บทความวิจัย (Research Article)

Optimization of minerals and plant growth regulators for micropropagation of strawberry 'Pharachatan 80'

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Abstract

Micropropagation is important for rapid multiplication of a wide range of nursery crops, including strawberry. Although most plant species or cultivars can grow on Murashige and Skoog (MS) medium, some display non-optimal growth. This study used response surface methodology (RSM) to study the effect of MS minerals on micropropagated strawberry growth and determine which of these minerals are critical for improving growth. *In vitro* growth of strawberry 'Pharachatan 80' was determined by varying five factors that included NH_4NO_3, KNO_3, mesos salts ($CaCl_2$, KH_2PO_4 and $MgSO_4$), minor elements, and EDTA-chelated iron. The effects of these five factors on plant quality, multiplication, shoot length, and leaf color were determined and validated. The results showed that modified MS as 1.0×NH_4NO_3, 0.5×KNO_3, 2.0×Mesos, 3.0× Minors and 2.0×Iron significantly improved growth of strawberry 'Pharachatan 80'. Finally, modified MS supplemented with 1 mg/L BAP was suitable for shoot multiplication.

Keywords: Micropropagation, mineral nutrition, response surface methodology, strawberry 'Pharachatan 80'

Introduction

Strawberry (*Fragraria annanassa*) which belongs to family Rosaceae is one of the world's most popular berry crops. The plant is grown worldwide both in commercial crop production and in-home gardens. Recent interest in strawberry and other berries for health and wellness is linked to their high levels of nutritional value such as vitamins A and C, fiber, and antioxidant activity [1]. Strawberry 'Pharachatan 80' is a favorite cultivar in Thailand because it grows fast, resists to pathogens and provides high yield with favorable test. Plant tissue culture system is used for strawberry nursery crop production in many countries including Thailand. Most research in plant tissue culture technique have focused on plant growth regulators for improving shoot multiplication and determining plant development. However, one of the most important factors in growth medium is mineral nutrition. Mineral nutrients, which are essential for growth and development, are the major components of plant growth media. These nutrients are essential for growth and developments as they are constituents of essential molecules such as proteins, nucleic acids, and parts of cell structures. Moreover, some of nutrients affect growth and development

by activating enzymes or functioning as co-enzymes or cofactors [2-3]. Despite their importance, very little information is available on *in vitro* mineral nutrition.

There are many approaches to modify growth medium by changing mineral nutrient concentrations, including the triangular method [4] or the traditional (factorial) approach [5-7]. Murashige and Skoog (MS) medium is commonly used in plant tissue culture because it has all essential mineral salts needed for plant growth and development. However, mineral concentration of each components used in this formulation might not be suitable for every genotypes or species [8]. Modifying or adjusting the concentrations of MS mineral components has been studied for many years to develop individual micropropagated plants because each plant may have specific mineral requirement. Murashige and Skoog (1962) studied one or two mineral components at one time with several nutrients and required many experiments with several treatments [8]. Recent studies on the effects of mineral nutrients on plant growth and development have applied computer or statistical approaches to optimize and understand the complexity of mineral nutrition with multiple interactions. One of these approaches, Response Surface Methodology (RSM), has been applied to model or optimize the mineral components or other factors for *in vitro* plant growth [9-13]. Optimization of mineral nutrients in strawberry micropropagation in Thailand has not been investigated yet. Therefore, the objective of this study was to optimize MS mineral salts in culture medium for micropropagation of strawberry 'Pharachatan 80' and test the effects of plant growth regulators on plant growth and multiplication.

Material and Method

Plant materials and establishment of shoot cultures

For establishment, the stolons of the strawberry 'Pharachatan 80' grown in greenhouse were used. Sterilized shoots of strawberry 'Pharachatan 80' were grown on MS medium [8] supplemented with 1.0 mg/L N6-benzylaminopurine (BAP) (Acros Organics 226410050), 30 g/L sucrose, 8 g/L agar (UnionSci Lot2P6000430) at pH 5.7. Shoot cultures were grown in bottles with 30 mL of medium and transferred to fresh medium every 4 weeks until there were sufficient plant materials. All plants were grown at 25^{O}C and a 16 h photoperiod with 2,000-3,000 Lux.

Optimization of MS minerals using RSM experiment

At the beginning, the experimental design was conducted as a 5-factor response surface design where the design points or treatments (combinations of the 5 factors) were selected using RSM software [9]. The mineral components in MS formulation consist of compounds as listed below **(Table 1).** The 5 mineral nutrient factors were based on MS mineral salts and each factor was varied over a range of concentrations expressed as relative concentration to MS medium (1x represents the basal MS concentration) **(Table 1)**. The MS-stock based 5-factor design had a total of 34 treatments. The treatments included 22 model points, 5 lack-of-fit points, 5 replicated points, and 2 MS medium controls of two blocks for pure error estimation a set of treatment combinations (34 treatments) **(Table 2)**. Each treatment had 4 plantlets placed individually in 4 bottles. Shoot tips, approximately 1.0 cm in initial length, were cultured on these 34

treatments. Shoots were cultured in the same medium at three-week intervals and harvested after 12 weeks. Graphical models for each response were extrapolated by modeling a graph of the response as a function of 2 factors while holding the others constant. Quality ratings were assigned to each plant using a numeric scale of 1 (poor quality with almost dead), 2 (moderate quality with some pale leaves but not tall stems) and 3 (good quality with green leaves and tall stems). Quality was also evaluated based on the length of the longest shoot measured in mm (from base to shoot tip). The numbers of shoots were counted, and leaf color was measured using The Soil Plant Analysis Development (SPAD) chlorophyll meter or SPAD meter.

Later, the validation test was conducted from optimization in RSM using the data from the first experiment **(Table 3)**. Six formulations with various MS mineral component concentrations were selected based on desirability (Table 3). Strawberry shoots were grown on these six treatments compared to MS medium (control). After 4 weeks, plant growth was evaluated for overall quality scores which were assigned to each plant using a numeric scale of 1-3 as listed above, shoot length, shoot number, leaf color, rooting scores on a scale of 1 (no root), 2 (1 or 2 roots) and 3 (more than 3 roots with height more than 1 cm) and root length.

The effect of plant growth regulators on shoot quality and multiplication

In this experiment, shoots were cultured on standard MS and modified MS formulation 3 (1.0×NH_4NO_3, 0.5×KNO_3, 2.0×Mesos, 3.0×Minors and 2.0×Iron) called as modified MS selected from previous Optimization of MS minerals using RSM experiment (Table 3). Both MS and modified MS formulation 3 were also supplemented with two types of plant growth regulators (PGR), 6 Benzylaminopurine (BAP) (Acros Organics 226410050) and Kinetin (Kn) ((6-Furfurylaminopurine) Sigma-Aldrich #48130). The concentrations of each were applied separately at 1, 2 and 4 mg/L including combination of 1 mg/L of both PGR. MS medium without PGR was the control treatment. After 4 weeks of culture, plant growth and multiplication were evaluated as described previously.

The experiment was set up as a completely randomized design with 4 shoots per replication and 5 replications per treatments. The results were subjected to an analysis of variance (ANOVA) and Duncan's multiple range tests using Statistical Package for the Social Sciences or SPSS Statistics 24.

Table 1 The 5 factors used to construct the 5-dimensional design space, their component MS salts, and concentration range expressed as × MS levels.

Factors	MS Salts	Range of relative concentration (x MS levels)
1	NH_4NO_3	0.5 – 3.0
2	KNO_3	0.5 – 3.0
3	$CaCl_2{\cdot}2H_2O$	0.5 – 3.0
	KH_2PO_4	
	$MgSO_4$	
4	$MnSO_4{\cdot}H_2O$ $ZnSO_4{\cdot}7H_2O$	0.5 – 3.0
	$CuSO_4{\cdot}5H_2O$	
	KI	
	$CoCl_2{\cdot}6H_2O$	
	H_3BO_3	
	$Na_2MoO_4{\cdot}2H_2O$	
5	$FeSO_4{\cdot}7H_2O$	0.5 – 3.0
	Na_2EDTA	

Table 2 MS-stock based five-factor design including 22 model points, 5 lack-of-fit points, and 5 replicated points, and 2 MS medium controls (34 treatments).

Block	Run	A: NH_4NO_3	B: KNO_3	C: Mesos	D: Minors	E: Iron
		relative concentration (x MS levels)				
1	1	2.1	1.2	0.5	3.0	0.5
1	2	0.5	3.0	3.0	1.6	3.0
1	3	3.0	3.0	0.9	2.2	1.2
1	4	1.1	3.0	0.5	2.9	3.0
1	5	3.0	3.0	3.0	0.6	0.5
1	6	0.5	0.5	1.1	0.5	3.0
1	7	1.0	0.5	3.0	3.0	3.0
1	8	2.8	0.6	1.4	0.5	0.5
1	9	1.3	3.0	2.8	3.0	0.5
1	10	0.6	1.4	3.0	2.2	1.8
1	11	0.5	2.5	0.5	1.0	0.5
1	12	3.0	3.0	3.0	3.0	3.0
1	13	3.0	2.0	0.5	0.5	3.0
1	14	3.0	1.0	3.0	2.5	1.0
2	15	2.5	2.4	0.5	0.5	0.5
2	16	1.6	3.0	1.6	0.5	2.1

Block	Run	A: NH_4NO_3	B: KNO_3	C: Mesos	D: Minors	E: Iron
		relative concentration (x MS levels)				
2	17	0.5	2.0	0.5	1.4	2.4
2	18	1.6	0.5	0.5	1.6	1.9
2	19	1.9	1.8	1.9	1.7	1.9
2	20	1.9	1.7	1.9	1.8	3.0
2	21	1.9	1.7	1.9	1.8	3.0
2	22	1.9	1.8	1.9	1.7	1.9
2	23	1.0	3.0	0.5	3.0	0.5
2	24	3.0	2.7	2.6	2.4	0.6
2	25	1.6	0.5	0.5	1.6	1.9
2	26	3.0	0.5	1.0	3.0	3.0
2	27	1.6	3.0	1.6	0.5	2.1
2	28	3.0	0.5	3.0	0.5	3.0
2	29	0.5	0.5	2.2	2.2	0.5
2	30	0.9	1.4	3.0	0.5	1.2
2	31	0.5	1.8	1.5	3.0	1.9
2	32	0.5	1.8	1.5	3.0	1.9
1	33	1.0	1.0	1.0	1.0	1.0
2	34	1.0	1.0	1.0	1.0	1.0

Table 3 Six modified MS formulations and control based on optimization of RSM. Treatment 3 (in bold) was used for testing the effect of plant growth regulators.

Formulation	A: NH_4NO_3	B: KNO_3	C: Mesos	D: Minors	E: Iron	Desirability
1	0.5	0.5	2.0	0.5	2.0	0.894
2	0.5	0.5	2.5	0.5	1.5	0.871
3	**1.0**	**0.5**	**2.0**	**3.0**	**2.0**	**0.85**
4	1.5	0.5	1.0	3.0	2.0	0.719
5	3.0	2.5	2.0	0.5	0.5	0.405
6	3.0	0.5	1.5	0.5	1.0	0.392
7	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**	**Control**

Results

Optimization of MS minerals using RSM experiment

The effects of each component were shown as color contour plots of the regions in the 5-factor design space **(Figure 1)**. The concentrations of both KNO_3 and NH_4NO_3 were indicated on axes for two dimensions of factors while the other factors were set as actual factors which are adjustable. In terms of overall quality, the response model showed that shoots grown on MS had overall quality scores of about 2.1. By contrast, Shoots grown on medium which had low nitrogen sources and minors with high mesos and iron twice had higher overall quality scores (Figure 1A-B).

Length of shoots grown on a basal medium of 1.0×MS but with reduced KNO_3, was increased compared to the 1.0×MS with 1.0×KNO_3 **(Figure 1C)**. Adapting using RSM showed that shoots grown on medium with 2.0×mesos, 1.0×minors and 1.0×iron, had higher shoot length when also grown on medium with 1.0×KNO_3 and NH_4NO_3 **(Figure 1D)**.

Shoots grown on 2.0×mesos, 2.0×minors and 3.0×iron medium with low NH_4NO_3 and low KNO_3 had higher shoot numbers compared to shoots grown on MS alone **(Figure 1E-F)**. Finally, shoots grown on 2.0×mesos, 0.5×minors and 1.0×iron medium showed greater leaf color scores when grown on 1.5×KNO_3 and reduced NH_4NO_3 (Figure G-H). Based on above data, six formulations were conducted and tested in validation experiment.

For the test of validation, the best quality, shoot number, shoot length, rooting scores, and root length compared to MS were obtained from Formulation 3 (1.0×NH_4NO_3, 0.5×KNO_3, 2.0×Mesos, 3.0×Minors and 2.0×Iron) and Formulation 4 (1.5×NH_4NO_3, 0.5× KNO_3, 1.0×Mesos, 3.0×Minors and 2.0× Iron; Figure 2 A-C).

The effect of plant growth regulators on shoot quality and multiplication

The result showed that growing strawberry shoots on modified MS without PGRs had high overall quality scores and shoots did not grow well at any concentration of added PGR (BAP or Kn). However, shoots on standard MS medium and supplemented with between 1, 2 and 4 mg/L Kn had high overall quality scores (Figure 3A). For multiplication, shoots grown on both standard and modified MS had their highest shoot numbers when supplemented with 1 mg/L BAP (Figure 3B). Like overall quality, the highest shoot lengths were obtained by shoots grown on standard MS and modified MS without PGRs (Figure 3C). While shoots grown on both standard and modified MS had highest color scores when supplemented with all concentrations of Kn. There was not any significant effect of nutrients and PGRs according to statistical analysis (Figure 4A). Shoots grown on standard or modified MS with all concentrations of Kn had high scores of rooting, like shoots grown on standard and modified MS without PGRs (Figure 4B). Finally, shoots grown on modified MS supplemented with 1 mg/L Kn had longer root compared to other treatments **(Figure 4C).**

Discussion

This study investigated the effects of *in vitro* Murashige and Skoog (MS) mineral salts and plant growth regulators (PGRs) on the growth and development of the strawberry cultivar ‘Pharachatan 80’ using response surface methodology (RSM). The model response showed distinct effects of each group of MS salts on plant growth and quality. Increased mesos, minor and

iron with reduced nitrogen sources resulted in better growth (Figure 1). A study of micropropagation in bromeliads indicated that increased calcium and copper with reduced iron resulted in the best growth [14]. Reed *et al*. (2013) reported that increasing mesos was the key factor for improving overall quality and growth of micropropagated pear in germplasm collection [10]. Similarly, Poothong and Reed (2014) investigated the effect of MS mineral and found that mesos and nitrogen were driving factors for improving growth appearance in *Rubus* [12]. Many studies showed that individual species had different mineral nutrition requirement. Mineral availability and their uptake in an *in vitro* culture system vary greatly compared to the complex buffer system of soil culture. This is because shoots or plants *in vitro* usually lack roots in the initial culture. Consequently, mineral acquirement depends on rate of diffusion and osmosis. When the MS medium is used for the *in vitro* culture of some plants for a period, they have showed abnormal growth, stunting, hyperhydricity, necrosis, and discoloration at various levels, depending on the species or genotype. Many hazelnut cultivars require more minor nutrient component in DKW formulation especially increased amounts of boron, molybdenum, and zinc [15]. El-Hawaz *et al*. (2016) observed the effects of low ammonium in culture medium and investigated the optimization using RSM of MS mineral salts including plant density on the subsequent growth after acclimatization in two genotypes of *Curcuma longa* L. [16]. They found that shoots grown on modified low ammonium medium and high potassium nitrate with high plant density had increased growth during acclimatization [16]. Improving plant appearance and growth by optimizing mineral nutrients in culture medium helps to increase the efficiency of micropropagation techniques in several plants [17]. Another protocol for medium optimization is to adapt mineral nutrients in culture medium using mineral concentrations in plant tissues as baseline. Goncalves *et al*. (2005) optimized the macronutrient concentration using MS as basal medium for rooting in micropropagated Carob tree [18]. The results showed that new formulated medium called Carob medium adapted based on the concentrations of macronutrients in leaves from mature tree and young micropropagated plants could increase the rooting frequency up to 80% [18]. The Carob medium had low nitrogen and potassium with twice calcium and magnesium [18]. Moreover, shoots grown on the new formulation did not have any apical necrosis [18]. Bouman and Tiekstra (2005) found that Cymbidium adapted medium (CAM) and Gerbera adapted medium (GAM) using calculated concentration based on mineral contents in healthy laves were suitable for growing Cymbidium and Gerbera relatively compared to other basal media [19]. In CAM, the concentration of calcium, phosphorus and magnesium including sulfur were approximately higher compared to half MS [19]. Similarly, the concentrations of those minerals in GAM were approximately higher compared to MS medium, this showed that gerbera needed more calcium, phosphorus and magnesium including sulfur for improved growth [19]. The results of this study showed that RSM could be applied to optimize mineral components in culture medium of strawberries and modified MS medium slightly increased growth (Figure 1 and 2). In strawberry micropropagation, BAP is the most common cytokinin used for multiplication. Sakila *et al* (2007) reported that the highest shoot multiplication of strawberry was produced in MS

supplemented with 1.5 mg/L BA + 0.5 or 0.1 mg/L Kn [20]. While Ashrafuzzaman *et al.* (2013) showed that the highest shoot multiplication with highest length of strawberry was obtained from shoots grown on MS with 0.5 mg/L BAP [21].

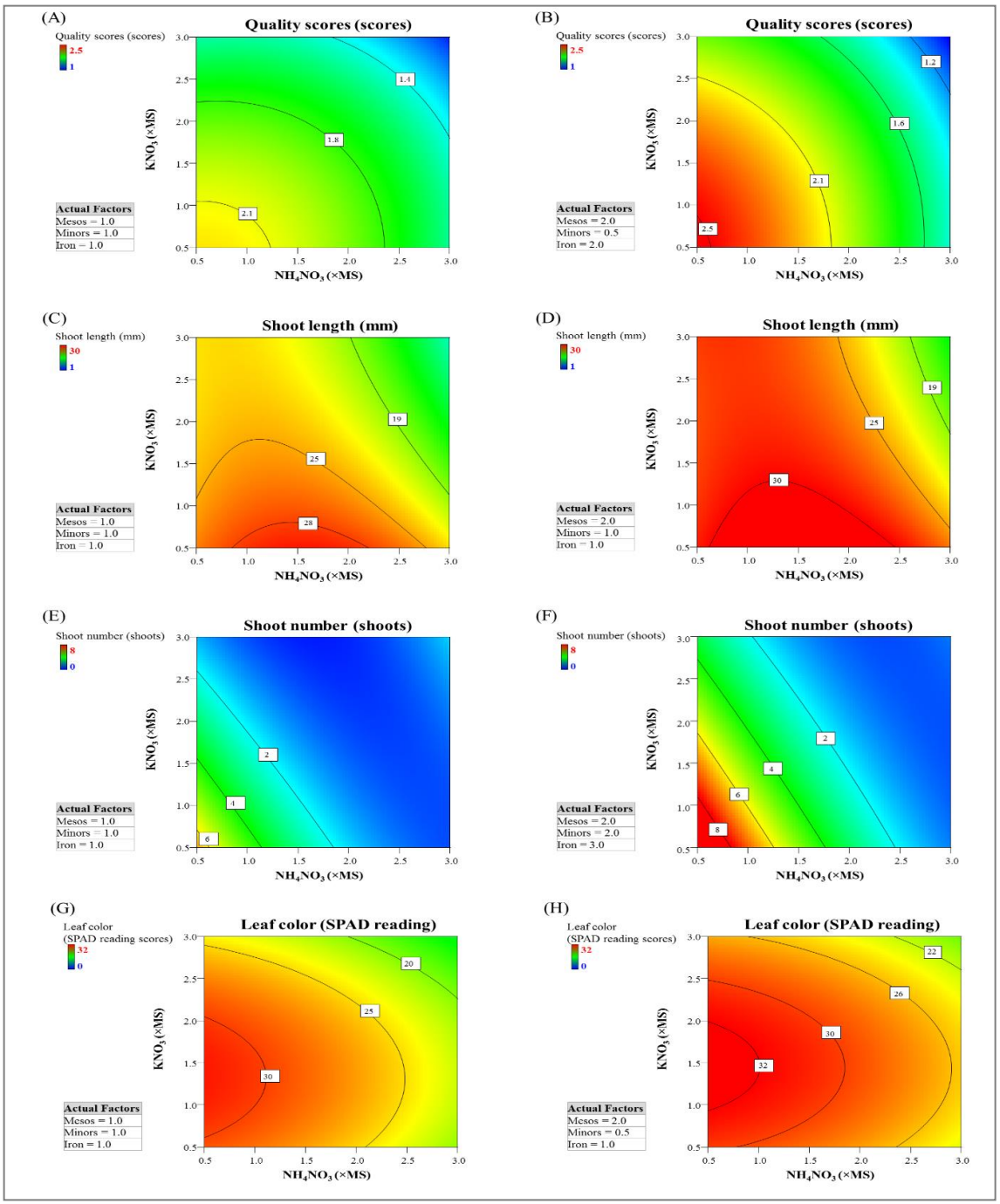

Figure 1 The projected graphs of mineral effects on overall quality (Rated scores 1 poor-3 good) of strawberry 'Pharachatan 80' (A-B), Shoot length (C-D), shoot number (E-F) and leaf color (G-H). Greatest response is in red, median in yellow-green and least in blue. Mean rating responses displayed in boxes. The effects of each component were shown as color contour plots of the regions in the 5-factor design space. The concentrations of both KNO_3 and NH_4NO_3 were indicated on axes for two dimensions of factors while the other factors were set as actual factors which are adjustable.

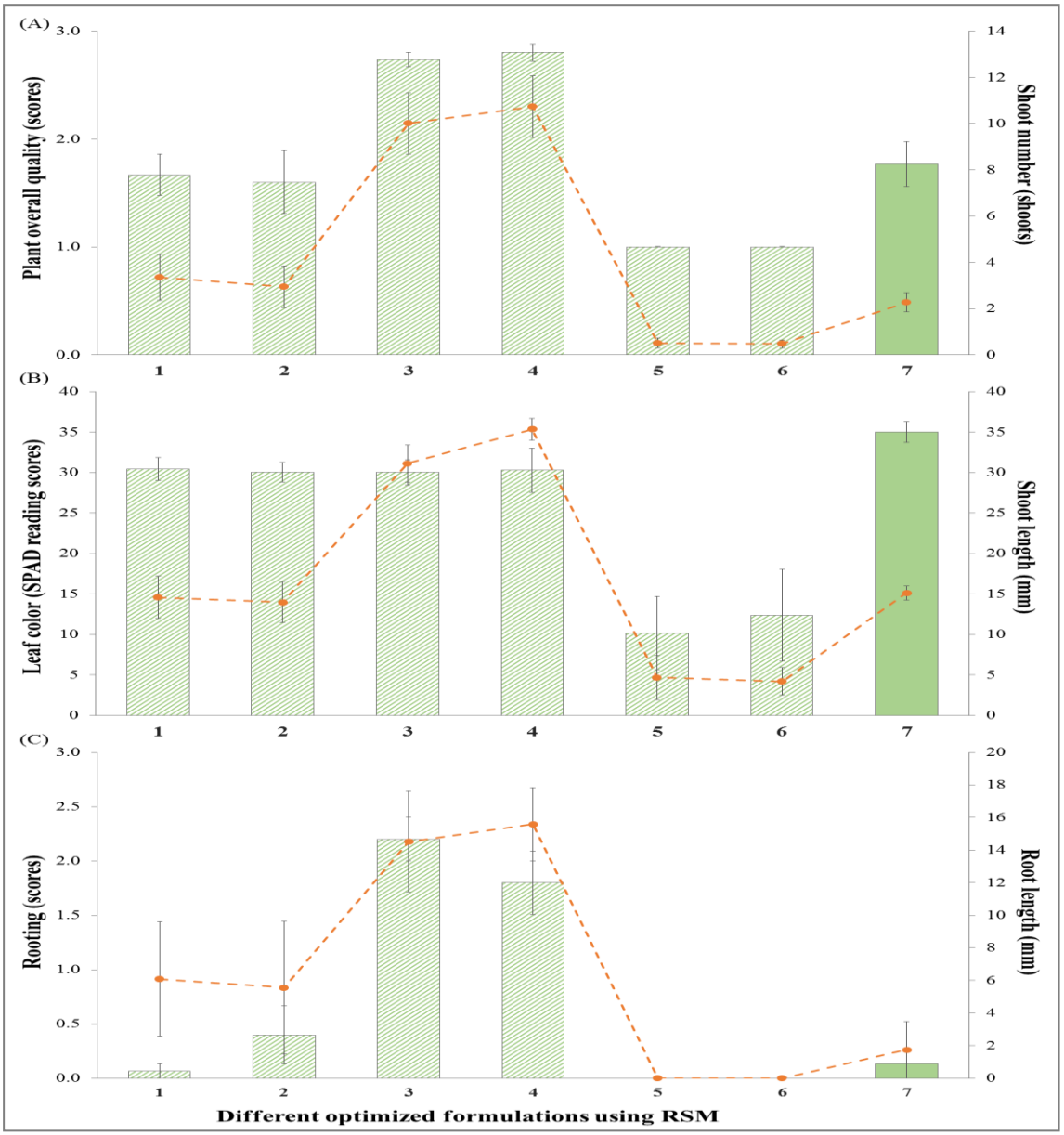

Figure 2 The effect of optimized media on plant growth appearance (A) overall quality representing in green boxes and shoot number representing in orange dots , (B) leaf color representing in green boxes and shoot length representing in orange dots and (C) rooting representing in green boxes and root length representing in green boxes.

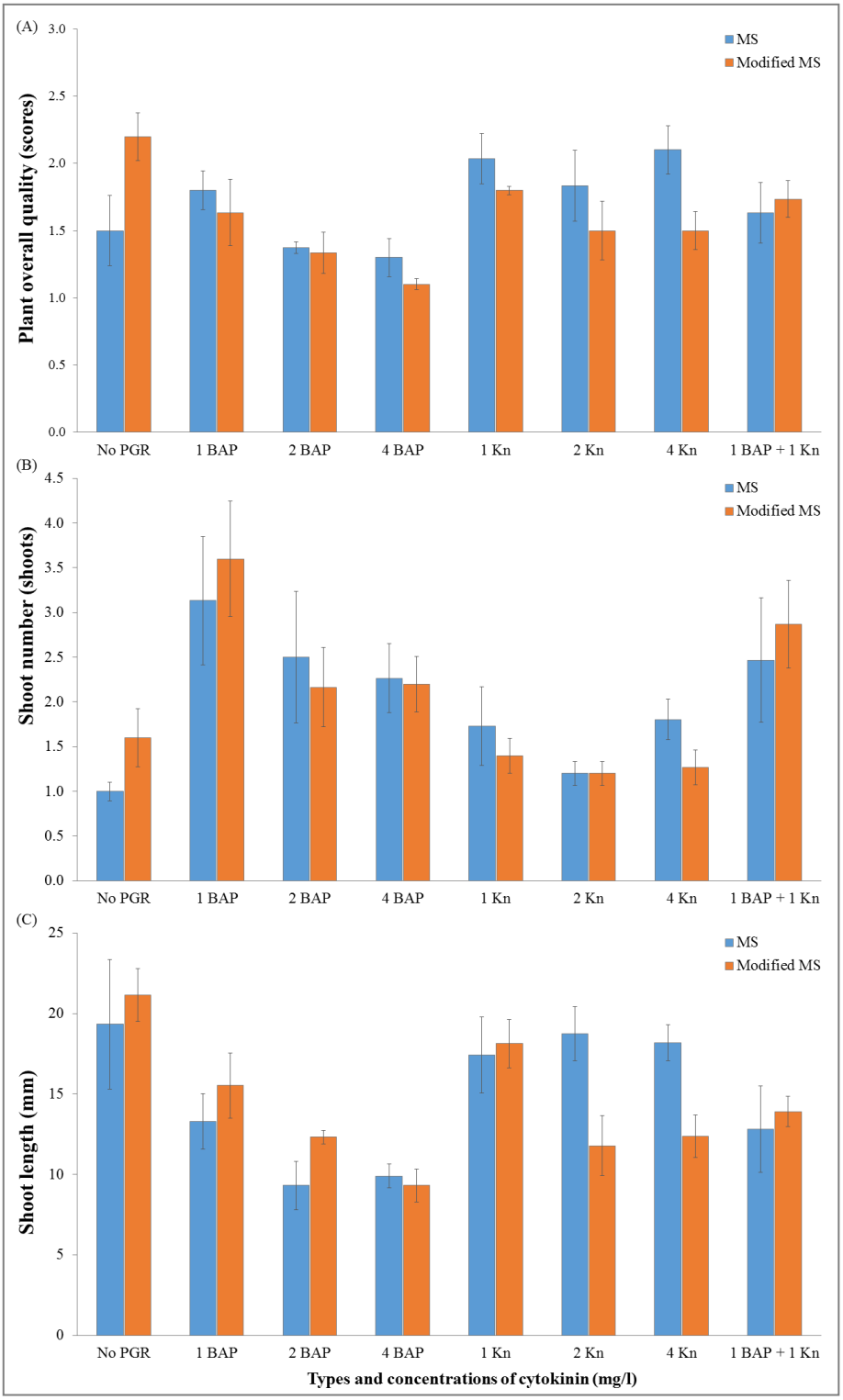

Figure 3 The effect of medium formulation and plant growth regulators on growth appearance (A) overall quality, (B) shoot number, and (C) shoot length. No PGR representing as control. Modified MS was modified MS formulation 3 (Table 3). Modified MS referred to 1.0×NH_4NO_3, 0.5×KNO_3, 2.0×Mesos, 3.0×Minors and 2.0×Iron.

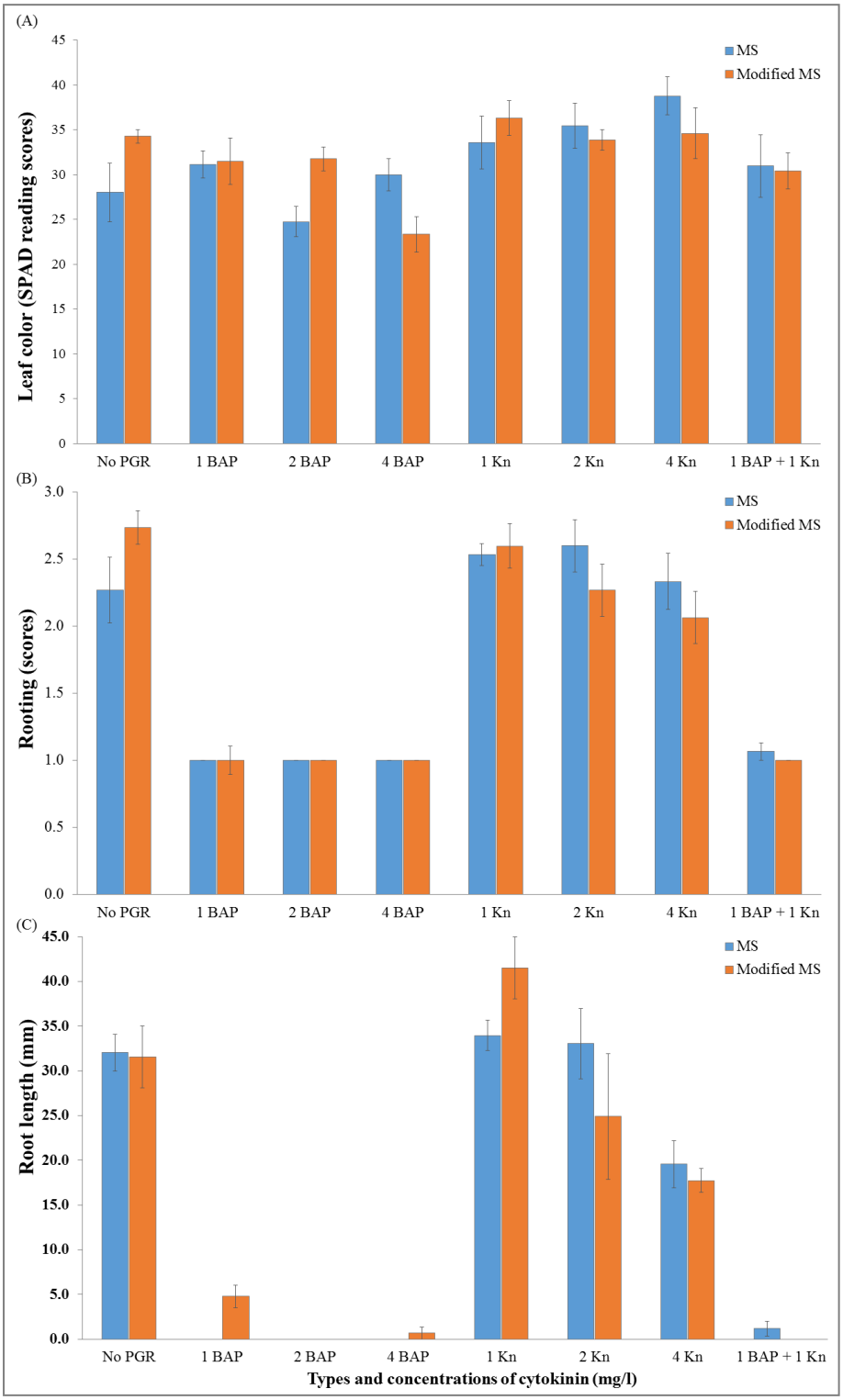

Figure 4 The effect of optimized media based on minerals and plant growth regulators on growth appearance (A) leaf color, (B) rooting and (C) root length. No PGR representing as control. Modified MS was modified MS formulation 3 (Table 3). Modified MS referred to 1.0×NH4NO3, 0.5×KNO3, 2.0×Mesos, 3.0×Minors and 2.0×Iron.

In conclusion, modified MS (1.0×NH_4NO_3, 0.5×KNO_3, 2.0×Mesos, 3.0×Minors and 2.0×Iron) supplemented with 1 mg/L BAP was suitable for shoot multiplication. Moreover, the response surface methodology (RSM) was more useful for reducing treatments compared to factorial experimental designs. Although the assessment of mineral effects on *in vitro* plant growth is very complicated, this approach is a significant step in determining which factors are keys to experimental designs for micropropagation of strawberry 'Pharachatan 80'.

Acknowledgements

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