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Effect of *In Vitro* Calcium Fortification on Regeneration and Microtuberization of Three Selected Irish Potato Varieties

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ABSTRACT

Potato is an important tuber crop that has a unique role in the food security of sub-Saharan Africa. Potato production faces several challenges, including limited sources of clean seed and the occurrence of nutrient deficiencies in plants. The availability of clean and viable seeds is the most prevalent challenge which necessitates the development of new strategies for enhancing production. Understanding the link between *in vitro* nutrient enhancement and seedling vigor in field conditions can assist in mitigating the adverse effects of these challenges after micro plant transplanting. *In vitro* experiments were set up to investigate the effects of calcium (Ca) fortification on several potato varieties, i.e. 'Shangi', 'Unica', and 'Dutch Robjyn' regeneration and microtuberization. Cuttings were subcultured onto modified MS media containing five levels of $CaCl_2$ 8.8 g L^{-1}, 10.4 g L^{-1}, 12 g L^{-1}, 13.6 L^{-1} , and 15.2 g L^{-1}. Microtubers were initiated on media with 60 g L^{-1} brown sugar and 6 mg L^{-1} 6-Benzylaminopurine. The experiment was set up in a completely randomized design with three replications. The regeneration capacity of the tested varieties differed significantly, as evidenced by shoot ($p=0.0002$) and root count ($p=<.0001$). The response of plants to fortification was variably dependent. The application of 10.4-13.6 g L^{-1} led to a significant increase ($p<0.05$) in root count in all three varieties. Furthermore, 13.6 g L^{-1} CaCl2 led to an increase in root-zone and mid-stem Ca content by 45%, 202%, and 165 % in 'Shangi', 'Unica', and 'Dutch Robjyn', respectively, compared to the control. The 'Dutch Robjyn' and 'Shangi' performed optimally in terms of regeneration and microtuberization, compared to 'Unica', under the effect of 10.4-13.6 g L^{-1} $CaCl_2$. The results confirmed the optimization of MS regeneration by Ca enhancement as a potential technology for scaling up the production of clean quality seeds.

Abbreviations: 6-Benzylaminopurine (BAP), Murashige and Skoog (MS)

Introduction

Irish potato (*Solanum tuberosum*) is the third most important food crop in sub-Saharan Africa, after rice and maize (Kadaja and Tooming, 2004). Potatoes are highly nutritive because of their rich contents of vitamin C, potassium, and dietary fiber (Beals, 2019). Potato is cultivated as a subsistence crop in Kenya, and, thus, plays a key role in alleviating poverty and improving food security in Kenya (Ayieko et al., 2006; Kiama et al., 2006). In Kenya, 'Shangi', 'Dutch Robjyn', and 'Unica' are popular table potato varieties and can be used for producing crisps as well (NPCK, 2019). Despite the many benefits associated with potato production in Kenya, the industry is faced with an inadequate supply of clean and quality

potato seeds to farmers (Muthoni et al., 2013; Karanja et al., 2014). The bulk of potato seeds in Kenya is produced mainly through informal systems. These usually involve the propagation of farmer-selected seeds that are unfortunately characterized by seed degeneration caused by pathogen build-up in the field and over time (Riungu, 2011; Kaguongo et al., 2013). In addition, relevant institutes and organizations can produce only 1% of certified clean seeds that make 20,000 plantlets a year. Nonetheless, at least 10% of clean seed is required to significantly enhance potato production in the country from two to three million tons to ten million tons (Gildemacher et al., 2009) presenting a challenge to efforts addressing food security. To boost clean seed production, various efforts have been aimed at developing and adopting new multiplication techniques such as micropropagation.

Potato micropropagation entails the rapid multiplication of stock plant material for the mass production of clean seed potatoes. The efficiency of micropropagation can be enhanced greatly through the modification of the culture media to include or enhance nutrients that promote plant regeneration and vigor while shortening the regeneration period. Furthermore, efforts have involved conferring tolerance to regenerated micro plants against abiotic and biotic stress after having the plants established under greenhouse or field conditions. Meso-nutrients ($CaCl_2.2H_2O$, KH_2PO_4, $MgSO_4$) are a group of minerals (mesos) in MS medium and play an influential role during plant growth *in vitro*. (Wada et al., 2013; Hunková et al., 2020) found that enhancing the amounts of mesos dramatically improved pear, blackberry, and blueberry micropropagation efficiency, respectively, by producing high-quality shoots and contributing to greater production of high-quality plantlets.

As an essential macronutrient, Ca is involved in a wide range of physiological processes such as promoting disease resistance to pathogens through the strengthening of cell wall structures and maintaining membrane stability (White and Broadley, 2003; Gilroy et al., 2016). Ca supplementation reportedly promoted potato microtuberization and micropropagation (Habib et al., 2004) as well as potato tuber yield in field conditions. It increased the postharvest shelf-life of horticultural produce, and improved tolerance to diseases such as bacterial wilt (Cybulska et al., 2012; Jiang et al., 2013). Potato varietal response to Ca fortification under *in vitro* conditions is not well documented, however. Ca fortification protocols for optimal potato microtuberization have not been properly investigated and optimized. Thus, the current study aimed at optimizing Ca fortification in three potato varieties to enhance shoot growth, root growth, microtuberization, and micro-plant vigor.

Materials and Methods

Plant materials

Virus-free, one-month-old micro plants of *Solanum tuberosum* varieties, i.e. 'Shangi', 'Dutch Robjyn', and 'Unica' were sourced from Kenya Agricultural and Livestock Research Organization (KALRO) Tigoni, Limuru, Kenya. The micro plantlets were cultured on hormone-free Murashige and Skoog (MS) medium with about 5-6 nodes, emergent shoots, and roots in each jar. The plantlets were transported in aseptically sealed plastic containers to Jomo Kenyatta University of Agriculture and Technology, Juja. They were stored in a culture room at 20°C for 3 days before sub-culturing them to ascertain that the cultures were pathogen-free.

Explants

Single node cuttings (12-15 mm with one axillary bud) of 1-month-old micro plants of the three Irish potato varieties were used for the regeneration experiment at the Institute of Biotechnology Research Laboratory (IBR) of Jomo Kenyatta University of Agriculture and Technology. The node cuttings were excised from the stem section of the micro plants on sterile paper using sterile blades. They were subcultured immediately onto culture jars containing freshly prepared MS medium.

Medium and growth conditions for shoot, root, node, and leaf growth

The three varieties ('Shangi', 'Dutch Robjyn', and 'Unica') were tested for regeneration responses of shoots, nodes, leaves, and roots under five levels of $CaCl_2$ (8.8 g L^{-1}, 10.4 g L^{-1}, 12 g L^{-1}, 13.6 g L^{-1}, 15.2 g L^{-1}) (Table 1). The samples were laid out in a completely randomized design with 3x5 factorial combinations. MS media contained five different $CaCl_2$ (hereafter Ca) concentrations in 300 ml glass culture jars, vitamins, sucrose (3%), 10 ml L^{-1} myo-inositol, and 0.2% gelrite. The pH value of culture media was adjusted to 5.8 before autoclaving at 121°C and 15 kg cm^{-1}. The cultures were maintained in a growth chamber at 21 ± 2°C at 2500 lux and were examined weekly for 4 weeks.

Microtuberization

Single node cuttings (12-15 mm long) obtained from *in vitro* regenerated plantlets were cultured on 20 mL of solid medium containing vitamins, 60

g L^{-1} sucrose, and 6.0 mg L^{-1} 6-Benzylaminopurine in 300 mL glass jar. The cultures were maintained in a growth room at 21± 2 °C in complete darkness. Assessments were done weekly and the microtubers were harvested after formation.

Table 1. Description of treatments used in the study

Treatment	$CaCl_2$ (g L^{-1})
T_0 (Control)	8.8 g L^{-1}
T_1	10.4 g L^{-1}
T_2	12 g L^{-1}
T_3	13.6 g L^{-1}
T_4	15.2 g L^{-1}

Data collection

Data on regeneration capacity (shoot, root, functional leaves, and node count) were assessed 28 days after culture. Data on microtuber count per cutting and the number of days to tuberization were counted. The fresh weight of harvested microtubers per cutting was measured. The number of days required for *in vitro* tuberization was recorded as a visible swelling of microtubers.

Nutrient analysis

Five micro plants were randomly selected from each of the three varieties ('Shangi', 'Dutch Robjyn', and 'Unica') and each of the five different Ca treatment groups. The samples were deflasked and rinsed under running tap water until all traces of culture media were removed. The micro plants were patted dry and left to air dry at ambient room temperature. The fresh weight of the micro plants was recorded, and then the mid-stem and root section was excised from each of the micro plants. The samples were weighed and the plant materials were oven dried at 70 °C in an oven for 24 hours. Oven-dried mid-stem and root zone samples were ground in a pestle and mortar. The samples were digested and analyzed for total calcium (Ca), magnesium (Mg), iron (Fe), and zinc (Zn) according to Welz and Sperling (2008). Ca, Mg, Fe, and Zn were assessed using an atomic adsorption spectrophotometer (Shimadzu AA-7000 series, Shimadzu Corporation, Japan).

Statistical analysis

All data were subjected to a two-way analysis of variance (ANOVA) using the GLM procedure in SAS (University Edition, SAS®, Version 9.4, SAS Institute Inc, Cary, NC, USA) (Cody, 2017). The mean values of the treatment groups were separated by Tukey's HSD (Honestly Significant Difference) test ($p \leq 0.05$).

Results

Shoot count, 28 days after culture

Variety-dependent differences were significant ($p<0.05$) in shoot count among Ca-enhanced MS media treatments. For 'Unica', significant differences in shoot count ($p<0.01$) were observed among the five treatments. MS media 8.8 g L^{-1} Ca (T0) had the highest number of shoots (5.57±1.21) compared to all other treatments. MS media containing 12 g L^{-1} Ca (T2) and 15.2 g L-1 Ca (T4) produced the lowest number of shoots (1.14±0.14) (Table 2) in 'Unica'. By contrast, shoot numbers in varieties 'Shangi' and 'Dutch Robjyn' exhibited no significant differences among the five treatment levels with shoot numbers for both varieties ranging between 1.4 to 4.1 shoots. Nonetheless, 10.4 g-12 g L^{-1} Ca promoted twice the number of shoots for 'Shangi' compared to the control. 'Unica' under T0 significantly outperformed (5.57±1.21) 'Shangi' (4.14±0.86) and 'Dutch Robjyn' (2.29±0.36) in shoot regeneration capacity.

Number of roots at 28 days after culture

Ca treatments had variable effects on the number of roots in each of the three potato varieties depending on the Ca concentration in the media. In all five treatments and three potato varieties, significant differences ($p<0.05$) occurred in the number of roots, 28 days after culture. In 'Unica', root regeneration varied per treatment, with T4 significantly promoting ($p=<.0001$) root regeneration, making 1.3 times more roots than the control, with the number of roots ranging between 5.1-15 roots (Table 2). For 'Shangi', T2 significantly ($p=0.0003$) caused the production of roots (9.86±0.70) twice as much as the control (4.86±0.83). T_1 significantly ($p=<.0001$) produced roots, 1.2 times the number of roots in 'Dutch Robjyn' (13.29±0.89), compared to the control (10.43±0.81). The application of 8.8 g-10.4 g L^{-1} Ca best promoted root regeneration for 'Dutch Robjyn' while 10.4 g-13.6 g L^{-1} Ca significantly promoted root growth in 'Shangi'. Under the effect of T4, 'Unica' (15.00±0.79) significantly outperformed 'Shangi' (9.86±0.70) and 'Dutch Robjyn' (13.28±0.89) in root regeneration capacity (Table 2). Overall, the number of roots regenerated by 'Shangi' ranged between 4.8-9.8 while 'Dutch Robjyn' and 'Unica' regenerated 3.4-13.2 and 5.1-15 roots, respectively (Table 2). Plantlets in MS media containing 10.4 g L^{-1} $CaCl_2$ (T_1) and 15.2 g L^{-1} (T4) had the highest significant difference in the number of roots among the three varieties.

Table 2. Effect of MS media calcium enhancement on *in-vitro* growth of 'Shangi', 'Dutch Robjyn', and 'Unica' potato varieties 28 days after culturing (DAC)

Variety	Treatment	Number of shoots		Number of roots		Number of nodes		Number of leaves	
Shangi	T_0	2.71±0.47	a	4.86±0.83	b	7.00±0.72	a	7.43±0.72	a
	T_1	4.14±0.86	a	6.14±0.67	b	10.29±1.90	a	10.14±1.98	a
	T_2	4.14±0.63	a	9.86±0.70	a	9.71±0.64	a	10.00±0.69	a
	T_3	2.43±0.30	a	7.00±0.58	b	8.00±0.58	a	7.86±0.501	a
	T_4	2.86±0.55	a	6.86±0.59	b	9.29±0.52	a	9.71±0.64	a
	p value	0.1311		0.0003		0.1686		0.2352	
Dutch Robjyn	T_0	2.14±0.34	a	10.43±0.81	ab	10.86±0.67	ab	11.29±0.61	ab
	T_1	1.71±0.18	a	13.29±0.89	ab	12.71±0.52	ab	12.71±0.52	a
	T_2	1.86±0.34	a	7.86±0.94	bc	9.71±0.75	ab	9.86±0.83	ab
	T_3	2.29±0.36	a	5.14±0.80	cd	10.14±1.20	ab	10.29±1.23	ab
	T_4	1.43±0.30	a	3.43±0.65	d	8.43±0.84	b	8.43±0.84	b
	p value	0.33		<.0001		0.0158		0.016	
Unica	T_0	5.57±1.21	a	11.14±1.49	b	10.14±1.06	a	10.43±1.13	a
	T_1	1.43±0.30	b	5.14±0.63	c	6.71±0.71	b	6.86±0.70	b
	T_2	1.1±0.14	b	12.57±0.53	ab	8.14±0.46	ab	8.43±0.37	ab
	T_3	1.29±0.18	b	6.00±0.31	c	7.29±0.42	b	7.29±0.47	b
	T_4	1.14±0.14	b	15.00±0.79	ab	8.86±0.40	ab	8.86±0.40	ab
	p value	<.0001		<.0001		0.0085		0.0071	

T_0=8.8 g L^{-1} Ca_2; T_1=10.4 g L^{-1} Ca; T_2=12 g L^{-1} Ca_2; T_3=13.6 g L^{-1} Ca; T_4=15.2 g L^{-1} Ca. Values indicate means±SE (n=7). Mean values in the same column, followed by the same letters, are not significantly different for each variety (Tukey's HSD test, $p \leq 0.05$).

Number of nodes at 28 days after culture

The application of 8.8 g-13.6 g L^{-1} Ca (T0-T3) had significant effects ($p<0.05$) on the number of nodes produced in varieties 'Unica' and 'Dutch Robjyn' (Table 2). T_1 significantly ($p=0.0158$) produced nodes, 1.1 times more than the nodes in 'Dutch Robjyn' (12.71±0.52) compared to the control (10.86±0.67). Meanwhile, the application of 8.8 g-13.6 g L^{-1} Ca promoted node regeneration (8.4-12.7) in 'Dutch Robjyn'. 'Unica' showed a significant difference in the number of nodes produced in response to the different Ca levels in MS media, 28 days after culturing (Table 2). T0 promoted the highest node number for 'Unica' (10.14±1.06). There was no significant increase in node number for 'Shangi', with T_1 promoting a higher number of nodes (10.29±1.90) 28 days after culture, compared to the other treatments. The mean node count ranged between 7.0-10.29 nodes per cutting in T_0-T_4 treatments.

Number of leaves, 28 days after culture

The effect of different Ca levels on the number of leaves was variable with significant differences in total leaf number for 'Dutch Robjyn' and 'Unica' varieties. In response to T_1 (10.4 g Ca), 'Dutch Robjyn' plantlets had a significantly higher ($p=0.016$) number of leaves, 1.1 times higher than that of the control (8.8 g L^{-1} Ca). The number of leaves regenerated by 'Dutch Robjyn' ranged between 8.4-12.7 leaves with T_1-T_3 significantly increasing leaf number. In contrast, 'Unica' plantlets produced a significantly higher number of leaves (10.43±1.13) under the control treatment (T_0) compared to the other treatments (Table 2). Leaf count ranged between 6.8-10.4

leaves per plantlet.

No significant difference was observed in the number of leaves in 'Shangi' plantlets in all five treatments (Table 2). The number of leaves ranged between 7.4-10.1 in 'Shangi' plantlets, with 10.4 g-15.2 g L^{-1} Ca promoting a higher number of leaves compared to the control (7.43±0.72).

Days to microtuber formation, number of tubers and tuber weight

Single nodal cuttings (SNC) of 'Shangi', 'Dutch Robjyn', and 'Unica' were subcultured onto a tuberizing MS medium, modified with $CaCl_2$ at different levels, and exhibited significant differences ($p<0.05$) in tuber weight and days to tuberization, depending on the variety, whereas the tuber count did not differ significantly.

The enhanced root zone of Ca had significant effects on the number of days to tuber formation (Table 3). Tuber initiation was overall irregular under conditions of *in vitro* Ca fortification. The tuberization process began with the formation of a single stolon in all three varieties under the effects of T_0, T_2, T_3, and T_4, subtending over the surface of the nutrient media as aerial stolons. T_1 led to 54%, 59%, and 43% formation of sessile microtubers at the axillary buds of explants in 'Shangi', 'Dutch Robjyn', and 'Unica' (Fig. 1). In 'Shangi', T_1 and T_2 promoted early tuberization (21.7-22.6 days compared to the control (8.8 g L^{-1} Ca). T_0, T_2, and T_3 also promoted earlier tuberization in 'Dutch Robjyn' (26.1-32.0 days) compared to T_1 (55.43±2.47) and T_4 (47.13±3.36). In 'Unica', T_2 (12 g L^{-1} Ca) completely inhibited microtuberization.

Fig. 1. From left to right: sessile microtubers in explant cuttings of 'Dutch Robjyn', 'Shangi', and 'Unica' subcultured in the T_1 treatment.

T_1 (10.4 g L^{-1} Ca) greatly reduced the number of days to tuber initiation by 16.83 days in 'Unica' compared to the control. Consequently, microtuber initiation was significantly delayed ($p=<.0001$), and 29 more days were required to form a microtuber, compared to the control in 'Dutch Robjyn' when the culture medium was fortified with Ca at 10.4 g L^{-1} Ca (Table 3). T_2 (12 g L^{-1} Ca) caused a significantly variable effect ($p<.0001$) on the tuber weights of the three varieties (Table 3). Additionally, T_2 increased tuber weight in 'Dutch Robjyn' by 2.3 times compared to the control.

Using 10.4 g-15.2 g L^{-1} Ca in the tuberizing medium had variable effects on the number of tubers produced in 'Shangi', 'Unica', and 'Dutch Robjyn' varieties. Each SNC in all three varieties and across the five treatments produced at least one microtuber (Table 3). Using 12 g-13.6 g L^{-1} Ca caused an overall increase in the number of microtubers in 'Dutch Robjyn', with T2 (12 g L^{-1} Ca) increasing the tuberization response by 25% compared to the control. In 'Shangi', T1 and T2 promoted the tuberization response by 150% and 100% respectively compared to the control with T_1 (10.4 g L^{-1} Ca) promoting the highest number of microtubers. In 'Unica', 10.4 g-15.2 g L^{-1} Ca inhibited microtuber generation, with T_2 (13.6 g L^{-1} Ca) inhibiting microtuber formation by up to 100% compared to the control (T0). However, T_2 promoted the growth of etiolated shoots.

Table 3. Effect of 5 different Ca levels on the number of days to tuberization, tuber count, and tuber weight in 'Shangi', 'Dutch Robjyn' and 'Unica' varieties

Variety	Treatment	Days to tuberization			Number of microtuber per cutting			Tuber fresh weight (mg)		
Shangi	T_0	43.50	±3.50	a	1.00	±0.00	a	38.00	±0.00	c
	T_1	22.60	±1.47	b	1.00	±0.00	a	26.20	±0.00	d
	T_2	21.75	±0.48	b	1.00	±0.00	a	29.50	±0.00	d
	T_3	42.00	±1.00	a	1.00	±0.00	a	43.50	±0.00	b
	T_4	49.67	±1.67	a	1.00	±0.00	a	57.33	±0.00	a
	p value	<.0001						<.0001		
Dutch Robjyn	T_0	26.13	±1.23	b	1.00	±0.00	a	14.50	±0.00	b
	T_1	55.43	±2.47	a	1.00	±0.00	a	16.71	±0.00	b
	T_2	32.00	±1.33	b	1.00	±0.00	a	34.00	±0.00	a
	T_3	25.89	±2.37	b	1.00	±0.00	a	17.33	±0.00	b
	T_4	47.13	±3.36	a	1.00	±0.00	a	18.13	±0.00	b
	p value	<.0001						<.0001		
Unica	T_0	38.50	±2.25	c	1.00	±0.00	a	29.83	±0.01	a
	T_1	21.67	±0.33	d	1.00	±0.00	a	23.67	±0.00	a
	T_2	*			*			*		
	T_3	94.00	±3.00	a	1.00	±0.00	a	21.00	±0.00	a
	T_4	57.33	±1.86	b	1.00	±0.00	a	13.33	±0.00	a
	p value	<.0001						0.224		

T_0=8.8 g L^{-1} Ca_2; T_1=10.4 g L^{-1} Ca; T_2=12 g L^{-1} Ca; T_3=13.6 g L^{-1} Ca; T_4=15.2 g L^{-1} Ca. Data indicate mean values±SE (n=10). Mean values in the same column followed by the same letters are not significantly different for each variety (Tukey's HSD test, $p \leq 0.05$). (* indicates that the treatment failed permanently in generating a microtuber.)

Total Ca

An increase in total Ca levels occurred in plantlets from the three varieties with the highest (9.59 mg g^{-1} DW) and lowest (2.09 mg g^{-1} DW) Ca levels observed in 'Shangi' and 'Unica' varieties, respectively. T3 (13.6 g L^{-1} Ca) caused increases of 45% and 202% in total Ca in the root and mid-stem section of 'Shangi' and 'Unica' varieties, respectively, compared to the control. T_3 also caused a 165% increase in total Ca recorded in 'Dutch Robjyn', compared to the control plantlets (Table 4).

Table 4. Total Calcium (Ca2+) content in root and mid-stem section of calcium-fortified 'Shangi', 'Dutch Robjyn' and 'Unica' under five Ca treatments levels

	Calcium (Ca^{2+}) content (mg/g DW)				
Variety	**8.8 g L^{-1} Ca**	**10.4 g L^{-1} Ca**	**12 g L^{-1} Ca**	**13.6 g L^{-1} Ca**	**15.2 g L^{-1} Ca**
Shangi	6.60	5.74	6.41	9.59	8.15
Dutch Robjyn	2.94	6.70	6.99	7.79	5.27
Unica	2.09	3.23	6.00	6.31	4.00

Mean values of 5 plants after oven drying for 24 hrs. Milligrams $gram^{-1}$ dry weight (mg g^{-1} DW).

Total magnesium, iron, and zinc

The magnesium content was lowest in the root sections of 'Shangi' (0.04 mg g^{-1} DW) and 'Dutch Robjyn' varieties (0.24 mg g^{-1} DW) under treatments T_1 (10.4 g L^{-1} Ca) and T3 (13.6 g L^{-1} Ca) respectively (Table 5). Meanwhile, T3 caused the highest magnesium content in the root and mid-stem section of 'Unica' (1.99 mg g^{-1} DW), amounting to an increase of 1.6-fold, compared to the control. T_2 (13.6 g L^{-1} Ca) caused the iron content in the root and mid-stem section to increase by 210% and 48% in 'Shangi' and 'Dutch Robjyn' respectively, compared to their respective control groups. T_4 (15.2 g L^{-1} Ca) caused a 133% increase in iron content in 'Unica', compared to the control.

Higher dosages of Ca (10.4 g-15.2 g L^{-1} Ca) also increased the iron content in the 'Unica' by all treatments (0.76 mg g^{-1} DW on average). Maximum peak values of 0.52 mg g^{-1} DW in 'Shangi' and 0.58 mg g^{-1} DW in 'Dutch Robjyn' were observed under the effect of T2 (13.6 g L^{-1} Ca) in terms of iron content. Using 13.6 g L^{-1} Ca led to an increase in total Zn2+ content in the mid-stem and whole root section, by 40% and 118%, in 'Shangi' and 'Unica', respectively. T2 increased the total Zn2+ content by 70% in 'Dutch Robjyn'. Specifically, this occurred in the mid-stem and whole root section, compared to the control (Table 5).

Table 5. Magnesium, zinc, and iron content in the root and mid-stem section of calcium-fortified 'Shangi', 'Dutch Robjyn', and 'Unica' under five Ca treatments levels

Variety	Treatment	Total Mg^{2+} (mg g^{-1} DW)	Total Fe^{2+} (mg g^{-1} DW)	Total Zn^{2+} (mg g^{-1} DW)
Shangi	T_0	0.08	0.17	0.20
	T_1	0.04	0.27	0.20
	T_2	0.35	0.52	0.16
	T_3	0.14	0.36	0.28
	T_4	0.12	0.17	0.10
	mean	0.14	0.30	0.19
Dutch Robjyn	T_0	0.37	0.39	0.22
	T_1	0.34	0.56	0.24
	T_2	0.47	0.58	0.37
	T_3	0.24	0.22	0.18
	T_4	0.31	0.44	0.20
	mean	0.35	0.44	0.24
Unica	T_0	1.20	0.45	0.48
	T_1	1.32	0.65	0.27
	T_2	1.49	0.72	0.57
	T_3	1.99	0.95	1.05
	T_4	0.59	1.05	0.95
	mean	1.32	0.76	0.67

T_0=8.8 g L^{-1} Ca; T_1=10.4 g L^{-1} Ca_2; T_2=12 g L^{-1} Ca; T_3=13.6 g L^{-1} Ca_2; T_4=15.2 g L^{-1} Ca. Mean values of 5 plants after oven drying for 24 hrs.

Discussion

The current study showed a powerful tool for the production of adequate, clean, and high-quality potato planting materials that may ultimately boost seed potato production. This can contribute to the goal of producing up to 10 million table potatoes per year in Kenya (Mbego, 2019). Currently, formal seed potato production systems are unable to meet the high demand for clean certified seed due to both technical and financial constraints making farmers rely on farm-saved seeds, often marked by high levels of seed degeneration (Muthoni et al., 2014; Joseph et al., 2015). Furthermore, a previous study revealed variable effects of Ca fortification, varietal differences, and their interactions on Irish potato shoots, roots, nodes, and leaf number, days to tuberization, tuber count, and micronutrient concentration. Different potato varieties exhibited unique characteristics, consequently presenting variety-specific management challenges (Muleta and Aga, 2019).

Ca is an essential macronutrient with significant effects on plant morphology, plant defense against pathogens, and plant response to abiotic stresses (Zhang et al., 2014; Kumar et al., 2015) In the present study, root regeneration and *in vitro* agronomic fortification was proved under enhanced root zone Ca conditions (>8.8 g L^{-1} Ca). $CaCl^2$ is categorized as a Meso nutrient for *in vitro* propagation. It usually plays an especially influential role in promoting shoot growth and quality not only as a deterrent to poor *in vitro* growth but also as a contributor to overall shoot quality (Reed et al., 2013; Lekamge et al., 2021) during *in vitro* culture. Calcium is a key component of media developed for Irish potato (Ozgen et al., 2011), diverse pear germplasm (Reed et al., 2013), and bromeliads (Aranda-Peres et al., 2009).

Supplemental Ca (>8.8 g L^{-1} Ca) in the root zone was found to promote the growth of apical shoots, especially in 'Dutch Robjyn' and 'Unica'. The application of 10.4 g-15.2 g L^{-1} Ca strongly promoted the growth of one apical shoot for the 'Unica'. This was consistent with previous studies that showed how supplemental Ca promoted dominant apical shoot growth (Ozgen et al., 2011; Machado et al., 2014). A relevant study reported a constraint that was associated with the tissue culture of Irish potatoes, revealing shoot tip necrosis that resulted in the death of apical shoots, loss of apical dominance, and the formation of auxiliary shoots (Ibrahim et al., 2016). Srikum et al. (2018) found that 880 mg L^{-1} $CaCl_2$ levels in the culture medium decreased shoot necrosis in *Elsholtzia stachyodes* and resulted in 100% shoot induction. This study confirmed that the addition of Ca in the form of $CaCl_2$ strongly promoted apical shoot growth.

In addition, supplemental Ca also significantly promoted ($p \leq 0.05$) root growth in the three varieties, namely, 'Shangi', 'Dutch Robjyn', and 'Unica'. Calcium usually plays a role in promoting primary root development and maintaining root hair growth by influencing the direction of microtubule growth (Bibikova and Gilroy, 2009; Leitão et al., 2019). Calcium can have an impact on primary root development by modulating auxin accumulation, transport, and signaling (Zhang et al., 2020). Lekamge et al. (2021) found that supplemental Ca caused extensive *in vitro* root growth in 'Okhotsk Chip' cultivar.

In each variety, each SNC across all five treatments produced a maximum of one microtuber. The present findings corroborate those of Hossain et al. (2019) who found that plantlets of 'Asterix', 'Granola', and 'Diamant' cultivars produced 1 microtuber even with the use of BAP (known to promote tuberization) and gibberellin scrubbers. Arvin et al. (2005) found an increase in Ca concentration that multiplied the microtuber count in cultivars 'Bintje' and 'Russet Bank'. Microtuberization is influenced by, but not specifically dependent on, mineral nutrition. Simon (2016) found significant varietal differences ($p \leq 0.05$) in the tuber count of four Irish potato varieties in three tuber sizes in Ethiopia. The results from the current study confirmed these previous studies since the varietal factor significantly affected the tuber weight ($p = < 0.0001$) and days to tuberization ($p = < .0001$). 'Shangi' formed a microtuber 21.7-43.5 days after culture initiation, with 13.6 g L^{-1} $CaCl_2$ resulting in the shortest duration to a single tuber formation (50% decrease in days to tuber formation), compared to the control. The applicaiton of 10.4 g L^{-1} $CaCl_2$ caused a 18.18% increase in Ca, compared to the control, when the tuberizing media influenced the formation of sessile microtubers for the three varieties. This might indicate that T_1 promoted a strong tuberization stimulus in the explants of the three varieties (Seabrook et al., 1993).

Supplemental Ca (10.4 g-15.2 g L^{-1}) greatly inhibited tuber production in 'Unica' by up to 100%. In addition, supplemental Ca (10.4 g L^{-1}) reduced the number of days to a single tuber formation by up to 43%, compared to the control. However, 12-15.2 g L^{-1} Ca significantly lengthened the number of days for a single tuber to be formed. This could potentially be linked to the secondary messenger role that exogenous Ca plays in regulating nitrogen metabolism (Xing et al., 2021). Excess Ca (>8.8 g L^{-1} Ca) potentially

inhibited N absorption and utilization in 'Unica', a variety bred for nitrogen use efficiency, thereby triggering early tuberization, similar to previous results found by Zakaria et al. (2008) who reported a higher number of microtubers when reducing nitrogen levels. For the three varieties and across all treatments, a low tuber weight occurred. Most microtubers were characterized by tuber weights in the range of 10-57 mg. Hossain et al. (2019) reported lower microtuber weights for potato varieties 'Asterix', 'Granola', and 'Diamant' compared to their control. This observation was attributed to the use of BAP (to trigger tuberization) and chlorocholine chloride. Additionally, excess Ca (>8.8 g L^{-1} $CaCl_2$) significantly reduced the number of shoots produced by 'Unica'.

The results showed that Ca content in the mid-stem and root zone sections of the three potato varieties increased in response to higher Ca content in the regeneration media. The highest content occurred in response to 13.6 g L^{-1} $CaCl_2$ in the culture medium (in 'Unica' by 202%, 'Shangi' by 45%, and 'Dutch Robjyn' by 165%). These results are consistent with previous studies that found how an increase in exogenous Ca contributed to higher microplant Ca content and promoted microplant quality (Sarkar et al., 2005). Excess Ca is known to harm the uptake of Mg, Zn, and Fe which were evaluated in this study.

The results revealed a marked decrease in Mg content in 'Dutch Robjyn' and 'Shangi' and an increase in 'Unica' (Table 5) consistent with previous results reported by Kozai et al. (1995) who found that increasing the exogenous Ca alone negatively influenced the magnesium uptake and limited the growth of *Solanum tuberosum* L. Mg contributes to an increase in leaf development by increasing chlorophyll content, thereby increasing photosynthetic efficiency (Rodrigues et al., 2021). Nonetheless, a decrease occurred in leaf count in 'Unica' under treatments with (>8.8 g L^{-1} $CaCl_2$). Iron, a micronutrient affecting plant metabolism, was found to be lowest in 'Shangi' (0.30 mg g^{-1} DW on average), consistent with previous values reported by Evelyne et al. (2021) who found that 'Shangi' had the lowest iron content compared to 'Dutch Robjyn' and 'Unica'.

Zinc, as a divalent cation (Zn^{2+}), usually plays a functional and structural role in enzymatic reactions (Marschner and Marschner, 1996). 'Unica' had the highest zinc concentration (0.67 mg g^{-1} DW on average) in the root and mid-stem section while 'Shangi' caused the lowest zinc concentration (0.19 mg g^{-1} DW on average). These values differed from those reported by Evelyne et al. (2021) who indicated that 'Dutch Robjyn' had the lowest zinc content compared to 'Unica' and 'Shangi'. This could be attributed to environmental and cultural differences under which the sampled Irish potato varieties were grown.

In conclusion, supplementing Ca as $CaCl_2$ significantly improved the performance of 'Shangi', 'Dutch Robjyn', and 'Unica' *in vitro*, achieving earlier and rapid shoot regeneration in 'Shangi', 'Dutch Robjyn' and rapid root regeneration in 'Unica', compared to conventional MS media formulations. Overall, 13.6 g L^{-1} Ca resulted in the best Ca-enriched plantlets of 'Unica', 'Shangi', and 'Dutch Robjyn'. Furthermore, 10.4 g^{-1}3.6 g L^{-1} Ca optimally promoted root and shoot growth in 'Dutch Robjyn' but also delayed tuberization. Shoot growth, root growth, and tuber count in 'Shangi' was promoted by 10.4 g-13.6 g L^{-1} Ca. In addition, 'Shangi' could be labeled as a potential 'calcium packer' due to its ability to take up larger amounts of Ca even in conventional Ca levels in MS media. The application of 8.8 g L^{-1} Ca triggered early tuberization in 'Unica' while 13.6 g-15.2 g L^{-1} Ca promoted rooting and resulted in the highest zinc and iron contents. Ca enhancement provided an optimal state of early plant regeneration and *in vitro* agronomic fortification in addition to the experimental provision of an *in vitro* Ca fortification protocol. Supplemental Ca influenced the mineral uptake rates of magnesium, iron, and zinc. The current study demonstrated that the quantitative increase in Ca within the MS media generated a variety-dependent response while promoting shoot and root growth.

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Conflict of interest

The authors indicate no conflict of interest for this work.

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