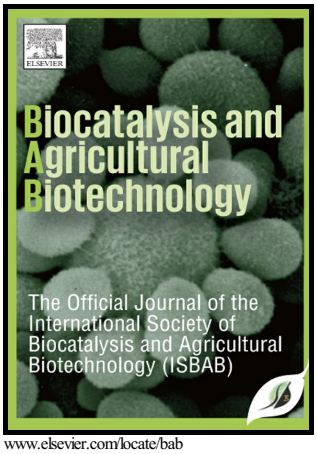

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Molecular and biochemical analysis of supplementation of calcium under *invitro* condition on tuberization in potato (*Solanum tuberosum* L.)

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Abstract

Calcium plays an important role in plant physiology and various plant cell signaling pathways and alters several biochemical processes by activating specific enzymes. Cytosolic calcium (Ca^{2+}) regulates the activity of Ca^{2+} dependent protein kinases, which phosphorylate various key metabolic enzymes. In this study, expression of the calcium (Ca^{2+}) dependent proteins, calmodulin (CaM1) and Calcium dependent Protein Kinase (StCDPK), as well as the other important tuberizing pathway enzyme, the lipoxygenase (LOX; EC 1.13.11.12) were studied upon Ca^{2+} application to the single node segments of potato used for tuberization studies. Calcium at higher levels (9 mM) significantly improved the tuber number, growth and tuber yield under *in vitro* conditions. The expression level of the CaM1 and StCDPK were significantly higher in stolons and initial tubers showing positive correlation with supplemental Ca^{2+} and tuberization response. Similar trends were also observed with LOX enzyme activity, which increased by 18% and 25% with the supplementation of Ca^{2+} at 6 and 9 mM, respectively to the tuber induction medium, when compared to explants

tuberizing on the medium without the Ca^{2+} control. Here we report the increase in tuberization, tuber growth and tuber yield with the supplementation of Ca^{2+} in tuberization induction medium could be attributed to the increased expression of the Ca^{2+} dependent proteins and enhanced lipoxygenase activity.

Keywords: Calmodulin; Lipoxygenase; *Solanum tuberosum* L; StCDPK; Tuberization

1. Introduction

A series of morphological and biochemical processes takes place at stolon tip during the tuber development in potato. These processes are synchronized by differential gene expression in stolon tips (Hannapel et al., 1991; MacLeod et al., 1999; Kloosterman et al., 2005; Asun et al., 2007). Numerous genes known to be entirely expressed at the time of tuberization have been recognized, which include *S-adenosylmethionine decarboxylase* (Taylor et al.,1991), *tubulins* (Taylor et al., 1991), *MADS box* genes (Kang. et al., 1996), *acyl* carrier protein *thioesterase* (Macleod et al., 1999), and *lipoxygenases* (LOXs) (Kolomiets et al., 2001; Kolomiets et al., 1996; Royo et al.,1996). It has been clearly established that CDPK is ubiquitous in plants which contain a protein kinase catalytic domain and a Ca^{2+}- requisite regulatory domain analogous to the calmodulin (CaM) (Lee at al. 1995; Kim et al. 2009). These kinases and CaM are reported to play an important role in conversion of stolen to tuber. The CaM antagonists were found to inhibit the expression of CDPKs and tuberization process (Harper et al., 1991; Jackson 1999; Cheong et al., 2003) which proves their role in potato tuberization. Earlier assumption and experiments have also suggested the involvement of CDPK in the events leading to tuber formation (Balamani et al., 1986; Macintosh et al., 1996). Potato *StCDPK1* encoding a calcium-dependent protein kinase (CDPK) was transiently induced upon tuberization in swelling stolons (Raices et al., 2001) and *StCDPK1* mRNA is localized in the apical

dome of tuberizing stolon tips (Raices et al., 2003, Gargantini et al., 2009).

Plant lipoxygenases (EC 1.13.11.12) belongs to class dioxygenases and involved in the oxygenation of polyunsaturated fatty acids, linolenic and linoleic acids, to produce 13- and 9-monohydroperoxides, respectively. The 13-monohydroperoxides are precursors of biologically active compounds like traumatin, jasmonic acid (JA), and methyl jasmonate, which act as hormone like regulatory and defense-related roles in plants (Siedow et al.,1991; Sarkar 2008). The LOX-derived metabolites such as JA, methyl jasmonate, and tuberonic acid (TA) include hormone-like properties and show strong tuber inducing activity under *in vitro* conditions. So far, LOXs are the only proteins that are reported to have direct role in tuberization of potato.

Calcium (Ca^{2+}) is reported to impart a major role in physiology and many plant cell signaling pathways including a transitory increase in cytosolic Ca^{2+} levels (Koda et al., 1992, Poovaiah et al., 1995; Raices et al., 2001, 2003; Reddy et al., 2004; Du and Poovaiah 2005). Calcium (Ca2+) signaling, which can be activated within seconds or minutes in response to quite diverse sets of stimuli (Harper et al. 2005). Increase in free cytosolic Ca^{2+} concentration is one of the initial events in the transduction of signals which may change various biochemical processes in plants by altering the activity of particular enzyme (Balamani et al., 1986; Upadhyaya et al., 2013). However, several reports have suggest that the tuberization is inhibited under invitro conditions by application of Ca^{2+} chelator such as EGTA or Ca^{2+} ionophores such as A 23287. The tuberization was again restored by addition of $CaCl_2$ to the medium. Experiments by Poovaiah et al. (1996) suggested that Ca^{2+} and CaM is involved in the tuberization process though gibberellic acid (GA) metabolism. Also, studies on barley endosperm during seed germination indicated an evidence for the modulation of GA by Ca/calmodulin pathway (Bush et al., 1996).

A few other studies demonstrated the influence of supplemental Ca2+ on tuberization of potato under field conditions (Ozgen et al., 2000, 2003, 2006; Ozgen and Palta, 2004; Chang et al., 2007) though the exact molecular mechanism of its influence was not studied. Present study was undertaken to investigate the biochemical and molecular mechanism underlying tuberization in potato influenced by Ca^{2+} supplementation under *in vitro* conditions.

2. Materials and Methods

2.1. In vitro tuberization with supplemented Ca^{2+} and/or Calcium Chelators/Antagonists

In vitro tubers were initiated from single node segments collected from 5 week old *in vitro grown* shoots of potato (*Solanum tuberosum* L. cv. Kufari Jyoti) regenerated on MS (Murashige & Skoog, 1962) medium containing sucrose (3%, w/v). Single node segments were cultured in plant culture dishes (120 mm Dia x 120 mm Ht) containing 50 ml of tuber induction medium (MS without Ca^{2+}) + sucrose (7%, w/v) and 0.8 % phyto agar supplemented with Ca^{2+} in the form of $CaCl_2$ (3 – 12 mM). Cultures were incubated at 19±2°C temperature and 60% relative humidity under continuous dark. Single node were also inoculated on tuber-inducing medium [MS+sucrose (7 %, w/v) and 0.8 % phyto agar] supplemented with $CaCl_2$ and the Ca^{2+} chelator, 1-2-bis(o-aminophenoxy) ethane-N,N,N,N′-tetraacetic acid (BAPTA; Sigma-Aldrich, St. Louis, MO, USA; dissolved in water) or $LaCl_3$ (inhibitor of Ca^{+2} import across membrane). MS medium devoid of Ca2+ served as control. Observations on tuber induction, growth, and yield were taken after 30 days culture. Each treatment contained 50 explants, and the experiment was repeated thrice with a total of 150 explants.

The treatment contained a total of 150 explants and the experiment was repeated three times and the samples were collected separately till 30 days depending

on the sample type. Stolons develop first hence collected first and so on. Observations on tuber initiation, tuber growth and tuber yield were taken till 30 days after initial culture.

2.2. Expression of CaM1, StCDPK and LOX genes

2.2.1. RNA isolation

Total RNA from stolons and tuber tissues treated with different Ca^{2+} treatments at four developmental stages of tuberization (Ulloa et al, 1997) was isolated using Trizol reagent (Invitrogen, USA) following the manufacturer's instructions.

2.2.2. Expression Analysis of Calcium Signaling Pathway Genes by qPCR

First strand cDNA was synthesized using SuperScript First Strand Synthesis Kit (Invitrogen, USA) following manufacture's protocol. About 5 µg of total RNA was taken in a nuclease free microcentrifuge tube and 1 µl dNTP mix and 1 µl Oligo-dT primer were added. Volume of the mixture was made up to 10 µl with sterile DEPC-treated water and the mixture was incubated at 65˚C for 5 min. After cooling the mixture to RT, 2 µl of 10X SuperScript™ Reverse Transcriptase buffer (Invitrogen, USA), 40U RNase inhibitor, 1 µl of 0.1 M DTT, 2 µl of 25 mM $MgCl_2$ and 50U of SuperScript™ Reverse Transcriptase enzyme. The mixture was gently mixed and incubated at 42˚C for 50 min.

. Real-time quantitative PCR (qPCR) was performed using a real-time PCR detection system (Applied Biosystems, USA). For the amplification of the RT-PCR products, SYBR Green Supermix (Applied Biosystems, USA) was used according to the manufacturer's protocol in a final volume of 25.0 µl. The real-time PCR was programmed to 95 °C 2 min, 40 cycles (95 °C 30 s, 55 °C 40 s, 72 °C 45 s), 72 °C 10 min, followed by a melting curve program 55 to 95 °C in increasing steps of 1.0 °C. All reactions were performed in triplicate. The mRNA levels for each cDNA probe

were normalized with respect to the actin mRNA level. The following primer pairs, with product sizes between 175 and 200 bp were used: CaM1 (FJ943935; F: 5′-GGATGGCGATGGCTGTATTA-3′and R: GTTCCTCCTCAGAATCAGTGTC-3′), StCDPK (AF115406; F: 5'-ACCAGGCAAGCTCTTCTAATAC-3' and R:5'-GTTTCGACCGGAGAACTTCA-3′), and LOX (X79107; F: 5′-CATGGAGGACTGATGAAGAA-3′ and R: 5′-CCGTTAGTCCATCCAGCTTATC-3′).

RT-PCR was also done with a similar set of primers. The cDNA (1 µl) thus synthesized from stolons and tuber tissues from different Ca^{2+} treatments were taken as template for PCR. RT-PCR reactions were performed in a thermal cycler (BioRad, USA) in a 25 µl reaction mixture containing 0.3 U of *Taq* DNA polymerase (Invitrogen, USA) in 1X buffer, 50 ng of template DNA, 400 µM of each dNTPs (Invitrogen, USA) and 10 pmol of each primer. PCR was performed using the following program: Initial denaturation at 94^{o}C for 3 min, followed by 35 cycles of denaturation at 94^{o}C for 30 s, annealing at 45, 52 and 57^{o}C, respectively for *CaM1*, *StCDPK* and *LOX* for 45 s and extension at 72^{o}C for 1 min with a final extension at 72^{o}C for 5 min. Actin mRNA served as loading control. Reaction products were separated on 1% agarose gel stained with ethidium bromide and visualized under UV light.

2.3. Estimation of LOX enzyme activity

Lipoxygenase activity from stolon and tubers was estimated using the method described earlier Gökmen et al, 2002. The stolons and tubers collected from *in vitro* cultures of potato inoculated for tuberization was used for enzyme extraction. Enzyme extraction was carried out by homogenizing 100 mg of frozen tissue with 500 µl of water at 4°C with a mortar and pestle. The slurry was filtered through three layers of

cheesecloth and centrifuged at 15000 g for 30 min. The supernatant was used as the crude enzyme extract. The protein determinations were carried out using the dye-binding method of Bradford (1976). A standard curve was made using BSA in the concentration range 1 - 50 μg/ml, in which a linear response was observed. The substrate solution was prepared by mixing 157.2 μl of pure linoleic acid (L1268, Sigma, USA), 157.2 μl of Tween-20 and 10 ml of deionized water. The solution was clarified by adding 1 ml of 1 N NaOH and diluted to 200 ml with 0.1 M potassium phosphate buffer (pH 6.0), giving a 2.5 mM final concentration of linoleic acid.

Substrate solution (29 ml) was transferred into a 100 ml flask placed in a temperature controlled water bath set at 30°C. The substrate solution was aerated by a gentle stream of air for 2 min and the reaction was started by adding 1.0 ml of crude enzyme extract into the flask. The aliquots of 1.0 ml from the reaction medium were transferred into glass tubes containing 4.0 ml of 0.1 N NaOH at time intervals of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 and 5.0 min. The use of 0.1 N NaOH stops the enzymatic reaction and ensures the optical clarity by the formation of the Na-salt of unreacted linoleic acid. Blank solution consisted of 1.0 ml of substrate solution and 4 ml of 0.1 N NaOH. One unit of LOX activity was defined as an increase in absorbance of 0.001 at 234 nm min^{-1} mg^{-1} of protein under assay conditions. A double beam spectrophotometer with 1.0 cm path-length cuvette was used.

3. Results

3.1. In vitro tuberization with supplemented Ca^{2+}

Supplementation of Ca^{+2} in tuber initiation medium considerably enhanced the tuberization efficiency in terms of tuber growth, number and yield under *in vitro* conditions as compared to the control devoid of supplemented Ca^{+2}. Tuber induction was observed on the nodal segment explants, in the tuberization medium

supplemented with Ca^{+2} within 4-5 days of culture. The *in vitro* tuber number, growth and tuber yield showed an increasing trend with an increase in the concentration of Ca^{2+} in the medium, up to 9 mM. Overall yield (127.2 g), tuber number and average tuber weight (0.82 g) were maximum at 9 mM of Ca^{2+} (Table 1). The increase in tuber yield was around 300% from explants cultured on MS medium supplemented with Ca^{2+} (9 mM) as compared to the control (Fig 1.0). Further increase in Ca^{2+} concentrations in tuber induction medium showed a reduction in tuber growth as well as yield. In our experiment, single nodal segments were also treated with the Ca^{2+} chelator BAPTA (5 and 10 mM), or the Ca2+ antagonist $LaCl_3$ (5 and 10 mM), prior to the application of $CaCl_2$ in tuber induction medium. It was observed that $LaCl_3$ and BAPTA inhibited tuberization in terms of delayed tuber induction and poor growth (data not shown).

3.2. Expression analysis of calcium signaling pathway

Expression of tuberization specific gene (LOX) and two Ca^{2+} sensors/binding proteins, such as calmodulin (CaM1) and calcium-dependent protein kinase (StCDPK), were analyzed in relation to supplementation of Ca^{+2} in tuberization medium. Real-time PCR results indicated that the expression of Ca^{2+}-dependent genes, CaM1 and StCDPK1, and also LOX were comparatively higher in stoloniferous shoots grown on tuber induction medium-supplemented Ca^{2+} ($CaCl_2$ at 9 mM) (Fig. 2 A), however**,** their expression showed decreasing pattern in initial (Fig. 2 B) and developing tubers (fig 2 D). Increase in number of tubers and tuber growth is corroborated with enhanced expression of Ca^{2+}-dependent proteins in stoloniferous shoots ready for the tuberization.

These results were further established by analyzing the transcript expression levels of these genes by RT-PCR where expression levels of CaM1, StCDPK1, and LOX in stoloniferous shoot tips were compared with application of Ca^{+2} and control. The

transcript expression level of these genes was comparatively higher in stoloniferous shoots on the tuberization medium supplemented with Ca^{+2} than the control single node explant tuberizing on the medium without Ca^{+2}. Further, their expression level absolutely correlated with varying concentrations of Ca^{2+} in the tuber induction medium (Fig 3 A, B & C).

In order to test the role of CaM1 and StCDPK1 overexpression in response to $CaCl_2$ application is dependent on Ca^{2+}, the single node segments were treated with the Ca^{2+} chelator BAPTA (5 and 10 mM), or the Ca^{2+} antagonist $LaCl_3$ (5 and 10 mM), before application of the $CaCl_2$. It was observed that $LaCl_3$ and BAPTA reduced the expression of CaM1 or StCDPK1 which was also clear in semiquantitative RT-PCR (Fig. 2 D) and RT-PCR analysis (Fig 3 D) in the stoloniferous shoots and, subsequently, in initial tuber tissues. It was also observed that BAPTA or LaCl3 application to single node segments drastically reduced the tuberization.

3.3. Enzyme activity of LOX in tuber tissues

LOX enzyme assay also showed enhanced activity under the varying concentrations of Ca^{2+}. The enzyme activity was estimated by spectrophotometric monitoring of the formation of hydroperoxides as the increase in absorbance at 234 nm due to the presence of a conjugated hydroperoxydiene moiety. LOX activity increased with the addition of Ca^{2+} in tuber induction medium and its activity further increased with the increase in the concentration of Ca^{2+}. The enzyme activity was maximum ($66.1x10^3$ units mg^{-1} of protein min^{-1}) in stolon tips formed from single node explants cultured on medium with Ca^{2+} at 9 mM (Fig 4 A). An increase in the LOX activity in stolon tips was 18% and 25% with the supplementation of Ca^{2+} at 6 and 9 mM, respectively to the tuber induction medium as compared to control tuberizing on Ca^{2+} free medium. In addition, the LOX activity showed a decreasing trend with tuber maturity. Matured tubers showed lower LOX activity at all the

concentration of Ca^{2+} applied in the experiment.

4. Discussion

In this study it was observed that addition of Ca^{2+} in tuber induction medium significantly improved the period of tuberization induction, tuber number, growth and tuber yield with an optimum concentration being at 9 mM. The period of tuber induction reduced to 4-5 days from 8-10 days in control. Reduction in tuberization induction period and boost in potato yield was found to be associated with increased expression of the Ca^{2+} dependent genes and *LOX* mRNA transcript. Our results indicated constructive role of these genes and proteins in enhancing potato tuber induction, tuber development and yield. Increased tuber size and tuber number contributed to higher *in vitro* potato yield. Our findings are in conformity with earlier experiments with potato, which showed an increased tuber size and yield by application of Ca^{2+} to the potato fields [Chang et al 2006]. However, these studies could not provide the molecular mechanism for increased tuber yield with Ca^{2+} application. Earlier, Balamani et al. (1986) took single node cutting of potato (*Solanum tuberosum* L.) for *invitro* tuberization studies with the application of $Ca^{2+}$and found that Ca^{2+} act as moderator of the tuberization signals. A temporary increase in calmodulin mRNA expression level in stolon tips was also reported by Jena et al (1989) during tuberization studies in potato. We used single node segment to explore the possible molecular machinery important in increasing tuber growth and yield in potato mediated by cytosolic Ca^{2+} in *in vitro* conditions. Free cytosolic Ca^{2+} in plant cells can be increased by several external sources including Ca^{2+} addition in growth medium. This increase in concentration of free Ca^{2+} is primary event in transduction of several signals which ultimately alter biochemical events in plants via changing the activity of particular enzyme. In earlier studies, a correlation was found among the Ca^{2+}-dependent phosphorylation of CaM and CDPK proteins, CDPK

activity, and the morphogenetic events which take place during tuberization (Takezawa et al., 1995; Macintosh et al., 1996). In this study, we observed a strong positive correlation between the expression levels of the two Ca^{2+} dependent proteins (*CaM1* and *StCDPK*) and LOX activity with *in vitro* tuberization response. A change was observed in the gene expression in stolon tips and initial tubers in the tuberization process (Hannapel1991; Taylor et al. 1992).

It was also observed that the application of calcium chelators BAPTA and calcium antagonists $LaCl_3$ decrease the expression of Ca^{2+} signaling pathway genes and also reduced and delayed the *in vitro* tuberization and growth promotion. This proved that calcium signaling pathway is dominant in the tuberization and further growth (Upadhyaya et al, 2013). It is established fact that the Ca^{2+} is a second messenger in several plant signaling pathways, adding extracellular stimuli to intracellular and whole-plant responses (Ozgen et al, 2005). Concentration of cellular Ca^{2+} is tightly regulated, and a little variation in its concentration changes the signaling and protein activation. Ca^{2+} cannot be synthesized or degraded, thus its level at a certain time and location depends on the balance between entry and efflux processes. In eukaryotic cells, numerous stimuli change the pool of Ca^{2+} to activate characteristic changes in the cytoplasmic Ca^{2+} (Pelacho et al., 1991).

Potato tubers originate from stolons that are lateral shoots borne, generally, at the basal nodes of the plant. There is an enlargement of pre-existing pith cells from sub-apical portion of the stolon, which is a specialized shoot. During the tuberization process, soluble CDPK activity is expressed highly in stolons at the time of the morphogenesis which confirm a possible role of the protein kinase. It was also suggested that a calcium-dependent protein kinase activity (CDPK) could be involved in the events leading to tuber formation (Macintosh et al. 1996; Gargantini et al., 2009; Nookaraju et al., 2012) and Potato *StCDPK1*, an active CDPK was found to be

differentially expressed in swelling stolon's (Raices et al., 2001). Another study suggested that both phosphatase and kinase activities induced at the onset of tuberization (Macintosh et al. 1996; Ulloa et al. 1997; Batistic and Kudla, 2004). These studies confirmed that StCDPK is an important mediator in the signal transduction pathways triggered during tuber development. Increased tuber growth and yield with the supplementation of Ca^{2+} in the present study could be attributed to the increased phosphorylation of tuberization specific enzyme by enhanced expression of the Ca^{2+} dependent genes, the *CaM1* and *StCDPK*. Recently, Gargantini et al. (2009) reported higher expression of *StCDPK1* in swelling stolons which played a key role in GA signaling during tuberization. It was also suggested that *StCDPK* may act as a stimulus-response coupler in calcium-regulated processes during tuberization in potato. Changes in concentrations of cytoplasmic Ca^{2+} could be major factor in the control of CDPK activity. The activity of *NtCDPK* was reported to be increased by the application of Ca^{2+} in case of tobacco (Yoshihara et al.,1996).

The enzyme activity of LOX is also correlated with its increased transcript expression as a function applied Ca^{2+} to the tuber initiation medium. Increased tuber yield and number in this study could be credited to the enhanced synthesis of LOX derived metabolites which played a central role in tuberization (Koda 1992; Yoshihara et al. 1996; Nam et al. 2008). However, enhancement in the activity of LOX might be partially due to increased phosphorylation by *StCDPK* or transcriptional activation of LOX by *CaM1* (Kolomiets et al., 2001; Nam et al., 2005). Yoon et al. (1999) also reported that the CDPK is also involved in the biosynthesis of methyl jasmonate (MJ) in alfalfa which is a LOX derived metabolite reported to play a major role in potato tuberization (Koda Y 1992; Yoshihara et al 1996). Thus, it is possible that Ca^{2+} and Ca^{2+} dependent proteins might play an important role in signal transduction pathway triggering tuber formation and further development. Further,

immune-histological and *in situ* phosphorylation studies are necessary to correlate the metabolic state with early molecular events in tuber formation as a function of Ca^{2+} supplementation.

In conclusion, our results showed increase in expression of the CaM1 and StCDPK in stolons, initial tubers showed positive correlation with supplemental Ca^{2+} as well as in tuberization response; hence, the CaM1 and StCDPK may be an important components of the cell signaling cascades triggered during stolon initiation, stolon to tuber transition and tuber development. *StCDPK* are also involved in GA-signaling and there by controlling potato tuberization. Supplemental Ca^{2+} (at 9mM concentrations) regulates the expression of LOX which finally regulates the tuberization process in potato.

Conflict of interest

The authors have declared no conflict of interest.

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Legends to figures

Fig. 1 Influence of external application of $CaCl_2$ on in vitro tuberization in Potato cv. Kufari jyoti nodal segments used for in vitro tuberization studies. Early tuber formations through the subapical swelling of the nodal region in the treated explants were observed. T= treated, C= control

Fig.2. Quantitative real time PCR analysis of CaM1, StCDPK and LOX gene expression profiling during *in vitro* tuberization of potato with application of $CaCl_2$. The cDNA was normalized in dependence of the transcript level of actin mRNA. S0- single node segment explants, C0- no Ca^{2+}, C1- Ca^{2+}at 6 mM, C2- Ca^{2+}at 9 mM. Different letters in each column indicate significant differences ($p \leq 0.05$) in relative gene expression between treatments after Tukey's test (n=3)

The gene expression in (A) Stolon's, (B) Initial tubers, (C) developing tubers (D) reduced expression of the gene with Ca+ chelators/antagonists

Figure 3. Expression levels of *CaM1*, *StCDPK* and *LOX* genes in stolon and tuber tissues of potato during tuber development. (A) Stolons, (B) Initial tubers, (C) Developing tubers (D) expression level of the gene with Ca+ chelators/antagonists
S0 – single node segments, C0 – no Ca^{2+}, C1 - Ca^{2+} at 6 mM and C2 - Ca^{2+}at 9 mM; basal medium used: MS (without Ca2+) + sucrose (7%, w/v).

Fig. 4. Lipoxygenase activity in stolon and tuber tissues of potato during *in vitro* tuber development. Values are mean ± SE (n=3). Lox activity in (A) Stolons (B) Initial tubers (C) Developing tubers (D) mature tubers S0 – single node segments; C0 – no Ca^{2+}; C1 - Ca^{2+} at 6 mM; C2 - Ca^{2+} at 9 mM; basal medium: MS (without Ca^{2+}) + sucrose (7% w/v).

Table 1. Influence of Ca^{2+} on *in vitro* tuber yield in potato cv. Desiree

Ca^{2+} (mM)	No. of stolon inducing tubers	Days to tuber initiation	Av. tuber size (mm)	Av. tuber wt. (g)	*Av. Tuber yield (g)
0	52	12-14	2.30±0.01	0.32±0.06	21.6±0.54
3	114	8-9	5.41±0.04	0.70±0.03	87.2±0.16*
6	129	5-6	7.10±0.03	0.76 ±0.12	94.2±0.17*
9	141	4-5	6.85±0.05	0.82±0.04	127.4±0.31
12	98	8-9	3.42±0.02	0.65±0.09	64.2±0.46*

Basal medium: MS (without Ca^{2+}) + sucrose (7%, w/v)
Values are mean ± standard error of three repeated experiments. No. of explants inoculated per treatment: 50. Average tuber yield was calculated per treatment co ntaining 50 explants. Values represent mean ±SE and asterisks (*) indicate sign ificant differences ($p \leq 0.05$) in the values

Highlights

- Calcium at higher levels (9 mM) significantly improved the tuber number, growth and tuber yield under *in vitro* conditions.
- Increase in expression of the CaM1 and StCDPK in stolons and initial tubers

showed positive correlation with supplemental Ca^{2+} and tuberization response.

- Enhancement in tuberization, tuber growth and tuber yield with the supplementation of Ca^{2+} in tuberization induction medium is attributed to the increased expression of the Ca^{2+} dependent proteins and enhanced lipoxygenase activity.