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EFFECT OF VARIOUS CONCENTRATIONS OF CALCIUM CHLORIDE ON CALLUS GROWTH AND POTASSIUM NUTRITION OF CALLI CULTURES OF POTATO (*SOLANUM TUBEROSUM*)

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Abstract

Effects of various concentrations of calcium chloride on callus growth and potassium nutrition of calli cultures of Potato (*Solanum tuberosum*) cv. Cardinal were studied. Calli cultures of Potato were treated with Calcium chloride in 5 different concentrations (0.1; 1; 3; 6; 9mM). Calcium chloride treatments had significant effect on almost all parameters studied. Maximum relative growth rate (0.711 week^{-1}) and water content (94.872%) were recorded at 6mM Calcium chloride. The maximum K^+ accumulation in dried matter (695.568 µmoles g^{-1} Dry Wt.) at 3mM Calcium chloride and maximum Ca^{++} accumulation in dried matter (450.83 µmoles g^{-1} dry wt.) at 9mM Calcium chloride were found. The minimum relative growth rate (0.376 week^{-1}), water content (62.611%), K^+ accumulation in dried matter (485.98 µmoles g^{-1} dry wt.) and Ca^{++} accumulation in dried matter (187.367 µmoles g^{-1} dry wt.) were recorded at 0.1mM Calcium chloride. It is concluded from the experiment that better results were obtained from 3-6mM Calcium chloride treatment on Potato callus.

Introduction

Potato *(Solanum tuberosum,* L.) is an important commercial cash crop of the world. It is basically a cool season crop in origin and has been grown traditionally under conditions that prevail in the northern latitudes of Europe and America and in tropical highlands such as the Andean region in South America or the Hamalayan, Karakuram, Hindukush valleys of the Indo-Pak subcontinent. Now potato is successfully grown in tropical, sub tropical and temperate climate and is adapted to diverse socio-economic conditions (Malik, 1995).

Pakistan is the seventh largest potato producing country in the world. Although potato production in Pakistan has increased many folds but per hectare yield is far less than other parts of the world (Malik, 1995; Abbas *et al*., 2011). Potato is conventionally propagated through vegetative means and thus diseases could be easily transmitted from one generation to the other. More than 18 potato diseases were reported in the country. (Turkensteen, 1986; Irfan, 2005). Hence, tissue culture is considered the best technique of producing quality diseases free seed for maximizing potato production (Afrasiab & Iqbal, 2012a).

Adequate calcium is a critical aspect of the mineral nutrition of potatoes. Calcium is involved in both the structure and function of all plant cell walls and membranes. Inadequate supplies of calcium cause growth abnormalities like internal brown spot and hollow heart. Adequate calcium nutrition can also improve skin color in red potatoes. Abundant tissue calcium also increases the tubers' resistance to soft rot during storage and may improve the performance of seed potatoes (Waterer, 2005).

Calcium has role in cell signaling by acting as secondary messenger and maintains the integrity of plasma membrane. It plays a regulatory role in the balance of cation anion. Ca sensing proteins are involved in many cellular processes like cytoplasmic streaming, organelles and vesicles transport, microtubules dynamics, cell division, chromosome segregation, cell elongation, tip growth and morphogenesis (Reddy, 2001). Ca influence cellular pH and also act as a regulatory ion in the source sink translocation of Carbohydrates through its effects in cells and cell walls. Ca is needed for cell wall strengthening and provides protection against biotic and abiotic stresses (Hirschi, 2004; Aranda-Peres *et al.,* 2009). Likewise, Potassium is required for turgor build up in plants and maintains the osmotic potential of cells, which in guard cells governs the opening of stomata. It affects the cell extension and cell walls thickness and stability (Schroeder *et al*., 2001). K^+ plays role in enzyme activation, protein synthesis and photosynthesis (Mezei *et al*., 1995; Tariq *et al.,* 2011). High internal K^+ concentration can dampen extreme sudden environmental events like cold, frost, late season rains, high salt stresses and heat waves (Kant & Kafkafi, 2004, Iqbal *et al.,* 2011).

Soluble Ca can improve crop production. Ca affects the opening of K channels in leaves, especially guard cells by working as secondary messenger. Calcium increases ammonium, potassium and some other monovalents absorption through 'Viets Effect' (Fenn & Feagley, 1999; Jacobson *et al.,* 1960). It also makes the use of nitrogen more efficient (Fenn & Taylor, 1990). Membrane selectivity for K^+/Na^+ is maintained and root growth is stimulated (Cramer *et al*., 1987). For most crops supplemental calcium often alleviates the effects of sodium on membrane and plant growth (Rangel, 1992). More potassium present in both the roots and leaves of citrus rootstocks suggests that supplemental calcium maintain K/Na selectivity (Banulus *et al*., 1991).

Potato is one of the crop plants, on which tissue culture techniques have been applied with some success, to improve potato production by means of micro propagation, pathogen elimination, disease free seed potato production, and germplasm conservation (Hussey & Stacey, 1981; Afrasiab & Iqbal, 2012b)). *In vitro*, the growth is a function of mineral elements and organic components of the medium. Physiological and biochemical differences are fully expressed in callus

cultures (Saric *et al*., 1995; Naz *et al.,* 2011). How rapidly a tissue grows and the extent and quality of morphogenesis responses are strongly influenced by the type and concentration of nutrients supplied (Niedz & Even, 2007; Nas & Read, 2004). The competitive effect of Ca on K absorption, other stimulatory and inhibitory effects of Ca have been reported for short-term, excised-root experiments. The stimulatory effects of Ca have been attributed to the need to maintain the integrity of cellular membranes and of selective ion transport mechanisms by which K is actively absorbed.

Keeping in view the importance of potato and its effective propagation through tissue culture, it is selected for *In vitro* studies. The calli of these plants were cultured on the stress of calcium chloride salt in order to investigate the effect of different concentrations of calcium chloride on callus growth and to study the effect of calcium on potassium nutrition in calli cultures of potato.

Materials and Methods

An experiment "Effect of calcium chloride levels on callus growth and potassium nutrition of callus cultures of potato" was conducted at *In vitro* Laboratory, Institute of Biotechnology and Genetic Engineering (IBGE), The University of Agriculture, Peshawar, during September-December, 2008. Clones of potato (*Solanum tuberosum*) cultivar Cardinal were used in this study. The experiment was laid out in Completely Randomized Design (CRD). There were 5 treatments, each replicated 8 times.

Sterilization of explants: Petioles were used as explants, the cut explants of *Solanum tuberosum* cv. Cardinal were washed with clean water for 30 minutes. The Sodium hypochlorite (bleach) solution at a concentration of 5-10% was used for surface sterilization of these explants. The petioles were transferred with flamed forcep into a universal bottle $^2/_3$ filled with bleach. The lid was placed on the bottle. Universal bottle was shacked gently and then inverted every few seconds for 5 minutes. The solution was poured away leaving the petioles behind in the universal bottle. The petioles were washed with sterile water for 5 times. With flamed forcep, the petioles were transferred to the flasks containing Murrashige and Skoog (MS) medium (Murrashige & Skoog, 1962), observing sterile practices at all times. The flasks were labeled and incubated in the dark at 25°C for calli induction.

Callus induction: First, Calli were induced using the basal MS. Then equal quantity of callus was transferred to the MS media (Murrashige & Skoog, 1962) with the following modifications as treatments:

Treatment Treatments	Calcium chloride ($CaCl_2$) Concentration (mM)
T1	0.10
T2	1.00
T3	3.00
T4	6.00
T5	9.00

The following parameters were studied during the experiment

Relative growth rate (week^{-1}): Growth of calli was measured by the method of Shah *et al*., 1990. Pre-weighed wide necked conical flasks containing 30 cm^3 of culture medium with various concentrations of $CaCl_2$ were inoculated with similar quantities of callus, and the inoculated flasks were reweighed to obtain the initial fresh weight of the callus inoculums. The cultures were incubated at 25^{o}C for 28 days in the dark. The relative growth rate (RGR) of the callus was calculated as:

RGR week -1 = [in (final weight) – in (initial weight)]/4

Water content (%): Sample of known fresh weight was dried to constant weight at 60^{o}C in an oven and % water content in callus tissue was calculated as:

$$\% \text{ Water} = \frac{\text{Fresh wt.} - \text{Dry wt.}}{\text{Dry wt.}} \times 100$$

Determination of ionic content: Ionic contents (Ca^{++} and K^+) in callus tissues were determined by following method.

a. Acid digestion: Inorganic cytosolutes (Ca and K) in plant tissues were determined by acid digestion. Plant material (calli of potato) was taken and dried to constant dry weight at 60^{o}C in an oven. The weighed oven dried samples were digested in 10cm^3 boiling concentrated nitric acid (HNO_3) in Kjeldhal flasks and were left overnight on a low heat on a sand bath in a fume cupboard. The digested material was boiled rapidly to reduce the volume to approximately 2.5ml.

b. Atomic absorption spectroscopy: The volume of the cooled samples was made up to 25 ml in a volumetric flask with double distilled water; this diluted the concentration of HNO_3 to 10%. The standard cation solutions were prepared in 10% HNO_3 and Ions i.e. Ca^{++} and K^+ were analyzed with (Analyst 700) atomic absorption spectrophotometer.

Statistical analysis: Data were statistically analyzed by using the software MSTAT-C. One way ANOVA was used to test the significance of sources of variance, while LSD test (p = 0.05) was used to compare the differences among treatment means.

Results and Discussion

Relative growth rate (week^{-1}): Relative growth rate of potato calli increased significantly on increasing calcium chloride concentration to the growth medium from 0.1mM to 6mM at which growth rate reached to its maximum (0.711) followed by 0.67 at 3mM, but at high concentration of 9mM relative growth rate decreased to 0.54 that was almost equal to 0.56, the relative growth rate at concentration of 1mM $CaCl_2$. Least relative growth rate (0.37) was recorded at 0.1mM concentration (Table 1 & Fig. 1).

Table 1. Effect of $CaCl_2$ treatment on relative growth rate, Ca^{++} and K^+ concentration in dry callus tissues of potato.

Treatment (mM)	RGR (Week^{-1})	Ca^{++} (µ moles g^{-1} dry wt.)	K^+ (µ moles g^{-1} dry wt.)	Water content (%)
0.1	0.376c	187.367c	485.989c	62.611
1	0.559b	190.533c	495.398bc	82.684
3	0.674a	195.068c	695.568a	87.965
6	0.711a	301.314b	639.259ab	94.872
9	0.539b	450.834a	507.270bc	81.323
LSD values at α=0.05	0.1015	82.23	146.0	-

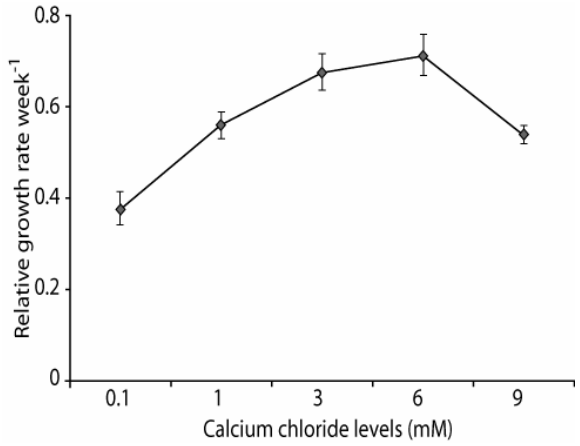

Fig. 1. The effect of various concentration of Calcium chloride on relative growth rate of calli cultures of *Solanum tuberosum* cv. cardinal. The data presented in the graph are means of eight replicates ± SE.

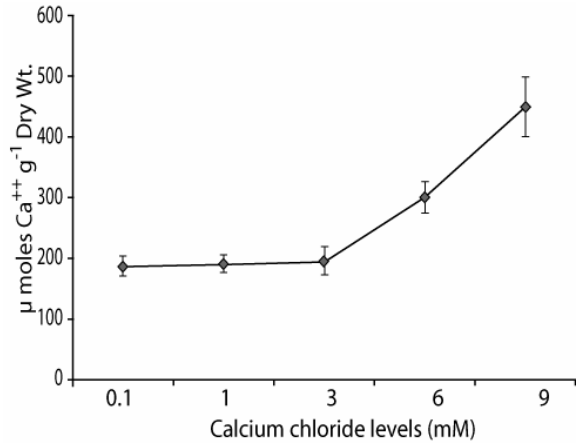

Fig. 2. The effect of various concentration of Calcium chloride on Ca content of dried calli cultures of *Solanum tuberosum* cv. cardinal. The data presented in the graph are means of eight replicates ± SE.

Callus fresh matter accumulation was proportional to the concentration of calcium nutrient in the growth medium. The results of this study are similar to those reported by Frett & Dirr (1986) working with *Petunia hybrida*. Those authors attributed that calcium effect on callogenesis to the decrease in lignifications of the cell wall caused by low level of calcium, facilitating callus initiation and growth *In vitro*. As the data reveals that increasing Calcium chloride to the growth medium from 0.1mM to 3mM significantly increased the relative growth rate of calli. This might be due to Ca/pectate interaction as a regulator of growth that dominates the requirement for calcium ions, and as a factor involved in the control of cell growth. Current results are also in agreement with the findings of Brewbaker & Kwack (1963), and Mascarenhas & Machlis (1964), who found a compelling interaction between calcium ion, the cell wall and cell growth and reported that calcium must be present in the medium to support pollen tube growth *In vitro.*

Increased growth rate of calli may be the result of calcium effect on plant hormone activity that affect growth *In vitro* as well, as action of each plant growth hormone can be altered by calcium salt. Similar results were reported by Montoro *et al*., (1995) that there was an interaction between the calcium and growth regulators in the medium, while working with calcium effect on *Hevea brasiliensis* calli. Further increasing of calcium chloride to the growth medium from 3mM to 6mM caused maximum increase in growth rate of calli. The present study results are in agreement with the research work of Arvin *et al*., (2005), who reported that the conventional calcium concentration in MS basal medium (3.0mM) is not enough for optimum growth of microtubers. Likewise, Arruda *et al.,* (2000) worked on anatomical and biochemical characterization of the calcium effect on *Eucalyptus urophylla* callus morphogenesis *In vitro.* They observed that lack of calcium supply caused a complete absence of a morphogenic process and tissue collapse. An increase in calcium concentration gave higher total protein and sugar contents, an increase in peroxidase specific activity and changes in the histological characteristics. He investigated that it was possible to verify that calcium stimulated globular somatic embryo formation at concentration of 6.62mM.

The current findings further reveal that 9mM calcium chloride in the growth medium caused a significant reduction in the relative growth rate of calli that showed toxic effect of high Ca content on calli growth. High calcium negatively affects the growth of calli. Such typical calcifugic behavior might be related to insufficient compartmentation or physiological inactivation of calcium (precipitation of calcium oxalate). Marschner (1995) associated these results to the inhibition of enzyme activities and magnesium uptake which is involved in protein synthesis and activation of enzymes closely related to cellular growth. Ahmad *et al*., (2009) also determined the relationship between callus growth and mineral nutrients uptake in salt-stressed Indica rice callus. They found that salinity stress increased the callus sodium, manganese and magnesium contents while potassium, calcium, and iron contents decreased. They concluded that reduction in callus relative growth rate was found to be inversely correlated with decrease in K^+, Ca^{2+}, and Fe^{2+} uptake and directly correlated with increased Na^+ and Mg^{2+} concentration in callus tissue.

Calcium ion (µmoles g^{-1} dry wt.): Ca content in the tissues (187.36 µmoles g^{-1} dry wt.), (190.53 µmoles g^{-1} dry wt.) and (195.06 µmoles g^{-1} dry wt.) were almost similar at concentrations of 0.1mM, 1mM and 3mM $CaCl_2$ respectively and increased significantly on increasing concentration of calcium chloride from 3mM to 9mM (Table 1). At 9mM calcium content in the tissues was maximum (450.83 µmoles g^{-1} dry wt.) followed by 301.3 µmoles g^{-1} dry wt. at 6mM and (195.06 µmoles g^{-1} dry wt.) at 3mM. Least calcium content (187.36 µmoles g^{-1} dry wt.) was observed at 0.1mM concentration of calcium chloride (Table 1 & Fig. 2).

The present research work shows that ionic concentration of calcium in the tissues of calli significantly increased from 187.36 to 450.83 with increasing calcium chloride level in the growth medium from 0.1mM to 9mM but the increase in Ca^{++} content was more pronounced at 6mM and 9mM. This indicates a direct link between Calcium chloride concentration in the medium and endogenous Ca^{++} content of the calli. These results are in agreement with the findings of Mc Guire (1984) and Arvin *et al*., (2005), who reported that increase in the concentration of calcium in nutrient solution supplied to the 2 cultivars of potato resulted in the tubers with increased concentration of calcium. Likewise, Lardet *et al*., (2007) showed that decrease in calcium concentration of the pre cultured medium led to a drop in callus calcium content in *Hevea brasilinsis*.

In the present piece of work it was found that elevation of calcium accumulation in the tissues resulted in increased growth rate of calli cultures of potato. Current study results are similar to those of Jackson *et al*., (1988) that callus growth is directly proportional to the Calcium concentration in the plant tissue. Similar results were found by Sarkar *et al*., (2004) who worked on the effect of different calcium levels on potato grown *In vitro* and found that calcium in excess of standard level (3.0mM) resulted in significant increase in calcium content in micro plants and improved calli growth by elevating Calcium chloride up to 6mM to the growth medium.

Low calcium content in the tissue (187.36 µmoles g^{-1} dry) at 0.1mM resulted in less growth of calli that might be due to the increase leakage of low molecular weight solutes from the cells of calcium deficient tissues while higher calcium accumulation (301.314 µmoles g^{-1} dry) at 6mM resulted in improved callus growth. For growth, cell wall loosening is required. Cell wall loosened when Ca^{++} concentration in cell wall is low. The Ca^{++} of the cell wall is released by action of auxins. Auxin present in the growth medium induces acidification of apoplasm and replaces the Calcium from the cross link of pectic chains, also activates calcium channels in the plasma membrane and leads to a transient increase in cytosolic free Ca^{++} concentration. Brummell & Maclachlan (1989) reported that this cytosolic free Ca^{++} stimulates the synthesis of cell wall precursors in the cytosol and its secretion into the apoplasm.

Potassium ion (µmoles g^{-1} dry wt.): Table 1 indicates that K^+ increased with increasing Ca in the medium and maximum K^+ content in the tissues was 695.5 µmoles g^{-1} dry wt. at 3mM $CaCl_2$ concentration and decreases to (639.2 µmoles g^{-1} dry wt.) that was followed by 507.2 µmoles g^{-1} dry wt. on increasing Calcium chloride level to 6mM and then 9mM respectively. K^+ accumulation in tissues i.e. 485.9 µmoles g^{-1} dry wt. and 495.3 µmoles g^{-1} dry wt., were almost similar at 0.1mM and 1mM respectively (Table 1 & Fig. 3). Over all effect of various levels of $CaCl_2$ on K^+ accumulation was significant.

Findings reveal that K^+ ion accumulation in the tissues increased and reached to its maximum value (695.56 µmoles g^{-1} dry wt.) with increasing calcium chloride concentration to the growth medium from 0.1mM to 3mM. This might be due to the calcium role in controlling membrane structure, function and permeability. Calcium between 0.1-6mM is considered to be necessary to maintain the integrity and selective ion transport of the plasma membrane. Our findings are similar to those of Hanson (1960) using roots of maize and Soya bean and showed that low extraneous calcium caused a marked decline in the ability of these tissues to absorb and retain solutes. Epstein (1961) also reported that calcium (0.1 to 1mM), but not magnesium, promoted the uptake of potassium and thus calcium ion imparts selectivity to the ion transport process. Lopez-Lefebre *et al*., (2001) found in tobacco that Ca had positive effect on N, Zn, Mn and B accumulation but not on K. On the other hand, Aranda-Peres *et al.,* (2009) reported that bromeliads exhibited significantly higher fresh and dry weight on cultural medium having more Ca concentration. They noted that leaf N, K, Zn, Mg and B increased as the Ca in the medium increased from 1.5 to 12mM.

Increase in the internal concentration of K ion in the presence of Calcium chloride parallels to the increase in growth capacity. Our research results are in agreement to the findings of Gozeal bin Hayyem (1987) who reported that internal concentration of K is a major factor that changes in the presence of calcium chloride and play a key role in determining growth capacity. Our results could also be related to the presence of K ions and other organic and inorganic anions that are the main solutes required in vacuole to promote cell expansion.

Larger reduction in internal potassium was caused at high level (9mM) of Calcium chloride in the medium and this was reflected in high degree of growth reduction. The reduction in internal K ion concentration could be related to either an interference with its uptake or its efflux from the cells. Marschner (1995) associated such reduction in growth not only to the toxicity of a given excess supply of nutrient but also to the deficiency of other nutrients causing an ionic unbalance among nutrients.

Water content (%): Maximum water content (94.872 %) was recorded at 6mM Calcium chloride followed by 87.965% at 3mM while minimum water content (62.611%) was observed at 0.1mM (Table 1 & Fig. 4).

According to the data for water content it can be speculated that up to 6mM Calcium chloride in the external medium loosens the cell wall material, resultantly there is a large cell volume for which more water is required to maintain turgidity of the cells while above this level (6mM), calcium may adversely affect loosening of cell wall and decrease expansion capacity ultimately cell volume reduced and less water accumulation in the cell occurred. Two thresh holds 0.1mM and 6mM below and above which cell wall adversely affect. Furthermore, the integrity of the plasma membrane is maintained by the presence of extra Ca leading to greater turgor pressure (higher water content) and nutrient retention in cells which produce greater growth potential in plants as reported by Lopez-Lefebre *et al*., 2001.

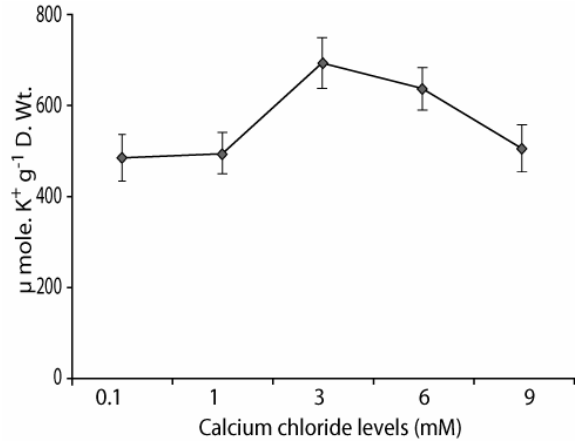

Fig. 3. The effect of various concentration of Calcium chloride on K content of dried calli cultures of *Solanum tuberosum* cv cardinal. The data presented in the graph are means of eight replicates ± SE.

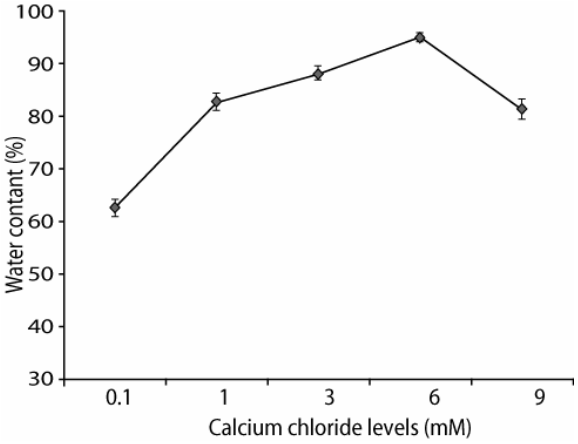

Fig. 4. The effect of various concentration of Calcium chloride on water content of calli cultures of *Solanum tuberosum* cv cardinal. The data presented in the graph are means of eight replicates ± SE.

Conclusion and recommendations: On the basis of results obtained, it is concluded that Calcium chloride treatment had significant effect on calli cultures of potato (Cv. Cardinal). Calcium chloride concentration from 3-6mM in the external medium enhanced K^+ uptake and beyond this, Calcium chloride adversely affected K^+ accumulation in tissue and ultimately callus growth was reduced. Hence, Calcium chloride concentrations from 3-6mM in MS medium are recommended to obtain maximum callus growth/biomass production. In addition, there is research need to explore more beneficial aspects of Calcium chloride treatments for micro propagation.

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