# Effect of Calcium Deficiency on Growth and Leaf Acid Soluble Proteins of Tomato

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## ABSTRACT

The effects of temporary Ca (Ca) calcium deficiency lasting 2, 3, 4 or 5 d were investigated on tomato plants at the 6-leaf stage, grown hydroponically under controlled conditions. With 2, 3 or 4 d of Ca deficiency, the dry weight of the tomato leaves, shoots or roots was not different from control. A significant decrease in tomato growth, of up to 70%, appeared on the fifth day. Some visual symptoms were observed on the tomato leaves. The phenomenon concerned was an irreversible mechanism that led to plant death after 12 d, even when Ca was added to the root medium after 2, 3, 4 or 5 d. This is the first report of such a rapid and drastic effect of an essential macronutrient. Moreover, Ca content in leaves during root deprivation showed a decrease in all plants, related to a remobilization toward the apex. Ca could be considered as a partly mobile element: the observation of the youngest leaf limbs by transmission electronic microscopy after 4 d of treatment showed disorganized tissues in a necrotic zone, due to wall impairment related to C deficiency. During temporary Ca deficiency, acid soluble proteins were analyzed in leaves (SDS PAGE electrophoresis / Maldi-TOF). After 4 d of Ca deficiency, protein induction in young leaves was revealed. Three proteins were identified as pathogenesis related proteins (PR-1, PR-3, PR-7) and a threonine deaminase precursor was also found. It was also the first time that pathogenesis related (PR) protein appearance has been shown to be related to Ca deficiency. The PR proteins are generally elicited by pathogen attack. This phenomenon seems to be calcium dependent because other mineral stresses, such as potassium (K) deficiency or sodium (Na) excess, did not reveal acid soluble protein changes. The retranslocation of Ca to young tissue could entail eliciting effects via wall fragments leading to a plant response similar to the response to pathogen attack.

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Consequently, activation by Ca deficiency of gene transcription coding for defense proteins could be hypothesized.

**Keywords**: tomato, Ca deficiency, mineral nutrition, pathogenesis-related proteins

## INTRODUCTION

Blossom-end rot is a common, nonpathogenic, physiological disorder that occurs on tomato crops as a result of climatic or cultural problems, particularly in greenhouse or soilless culture. Necrosis of the distal part of the fruit, due to membrane cell alteration, is a visual symptom of this disease. Blossom-end rot is related to calcium (Ca**)** deficiency in the fruit resulting from insufficient mass flow through the plant (Taylor and Locascio**,** 2004). This Ca disturbance affects fruits of various species and is known as bitter pit in apple, tip burn in strawberry, and watercore in melon (Bernadac et al., 1996). These physiological disorders result in deficiency of Ca being available to the maturing fruit, when damages appear. The development of this pathology is specific to Ca nutrition and may be related to the physiological roles of this macronutrient, which mainly plays a role in the strengthening cell walls (Grant et al., 1973; Morris et al., 1982) and in membrane stabilization and integrity (Jones and Lunt, 1967**).** Moreover, cytosolic Ca contributes, as a second messager (Hepler and Wayne, 1985; Trewavas and Gilroy, 1991), to signal transduction pathways in plant responses to different stimuli, like the defense response (Fluhr et al., 1994; Lee and Rudd, 2002**).** These stimuli enhance the low concentration of cytosolic calcium, leading to a $Ca^{++}$/Calmodulin complex that interacts with target proteins to activate cell responses (Heo et al., 1999; Yang and Poovaiah, 2002; White and Broadley, 2003). During a pathogen attack, a class of proteins called "pathogenesis related (PR) proteins" appear in the infected plants (Gianinazzi et al., 1970; Van Loon and Van Kammen, 1970). These PR proteins have specific properties. They are selectively extractable at low pH and they are highly resistant to the proteolytic enzymes. These two properties allow their presence in intercellular spaces. They are classified in families according to their molecular weights; to date, 14 families have been identified (Van Loon and Van Strien, 1999). Some works have shown that these PR proteins can also be synthesized in plants under abiotic stresses like osmotic stress, ethylene, heavy metal and UV-B radiation (King et al., 1999; del Campillo and Lewis, 1992; Cruz-Ortega and Ownby, 1993; Jung et al., 1995).

Calcium is taken up by the root and translocated through the root via an apoplastic pathway. Calcium translocation is maximal in the root apical zone where endodermal cells are not suberized. In the root basal zone, endodermal cells possess Casparian bands impermeable to water and ions (White, 2001). So, the Ca channel present in the endodermal outer and inner membrane allows Ca to be loaded to the xylem sap. Calcium flux increases in daytime because water

absorption is higher (Kitanoet al., 1999). Calcium transport is driven by transpiration, root pressure and cation-exchange adsorption sites in xylem vessels (Kirkby and Pilbeam, 1984; Ho and Adams, 1989; Mengel and Kirkby, 2001). Calcium is not mobile, that is to say that this element is not remobilizable between organs inside the plant (Loneragan and Snowball, 1969). So, Ca is excluded from phloem sap (Epstein, 1973). Calcium ions accumulate in the older organs of plants and are not retranslocated to youngest organs (Wallace et al., 1966). The major consequence appears when root uptake of Ca is interrupted. For tomato plants grown in soilless culture, a temporary Ca deficiency over a period of 5 d will entail plant death. To date, no visual symptoms have been observed on the aerial part, but the resumption of Ca supply is not sufficient for plant growth (Morard et al., 1996).

The aim of this study was to determine whether the death of tomato plants after a temporary Ca deficiency could be predicted by changes in the acid soluble proteins in the tomato leaf. In this paper, the following points were studied and discussed: (i) influence of Ca deficiency on growth and Ca content of tomato plants, (ii) effects of Ca deficiency on profile of leaf soluble proteins, (iii) relationship between Ca deficiency and leaf soluble protein patterns.

## MATERIALS AND METHODS

### Plant Material and Growth

The experiment was conducted on tomato plants (*Lycopersicon esculentum* Mill. cv. *Rondello*) grown hydroponically in an aerated non-circulating solution under controlled conditions (temperature 24 ± 0.5 °C/18 ± 0.5 °C day/night cycles; photoperiod 16 h under daylight fluorescent lamps providing 400 µmol $m^{-2}$ $s^{-1}$ [Philips 600W, Eindhoven, Netherlands] and 10 h darkness; relative humidity 70%) in a phytotron. Tomato seeds were germinated on filter paper moistened with distilled water at 25 °C in the dark for seven d. The plants were grown for two weeks in a PVC tank containing 20 L of nutrient solution and then were grown individually in a PVC tank containing 15 L of nutrient solution. The nutrient solution (pH 5.2) contained 5 mmol/L ($KNO_3$), 0.25 mmol/L ($Ca(NO_3)_2$), 9.5 mmol/L ($NaNO_3$), 2 mmol/L ($KH_2PO_4$,), 1.5 mmol/L ($MgSO_4$). Micronutrient concentrations were 268.6 µmol/L (EDTA-Fe), 8.9 µmol/L ($MnSO_4$), 24.1 µmol/L ($H_3BO_3$), 1.7 µmol/L ($ZnSO_4$), 3.9 µmol/L ($CuSO_4$), 0.1 µmol/L ($Na_2MoO_4$). The nutrient medium was oxygenated by artificial aeration for 4 m every 15 m. The volume of the nutrient solution was made up daily with de-ionized water and was replaced once a week. The tomato plants were grown to the 8-leaf stage.

## Treatment and Samples of Plants

In order to induce calci Ca um deficiency, the Ca content of the nutrient solution was reduced from 0.25 mmol/L (maintained in the control) to 0 mmol/L. The treatment was applied when the tomato plants had developed 6 leaves. The nutrient solution with 0.25 mmol/L of Ca was noted (+Ca) and the deficient nutrient solution (-Ca). The Ca deficiency durations tested were 2 d (-Ca2), 3 d (-Ca3), 4 d (-Ca4) and 5 d (-Ca5). Each treatment was replicated 3 times. In each one, the plants subjected to the deficiency treatment were compared to control plants fed on the control nutrient solution.

Before the start of treatment, each plant apex was marked with a PVC ring to distinguish new leaves from these present previously. Plants were harvested at the 8–leaf stage. Each plant was separated into roots (R), stems (OS, YS), old leaves (OL), young leaves (YL), new leaves (NL) and apex (A). The roots were washed for one minute in deionized water.

After the 2nd, 3rd, 4th or 5th day of treatment, the Ca supply was restored and the plants were transferred to a complete nutrient solution for 12 d.

## Analysis of Calcium Contents

Sample plants were assayed every day after transfer to the deficient nutrient solution. After drying at 80°C for 3 d and grinding, the mineral elements were determined after calcination at 550 °C. Calcium content was measured using high pressure ion chromatography with the Dionex® DX-100 (Ion pac Cs12A, Dionex Co, Sunnyvale CA, USA). The data were submitted to analysis of variance (ANOVA) followed by the T-test with a 5 % threshold.

## Extraction and Analysis of Leaf Acid Soluble Proteins

All operations were carried out on ice or at 4 °C. The procedure was one adapted from that of Christ and Mösinger (1989) in preliminary trials (Baboulène, 2003). Liquid-nitrogen-frozen leaf tissues were powdered using a chilled mortar and pestle. The powder was homogenized in an extraction medium consisting of 5 % acetic acid and 1 % 2-mercaptoethanol (pH 2.8) at a ratio of 0.750 to 1 buffer to fresh weight (V/W). The homogenate was centrifuged at 13000g for 30 min at 4 °C. The supernatant was separated and adjusted to neutrality with potassium hydroxide (KOH) (5 N) and then was recentrifuged as before. The soluble protein content of the supernatant was measured by the method of Bradford (1976) with BSA as a standard. The proteins were separated by denaturing SDS (12 %) polyacrylamide gel electrophoresis as described by Laemmli (1970). Protein bands were stained with 0.1 % Coomassie brilliant blue R-250. Molecular weight markers ranging from 14.4 to 97.4kDa (BioRad) were electrophoresed to estimate the molecular masses of the various proteins.

### Protein Identification with Proteomic Approach

The protein bands, which showed a different expression between the deficient and the control plants, were analyzed using the Maldi-TOF mass spectrometry process (Hillenkamp et al 1991). A band protein aliquot was extracted directly from the electrophoresis gel with a micro pipette. The protein was digested by the trypsin. So, the peptidic fragments were analyzed by Maldi-TOF and the peptide mass map obtained experimentally was compared to the theoretical peptide mass maps present in the MS-Bridge data banks.

## RESULTS AND DISCUSSION

### Influence of Ca Deficiency on Growth and Ca Content in Tomato Plants

Calcium deficiency was studied over a period of five d. The effects of 2 d (-Ca2), 3 d (-Ca3), 4 d (-Ca4) and 5 d(-Ca5) of Ca deficiency were measured on tomato plant growth. The dry matter of tomato leaves, shoots or roots was not modified by a Ca deficiency lasting up to 4 d (Table 1). In contrast, a significant decrease, of as much as 70%, in growth was measured on the fifth day of root Ca deficiency and a significant increase of aerial part/root ratio was observed in Ca deficient plants (Table 1). This result is in agreement with Tewari et al. (2004).

The variation of calcium content in different organs depends on the variation of the amount of dry matter or the amount of Ca, or both. To observe

**Table 1**

Effects of two (-Ca2), three (-Ca3), four (-Ca4) and five (-Ca5) d of calcium deficiency on dry weight (g) of root (R), old and young stem (S = OS+YS), old, young, new leaf and apex (L=OL+YL+NL+A) and aerial part PA (S+L)/ root (R) ratio of (+Ca) and (-Ca) tomato plants. Control plants are noted +Ca2, +Ca3, +Ca4 and +Ca5.

| Treatment | R | S | L | PA/R | Total Mass |
|---|---|---|---|---|---|
| **(+Ca2)** | 0.615 ± 0.07 a | 1.073 ± 0.38 a | 2.439 ± 0.64 a | 5.65 ± 1.00 a | 4.126 ± 1.10 a |
| **(-Ca2)** | 0.389 ± 0.14 a | 0.773 ± 0.28 a | 1.903 ± 0.73 a | 6.96 ± 1.78 a | 3.065 ± 1.11 a |
| **(+Ca3)** | 0.578 ± 0.06 a | 1.009 ± 0.30 a | 2.776 ± 0.09 a | 6.57 ± 0.56 a | 4.364 ± 0.42 a |
| **(-Ca3)** | 0.546 ± 0.04 a | 1.280 ± 0.42 a | 3.511 ± 0.85 a | 8.69 ± 1.64 a | 5.337 ± 1.30 a |
| **(+Ca4)** | 0.613 ± 0.12 a | 1.262 ± 0.37 a | 3.665 ± 0.46 a | 8.07 ± 0.60 a | 5.540 ± 0.93 a |
| **(-Ca4)** | 0.525 ± 0.11 a | 1.247 ± 0.19 a | 3.370 ± 0.79 a | 8.86 ± 1.47 a | 5.142 ± 1.06 a |
| **(+Ca5)** | 1.094 ± 0.11 **a** | 2.056 ± 0.23 **a** | 5.174 ± 0.27 ns | 6.64 ± 0.64 **b** | 8.324 ± 0.43 **a** |
| **(-Ca5)** | 0.557 ± 0.08 **b** | 1.249 ± 0.16 **b** | 3.994 ± 0.69 ns | 9.49 ± 1.48 **a** | 5.800 ± 0.90 **b** |

Each value is the mean of three samples ± SE. Values followed by the same letter are not statically different at $P<0.05$.

the effect of Ca deficiency on biomass accumulation and Ca amount more easily, data were expressed in percentages of variation of dry matter and Ca amount in different organs compared to control tomato plants at the beginning of the experiment.

A positive difference indicated an increase in the amount of dry matter or Ca in the organ. A negative value meant a decrease in the amount of Ca in this organ related to a translocation of Ca to another physiological sink. After 2 or 4 d of Ca deficiency, dry matter was not significantly different between deprived and control plants, except for +Ca4 FN8 leaf (Figures 1A, 2A). However, the variation of Ca amount indicated a decrease of Ca accumulation in some organs (Figures 1B, 2B). This decrease, observed first in (-Ca2; -Ca4) tomato roots, could be related to a translocation of Ca from apoplasmic stores and perhaps from the root surface to xylem sap. This phenomenon was also observed in old stem (OS) that conducted sap from the collar to 1-4 d old leaves, and young stem (YS) conducting from young leaves (YL) to the apex (A). This hypothesis is supported by results obtained in the same experimental conditions with excised tomato roots (Morard et al., 2000). Calcium increased slowly in old leaves (OL1,2; OL3; OL4), and decreased only in young leaves (YL5, YL6). This phenomenon cannot be explained if Ca is considered as a non-mobile element. Leaves are physiological sinks where calcium is accumulated with tissue ageing (Mengel and Kirkby, 2001). The large decrease in Ca amount, especially in young leaves, indicated that, in our experimental conditions, Ca should be considered as a partly mobile element. These data are new findings in the calcium nutrition of plants. In spite of this phenomenon, after 4 d of Ca root deficiency, this macronutrient quickly became insufficient to maintain its physiological roles, particularly in the cell walls and cell membrane structure of young, new leaves and apex. Moreover, the Ca deficiency entailed a decrease in tomato development, corresponding to 1 leaf less than control (FN9+A vs FN8+A), which appeared after 4 d.

At the end of 2 or 4 d of treatment, the Ca supply was restored for 12 d (-Ca2 +Ca12) and (-Ca4 +Ca12). No visual symptoms were observed on young leaves with 2 or 4 d of Ca deficiency (Fig 3 B-D). Surprisingly, visual symptoms of calcium deficiency appeared 12 d later even when Ca was resupplied in the nutrient solution (Figure 3 H). These leaf effects were curling and margin deformations of the blade, leading to necrosis of the growing tips and youngest leaves. These visual symptoms were the latest manifestation of an inner and irreversible mechanism that led to plant death. Consequently, a temporary calcium deficiency over a period of 4 d was the threshold of lethal irreversibility on tomato plants at 8-leaf stage. This shortness was specific to calcium. For instance, a temporary potassium deficiency was not lethal over a period of 10 d (Baboulène et al. 2001).

Changes of calcium concentration were measured in the nutrient solution during the last experiment. The results, expressed as root absorption, could also

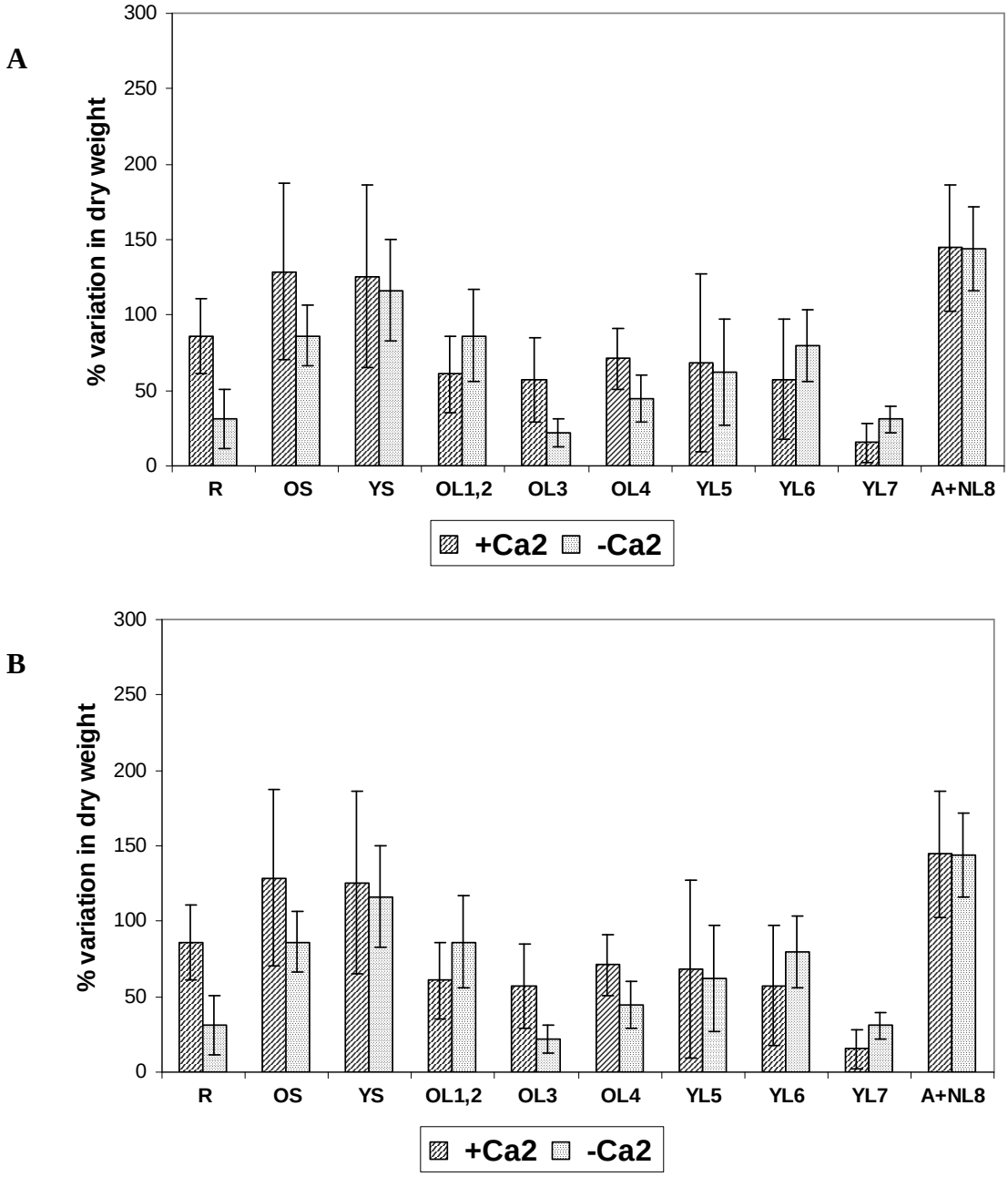


**Figure 1.** Variation (%) in dry weight (A) and Calcium amount (B) of different organs from tomato plants after two days of calcium deficiency compared to control tomato plant at the beginning of the experiment. Tomato plants deprived of calcium after 2 days are noted (-Ca2) and control plants are indexed (+Ca2). *R: root; OS: old stem; YS: young stem; OL: old leaf; YL: young leaf; NL: new leaf; A: apex.* Each value is the mean of three samples ± SE, (*: value statically different at $P<0.05$).

be considered as an indication of plant growth (Figure 4A). No calcium uptake was observed over a temporary Ca deficiency of 4 d (-Ca4 +Ca12, Figure 4B). This result was corroborated with the necrosis of root apices observed some d after the start of Ca deficiency (data not shown).

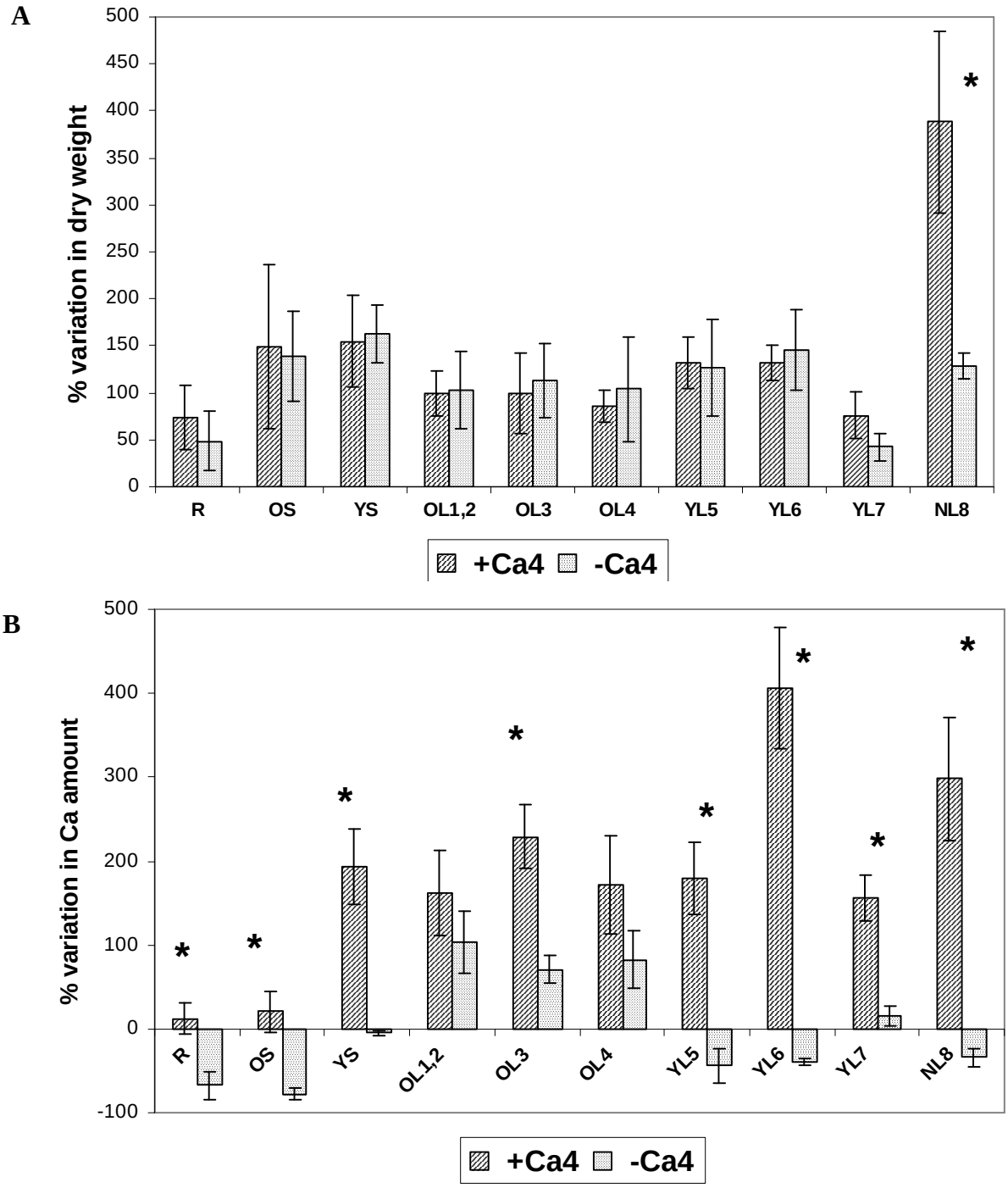


***Figure 2.*** Variation (%) in dry weight (A) and Calcium amount (B) of different organs from tomato plants after four days of calcium deficiency compared to control tomato plant at the beginning of the experiment. Tomato plants deprived of calcium after 4 days are noted (-Ca4) and control plants are indexed (+Ca4). *R: root; OS: old stem; YS: young stem; OL: old leaf; YL: young leaf; NL: new leaf; A: apex.* Each value is the mean of three samples ± SE, (*: value statically different at $P<0.05$).

### Effects of Calcium Deficiency on Pattern of Leaf Acid Soluble Proteins

There was no sign that the irreversible mechanism of tomato death over a period

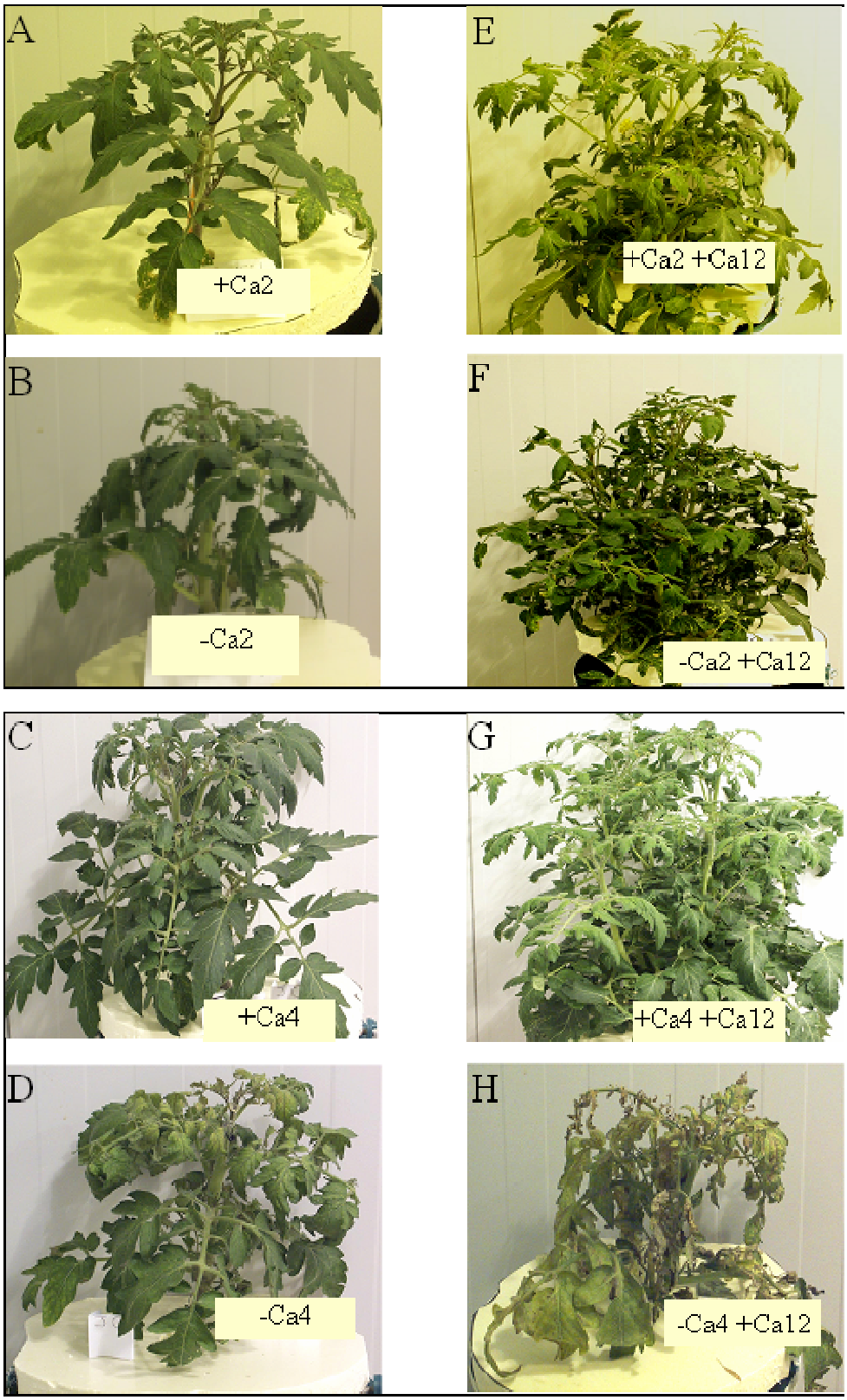


**Figure 3.** Photos of tomato plant after 2 (-Ca2) and 4 (-Ca4) days of calcium deficiency and after 12 (+Ca12) days of calcium resupply. Control plants are indexed +Ca2, +Ca4 and after 12 days of calcium resupply (+Ca2 +Ca12), (+Ca4 +Ca12).

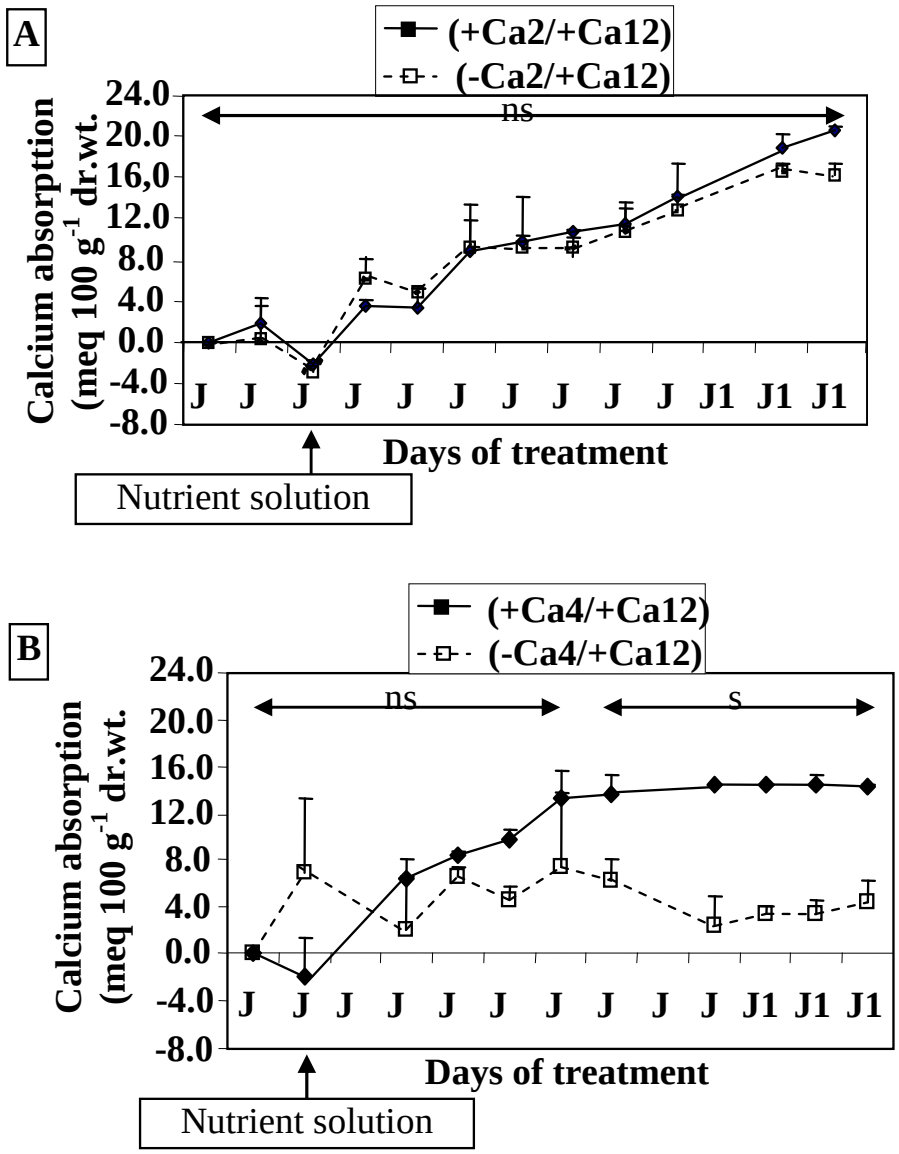


**Figure 4.** Influence of 12 d calcium resupply on absorption of calcium after 2 days (-Ca2 +Ca12) and 4 days (-Ca4 +Ca12) of calcium deficiency on tomato plants. Control plants are noted (+Ca2 +Ca12) and (+Ca4 +Ca12). Each value is the mean of two samples ± SE, ($P<0.05$).

of 4 d of Ca deficiency could be prevented. Acid soluble proteins were extracted from old, young and new leaves after 2, 3, 4 and 5 d of Ca deficiency. A significantly different pattern of SDS-PAGE gels was observed between deficient and control plants on d 4 d (Fig 5 A) and 5 d (Figure 5 B). Five soluble proteins appeared, highlighted by arrows and numbered (1) to (5). The signal intensity of protein bands 1, 3, 4 and 5 was higher in (-Ca4) and (-Ca5) deficient tomato plants than controls (Figure 5). These modifications of acid soluble protein patterns were detected on new, young and old leaves.

Band protein aliquots were extracted for identification. After trypsin digestion, peptidic fragments were analyzed by Maldi-TOF mass spectrometry. Each peptide map obtained was compared to the MS-Bridge data bank. These

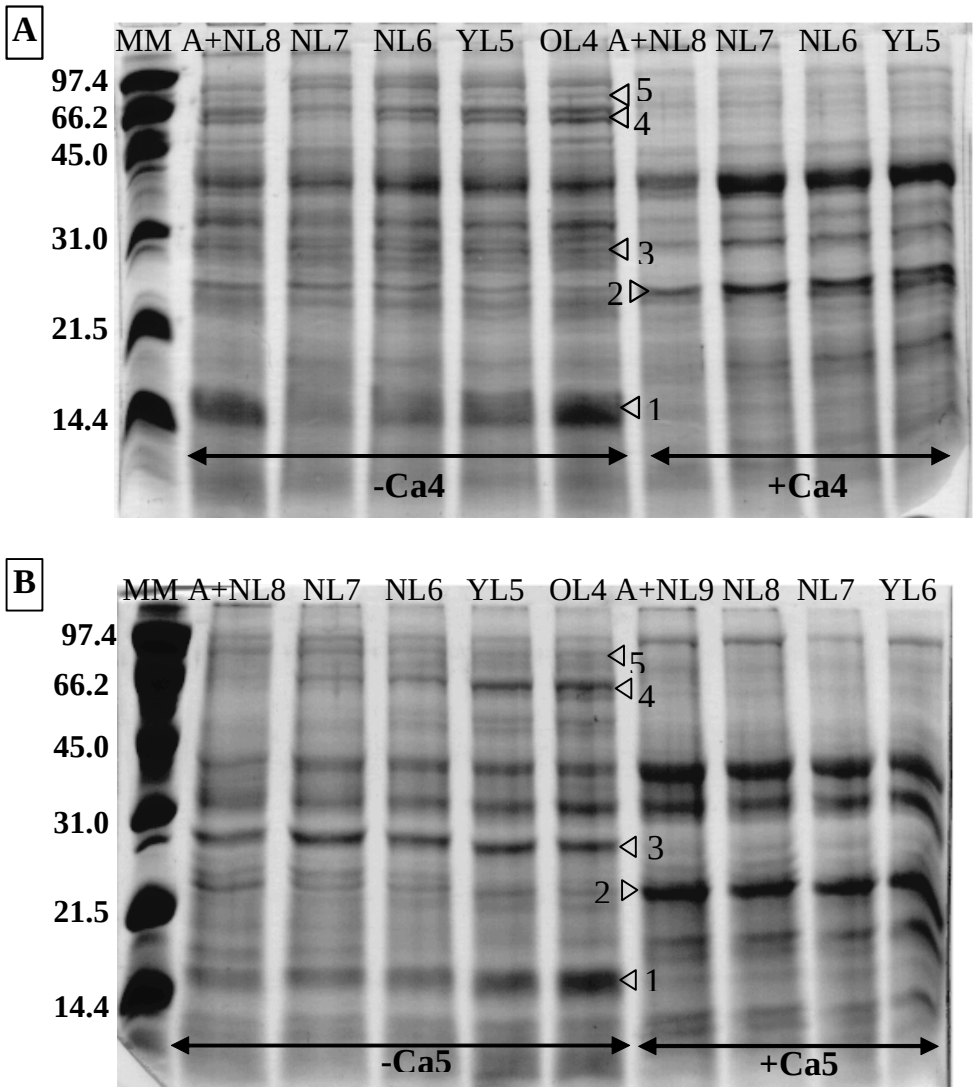


**Figure 5.** Gel SDS-PAGE of acid soluble proteins (pH 2.8) from tomato plants leaves extracted after 4 (A) or 5 d (B) of treatment. Arrows denote the position of proteins which expression is modified between (-Ca4, -Ca5) and (+Ca4, +Ca5) tomato plants. MM: Molecular weight markers [kDa]; A: apex; NL: new leaf; YL: young leaf; OL: old leaf.

results are schematically represented in Figure 6**.** Protein (1) was identified as protein P6 with a molecular mass of 17.520 kDa (pI 8.9), first sequenced by Van Kan (1992). This protein P6, an isomer of P14, belonged to the PR-1 family (Van Loon et al., 1994) largely synthesized in tomato plants in response to pathogenic attacks. Protein (2) was identified as PR-3(a) acidic endochitinase precursor (27.632 kDa and pI 5.9), first sequenced by Danhash et al. (1993). This protein belonged to the class II chitinase PR-3(a) (Van Loon et al., 1994). Protein (3) was characterized as photosystem II oxygen-evolving complex protein (OEE II) with a molecular mass of 23.00 kDa (pI 8.3), sequenced by Betts and Pichersky (1992). Protein (4) corresponded to threonine deaminase precursor (64.938 kDa and pI 5.3), first sequenced by Samach et al. (1991).

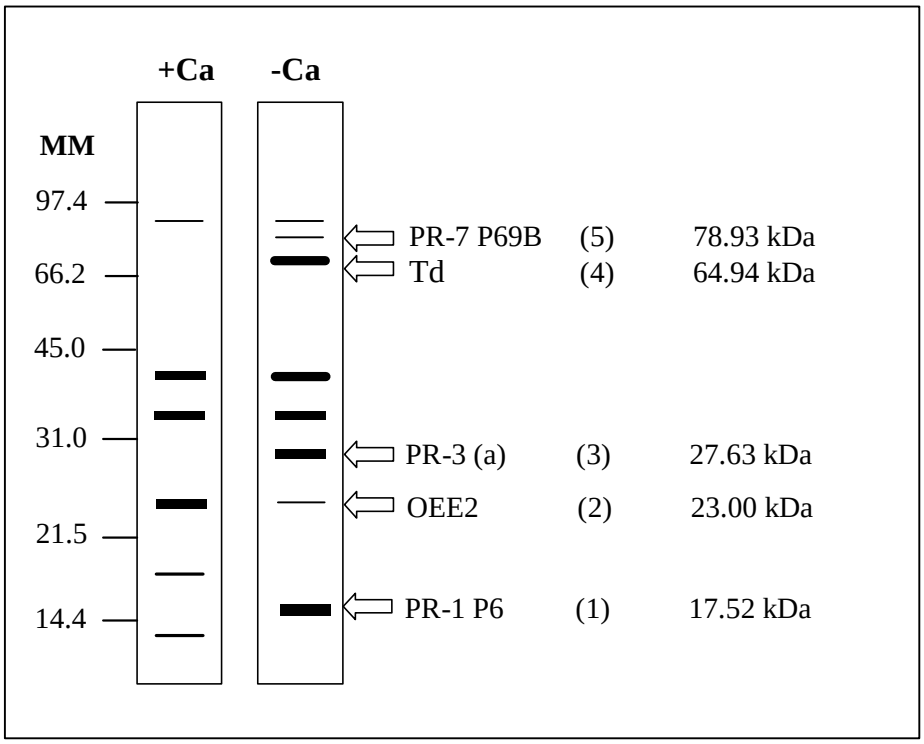


***Figure 6.*** Schematic representation of the effect of 4 or 5 d calcium deficiency on protein patterns on tomato leaves.

From sequence homologies, protein (5) was identified as PR-7 subtilisin-like proteinase precursor P69B (78.931 kDa and pI 6.3), first sequenced by Tornero et al. (1997). All these proteins were sequenced on tomato plants.

Under these experimental conditions, the effects of 4 or 5 d of Ca deficiency on the pattern of leaf acid soluble proteins can be schematized as in

**Table 2**

Presence (+) or absence (-) of protein in tomato leaves after 2,4 and 5 calcium deficiency days and after 12 days of calcium resupply. (↘) indicates a reduction of protein band intensity.

| Protein name | Calcium deficiency | | | | | | Calcium resupply | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 2 days | | 4 days | | 5 days | | 12 days | | | |
| | -Ca2 | +Ca2 | -Ca4 | +Ca4 | -Ca5 | +Ca5 | -Ca2 | +Ca2 | -Ca4 | +Ca4 |
| PR-1 (17.520 kDa) | - | - | + | - | + | - | - | - (?) | - | - (?) |
| PR-3 (27.632 kDa) | - | - | + | - | + | - | - | - | - | - |
| OEE2 (23.000 kDa) | - | - | +(↘) | + | +(↘) | + | - | - | - | - |
| Thréonine déaminase (64.938 kDa) | - | - | + | - | + | - | - | - | - | - |
| PR-7 (78.931 kDa) | - | - | + | - | + | - | - | - | - | - |

Figure. 5. The protein signal of Ca deficiency revealed mostly the expression and activation of plant defense proteins, which are induced upon attack by different types of pathogens. These homogeneous groups of proteins have been classified in 14 families of PR protein (Van Loon et al. 1994; Hoffmann-Sommergruber 2002). The three PR proteins (PR-1, PR-3, PR-7) are secreted and active in the apoplasm in relation to an attack by a pathogen. PR-1 protein is also induced by abiotic stress such as UV and ethephon (Christ and Mösinger 1989). The induction of 3 PR proteins related to Ca deficiency is shown here for the first time in the tomato plant. This phenomenon seems specific to mineral stress (Baboulène et al. 2001). In the same experimental conditions, K deficiency did not entail any change in the acid soluble protein pattern (data not shown).

Two other proteins were also expressed with Ca deficiency. Protein (4) is involved in photosynthesis. This result is corroborated by a decrease of chlorophylls in leaves of Ca deficient tomato plants (Schmitz-Eiberger et al. 2002). The last protein (5) appears to be implicated partly in the control of the biosynthesis of branched chain amino acids in plants. The relation with Ca deficiency is not known to date (Gilroy et al. 1993, Sanders et al. 1999).

## Relationship Between Ca Deficiency and Pattern of Leaf Soluble Proteins

At the end of the experiment, tomato plants were at the $8^{th}$ leaf development stage. The first question that comes to mind is why PR protein appeared in all leaves when visual symptoms were found only on new leaves (NL6-NL7). The first explanation is that the fall in the Ca amount was greater in young leaves and lesser in old leaves between 2 and 4 d of Ca deficiency (Figures 1B, 2B). After the exhaustion of root Ca destorage (Morard et al. 2000), Ca can be remobilized from these young leaves in relation with the roles and the location of this macronutrient in plant cells. Calcium content in cytoplasm is very low (around 1 µM) when this macronutrient was very high (about 10mM) in cell walls and in vacuoles (Marschner 1995). These indications consolidate the idea that the Ca deficiency would initially affect the Ca ions present in the wall and not intracellular Ca (Ho and Adams 1989). So, the Ca of the young leaves could be more easily remobilized than that of older leaves because the Ca sink (apex) is closer. Moreover, Ca ion is more bound to the lignified cell wall of old leaves. The Ca retranslocated to young tissue could be derived from extracellular space and cause the release of wall particles like pectin fragments. The works of Konno et al. (2002a, b) on carrot cells deprived of Ca showed an increase of ß-galactosidase activity. This phenomenon was correlated with solubilization of galacturonic acid-rich polysaccharides in the extracellular medium. The release of wall fragments like oligogalacturonides led to the increase of cytosolic Ca as secondary messenger resulting in the activation of the gene transcription coding for defense proteins against an external pathogen attack as eliciting action (Messiaen and Van Cutsem 1993, Honée et al. 1995, Seling et al. 2000). The

synthesis of antifongic PR-1, chitinase PR-3 and proteinase PR-7 could be a response of the tomato plant to wall degradation in relation to Ca retranslocation. Consequently, the changes in the soluble protein pattern in all leaves seem to indicate that tomato plants could not distinguish a Ca deficiency from a pathogen attack. In addition, Ca deficiency would affect only the apoplasmic pool because activation of defense proteins results from an increase of cytosolic calcium.

The second question is why only 7th-8th new leaves (NL7-NL8) developed the visual symptoms of Ca deficiency when the protein signal was lower. The role of Ca is the same in different leaf tissues but its physiological use is different. In young expanded leaves, cellular division and elongation are very intense and require, for a normal growth, a large amount of Ca ions to

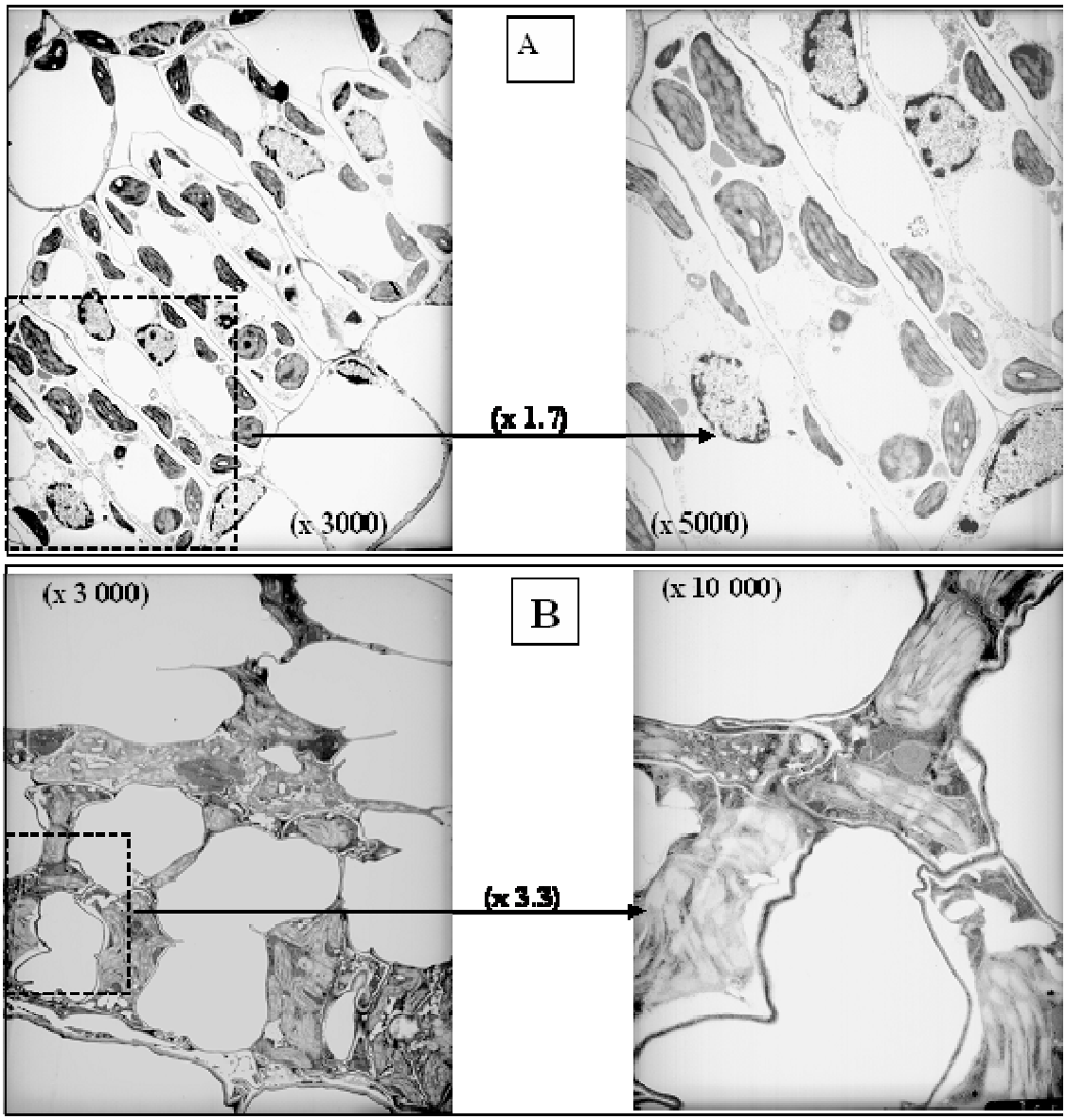


***Figure 7***. Observation by transmission electronic microscopy of limb cells in new leaves after 4 days of treatment: (A) plant control, (B) necrotic zone of calcium deficiency plant.

strengthen the wall structures. In the case of a total deficiency, the deformation of leaf blades and the apparition of necrosis (Figure 3) was related to a lack of Ca ions as binding between galacturonic acids and then to a bursting of the middle lamella under the effect of osmotic pressure. So, apopalasmic Ca is mobilized for this essential role, which explains its fall when diluted in new organic matter (Figure 1B, 2B). Calcium deficiency related to root deficiency is partly compensated by mature leaves translocation (YL 5-YL 6). But this phenomenon quickly becomes insufficient and induces the death of meristematic tissues 3-4 d later. Görlach et al. (1995) indicated that brown necrosis was mainly resulted from an accumulation of lignin via phenolic compound production. The transcription gene of phenylalanine ammonia-lyase (PAL) was activated in response to pathogen attack or to treatment with fungal elicitors.

The limb cells of the youngest leaves of tomato plants were observed by transmission electronic microscopy after 4 d of treatment. The cells of control leaves were regularly disposed and all organelles were distinct inside (Figure. 7A). In contrast to the controls, the photos of Ca deficient plants showed disorganized tissues due to wall impairment in the necrotic zone. The loss of wall and plasmic membrane integrity led to a leak of cytosol into intercellular spaces and thus to the formation of meats (Figure. 7B).

## CONCLUSIONS

Calcium deficiency induced a net decrease of dry weight versus control only after 4 d of Ca deprivation; the death of the plant appeared the fifth day of Ca deprivation;

The significant decrease of Ca content in young mature leaves indicated in these experimental conditions should be considered as a partly mobile element: this phenomenon was a new finding in the Ca nutrition of plants;

Calcium deficiency could be related, after 4 d, to the appearance of leaf soluble protein (Pathogen Related protein). As to the knowledge of the researchers, this was the first time that Ca deficiency has been related to the stress protein generally elicited by pathogen attack.

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