MS concentration, promoted shoot multiplication and shoot length. The optimal sucrose concentration was 3 or 4 % sucrose for shoot proliferation in *Lilium longiflorum* (Netto *et al*., 2005) as it is for many plants. However, Nhut *et al*. (2001) also found that reduced sucrose at 1 or 2% was suitable for shoot regeneration lily. In *Dendrobium nobile*, the highest multiplication and shoot elongation were obtained with high sucrose (6 %), which was twice the typical concentration used in other plants (Faria *et al*., 2004).

4. Conclusion

This study indicated that optimizing *in vitro* minerals especially nitrogen and mesos is critical for plant growth and development of red raspberry cultivars. Therefore, optimizing the MS salts provided better growth and increased the efficiency of micropropagation. The SPAD reading was suitable for evaluating greenness and chlorophyll content of red raspberry. Moreover, reduction of sucrose to 1.5% provided better shoot length and multiplication for 'Amity' red raspberry. The optimal medium for 'Amity' red raspberry was 0.5x MS nitrogen, 2x MS mesos, and 1.5 % sucrose.

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