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Original Article

In vitro mineral nutrition for improving growth and multiplication of stevia

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ABSTRACT

In vitro propagation is important for rapid multiplication of a wide range of nursery crops or medicinal plants, including stevia (*Stevia rebaudiana* Bertoni). The aim of this study was to investigate the effects of mineral salts on plant growth and development of stevia shoot cultures. Response surface methodology was used to design experiments by varying three factors: nitrogen salts (NH_4NO_3 and KNO_3), mesos salts ($CaCl_2$, KH_2PO_4 and $MgSO_4$) and minor elements (Zn-Mn-Cu-Co-Mo-B-I-EDTA-chelated iron). The concentrations of each factor were defined as relative concentrations compared to Murashige and Skoog (MS) concentrations (0.5–3.0 × MS). The effects were evaluated of these three factors on plant quality, multiplication, shoot length and leaf numbers. The minor elements were the most significant factors associated with shoot length and leaf numbers. Increasing minor elements above an MS level of 1 × and decreasing nitrogen tended to increase shoot length significantly. Increasing minor elements and nitrogen up to 3 × MS and increasing mesos to 1.5 × MS were required to improve leaf numbers. Two optimized media were compared to MS for growth characteristics, phenolics and antioxidant activity. One of the media was identified as significantly better than MS for growth, low phenolic production and low antioxidant response.

Introduction

Stevia (*Stevia rebaudiana* Bertoni) has been used commercially in a wide range of products in the food and beverage industry as a natural sweetener for many decades because it has stevioside and rebaudioside in its leaves (Prakash and Chaturvedula, 2018). This plant is commercially cultivated in Brazil, Paraguay, Central America, Thailand, Korea, China and India (Hossain et al., 2017). Although stevia can be propagated by using seed or stem cuttings, one of the most effective methods for propagation is *in vitro* clonal propagation. This technique can produce disease-free and uniform plants for large scale production. Consequently, an effective protocol of micropropagation is very necessary for stevia cultivation (Yucesan et al., 2016). Plant tissue culture medium is a major component of plant growth response which is composed of several factors such as minerals, organic compounds, plant growth regulators and a carbon source (Williams, 1993). The mineral nutrients play essential roles in plant growth and development (Anderson, 1980; Murashige and Skoog, 1962) but they have rarely been studied for stevia culture. Generally, the Murashige and Skoog (MS; Murashige and Skoog, 1962) formulation is used for *in vitro* culture of many plants. However, many studies have shown that some plants do not grow well on MS medium. In many plant species such as red raspberries, pear, soybean, rice or cotton, shoot cultures exhibiting non-optimal growth have been observed (Dantas et al., 2001; Greenway et al., 2012; Ružić et al., 2000). Stevia, which is a herbal plant used as a sugar substitute, has been propagated by seeds or cuttings. Nevertheless, the seeds have a low germination percentage and cuttings might result in non-uniform clones. Plant tissue culture can be applied for rapid clonal propagation and uniform plantlets. Most studies of *in vitro* clonal propagation via direct organogenesis in stevia have applied MS medium and focused on plant growth regulators rather than optimizing mineral nutrients (Gantait et al., 2015). Modifying mineral salts in the culture medium of stevia can provide an effective technique for improving micropropagation and commercial production for industry.

Determining the effects of *in vitro* mineral nutrients is very challenging and very complicated because changing one compound of nutrients can affect the uptake or availability of the others (Niedz and Evens, 2006; Williams, 1993). Shoot cultures of stevia on MS medium have small leaves with thin and too long stems showing

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poor shoot quality. Design of Experiments (DOE) software is one of useful tools for experiments and can now be used to design experiments that optimize these nutrients and model the effects of MS mineral nutrients. This design has been used with many plants such as red raspberry, pear, gerbera and hazelnut (Hand et al., 2014; Niedz and Evens, 2007; Niedz et al., 2014; Poothong and Reed, 2014, 2015, 2016; Reed et al., 2013a). Response surface methodology (RSM), which is one of the techniques applied in DOE, has been used to explore the relationships between several factors and one or more responses to achieve some goal for optimization as it allows a reduction of treatments or experiments, and also provides a prediction of the influential factors using mathematical models (Anderson and Whitcomb, 2005; Bradley, 2007). The aim of this study was to determine the optimal mineral components for stevia using RSM, and to study the effect of those minerals on the growth and development of stevia.

Materials and methods

Plant materials and establishment of shoot cultures

Stock cultures of the stevia used for this study were initiated from stock plants age 5 mth at the School of Agriculture and Natural Resources, University of Phayao, Thailand. The shoot tips were used as explants. After surface sterilization (using 10% chlorox with Tween20 for 10 min and rinsing with sterilized water three times), all shoots were grown on MS (Murashige and Skoog, 1962) medium with LS vitamins (Linsmaier and Skoog, 1965), 4.44 μM 6-benzylaminopurine (BAP), 0.49 μM indole-3-butyric acid (IBA), 30 g/L sucrose with 8 g/L agar (UnionSciences Lot:2P6000430; Chiang Mai, Thailand) with the pH at 5.7. Then, the media were autoclaved. Shoots were grown in glass bottles (225 g) closed with plastic caps with 30 mL of medium per container and transferred to fresh medium every 4 wk. All shoots were grown at 24 ± 1 °C and a 16 h photoperiod (2500–3000 lx intensity).

Nutrient optimization

The experimental design was a three-factor RSM design where the design points (combinations of the three factors) were selected using a modified D-optimal design using the software application Design Expert®8 (Design-Expert, 2010). These points were suitable for fitting a quadratic polynomial equation. Three mineral nutrient factors were based on MS salts: 1) NH_4NO_3, and KNO_3; 2) mesos ($CaCl_2 \cdot 2H_20$, KH_2PO_4, $MgSO_4.7H_2O$); and 3) micronutrients (B, Cu, Co, I, Mn, Mo, Zn and Fe-EDTA). Each factor was varied over a range of concentrations expressed in relation to the MS medium (where 1× was the MS concentration) as shown in Table 1. There were 10 model points, 5 lack-of-fit points and 5 replicated points either within or on the surface of the three-dimensional design space (Table 2). Then, cut stems containing 1 node, (about 1.0 cm) were cultured on a set of treatment combinations. Each treatment included four plantlets in each of five bottles ($n = 20$). Nodal segments were transferred to the same medium at 4 wk intervals and harvested after 12 wk.

For the second step, optimization points for predicting the most suitable concentrations of the three mineral components were conducted for validation testing. Two optimized combinations were selected and tested compared to MS (Table 3). Shoots were grown on different formulations of media. In the validation test, the experiments were conducted to empirically assess the capability

Table 1
Three factors used to construct the three-dimensional design space, their component Murashige and Skoog (MS) salts and concentration range expressed as ×MS levels.

Factor	MS salt	Range
Group 1	NH_4NO_3 KNO_3	0.5–3.0×
Group 2 (mesos)	$CaCl_22H_2O$ KH_2PO_4 $MgSO_4$	0.5–3.0×
Group 3 (minors)	$MnSO_4H_2O$ $ZnSO_47H_2O$ $CuSO_45H_2O$ KI $CoCl_26H_2O$ H_3BO_3 $Na_2MoO_42H_2O$ $FeSO_47H_2O$ Na_2EDTA	0.5–3.0×

EDTA = ethylenediaminetetraacetic acid.

Table 2
Murashige and Skoog (MS)-stock based on three-factor design with 10 model points, 5 lack-of-fit points and 5 replicated points and MS medium controls (Run 21) for pure error estimation.

Treatment designed runs[a]	Factor 1 NH_4NO_3	Factor 2 KNO_3	Factor 3 Mesos
1	0.50	1.33	0.50
2	3.00	0.50	1.75
3	0.50	0.50	3.00
4	1.75	3.00	1.75
5	1.33	2.17	3.00
6	1.33	2.17	3.00
7	1.33	0.50	1.33
8	2.17	3.00	0.50
9	0.50	3.00	2.17
10	3.00	1.75	1.75
11	1.13	1.75	1.75
12	1.13	1.75	1.75
13	0.50	3.00	0.50
14	1.33	0.50	1.33
15	3.00	3.00	3.00
16	3.00	1.75	0.50
17	1.13	1.75	1.75
18	3.00	0.50	0.50
19	3.00	1.75	1.75
20	3.00	0.50	3.00
21	1.00	1.00	1.00

[a] Runs 1–20 were assigned for treatment combinations and run 21 (MS point) was run as control.

Table 3
Optimized formulations of Murashige and Skoog (MS) levels using three-factor design for validation.

Factor	MS salt	Relative concentration expressed as × MS levels		
		Optimized Formulation 1 (OMS1)	Optimized Formulation 2 (OMS2)	MS control
Group 1	NH_4NO_3 KNO_3	1.5	2.2	1.0
Group 2 (mesos)	$CaCl_2{\cdot}2H_2O$ KH_2PO_4 $MgSO_4$	1.8	1.3	1.0
Group 3 (minors)	$MnSO_4{\cdot}H_2O$ $ZnSO_4{\cdot}7H_2O$ $CuSO_4{\cdot}5H_2O$ KI $CoCl_2{\cdot}6H_2O$ H_3BO_3 $Na_2MoO_4{\cdot}2H_2O$ $FeSO_4{\cdot}7H_2O$ Na_2EDTA	3.0	3.0	1.0

EDTA = ethylenediaminetetraacetic acid.

Table 4
Analysis of variance model terms, *p*-values (Prob. > *F*) and lack-of-fit statistics for the effects of Murashige and Skoog (MS) mineral salts on overall quality, shoot number, shoot length and leaf number of micropropagated stevia shoots.

Source	Overall quality	Shoot number	Shoot length	Leaf number
Model	0.1040	0.3221	0.0069*	0.0381*
A Nitrogen	0.0668	0.8011	0.2382	0.1693
B Mesos	0.9964	0.6556	0.4301	0.7884
C Minors	0.1810	0.0265*	0.0005*	0.0062*
AB	0.6221	0.0952	0.2269	0.1072
AC	0.1884	0.9517	0.5582	0.8895
BC	0.7757	0.4081	0.1264	0.7232
Lack of fit	0.9397	0.0941	0.8459	0.2784
Model type	Quadratic	Quadratic	Quadratic	Quadratic

*Significant at $p < 0.05$.

and usefulness of the predictive and proposed model via optimization. Points in the design space from numerical optimization were defined as optimized MS medium 1 (OMS1) and optimized MS medium 2 (OMS2) as shown in Table 3. Growth appearance was evaluated for data collection. In the validation test, total phenolic and the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of plants were investigated. Plant samples were prepared by grinding 10 mg of fresh sample in 70% ethanol. The solution was then kept as a stock solution. Samples were diluted to a desirable working concentration in water. 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).

Data collection and statistical analysis

Plant growth response data were taken from shoots grown on each treatment (Table 2). Response data (described below) used for statistical analysis were calculated from the mean of four shoots from four replications ($n = 16$). Four shoots were used from predetermined locations in the container for photographs ($n = 4$). Quality ratings were assigned to each shoot on a scale of 1 (poor quality), 2 (moderate quality) and 3 (good quality). Shoot and leaf numbers were counted, and the shoot length of the counted shoots was measured in millimeters (from base to shoot tip). Experimental design, analysis and graphics were conducted using the Design Expert®software (Stat-Ease Inc.; Minneapolis, MN, USA; Design-Expert, 2010). The best fitting polynomial regression model was obtained for each measured response. The data were analyzed using analysis of variance (ANOVA) and the *F* and *P* values of overall models used were tested at the significant level of $p \leq 0.05$ and at the highly significant level of $p \leq 0.0001$. For the validation test, plant growth response data were taken from shoots grown on each treatment (Table 3). Response data (described above) were calculated from the mean of four shoots from four replications ($n = 16$) and subjected to one-way ANOVA for mean comparison. The least significant difference was tested at $p < 0.05$ using the IBM SPSS statistical package (Version 24.0; IBM Corp.; White Plains, NY, USA).

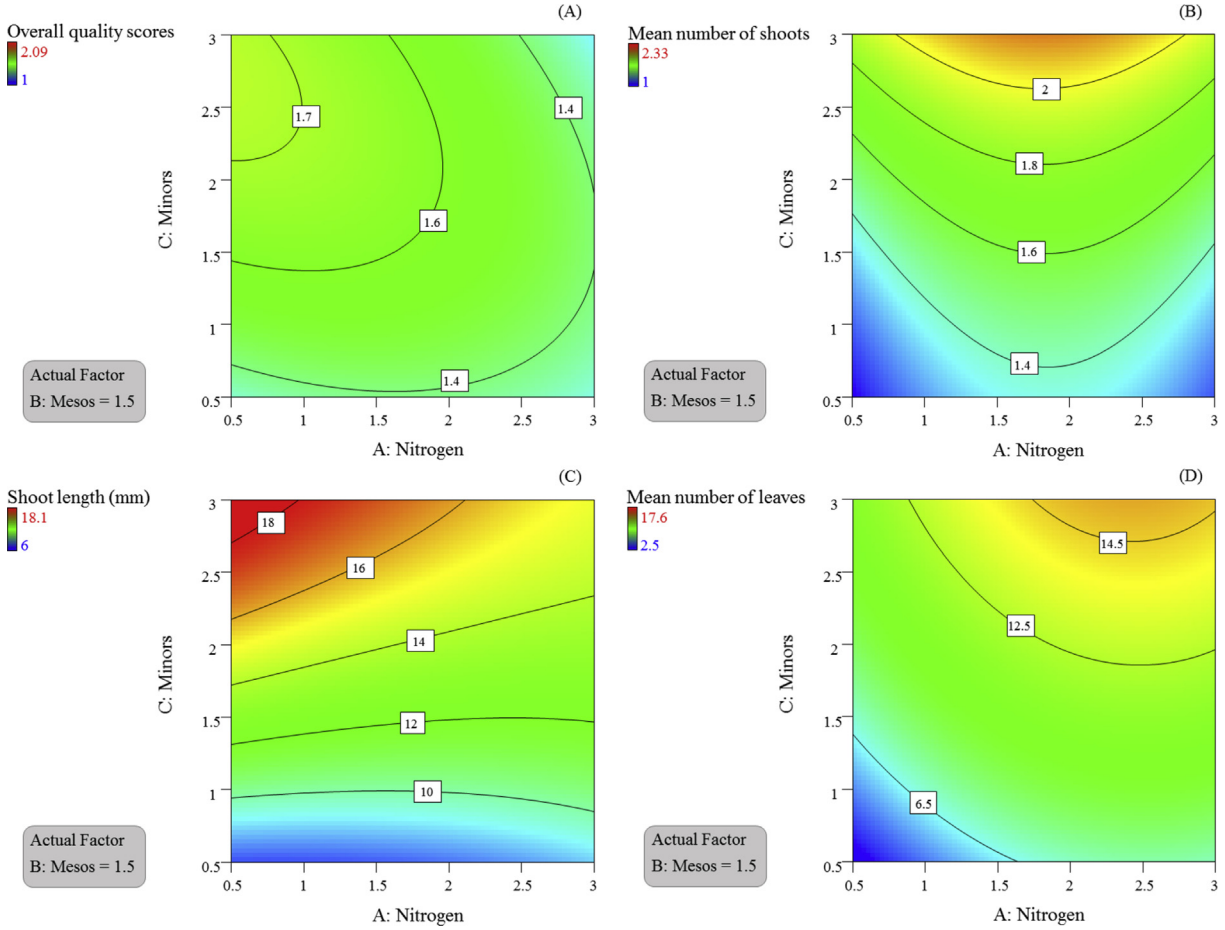

Fig. 1. Projected 3-D graph of mineral effects on shoot responses: (A) overall quality [scored 1 = poor (dark blue) to 3 = good (red)]; (B) mean number of shoots [low number (dark blue) to high (red)]; (C) shoot length [low shoot length poor (dark blue) to high (red)]; (D) mean number of leaves [low number (dark blue) to high (red)].

Results

The analysis of variance table shows the effects of three factors on the evaluated responses using RSM (Table 4). Projected 3-D graphs of the best response regions in the 3-factor design space are presented in Fig. 1. The response models were significant in shoot length and leaf number and the significant effects of the minors and nitrogen varied in both cases (Table 4 and Fig. 1).

Quality rating, shoot number, shoot length and leaf number

The quality rating (scored from 1 to 3) was a subjective measurement that represented the overall appearance of the stevia shoots. The models indicated that changes in any of the three factors were not significant ($p = 0.1040$). Shoot quality was slightly improved by increased levels of minor elements and by low nitrogen while high mesos decreased quality (Fig. 1A). Shoot numbers seemed to be influenced by high concentrations of minor elements over a range of nitrogen concentrations and low to moderate mesos (Fig. 1B). The optimal shoot number was 2–4 shoots per initial shoot. The models showed that producing a greater number of shoots required higher levels of minor elements (Fig. 1B). Increased nitrogen seemed to reduce shoot multiplication. For micropropagated stevia, the optimal shoot length was about 30 mm. Increased levels of minor elements and low nitrogen and low-to-moderate levels of mesos were significant for shoot length (Table 4). Minors at 3 × MS with half strength MS nitrogen provided longer shoots (Fig. 1C). Leaf numbers were influenced by high levels of minor elements and nitrogen with moderate levels of mesos (Table 4 and Fig. 1D). The optimal leaf number was 5–10 leaves per shoot. The extrapolated graph showed that shoots grown on medium with increased level of minor elements and nitrogen had greater leaf numbers (Fig. 1D). Based on the statistical analysis, the level of mesos did not have a significant effect on leaf number. However, mesos at 1.5 × MS concentration resulted in slightly greater leaf numbers (Fig. 1D).

Optimization of Murashige and Skoog mineral salts using response surface methodology

Two combinations provided from the software optimization using the desirability function of RSM were tested and compared to MS medium for validation. The results show that shoots grown on OMS1 and OMS2 had significantly improved quality (Fig. 2A), shoot numbers (Fig. 2B), shoot length (Fig. 3A) and leaf numbers (Fig. 3B) compared to plants grown on MS medium. The appearance of plants grown on the two optimized MS media showed greatly improved growth (Fig. 4). The phenolic contents and the DPPH radical scavenging activity of plants grown on the three media

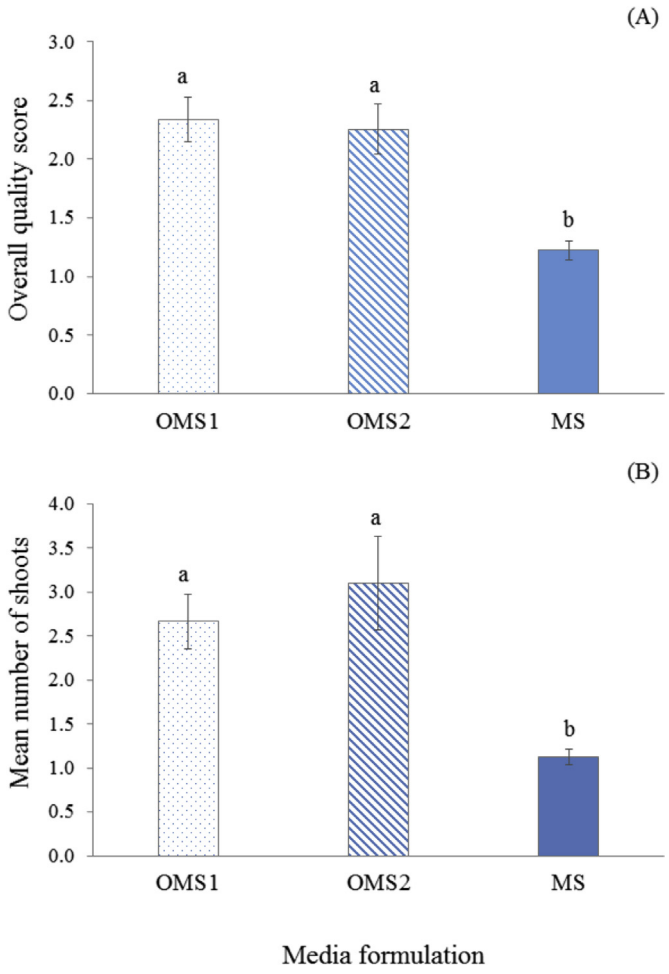

Fig. 2. Effects of optimized Murashige and Skoog (MS) medium on shoot growth: (A) overall quality scores; (B) mean number of shoots, where error bars indicate ± SE and different lowercase letters above columns indicate significant ($p < 0.05$) differences using a least significant difference test.

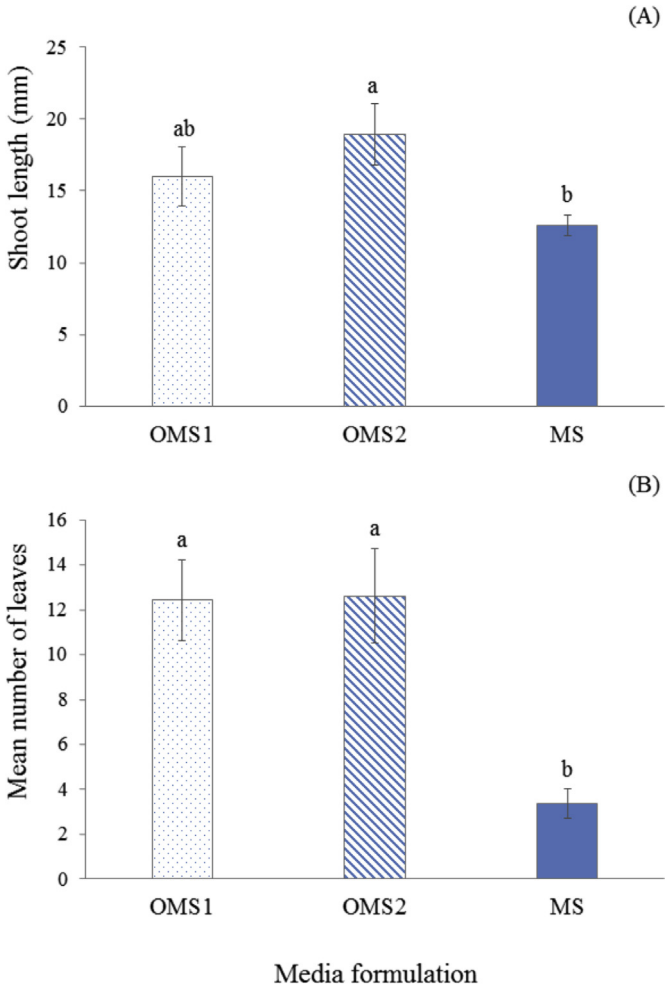

Fig. 3. Effects of optimized Murashige and Skoog (MS) medium on shoot growth: (A) shoot length; (B) mean number of leaves, where error bars indicate ± SE and different lowercase letters above columns indicate significant ($p < 0.05$) differences using a least significant difference test.

varied (Fig. 5). The plants grown on the OMS1 mineral formulation had a high phenolic content similar to MS but much higher than OMS2. The radical scavenging activity measured as Trolox equivalent antioxidant capacity (TEAC) was lowest for OMS2 while MS and OMS1 were significantly higher indicating that they were more stressed. OMS2 had low phenolics and low TEAC, indicating less stress overall. In addition, the shoot cultures on OMS2 had greater multiplication than the other two media (Fig. 4).

Discussion

The effects of mineral nutrients on *in vitro* growth of stevia were investigated using MS salts as the basal medium and applying RSM as to identify better alternative approaches. These mineral-based experiments are useful for understanding how *in vitro* nutrition affects plant growth and development. Micropropagation of stevia on the mineral nutrients of MS medium produced small shoots or poor shoot elongation (Fig. 4). Optimizing the mineral nutrients produced major changes in the morphology of shoots, with increased shoot length and greater multiplication. Leaves were healthy and expanded. These changes make it possible to economically micropropagate stevia lines.

Salts were grouped and used as the factors, so the effects of individual salts or ions could not be determined. However, the RSM approach in this study identified the driving factors for improving growth. The modeled responses using polynomial regression analysis from this study indicated that increasing minor nutrients to 3 × MS and decreasing nitrogen to 0.5 × MS with moderate levels of mesos should increase shoot length. This result was similar to the response of trailblazer red raspberry in a study modeling the effects of mineral nutrition and testing five factors (Poothong and Reed, 2014). That model showed that the best shoot length of trailblazer required moderate-to-high levels of mesos and low N and KNO_3 (Poothong and Reed, 2014). In micropropagated hazelnuts, the minor nutrients were also very important and caused a major difference in shoot growth and development (Hand et al., 2014). Although studying minor nutrient interaction was conducted using Driver and Kuniyuki Walnut medium (DKW: Driver and Kuniyuki, 1984) as the basal medium, those results clearly showed the effects of these minor elements on plant growth and

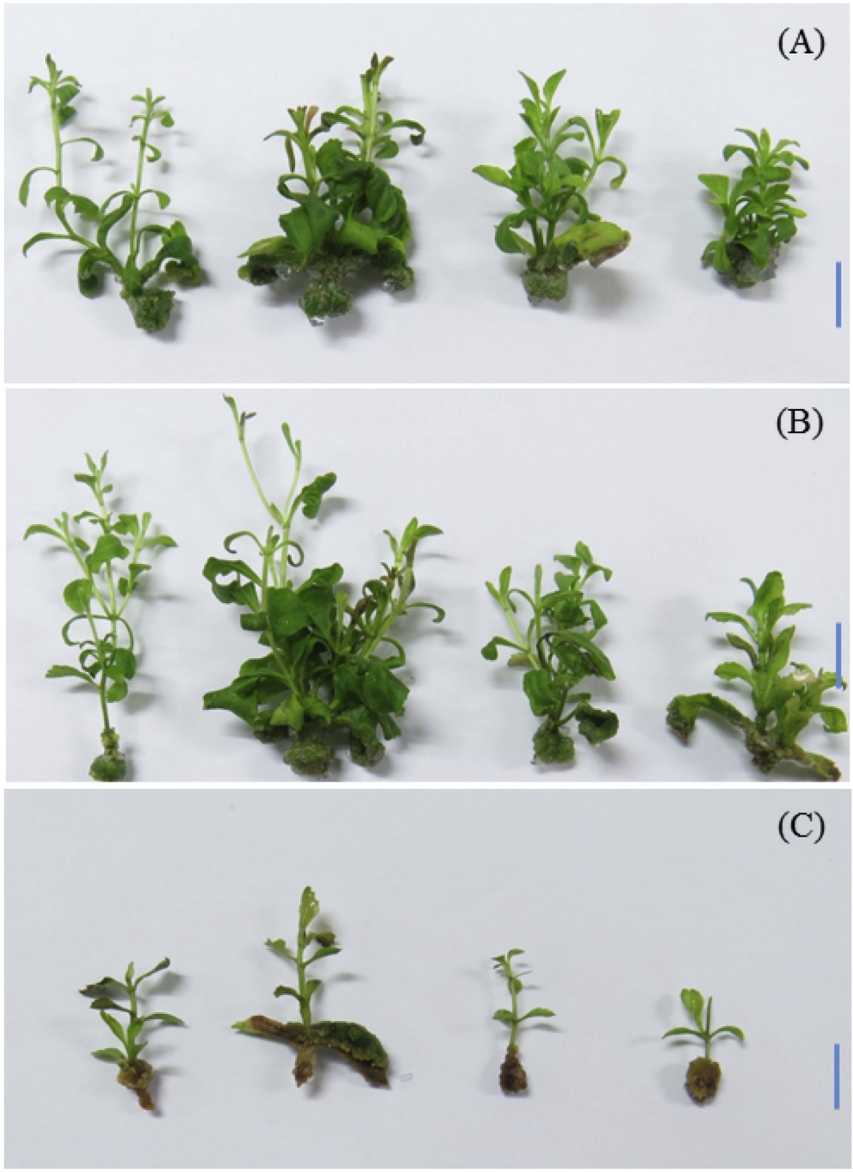

Fig. 4. Plant growth appearance of stevia shoots grown on different media formulation (see Table 3): (A) OMS1; (B) OMS2; (C) Murashige and Skoog, where scale bar = 1 cm.

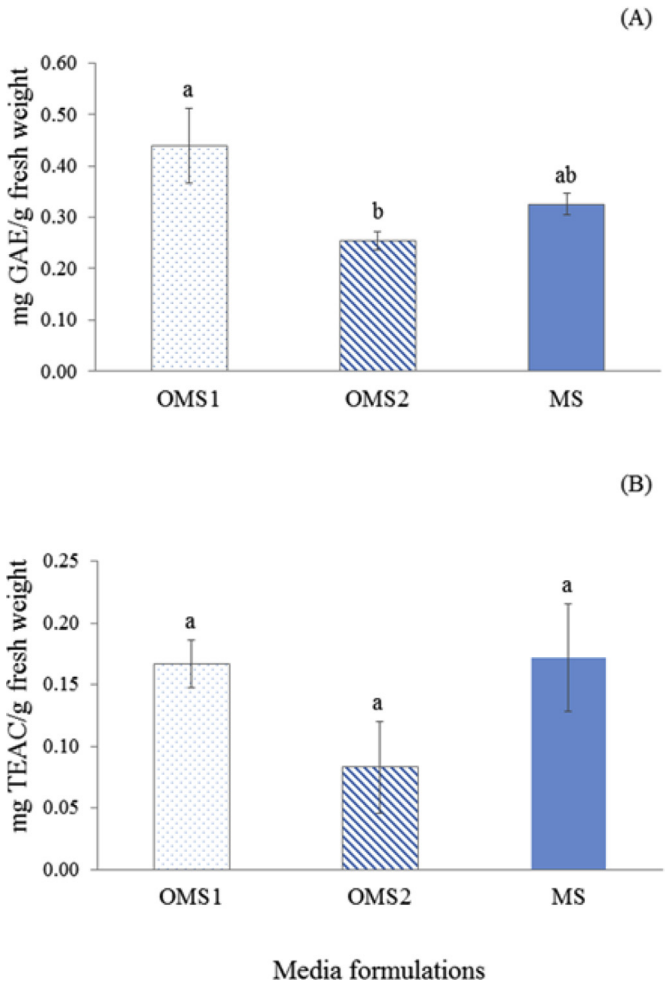

Fig. 5. Influence on shoots of media formulation (see Table 3) on: (A) total phenolic contents in stevia shoots; (B) antioxidant activity as 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity, where error bars indicate ± SE and different lowercase letters above columns indicate significant ($p < 0.05$) differences using a least significant difference test; TEAC = Trolox equivalent antioxidant capacity; GAE = gallic acid equivalents.

development depending on the genotype or cultivar (Hand et al., 2014). The minor element components in different culture media such as MS, DKW or Woody Plant Medium (WPM; Lloyd and McCown, 1980) are not identical. This could be interpreted as different plants needing various minor elements. Increasing minor nutrients was consistent with Hand and Reed (2014) who mentioned that 2 × DKW medium produced better overall quality of plants in most cultivars. The minor element concentration of twice DKW was much higher than that of MS. Based on the previous studies, increased $CuSO_4$ as 10 × and Na_2MoO_4 as 6.4 × using DKW salts as the basal medium also improved the quality and growth of hazelnut (Nas and Read, 2004). The results from the currents study indicated that the MS level of minor elements was not sufficient for stevia. Future study should investigate each component of MS minor element levels for a better understanding of the roles of *in vitro* mineral nutrition.

Most studies of *in vitro* clonal propagation of stevia applied MS as the basal medium and examined the effects of plant growth regulators for improved multiplication or regeneration from different tissues rather than testing the effects of minerals (Gantait et al., 2015). Nevertheless, Hwang (2006) investigated the effects of different medium formulation based on salts and found that MS formulation was the best compared to Gamborg's B5 medium (B5) (Gamborg et al., 1968), WPM and the medium used by Schenk and Hildebrandt (1972) for shoot regeneration and multiplication. The plant tissue culture study by Ibrahim et al. (2008) modified the MS mineral salts by adjusting the strength of concentrations. Shoot multiplication of stevia was improved on a medium with increased levels of micronutrient ($MnSO_4$, KI and CoCl) in the MS basal medium (Jain et al., 2012). Altering this factor also had significant effects on increased chlorophyll content and biomass in stevia leaves (Jain et al., 2012).

Minor elements are critical for growth and development because they are involved as cofactors for enzymes in plant metabolic processes. The current study found that increased levels of minor elements improved the shoot length and leaf number. Plants grown on test media with high levels of minor elements, high nitrogen and intermediate levels of mesos showed improved plant quality and growth appearance. Shoots from the two optimized media displayed very different profiles. The OMS2 shoots also had phenolic contents and DPPH radical scavenging activity that were lower than either OMS1 or MS. Both phenolic compounds and DPPH are widely used to investigate the relationship between antioxidant and scavenging activities and stresses. These results appeared to indicate less stress with the OMS2 medium. In *in vitro* cultures stress can result from limiting factors of nutrition, light and carbon dioxide content. These stresses result in plants growing poorly. If the culture medium is not suitable or has non-optimal mineral nutrients, plants will show stunted and abnormal growth along with higher amounts of reactive oxygen species or changes of metabolites (Poothong et al., 2017; Reed et al., 2013b).

The current study investigated the initial optimization of stevia mineral nutrient medium and showed that changes in the levels of minor nutrients had significant impacts on the growth of stevia shoots. In addition, the initial optimized medium (OMS2) provided an intermediate growth medium for continued optimization.

Conflict of interest

The authors declare that there are no conflicts of interest.

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