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Isolation and Characterization of a Soluble Phosphate Hydrolysing Activity from an *in vitro* Coffee Cell Line Grown in the Presence of Aluminum

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Abstract : As our interest is focused in obtaining a better knowledge of the plant phosphate metabolism and its interactions with Al ion, we used an *in vitro* coffee cell line (*Coffea arabica* L.) tolerant to aluminum ion to purify a protein with phosphate hydrolysing activity with the aim to analyze whether the enzyme can be contributing to the Al-tolerance in this coffee cell line. The protein was purified at least 138-fold; silver stain on a 10% SDS-PAGE detected a partially purified 30 kDa polypeptide which showed ability to hydrolyse phosphate either in native gels or soluble assays. The semipurified protein is able to hydrolyse sodium pyrophosphate (PPi-Na) and adenosine triphosphate (ATP) very efficiently, although P-serine (P-ser), P-Threonine (P-thr), P-Tyrosine (P-Tyr), Phytate, D-Myo-Inositol-1P (D-Myo Inos-1P) and the synthetic ρ-nytrophenyl phosphate (p-NPP) could also be used as substrates. Enzymatic activity of the EDTA-inactivated enzyme can be restored by Mg^{2+}, as well as other divalent cations such as Fe^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, Ca^{2+} and Mn^{2+}. In contrast, Al^{3+} could only partially reactivate the phosphate hydrolysing activity, which suggests that it is not a cofactor for this enzyme. When Al^{3+} was added to the Mg^{2+}-enzyme complex, it strongly inhibited the enzymatic activity either, exerting a negative effect over the enzyme or the substrate. Between a number of phosphohydrolase inhibitors, only KNO_3 was able to decrease the activity suggesting that this enzyme should be related with the V-ATPase protein family or with plant phosphatases.

Key words: *In vitro* coffee cell cultures, phosphate hydrolysing activity, plant protein purification, aluminum stress

INTRODUCTION

Aluminum is the most abundant metal in the earth´s crust. Under acidic conditions this metal can be found as free Al^{3+}, condition in which a wide range of negative effects are exerted on animal and plant cells (Yamamoto *et al*., 2002). The mechanisms through which aluminum exerts its toxicity have not been established yet (Kochian and Jones, 1997; Taylor *et al*., 2000; Yamamoto *et al*., 2002).

Aluminum ion is one of the major factors limiting plant growth and productivity in acid soils (De la Fuente *et al*., 1997; Takayuki *et al*., 2002). Although the molecular and biochemical basis of Al^{3+} toxicity are still far from being understood, some of the physiological consequences of Al^{3+} toxicity have been previously described. For example, it is well known that Al^{3+} inhibits root elongation in Al-susceptible species, by injuring the root apex (Kinraide, 1997; Martínez-Estévez *et al*., 2003). In contrast, it has been postulated that Al-tolerant species could have internal or external mechanism (s) which can help plants minimize the Al^{3+} uptake or detoxify the Al^{3+} within the symplast (Pellet *et al*., 1995, 1996).

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In tobacco cultured cells, Al^{3+} disturbs growth, an event that seems to be associated with malfunction of the mitochondrion, probably Al^{3+} interfers with the enzymatic activities of ATPase and ATP synthase because a depletion of ATP was observed (Hamilton *et al*., 2001; Yamamoto *et al*., 2002). These effects are very likely to be reflected in the phosphate metabolism which could, in turn, increase the concentration of inorganic phosphate (Pi) inside the cells. The hydrolysis of intracellular phosphate compounds acts as a source of inorganic phosphate (Pi) in plant cells (Hegeman and Grabau, 2001; Olczak and Watorek, 2000); it has been suggested that Pi can form complexes with the Al^{3+} ion, decreasing its toxicity (Rocha-Facanha and Orokova-Facanha, 2002).

The coffee plant generally grows in slightly acidic soils with high concentrations of Al ion. The way in which this plant cope with the challenge of aluminum toxicity is not understood, because in the field there are many environmental factors that makes the unraveling of interactions between coffee plant and Al ion a bigger task. The *in vitro* establishment of both Al-tolerant and Al-suceptible coffee cell cultures (Martínez-Estévez *et al*., 2001) and a characterization of these cultures was recently reported (Martínez-Estévez *et al*., 2003). As most of our interest is in the study of plant phosphate metabolism and its interactions with Al ion, we use the *in vitro* Al-tolerant coffee cell culture to purify a sodium pyrophosphate hydrolysing enzymatic activity; in order to analyse if this enzyme contributes in the ability of the Al-tolerant coffee cell line to survive to the Al^{3+} stress. In this study we describe the partial purification of the phosphate hydrolysing activity from the *in vitro* cultured Al-tolerant coffee cell line; characterization about the effects of Al ion exerts over this enzyme was conducted by *in vitro* assays.

MATERIALS AND METHODS

Coffee Cell Lines

This study was carried out during 2005-2006 in Yucatán, México. Aluminum-tolerant coffee cell line was cultured as described previously (Martínez-Estévez *et al*., 2001, 2003). This cell line has been grown continuously in a medium supplemented with 25 μM Al^{3+} and whose pH was adjusted to 4.3 prior to sterilization (Martínez-Estévez *et al*., 2003). Cell growth evaluation through the growth cycle was carried out by harvesting culture flasks every 2 days. Cells were vacuum filtered on sterile Whatman paper 3 mm; the resulting weight was quantitated and considered as fresh weight.

Extraction and Determination of Protein Concentration

Three grams of coffee cells were homogenized in a mortar in the presence of liquid nitrogen, until a fine powder was obtained. The powder was further homogenized in 1.5 mL of the extraction buffer (0.1 M Hepes, pH 6.9; 2 mM EGTA; 2 mM $MgSO_4$; 1 mM dithiothreitol; 30% sucrose; 10% glycerol; 1 μg mL^{-1} leupeptin; 1 μg mL^{-1} aprotinin; 1 mM Phenyl Methyl Sulphonyl Fluoride (PMSF) and 0.01% NaN_3). The homogenate was centrifuged at 16 000xg for 30 min and the supernatant (soluble fraction) was collected and stored at -70°C until use. The protein concentration was determined with the commercial Bradford (Bradford, 1976) dye-binding assay (Bio-Rad), using BSA fraction V (Sigma) as protein standard.

Protein Purification

Ten grams (fresh weight) of Al-tolerant coffee cells were harvested from culture flasks at day 16-18 of culture. Cells were homogenized as described above but powder was homogenized in 5 mL of the extraction buffer. The homogenate was centrifuged at 16. 000xg for 30 min and the supernatant was collected. The pellet was re-extracted with 5 mL of the extraction buffer and then centrifuged again at 16 000xg for 30 min. The supernatants were pooled and applied to a column packed with 40 mL of Sephacryl S-100 resin (Pharmacia). The column was eluted at 9 mL h^{-1} with the extraction buffer. Collected fractions were tested on native gels for PPi-Na phosphate hydrolysing activity and the

positive fractions were pooled and then applied onto a 10 mL hydroxyapatite column equilibrated with 5 mM Na_2HPO_4/5 mM NaH_2PO_4, pH 7.0, containing 50 mM NaCl and 1 mM dithiotreitol. The column was eluted at 35 mL h^{-1} using a linear gradient of phosphate buffer (5 to 250 mM pH 7.0 containing 50 mM NaCl and 1 mM dithiotreitol). Fractions with activity were mixed and then loaded onto a 10 mL DEAE-Sephacel column, equilibrated with 50 mM $NaHPO_4/NaH_2PO_4$, pH 7.0, containing 1 mM dithiotreitol and 5 mM NaCl. The column was eluted at 60 mL h^{-1} using a linear gradient of NaCl (5 to 1000 mM). Fractions containing phosphate hydrolysing activity were mixed and then loaded on a 10 mL Q-Fast Flow Sepharose column, equilibrated with 20 mM Hepes/Tris, pH 7.2, containing 2 mM EDTA and 1 mM dithiotreitol. The column was eluted at 45 mL h^{-1} using a linear gradient of NaCl (0 to 1000 mM). Positive samples were mixed and added with 10% glycerol (V/V), 2 mM EDTA, 1 mM dithiotreitol, 1 mM ATP, 0.5 mM $MgCl_2$, 1 mM EGTA and one half of a tablet of complete® plant protease inhibitor (Roche™). Samples were stored at -20°C until use.

Protein Electroelution

Fractions collected from the Q-Fast Flow column and containing the PPi-Na phosphate hydrolysing activity were pooled and then concentrated to 1 mL in an ultrafiltration cell (Amicon), using a YM-10 membrane. Concentrated protein was loaded on a preparative 10% polyacrylamide native gel and then electrophoresed at 100 V for 12 h at 4°C. At the end of the electrophoresis, a longitudinal slice (1 cm wide by 12 cm height) of the preparative gel was assayed for PPi-Na phosphate hydrolysing activity. The gel slice was aligned with the rest of the preparative gel and then the section with PPi-Na phosphate hydrolysing activity was located and excised. The gel slice was cut in small pieces and introduced in a dialysis bag (3000 Daltons pore exclusion size; Sigma). The bag was loaded with 1.5 mL of electroelution buffer (20 mM Tris; 150 mM Glycine) and subjected to electroelution at 35 mA overnight and at 200 mA at 4°C for 1 h. The electroeluted protein was collected from the dialysis bag and activity was re-evaluated in a native 10% polyacrylamide gel.

In-gel Evaluation of Phosphate Hydrolysing Activity

Protein samples (25 μg) were added with a modified Laemmli (Laemmli, 1970) buffer (without SDS and 2-mercaptoethanol), loaded on 10% polyacrylamide native gels and electrophoresed for 2 h in a miniprotean III electrophoresis chamber (Bio-Rad). After that, native gels were incubated with gentle agitation in 50 mM Tris-HCl, pH 7.4, containing 2 mM $MgCl_2$ and 2 mM PPi-Na, for 45 min at 37°C. The gels were washed twice with 50 mL of 50 mM Tris-HCl, pH 7.4 and 2 mM $MgCl_2$. Then the PPi-Na phosphate hydrolysing activity was revealed by incubating the gels in 0.5 M H_2SO_4 added with 40 mM ammonium molybdate and 85 mM ascorbic acid. Positive reactions developed a distintic blue signal on the gels. The reaction was stopped by washing the gels extensively in double distilled and deionized water. Soluble PPi-Na phosphate hydrolysis assays were also performed using 500 μL of a reaction mixture composed of 50 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, 2 mM PPi-Na and 0.1 μg of the electroeluted protein. Tubes were incubated at 37°C for 30 min and then mixed with Na-EDTA pH 8 at a final concentration of 20 mM to stop the reaction. Phosphate hydrolysis was quantified by the addition of 500 μL of a solution containing 0.5 M H_2SO_4, 40 mM ammonium molybdate and 85 mM ascorbic acid. After 30 min, the absorbance was determined at 700 nM.

Assays with Biological and Synthetic Substrates

Electroeluted protein (0.1 μg) was incubated in the presence of 2 mM $MgCl_2$ and 2 mM of each one of following substrates: phospho-serine (P-Ser), phospho-threonine (P-Thr), phospho-tyrosine (P-Tyr), adenosine triphosphate disodium salt (ATP), sodium pyrophosphate (PPi-Na), phytate (Phyt), D-myo-Inositol 1 phosphate (D-Myo-Ino 1-P) and p-nitrophenyl phosphate (p-NPP). These chemical compounds were used as individual substrates in each reaction. Reaction tubes were incubated at 37°C for 30 min and then assayed as described above.

Evaluation of the Effect of Different Concentrations of $MgCl_2$

The electroeluted protein was depleted of divalent cations by incubating the protein sample in 20 mM Na-EDTA for 60 min at 4°C. Then the EDTA was removed by centrifugation at 10 000xg in a microcon YM-10 (Amicon). The PPi-Na soluble phosphate hydrolysing assay was carried out in the presence of different concentrations of $MgCl_2$, as was described previously (Kuhn and Ward, 1998). In this case the PPi-Na concentration was maintained at 2 mM.

Evaluation of the Effect of Different Concentration of PPi-Na As Substrate

The assay was carried out with 0.1 μg of the electroeluted protein and different concentrations of PPi-Na as substrate. For this analysis, the $MgCl_2$ concentration was mantained at 2 mM. The released phosphate was determined as described by Ames (1966).

Effect of Al^{3+} on the Phosphate Hydrolysing Activity

Two minutes after the enzymatic reaction was started by the addition of PPi-Na, different concentrations of $AlCl_3$ were added to the reaction tubes. The enzymatic reaction was incubated at 37°C for 30 min and then stopped by the addition of Na-EDTA, pH 8.0 at a final concentration of 20 mM. The released phosphate was determined as described by Ames (1966).

Effect of Different Cations

Electroeluted protein depleted of divalent cations, was incubated independently with 2 mM of FeCl, $ZnSO_4$, Na_2MoO_4, $MgCl_2$, $CaCl_2$, $MnSO_4$ and $AlCl_3$. After incubation at 37°C for 5 min, the PPi-Na phosphate hydrolysis assay was performed during 30 min at 37°C. The phosphate release from PPi-Na was determined at 700 nm as described above. In this particular case, the assay for the aluminum ion was carried out at pH 7.4.

Effect of phosphohydrolase inhibitors on the phosphate hydrolysing activity

Phosphate hydrolysing activity of the electroeluted protein (0.1 μg) was evaluated in the presence of 10 mM $(NH_4)_6Mo_7O_{24}$, 1 mM Na_3VO_4, 100 mM KNO_3, 10 mM NaF, 2 mM NaN_3, or 5 mM butanedione monoxime (BDM), respectively. The assay was carried out for 30 min a 37°C and then stopped by addition of Na-EDTA, pH 8.0 at final concentration of 20 mM. PPi-Na phosphate hydrolysis was evaluated as described previously.

RESULTS

Cell Growth

The Al-tolerant coffee cell culture increased its fresh weight until day 16 of growth, reached the stationary phase of growth at day 18 and remained on this stage until day 32 of growth (Fig. 1A). The fresh weight acquired at the end of the growth curve of the Al-tolerant cell line was 7.2 g (Fig. 1A).

Determination of PPi-Na Hydrolysis on in Gel Native Assays and in Solution Assays

Soluble protein extracted from Al-tolerant cell line was assayed for its PPi-Na phosphate hydrolysing activity in 10% polyacrylamide native gels (Fig. 1B). In these assays, coffee cell line maintained a similar activity pattern along of the growth curve (Fig. 1B). However, when we analyzed the PPi-Na phosphate hydrolysing activity in a solution assay, the Al-tolerant cell line showed a high PPi-Na phosphate hydrolysing activity on days 16-18 of the culture (Fig. 1C). From that day on, this cell line showed a similar PPi-Na phosphate hydrolysis pattern.

Purification of the PPi-Na Phosphate Hydrolysing Activity

To purify the enzyme, the Al-tolerant coffee cells were harvested at day 16-18 of culture cycle, when the cells had maximum specific activity (Fig. 1C). Total soluble protein was fractionated through different chromatography steps (Sephacryl S-100; Hidroxyapatite; DEAE-Sephacel and Q-Fast Flow

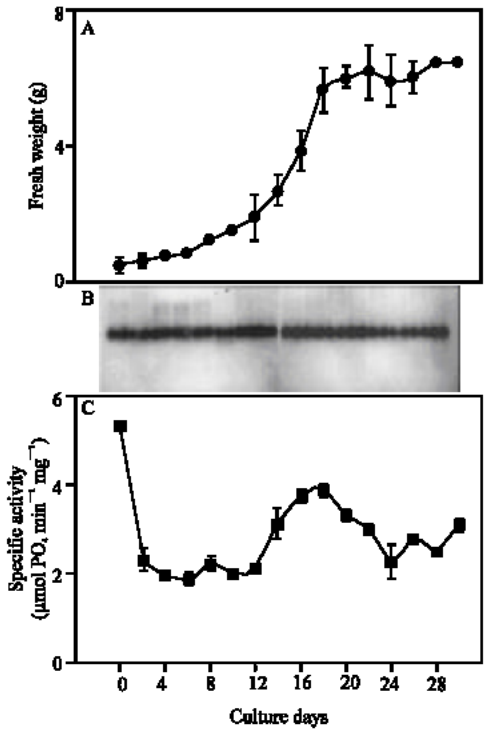

Fig. 1: Evaluation of PPi-Na-phosphate hydrolysing activity in native gels or in solution assays along the culture cycle of the Al-tolerant coffee cell line. Fresh weight for the aluminum tolerant coffee cell line was recorded along the culture cycle (A) such as was described in materials and methods. For the in native gel evaluation of the coffee PPi-Na-phosphate hydrolysing activity (B), soluble protein (25 µg) obtained through the culture cycle were subjected to electrophoresis in 10% polyacrylamide native gels. After protein electrophoresis gels were tested for the presence of the phosphate hydrolysing activity according to material and methods. For the evaluation of the in solution PPi-Na phosphate hydrolysing activity (C), the activity was measured using soluble protein (25 µg) obtained through the culture cycle. Phosphate released enzymatically was determined after 30 min at 700 nm in a DU-65 spectrophotometer (Beckman). Data are expressed as means±standard error of at least three independent determinations. Gel pictures were taken 3-5 min after stopping the reaction by washing the gel extensively in distilled/deionized water. Pictures are representative of the results obtained in at least three independent assays

Sepharose) and tested for the presence of PPi-Na phosphate hydrolysing activity. The fractions containing activity were pooled and an aliquot from each column elution was analysed to check for protein complexity on a 10% SDS-PAGE gel (Fig. 2A, lanes 3, 4, 5 and 6). After the last Q-Fast Flow column, the number of protein bands was still abundant (Fig. 2A, lane 6). Therefore, the protein was submitted to an additional purification step by running it on a preparative native gel and the areas of the gel that co-migrated with a band containing the PPi-Na phosphate hydrolysis activity, were excised and then electroeluted. A sample of the electroeluted material was analysed on a 10% SDS-PAGE gel and a major protein band of 30 kDa was observed after silver staining (Fig. 2A, lane 7). In addition, three minor contaminant bands with molecular weights of 25, 50 and 53 kDa were observed (Fig. 2 A, lane 7).

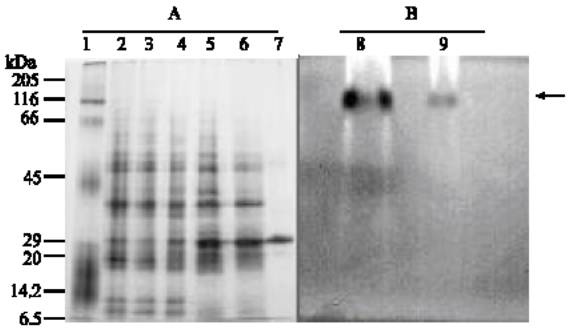

Fig. 2: Protein silver staining after each one of different steps of the purification process and in gel phosphate hydrolysing activity assay in the Q-Fast and electroeluted protein. From each one of the columns protein samples (4 μg) taken from the pooled fraction containing the PPi-Na-phosphate hydrolysing activity were subjected to electrophoresis in a 10% denaturing polyacrylamide gel and then silver stained (A). A protein sample taken from the pooled Q-Fast fraction (lane 8) and electroeluted protein (lane 9) were assayed for PPi-Na phosphate hydrolysing activity in an in native gel (B). Lanes were loaded with protein samples taken from total extract (2); Sephacryl S-100 (3), hydroxyapatite (4), DEAE-Sephacel (5) and Q-Fast Sepharose (6) columns. A sample from the electroeluted protein was also analyzed (7). Labels in the left of figure show the position of the protein molecular weight standards. Arrow in the right of panel B shows the positive spot for the hydrolysed PPi-Na

Assay of the Phosphate Hydrolysing Activity in the Electroeluted Protein

To corroborate that the protein that showed PPi-Na phosphate hydrolysing activity was recovered after electroelution, a native 10% polyacrylamide gel was loaded with a protein sample from the Q-Fast column eluate (before electroelution) and the sample after electroelution, followed by the PPi-Na phosphate hydrolysis assay. This assay showed that in both protein fractions (Fig. 2B, lane 8 and 9, respectively) the phosphate hydrolysing activity was recovered, showing a distinct spot of released inorganic phosphate on the gel (right arrow).

Protein Purification-fold

The purification table showed that after electroelution, the PPi-Na phosphate hydrolysing activity was purified about 138 fold with a yield of 0.125% and 0.058 mg of semi-pure protein was recovered (Table 1).

Use of Different Phosphorylated Substrates

Plant soluble enzymatic phosphate hydrolysing activities are highly unspecific as they can use a broad range of substrates *in vitro* (Hegeman and Grabau, 2001). To evaluate this coffee enzyme activity, we explored if it was capable of hydrolysing other natural or synthetic substrates, besides PPi-Na. The enzyme was able to hydrolyse substrates such as P-ser; P-thre; P-Tyr, D-Myo-Inositol-1-phosphate and ATP as efficiently as the PPi-Na itself; p-nitrophenyl phosphate (a synthetic compound broadly used as a phosphatase substrate; Olzack *et al*., 2003) was also an efficient substrate. Although, phytate, a natural hexakisphosphate, was hydrolysed in a less extent (Table 2), a chi-square statistical analysis at 0.05 of probability revealed not significant differences with the other tested substrates.

Table 1: Purification summary. Phosphate Hydrolysing activity from Al^{3+} tolerant coffee cell line

	Total protein (mg)	Specific activity (μmol PO_4 min^{-1} mg^{-1})	Purification fold	Yield (%)
Total extract	46.400	2.8	1.00	100.00
Sephacryl S-300	17.100	3.5	1.28	36.94
Hydroxyapatite	4.400	6.7	2.38	9.47
DEAE-Sephacel	0.830	26.4	9.42	1.79
Q-Fast Sepharose	0.570	40.6	14.47	1.23
Electroelution	0.058	388.7	138.48	0.125

Table 2: Coffee phosphate hydrolysing activity using different kind of phosphated-substrates. The enzymatic activity of the electroeluted protein (0.1 μg) was evaluated as described in materials and methods and the specific activity was determined measuring the release of phosphate from each one of the substrates. Substrates were used at 2 mM of final concentration. Data are expressed as means±standard error of three independent determinations

Substrates	Specific activity (umol PO_4 min^{-1} mg^{-1})
P-ser	18.83±3.08[b]
P-Threo	00.19±0.608[b]
P-tyr	19.90±0.07[b]
ATP	27.80±0.319[b]
PPi-Na	20.57±2.3[b]
Phytate	12.70±0.856[b]
D-Myo-Ino 1-P	19.40±0.856[b]
P-PO_4	19.86±0.425[b]

Pyrophosphate as Substrate

Pyrophosphate is an important cell bioproduct (Stitt, 1998; Farré *et al*., 2000; Casolo *et al*., 2002) and is also commercially available at low cost; therefore, it had certain advantages to continue the characterization of the activity using this compound. When the Mg^{2+} concentration was maintained at 2 mM, the enzyme hydrolysed PPi-Na efficiently at concentrations of 5 mM and below (Table 3A). However, increasing the PPi-Na to 10 mM decreased the phosphate hydrolysing activity (Table 3A). Statistical analysis at 0.05 of probability revealed that 10 mM of PPi-Na was the only one that produces a significant different result among all tested concentrations.

Dependence of the Magnesium Ion for Catalytic Enzymatic Activity

Mg^{2+} is an essential cation required for the activity of many animal and plant phosphohydrolases, such as the soluble inorganic pyrophosphatases (Mortain-Bertrand *et al*., 1996; Visser *et al*., 1998). To explore if the coffee phosphohydrolase require Mg^{2+} for its catalytic activation we tested the effect of different concentrations of Mg^{2+} on the enzymatic activity. We used conditions that maintained the PPi-Na at 2 mM. From 0.05 mM $MgCl_2$ and in increasing concentrations to up to 10 mM $MgCl_2$, the enzymatic activity increased from 18 to approximately 35 μmol of hydrolysed phosphate and no negative effects were observed even at 10 mM of Mg^{2+} (Table 3B).

Effect of Divalent Cations on the Phosphate Hydrolysing Enzymatic Activity

The activity of PPi-Na phosphohydrolase was analysed in terms of its ability to use different divalent cations. Fe^{2+}, Ca^{2+}, Mg^{2+}, Zn^{2+} Cu^{2+} and Mo^{2+} were able to reactivate the activity after its previous deactivation by incubation in 20 mM of EDTA. When Mn^{2+} and Al^{3+} were tested, we observed less activation of the phosphatase enzyme (Table 4). As expected, activation by Al^{3+} was at much less extent than the produced by all the other cations (Table 4). The chi-square analysis revealed that the reactivation of the phosphohydrolase enzymatic activity by Al^{3+} was statistically different from the all other tested cations. In addition, it was observed that when different concentrations of Al^{3+} were added to the enzymatic assay containing 2 mM of the substrates, Mg-PPi-Na and the active enzyme, Al^{3+} caused a statistically significant decrease on the specific activity of the protein with respect to the assays lacking of Al ion (Table 5).

Table 3: Effect of the PPi-Na and $MgCl_2$ on the phosphate hydrolysing activity

A: PPiNa (mM)	Specific activity (μmol PO_4 min^{-1} mg^{-1})
0.05	17.20±1.42[b]
0.1	17.60±0.21[b]
0.25	18.60±0.678[b]
0.5	18.64±0.45[b]
1	21.07±0.35[b]
2	22.09±0.35
3	23.05±0.212[b]
4	23.35±1.82[b]
5	20.90±1.89[b]
10	4.00±0.002[a]
B: $MgCl_2$ (mM)	Specific activity (μmol PO_4 min^{-1} min^{-1})
0.05	18.90±0.028[b]
0.1	20.26±0.81[b]
0.25	23.05±1.61[b]
0.5	24.57±1.14[b]
1	26.60±0.04[b]
2	27.80±2.89[b]
3	32.00±0.85[b]
4	31.70±1.36[b]
5	35.20±0.99[b]
10	34.04±2.17[b]

Electroeluted protein (0.1 μg) was incubated with 2 mM of $MgCl_2$ and increasing concentrations of PPi-Na (A), or incubated with 2 mM of PPi-Na and increasing concentrations of $MgCl_2$ (B). The enzyme activity was determined by the phosphate release from PPi-Na hydrolysis. Data are expressed as means±standard error of the three independent determinations. A chi-square statistical analysis at $p = 0.05$ was carried out to differences between different assays; a = statistically different, b = statistically not different

Table 4: Effect of cationic ions on the phosphate hydrolysing activity

Metallic ions	Specific activity (umol PO4 min^{-1} mg^{-1})
Fe^{2+}	18.80±0.912[b]
Zn^{2+}	18.89±1.48[b]
Co^{2+}	20.87±0.81[b]
Mo^{2+}	24.42±2.1[b]
Cu^{2+}	24.32±1.57[b]
Mg^{2+}	25.74±1.1[b]
Ca^{2+}	20.92±2.43[b]
Mn^{2+}	13.73±1.57[b]
Al^{3+}	7.75±0.424[a]

The partially purified enzyme was inactivated with EDTA and then incubated independently with 2 mM of each one of: Fe^{2+}, Zn^{2+}, Mo^{2+}, Cu^{2+}, Mg^{2+}, Ca^{2+}, Mn^{2+} metallic ions. In a separate experiment inactivated protein was incubated with 25 μM of Al^{3+} and then the enzymatic activity was measured. Data are expressed as means±standard error of three independent determinations

Table 5: Effect of the Aluminum ion on the phosphate hydrolysing activity.

($AlC l_3$) mM	Specific activity (μMol PO_4 min^{-1} mg^{-1})
0 (control)	28.50±1.2
0.0125	6.43±1.21[a]
0.025	6.84±0.64[a]
0.05	6.13±1.16[a]
0.1	8.05±1.75[a]
0.25	9.27±0.24[a]
0.5	10.33±1.31[a]
1	9.77±0.28[a]
2	7.75±0.32[a]
3	4.50±0.34[a]
4	4.40±0.42[a]
5	2.40±1.2[a]

Electroeluted protein (0.1 μg) was used to perform in solution PPi-Na-hydrolysing assays in presence of 2 mM $MgCl_2$, 2 mM PPi-Na and different concentrations of Al ion. Data are expressed as means±standard error of the three independent determinations. A chi-square statistical analysis at $p = 0.05$ was carried out to detect differences in efficiency of inhibition among the $AlCl_3$ concentrations; a = statistically different, b = statistically not different

Table 6: Effect of different phosphohydrolase inhibitors on the PPi-Na phosphate hydrolysing enzyme

Inhibitors	Specific activity (μmol PO_4 min^{-1} mg^{-1})
Control	17.05±0.24
KNO_3 (100 mM)	6.23±0.14[a]
BDM (5 mM)	18.40±1.25[b]
NaN_3 (2 mM)	18.44±1.07[b]
$Na_3(VO)_4$ (0.2 mM)	19.20±0.30[b]
NaF (10 mM)	15.70±0.07[b]
NH_4Mo_7 O_{24} (10 mM)	7.85±1.42[b]

Inhibitors of different protein phosphohydrolase activities were tested on the complex Mg-PPi-Na hydrolysing coffee enzyme. The released phosphate was determined as described in material and methods. Data are expressed as means±standard error of the three independent determinations. A chi-square statistical analysis at p = 0.05 was carried out to detect differences in efficiency of inhibition among the tested compounds; a = statistically different, b = statistically not different

Effect of Different Inhibitors of Phosphohydrolases on the Phosphate Hydrolysing Enzymatic Activity

Since phosphate hydrolysis has been associated with different kinds of cellular enzymes, we decided to use some specific inhibitors of individual phosphohydrolases to obtain some evidence about the kind of phosphohydrolase whose activity we had enriched. When butanedione monoxime (BDM; an inhibitor of the ATPase associated with the myosin molecule (Cheung *et al*., 2001), NaN_3 (inhibitor of mitochondrial ATPase) and vanadate (an inhibitor of P-type ATPases, including the plasma membrane H^+-ATPases; Soga *et al*., 2000) were used, we observed no effects on the phosphate hydrolysing activity (Table 6). This result suggests that this phosphohydrolase is neither associated with the ATPase activity nor with phosphate hydrolysis by ATPases related to the plasma membrane or mitochondria (Table 6). NaF, a compound that is used as inhibitor of pyrophosphatase activity in plant (Wang *et al*., 1986) and animal cells (Rodrigues *et al*., 2000), did not affected the activity under the tested concentrations (Table 6). In contrast, when KNO_3 (inhibitor of vacuolar ATPase (Dschida and Bowman, 1995) and $(NH_4)_6Mo_7O_{24}$ (inhibitor of plant phosphatases; Tso and Chen, 1997) were used, an important decrease in the phosphohydrolase enzymatic activity was observed. However, statistical analysis showed that only the inhibition of the phosphatase activity caused by KNO_3 was statistically different from the effect caused by all other tested compounds.

DISCUSSION

Recently, it was described that highly phosphorylated intracellular compounds can be used as a source of inorganic phosphate in plant cells (Hegeman and Grabau, 2001; Olczak and Watorek, 2000), which, in turn, can chelate Al^{3+}, thus decreasing its toxicity (Rocha-Facanha and Orokova-Facanha, 2002). In the present study, one Al-tolerant coffee cell line grown in the presence of 25 μM $AlCl_3$ (Martínez-Estévez *et al*., 2003) was evaluated for its *in vitro* cell growth. No negative effects of Al ion were found on the increase of fresh weight along the growth cycle (Fig. 1A). Its growth rate is similar than observed for an Al-susceptible coffee cell line established in our laboratory and cultured in an Al-free medium (data not shown). Results indicate that the culture tolerates well 25 μM Al^{3+} which is 50% of the lethal dose for the coffee susceptible cell line (Martínez-Estévez *et al*., 2003).

Since most of our interest was focused on analysing if the intracellular phosphate plays a key role in the survival of the Al-tolerant coffee cell line, we evaluated the PPi-Na phosphate hydrolysing activity along its culture cycle, no differences were found on in gel native assays (Fig. 1B); this result probably was due to capability of this technique since the in-gel PPi-Na phosphate hydrolysis assay just gives information about the presence or absence of enzyme activity. In contrast, a soluble assay revealed that the Al-tolerant cell line had maximum PPi-Na phosphate hydrolysing activity at days 16-18 of growth cycle (Fig. 1C). This difference on PPi-Na hydrolysing activity could be reflecting,

at least in part, the differences in enzymatic activities that might participate in the phosphate hydrolysis as a specific cellular response to the Al-presence on the cell culture medium. Alternatively, it could reflect differences in the phosphate metabolism during the growth of the Al-tolerant cell line. The fact that the maximum PPi-Na phosphate hydrolysing activity was observed when cells were in the exponential phase of growth would support this idea.

As we were interested to know if the reason for the maximum enzymatic activity of the PPi-Na phosphohydrolase observed during the exponential phase of growth of the Al-tolerant cell line could be a consequence of the direct interaction between Al^{3+} and enzyme, or it might be that the phosphohydrolase is never in contact with the Al ion and the increase in enzymatic activity occurs as an indirect effect of the cell response which translates it in an increase in its phosphate metabolism in order to cope with this metal.

To explore these possibilities the PPi-Na phosphate hydrolysing activity was isolated from Al-tolerant coffee cell line and its catalytic properties characterized on *in vitro* assays, both, in presence and absence of Al ion. According to the inhibitor sensibility the enzyme probably belongs to the phosphatase family ($NH_4Mo_7O_{24}$ sensitive; (Tso and Chen, 1997; Roym *et al*., 2003), or it may be related to the vacuolar ATPase (KNO_3 sensitive; Dschida and Bowman, 1995). Additionaly, phosphohydrolase activities, particularly enzymes that use PPi-Na as substrate, like pyrophosphatases, are usually modulated by the substrate or the product (Leigh *et al*., 1992; Kuhn and Ward 1998; Cirkovic *et al*., 2002). This coffee phosphate enzyme used PPi-Na as substrate in a range of 0.05 to 4 mM and at higher concentrations, the activity was clearly inhibited (Table 3A). This result suggested that the PPi-Na or free phosphate produced by the hydrolysis could be a negative modulator of this enzyme in the cell. It is noteworthy that in plant cells the PPi concentration ranges between 0.2 and 1 mM (Du Jardin *et al*., 1995), in which case, the results obtained on *in vitro* assays could be reflecting some characteristics of its physiological regulation.

It is well accepted that catalytic activity of the phosphohydrolase enzymes is strictly dependent of the Mg^{2+} ion. We tested the isolated protein in concentrations ranging from 0.05 to 10 mM and not negative effects on the enzymatic activity were observed (Table 3B). However, the addition of 20 mM of EDTA abolished the enzymatic activity (data not shown). Taking advantage of last finding, we evaluated the ability of different divalent cations to restore the enzymatic activity of the EDTA-inactivated protein. It was observed that the addition of Fe^{2+}, Zn^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+} and Mo^{2+} could restore the activity to a similar extent as the complex Mg-protein. In addition, it was observed that the addition of Al^{3+} ion to the Mg-PPi-Na-enzyme, caused a quick decrease on the phosphate hydrolysing activity (Table 5). This last result, together with the inability of Al^{3+} to restore the activity of the enzyme, strongly suggest that aluminum might de-activate the enzyme indirectly, perhaps through interaction with the PPi-Na substrate instead of displacing the Mg^{2+} from the enzyme. This is supported by the fact that the same effect was observed at lower concentrations of Al^{3+}.

It has been previously reported that when plant cells are exposed to aluminum stress, they release high concentrations of phosphate containing compounds and the conjugation of phosphate neutralizes the Al^{3+} toxicity (Rocha-Facanha and Okorokova-Facanha, 2002). For example, when Al-tolerant wheat seedlings were subjected to a stress of 25 to 100 μM of Al^{3+} (moderate to strong Al-stress), an increase of V-ATPase and the F1-F0-ATPase activities was observed (Hamilton *et al*., 2001). In both cases, ATP was hydrolysed and phosphate released. The coffee PPi-Na phosphate hydrolysing activity was able to de-phosphorylate many compounds efficiently *in vitro*, although ATP was its best substrate (Table 2). An additional level of specificity could occur by *in vivo* control at the subcellular or time levels, such that the enzyme could dephosphorylate multiple substrates at physiological pH. If this was the case, it could also be involved in the regulation of the coffee cell phosphate pool level in order to cope with the aluminum. Olzack *et al*. (2003) proposed such a physiological role for a novel metallophosphatase, diphosphate nucleoside-specific phosphohydrolase (PPD1) from *Lupinus luteus* seeds which cleaved pyrophosphate bonds with an optimum pH of 6.25.

Clearly, much work is still needed to gain a better understanding of this enzyme and its function in coffee cells submitted to aluminum ion. For this reason, we plan to produce antibodies against this protein and obtain the protein sequence in order to have a better idea about its possible biological meaning. The cDNA sequence will help to clarify if it is involved in contending with the stress produced by the Al ion and/or overcome its detrimental effect on plant cells. This will be particularly useful for a better yield of the coffee crops growing on acid soils.

ABBREVIATIONS

EDTA : Ethylenediaminetetracetic acid, disodium salt;
EGTA : Ethylene glycol bis (β-aminoethyl) tetraacetic acid;
Na_2HPO_4 : Monobasic anhydrous sodium phosphate;
NaH_2PO_4 : Dibasic anhydrous sodium phosphate.

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