Effect of enhanced CaCl₂, MgSO₄, and KH₂PO₄ on improved in vitro growth of potato

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Abstract  Potato (Solanum tuberosum L.) is a major global food crop. Contemporary potato production largely utilizes micropropagation to produce healthy seed potatoes. The micropropagation of potatoes is widely achieved through nodal explants using the conventional Murashige and Skoog (MS) medium. Currently, effective culture media that can facilitate rapid propagation are increasingly required for new cultivars that have been developed to possess improved traits. In this study, we evaluated the effect of enhanced meso nutrients (CaCl₂·2H₂O, MgSO₄, and KH₂PO₄) in MS medium on the growth of S. tuberosum. The cultivars used in this study were representative of Japanese, European, and Peruvian lines. Enhanced meso nutrients improved the overall quality of all cultivars, as indicated by longer shoots and larger leaves with dark color, compared with MS medium only. Shoots grown on enhanced mesos were approximately 1.5 times longer than on MS medium. Quantitative ion analysis revealed that plantlets with improved shoot length and leaf quality in most cultivars had increased calcium, magnesium, potassium, and phosphorus uptake than plantlets on MS medium. The results suggest that the reduced iron uptake on 3.0× MS, compared with 2.0× or 2.5× MS mesos, reduced plant growth. This study revealed for the first time that mesos concentrations higher than MS medium concentrations, complemented by enhanced calcium, magnesium, potassium, phosphorus, and iron uptake, play a significant role in improving the in vitro growth of potato.

Key words: efficient propagation, in vitro, Solanum tuberosum L.

Introduction  Potato (Solanum tuberosum L.) is a major global food crop with an annual average production of 3.7×10⁸ tons (FAO 2018). Being an important source of carbohydrates, minerals, and several vitamins, potatoes are considered to have great potential to address global hunger. Conventional potato production systems are characterized by the use of vegetative propagation for seed potato production, in which only 4–15 potatoes are obtained from one tuber; hence, the efficiency is significantly low (Naik and Karihaloo 2007). Furthermore, vegetative propagation allows systemic viruses to accumulate in successive generations (Simmonds 1997). Contemporary potato production avoids these drawbacks by deploying micropropagation during the initial stages of seed potato production. Healthy plantlets for microtuber or minituber production, leading to high-quality seed potatoes, can be obtained using the micropropagation of virus-free shoots (Naik and Buckseth 2018). Nodal cuttings have long been recognized as a means of achieving large quantities of healthy in vitro plantlets with high genetic quality (Dodds 1988; Struik and Wiersema 1999). The potato production sector is significantly dynamic, with numerous breeding attempts to improve cultivars that possess higher yields and resistance to extreme environmental conditions, pests, and diseases. Therefore, there is a need for efficient propagation systems that can achieve potato micropropagation significantly faster.

Murashige and Skoog (MS) medium (Murashige and Skoog 1962) has been widely used as a conventional growth medium (Amirouche et al. 1985; Hussey and Stacey 1981; Roca et al. 1978; Stace-Smith and Mellor 1968; Struik and Wiersema 1999) for nodal explants. Although the significance of mineral nutrients in in vitro plant propagation is well recognized (Niedz and Evens 2007; Ramage and Williams 2002), research on the medium constituents aimed at improving and accelerating S. tuberosum growth has been limited. Evans (1993) and...
Zarrabeitia et al. (1997) studied the effects of varying concentrations of nitrogen in MS medium and found that reduced nitrogen amounts in the medium can improve the growth of certain potato genotypes. Kozai et al. (1995) found that the volume and initial strength of MS medium affect the in vitro growth, photosynthesis, and ion uptake of potato plantlets. They revealed that the ion uptake rates, including those of phosphorus, potassium, calcium, and magnesium, increased with increasing volume and strength of the medium. One particularly studied mineral nutrient in terms of potato in vitro propagation is calcium (Habib et al. 2004), where it has been found that increased calcium levels (15 mM) in the medium caused significantly greater shoot fresh and dry weights. The MS medium was initially developed for tobacco callus culture (Murashige and Skoog 1962), and it is recognized that established medium formulations, including MS medium, could require optimization for shoot growth of different species other than the growth of the originally intended tissue (Adelberg et al. 2010; Ramage and Williams 2002). Plant culture media are primarily classified as either macro nutrients (nitrogen, potassium, calcium, phosphorus, magnesium, sulphur), or micronutrients (iron, nickel, chlorine, manganese, zinc, boron, copper, and molybdenum). Niedz and Evans (2006) and Reed et al. (2013a) identified mesos (CaCl₂·2H₂O, KH₂PO₄, MgSO₄) in MS medium minerals (Murashige and Skoog 1962) which has been found to dramatically improve the shoot quality. Currently, mesos component of culture media is being recognized as one of the most influential groups of minerals for in vitro plant growth (de Carvalho et al. 2018; Poothong and Reed 2014; Reed et al. 2013a). However, it was mainly used to improve the growth of plants with poor in vitro growth.

This study demonstrated that improved growth in the in vitro propagation of potato could be achieved using enhanced mesos (CaCl₂·2H₂O, MgSO₄, and KH₂PO₄). We show that calcium, magnesium, phosphorus, potassium and iron uptake play a significant role in improving the in vitro growth of potato.

Materials and methods

Plant material and establishment of shoot cultures

Six virus-indexed *Solanum tuberosum* L.; four Japanese cultivars, ‘Sayaka’ (Pentland Dell×R392-50), ‘Nishiyutaka’ (Choukei 65), ‘Inca-no-hitomi’ (seeds of ‘Inca-no-mezame’), ‘Okhotsk Chip’ (Atlantic×ND860), European cv. 261 (B57082-3) and Peruvian cv. 325 (W780N65-1) were used in this study. Cultures were grown on MS medium at five meso nutrient concentration levels: 1.0×, 1.5×, 2.0×, 2.5× and 3.0×MS (Table 1) with 30 g l⁻¹ sucrose and 8 g l⁻¹ agar at pH 5.8. Stem explants of approximately 1.5 cm length were inoculated on 10 ml of medium in culture tubes. Three subsequent stem explant cultures were carried out on a similar medium at three-week intervals. All explants were grown at 20±1°C under a 16-h photoperiod with 70–90 µmol m⁻² s⁻¹ irradiance provided by cool white fluorescent bulbs. Culture tubes were randomly rearranged on a growth chamber shelf throughout the experimental period.

Data collection

The growth characteristics of plantlets were evaluated during the final culture interval. The longest shoots of each plantlet were measured to evaluate shoot growth. At the end of the above period, all plantlets from each concentration treatment were harvested, the medium remaining on the roots was removed, and fresh and dry weights of plantlets were measured. Table 1 shows the concentrations of mineral salts in MS medium and MS with increasing mesos concentrations.

| MS salt                          | Concentration (mg l⁻¹) |
|----------------------------------|------------------------|
| Ammonium nitrate                 | 1650                   |
| Potassium nitrate                | 1900                   |
| Calcium chloride dihydrate       | 440 660 880 1100 1320 |
| Potassium dihydrogen phosphate  | 170 255 340 425 510   |
| Manganese (II) sulphate pentahydrate | 24.1                   |
| Zinc sulphate heptahydrate       | 8.6                    |
| Cupric sulphate pentahydrate     | 0.025                  |
| Boric acid                       | 6.2                    |
| Potassium iodide                 | 0.83                   |
| Sodium molybdate dihydrate       | 0.25                   |
| Cobalt chloride                  | 0.025                  |
| Ferrous sulphate heptahydrate    | 27.8                   |
| Sodium ferric ethylenediaminetetraacetate | 37.3 |
| Magnesium sulphate heptahydrate  | 370 555 740 925 1110  |
| Thiamine                         | 0.1                    |
| Myo-inositol                     | 10                     |
| Nicotinic acid                   | 0.5                    |
| Pyridoxine                       | 0.5                    |
| Glycine                          | 2                      |
rinsed thrice with distilled water, and each plantlet’s fresh weight was recorded. Ten stem explants were subjected to each concentration treatment, with three replications.

Leaf quality was assessed based on leaf size and colour. Leaf size was rated on a scale of 1 to 3 with respect to the leaf width: 1, small (width < 10 mm); 2, moderate (width 10–15 mm); and 3, large (width > 15 mm). A portable Soil-Plant Analysis Development (SPAD) 502 chlorophyll meter (Minolta Camera Co. Ltd., Tokyo, Japan) was used to measure chlorophyll content of the second leaf from the top of the shoot (Hand and Reed 2014).

**Quantitative ion analysis**

After obtaining the fresh weight, the plantlets were oven-dried at 70°C for three days for dry weight measurements. They were subsequently burnt at 600°C for 2 h in a muffle furnace. Ash samples were dissolved in 10 ml of 0.1 N HCl after cooling. Filtered supernatants were quantitatively analyzed using an inductively coupled plasma emission spectrometer (ICPS 7510; Shimadzu, Kyoto, Japan).

**Statistical analysis**

The experimental design was a completely randomized. Data were analyzed using a one-way ANOVA. (Rstudio 2015). Tukey’s multiple comparison test was performed as a post hoc test for mean separation.

**Results**

**Shoot growth and leaf quality**

Significant improvements in shoot elongation were...
achieved by all potato cultivars on enhanced mesos, compared with 1.0×MS. Cultivars ‘Nishiyutaka’ (Figures 1A, 2) and ‘Okhotsk Chip’ (Figure 3A) had significantly increased shoot elongation on both 2.0× and 2.5×MS. Cultivars ‘Sayaka’ and 325 achieved significant shoot elongation on 2.0×MS, while cv. 261 had significantly

Figure 3. Shoot length, dry weight, leaf size, and leaf colour of ‘Okhotsk Chip’ on increasing mesos concentrations. Data represents mean±standard error (SE). Different lowercase letters represent statistically significant differences (p≤0.01 for shoot length; p≤0.05 for dry weight, leaf size and leaf colour), n=10.

Figure 4. Samples of plantlets of ‘Sayaka,’ ‘Inca-no-hitomi,’ cv. 261, cv. 325 and ‘Okhotsk Chip,’ cultured on the following media: A; C; E; G; I: 1.0× Murashige and Skoog (MS), B: 2.0×MS, D: 2.0×MS, F: 2.5×MS, H: 2.0×MS, J: 1.5×MS, K: 3×MS.

Table 2. Mesos concentrations that produced higher average shoot and root elongation of cv. 428, compared with MS medium.

| Mesos concentration | Average shoot elongation (cm)±SE | Average root elongation (cm)±SE |
|---------------------|----------------------------------|--------------------------------|
| 1.0×MS              | 2.96±0.33                        | 9.02±0.77                      |
| 1.5×MS              | 7.44±0.46**                      | 9.20±0.71                      |
| 2.0×MS              | 4.71±0.46**                      | 9.76±0.96                      |
| 2.5×MS              | 5.02±0.34**                      | 9.34±0.47                      |
| 3.0×MS              | 1.99±0.17                        | 10.49±0.58                     |

** average value significantly different from the MS medium at p≤0.01, n=10.
longer shoots on 2.5×MS, compared with 1.0×MS (Figure 4). Plantlet evaluation revealed that shoot elongation of ‘Inca-no-hitomi’ achieved on 3.0×MS was significantly higher than that on 1.0×MS after two weeks. Results indicated that potato cultivars achieved significant shoot elongation at mesos concentrations ≥2.0×MS. However, ‘Nishiyutaka,’ 261, 325, and ‘Okhotsk Chip’ exhibited a decrease in shoot elongation at 3.0×MS (Figures 1A, 3A). In addition, ‘Okhotsk Chip’ showed extensive root growth, in contrast to their poor shoot growth on 3.0×MS (Table 2; Figure 4K). Plantlet dry weight also increased on enhanced mesos, relative to 1.0×MS. Mesos concentrations at 2.5×MS produced the greatest biomass in ‘Nishiyutaka’ and was significantly greater than on 2.0×MS and 3.0×MS (Figure 1B).

Leaves with a SPAD value between 45 and 55 were considered to have the optimum leaf quality. All cultivars required enhanced mesos to produce the optimum leaf quality. Mesos concentrations of 1.5× and 2.5×MS resulted in optimum leaf quality for ‘Nishiyutaka’ (Figure 1C, D). The optimum leaf quality of ‘Sayaka’ and ‘Okhotsk Chip’ (Figure 3C, D) were attained on 2.0×MS.
mesos. The optimum leaf quality of cvs. 261, 325, and ‘Inca-no-hitomi’ were attained on 2.5×MS mesos.

The study further revealed that a mesos concentration of 2.0×MS affected shoot multiplication of ‘Nishiyutaka’ (Figure 2C). However, the leaf size and colour achieved on 2.0×MS were significantly lower than those on 2.5×MS (Figure 2C, D). The occurrence of multiple shoots might have lowered the leaf quality in plantlets on 2.0×MS.

Quantitative ion analysis
A difference in ion uptake by plantlets on enhanced mesos was observed. Calcium uptake by plantlets increased in the enhanced medium - calcium uptake by ‘Nishiyutaka’ on 2.5×MS mesos was 1.5 times greater than on 1.0×MS (Figure 5A). Plantlets of cvs. 261 and ‘Okhotsk Chip’ (Figure 6A) exhibiting improved growth on 2.5×MS mesos had calcium content double that on 1.0×MS. The enhanced mesos at 2.0×MS improved the growth of cv. 325, with a 1.4-times increase in calcium uptake.

Increased magnesium and potassium uptake improved shoot elongation and leaf quality of ‘Nishiyutaka’, 261, 325, and ‘Okhotsk Chip’ compared with 1.0×MS (Figures 5B, C, 6B, C). Phosphorus was also among the minerals of which the content in plantlets grown on enhanced mesos showed an overall increase (Figures 5D, 6D). All cultivars exhibited an increase of 1.3–1.5 in phosphorus uptake when grown on enhanced mesos, compared with those grown on 1.0×MS. The iron uptake by cultivars increased on media with enhanced mesos. However, iron uptake remarkably decreased in all cultivars on 3.0×MS. The iron uptake on 3.0×MS was even lower than that on 1.0×MS in ‘Nishiyutaka’, 261, ‘Okhotsk Chip’, and ‘Inca-no-hitomi’.

Genotypic differences in mineral uptake were observed during this study (Figures 5, 6). Cultivar ‘Inca-no-hitomi’ showed considerably lower uptake rates of calcium than the other cultivars.

Discussion
Micropropagation is a part of the present-day seed potato production systems, in which in vitro multiplication through nodal cuttings is a major phase (Tadesse et al. 2001). The wide application of MS medium as a standard growth medium for potato suggests that MS medium is an optimal medium for its micropropagation. However, MS medium was initially developed for the rapid growth of tobacco callus cultures; therefore, improvements may be necessary to propagate potato efficiently. Even though a number of studies have investigated the effects of medium constituents on the in vitro growth of potato plantlets, new media have not been developed that significantly improves the propagation growth of potato; MS medium is still used as a standard.

Minerals play a significant role in plant morphogenesis (Ramage and Williams 2002). Regulation of plant tissue growth by determining the mineral nutrient relationships and calculating the specific media formulations to achieve predefined growth levels has been discussed (Niedz and Evens 2007). Many researchers attempted to change mineral nutrients in culture media to achieve successful growth of slow-growing and recalcitrant species and overcome physiological disorders. The mesos component (CaCl2·2H2O, KH2PO4, MgSO4) was an influential factor in the plant quality of Pyrus (pear) species (Reed et al. 2013a). Increased mesos in MS medium helped overcome undesirable physiological responses of Pyrus species and achieve improved growth (Reed et al. 2013b). The overall quality and shoot length of hazelnut cultivars were improved by increased calcium and mesos (MgSO4 and KH2PO4) in Driver and Kuniyuki Walnut (DKW) medium (Hand et al. 2014). Compared with trees such as pear and hazelnut, potato propagation occurs over a shorter period, so it was thought that shoot propagation might not be improved with an enhanced meso medium. However, potato cultivars from Japan, Peru, and Europe exhibited improved growth on enhanced mesos concentrations (Figures 2, 4). An effective mesos concentration level for all cultivars was ≥2.0×MS (Table 3).

The plantlets’ calcium uptake with improved shoot elongation and dry weight was ≥1.5 times greater on enhanced mesos (Table 1), than uptake on 1.0×MS in all cultivars (Figures 5A, 6A). Shoot fresh and dry weight of S. tuberosum (in six cultivars and two wild species)

| Table 3. Average shoot length of all cultivars on the MS medium and increasing mesos concentrations. |
|----------------------------------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|---------------------------------- |
| Mesos concentration | 'Sayaka' | 'Nishiyutaka' | 'Inca-no-hitomi' | cv.261 | cv.325 | cv.428 |
| 1.0×MS | 2.06±0.38 | 3.26±0.25 | 5.65±0.58 | 4.4±0.41 | 4±0.48 | 2.96±0.33 |
| 1.5×MS | 3.24±0.11 | 3.38±0.45 | 6.15±0.65 | 4.13±0.3 | 4.9±0.51 | 7.44±0.46** |
| 2.0×MS | 3.12±0.24* | 5.84±0.61* | 6.75±0.67 | 6.05±0.7 | 6.9±0.62* | 4.71±0.46** |
| 2.5×MS | 2.9±0.42 | 5.66±0.49* | 6.18±0.38 | 6.22±0.32** | 5.9±0.4 | 5.02±0.34** |
| 3.0×MS | 2.1±0.28 | 4.2±0.13 | 5.52±0.38 | 5.63±0.33 | 5.05±0.74 | 4.99±0.37 |

Shoot elongation were evaluated at the end of a three-week culture period. * average value significantly different from the MS medium at p≤0.05, n=10. ** average value significantly different from the MS medium at p≤0.01, n=10.
have been significantly improved on MS medium with a calcium concentration of 15 mM, compared to that of 5 mM (Habib et al. 2004). However, significant improvements in shoot length have only been achieved by the two wild species (S. microdontum and S. kurzianum) during their study.

Improved shoot elongation and leaf quality was achieved by the potato cultivars, with greater uptake of magnesium seen as a minimum 1.2-fold increase on enhanced mesos, compared with the uptake on 1.0×MS (Figures 5B, 6B). Magnesium concentrations in MS were inferred as too low for Hemerocallis shoot cultures, especially those grown on high sucrose and in high density (Adelberg 2010). Deficiency of magnesium in standard MS has also been evident in Curcuma longa L. (turmeric) (Adelberg et al. 2013). The effect of magnesium on in vitro propagation of potato do not have clear data (Hussey and Stacey 1981).

Potato plantlets required increased calcium and magnesium uptake from 1.0×MS to improve shoot elongation and leaf quality. Furthermore, the uptake of phosphorus by plantlets on enhanced mesos was at least 1.2 times greater than that on 1.0×MS (Figure 5D) and was significant in 'Okhotsk Chip' (Figure 6D). The phosphorus may not be adequately supplied by standard MS medium for Hemerocallis genotypes and Curcuma longa L., respectively (Adelberg et al. 2010, 2013). The uptake of phosphorus by potato plantlets on MS medium with a phosphorus concentration of 0.5×MS was greater, when compared to that of 0.05×MS (Sausen et al. 2020).

Iron is an essential micronutrient with significant effects on plant morphology and metabolism (Rout and Sahoo 2015). In pear cultures, high iron concentrations caused stunted shoots (Reed et al. 2013a), and iron concentrations in standard MS with high mesos contributed to shoots without physiological disorders (Reed et al. 2013b). Iron uptake by pear was lesser on increased mesos than on MS, suggesting that more iron may not necessarily contribute to leaf colour if adequate amounts are available (Wada et al. 2015). Low iron concentrations (0.5×MS) resulted in the best overall quality, shoot length, and multiplication of red raspberries (Poonthong and Reed 2014). We revealed a marked decrease in iron uptake by the plantlets grown on 3.0×MS than on 2.5×MS (Figures 5E, 6E). This decrease paralleled that in shoot elongation in 'Nishiyutaka,' '261,' 325, and 'Okhotsk Chip' cultivars on 3.0×MS. (Figures 1, 3). However, calcium and magnesium uptake continued to increase on 3.0×MS. Phosphorus uptake also continued to increase on 3.0×MS in cvs. 261, 325, 'Okhotsk Chip,' and 'Inca-no-hitomi.' It could be concluded that the improved growth of potato plantlets was influenced by enhanced iron uptake. Therefore, we demonstrated the importance of iron uptake.

‘Inca-no-hitomi’ has a rapid growth rate, and evaluation of growth requires shorter time intervals. The mineral analysis revealed that ‘Inca-no-hitomi’ required approximately 1.5-times more potassium than that in 1.0×MS for significant shoot elongation. Fast-growing radishes require higher potassium concentrations than slower-growing barley and ryegrass (Woodhouse et al. 1978). The potassium uptake by ‘Inca-no-hitomi’ during our study suggests a potential relationship between the greater uptake rate and the cultivar’s rapid growth.

Increased calcium, magnesium, phosphate, and iron uptake improves propagation growth. However, it was suggested that calcium, magnesium, phosphate, and potassium uptake increased, whereas iron decreased, suppressing shoot growth and root development. Therefore, our results suggest that enhanced mesos can improve the growth of potato, in which iron plays a significant role.

Calcium is essential for plant cell proliferation and growth (Hepler and Wayne 1985). However, growth is limited by increasing only the calcium component (Kozai et al. 1995). Magnesium contributes to increased chlorophyll content (Hermans et al. 2004; Marschner 2011). Phosphorus is essential as a structural component of nucleic acids, phospholipids, and ATP. Phosphate is often a limiting factor in tissue culture media, and the standard MS concentrations may not adequately supply growing shoots (Adelberg et al. 2010; Williams 1993). Potassium is vital for plant growth and metabolism (Leigh and Wyn Jones 1984). Magnesium, potassium, and iron are also crucial for plant cell proliferation and growth. However, even if each component was used alone and its medium component was increased, only a small difference would be observed in the rapid growth (Kozai et al. 1995). Each mineral contributes significantly to plant growth, but it is thought that this is because other minerals are deficient as the plant grows.

Results of our study demonstrate the cumulative effect of the three mesos components, CaCl₂, MgSO₄, and KH₂PO₄ in achieving an optimum state; increased concentrations of these three mesos influenced the mineral uptake rates of rates of iron and potassium, like calcium, magnesium and phosphorus. It was shown that even if the uptake of calcium and magnesium increases, the uptake of iron decreases and the shoot propagation changes, which is a characteristic tendency for the uptake of calcium, magnesium and phosphate for cell proliferation and plant growth. Therefore, the results further suggest that iron and potassium play an essential role in increased growth of potato.

We show that the quantitative uptake of minerals (calcium, magnesium, phosphorus, potassium, and iron) by plants determines the shoot elongation, leaf quality, and root development of potato.
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