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Full Length Research Paper

Purification and characterization of a peroxidase present in xilopodium exsudates of umbu plants (*Spondias tuberosa* A.)

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Umbu plants are drought resistant trees which are able to store water and several other substances into its adapted root, named xylopodium. The exsudate from xilopodium is a natural solution rich in salts, sugars and a little concentration of proteins. In this work, we report the purification of a peroxidase (POX) from umbu xilopodium exsudate by direct extraction from polyacrylamide electrophoresis gels. Umbu POX showed optimum activity at pHs around 6.0 to 7.0 and high thermal resistance after incubation at 70°C for 6 min. POX activity present in crude extracts was more heat-resistant than in its purified form. When assayed with metal ions, umbu POX activity was shown to be inhibited by Mn^{2+} and stimulated by Ca^{2+} and Mg^{2+}; it was also inhibited by sodium azide (concentrations higher then 1 mM) and not inhibited by either EDTA or tropolone. POX Km values for guaiacol and methylcatechol substrates were 6.83 and 22.25, respectively. The enzyme is proposed to be a guaiacol peroxidase and was seen to be located at higher concentrations in the outermost layers of xylopodium tissue, such as the endoderm.

Key words: Root enzyme, tissue brownig, guaiacol peroxidase, oxidative metabolism.

INTRODUCTION

The genus *Spondias* comprises some producing species of exotic edible-fruit of high commercial value in several tropical countries, such as mombin (*Spondias mombin*) and siriguela (*Spondias purpurea*). Belonging to the

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Abbreviations: APX, Ascorbate peroxidase; **CAT,** catalase, **GPX,** guaiacol peroxidase; **HRP,** horseradish peroxidase; **LiP,** lignine peroxidase; **POX,** peroxidase; **MnP,** manganese peroxidase; **SOD,** superoxide dismutase.

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genus, *Spondias tuberosa* is the only species that has the special ability to form xilopodium which is a modified root, which often possesses dormant meristematic tissues, able to promote vegetative reproduction and functions in the storage of water and nutrients, ensuring survival of the plant during the dry season. These are also important adaptations for plants growing in environments prone to frequent fires (Braga et al., 2006). People from arid regions of Brazil make candy bars from umbu xilopodium and it is used as food and also it is still restricted to this region. Although, there are a work in incipient phase to production of pickes of xilopodium with economic pontential to exportation (Cavalcanti et al., 2004). The browning color atributed to this product may be related to oxidation of phenols by poliphenoloxidase or peroxidase, a common trouble viewed in food processing (Mousavizadeh and Sedaghathoor, 2011). Due to the formation of xylopodium and other characteristics, umbu plants are highly resistant to drought (Silva et al., 2009). A unique umbu tree is able to grow down into its roots near to two tons of xylopodium (Cavalcanti and Resende, 2006). Plants that exhibit comparably high resistance to climatic stress have been shown to possess relatively higher enzymatic antioxidants levels, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and GPX (guaiacol peroxidase) than susceptible varieties. *S. tuberosa*, despite being a dry resistant tree, has not been yet investigated in this sense, and no information about enzymes related to this property is available currently (Csiszár et al., 2007). Peroxidases are present in several organisms from many species including bacteria, fungi and animals (Hiraga et al., 2001). They are enzymes that have a reactive group formed by either heme or porphyrin groups involved in catalysis and are able to oxidize several organic compounds through the transference of electrons to hydrogen peroxide.

Plant peroxidases belong to the class III of peroxidases and form a large multigenic family with molecular weights varying from 30 up to 150 kDa (Bakalovic et al., 2006; Cosio and Dunand, 2008). Class III peroxidases have variable domain but conserved motifs position in the structures; it is due to the catalytic function preservation, associated always to presence of a heme group with an iron ion interaging with histidine and phenylalanine amino acids residues in conservaed position (Veitch, 2006). Ions Ca^{+} estabilished the POX structure but are interacting directly in the active site of enzyme but can interfier in motifs conformation and enzymatic specificity. Several class III peroxidases are glycosylated in many residues and it confers stability to high temperature and pH variation (Mathé et al., 2010). Plants can express several peroxidases involved in different physiological process such as defense mechanisms acting against pathogens (Kuzaniak and Sklodowska, 2005; Almagro et al., 2009), hormone regulation (Gutiérrez et al., 2009), lignin biosynthesis, oxidation of lignin precursor phenols (Fagerstedt et al., 2010), and responses to abiotic stresses including UV radiation and drought (Kim et al., 2007; Krishnamurthy et al., 2009). The relationship between peroxidase and water stress is related to protection of plant tissues against the oxidative stress built up under this condition (Reddy et al., 2004).

Although, peroxidase performs several roles in plant metabolism, it is an undesirable molecule in food industry due to properties of plant tissues browning plant tissues. However, its isolated form, peroxidases are among the most useful enzymes in biotechnology, since several industrial processes can be performed by this kind of enzyme, such as soil detoxification and bioremediation of waste waters contaminated with phenols, cresols and chlorinated phenols.

Lignin peroxidase (LiP) and manganese peroxidase (MnP) may be successfully used for biopulping and biobleaching in paper industry (Hamid and Rehman, 2009). Peroxidase-based biosensors and bioelectrosensors have found use in analytical systems for example as components of lab and medical diagnose kits, such as those employed for ELISA technique (Shahriar and Elham, 2013). Thus, the search for new peroxidases is no strictly aimed to the understanding of their involvement in physiological processes, but it is also devoted to the discovery of new molecular tool available for a diverses purposes. The roles of plant peroxidases have been under investigation for a long time. Horseradish root POX was the first one to be isolated and characterized in the 1940's (Poulos, 2010), and until today some aspects about its function are not completely understood. In this work, we isolated and characterized a plant peroxidase from umbu xylopodium exsudate.

MATERIALS AND METHODS

Plant materials

Six months-old naturally occurring umbu (*S. tuberosa*) plants, propagated from seeds, were used in the experiments (Figure 6A). Plant xilopodium were removed, cut into pieces around one inch in size and ground in liquid nitrogen with a chilled mortar and pestle until obtaining a fine powder. Approximately 100 g of this material was centrifuged (30 min, 8000 x *g*, 4°C), yielding approximately 50 mL of supernatant (xylopodium exsudate). Exsudates were then stored at - 80°C before use in subsequent analysis.

Peroxidase purification

The purification of umbu peroxidase was performed by direct extraction from an electrophoretic gel using a combination of protocols (Retamal et al., 1999; Scheer and Ryan, 2001) with some adaptations. The method was initiated with the preparation of a polyacrylamide gel (main gel), with dimensions of 150 × 130 × 1.5 mm (height × width × thickness, respectively). Subsequently, the gel was made by containing a unique lane to load a volume of 6 mL of sample, and native electrophoresis was performed in order to preserve the original properties of the enzyme. Running was performed at a constant current of 30 mA. After running, the gel sectioning was performed by successive horizontal slicing, from the

top to the bottom of the gel with two millimeters width for each slice. The slices were separately ground with 10 parts of distilled water in a microfuge tube (1500 µL) using a micropotter. The material was then centrifuged at 1400 x *g* for 2 min and the supernatant was removed with a micropipette. The activity of each POX sample was measured. The three more active fractions (with 2.5, 2.7 and 2.9 cm of mobility) were defined as purified umbu peroxidase (umbu POX fractions), and stored at - 80°C.

Protein dosage

Protein concentrations of xylopodium exsudates were determined by the Bradford method (1976). Xylopodium exudates (100 µL) were added to 700 µL of distilled water and 200 µL of commercial Bradford reagent. A blank was prepared by adding distilled water in place of the xylopodium exsudate. The measurement was taken at 595 nm. The standard curve for protein determination was developed using a series of known concentrations of bovine serum albumin.

Peroxidase activity determination in solution and in gel

The peroxidase activity was determined by colorimetric assay (Janda et al., 2003) through the measurement of changes in absorbance at 470 nm. In a tube, 8 mM guaiacol, 16 mM hydrogen peroxide, 100 mM phosphate buffer pH 7.0 and 20 µL of xylopodium exsudate were combined in a final reaction volume of 2 mL. A blank was prepared by replacing xylopodium exudates by an equal volume of distilled water. After 5 min of reaction, absorbance readings were taken at 470 nm. The measurements were expressed as units of enzyme activity per mg protein (EU / mg) (specific activity), and one unit was arbitrarily defined as the amount of enzyme which caused a change of 0.01 absorbance per minute. The staining of active isolated POX fractions on SDS-PAGE gels was performed by immersing the gels in the same reaction medium described above, until bands were visible. Stained gels were washed with distilled water and after photographed (Cesarino et al., 2012).

Thermal inactivation of umbu POX

To determine the thermal stability of the enzyme, umbu POX was incubated in a water bath at temperatures of 60, 70, 80 and 90°C for 0, 1, 2, 4, 6 or 8 min. The heat treatment was stopped by transferring samples to ice (0°C). The control time (0 min) was referred to as the sample maintaining 100% of initial activity. Three independent activity measurements at each heating time were taken and the average value was used for determination of the percentage of inhibition by thermal inactivation. The enzyme activity values were expressed as percentage of relative activity, referring to the unheated control. To evaluate the effect of xylopodium exsudate medium on POX activity preservation, a comparative thermal inactivation test was made, comparing those activities from purified POX and from crude xylopodium esxudate extraction. The samples were assayed at temperatures of 40, 50, 60, 70, 80, 90 and 100°C, and incubated for 5 min. The following procedures were as described above.

Effect of pH on umbu POX activity

The influence of pH on umbu POX activity was analysed by measuring the enzyme activity towards guaiacol and hydrogen peroxide, except by the variation of pH buffering systems, which were 100 mM phosphate (pH 3.0 up to 6.5) and Tris-HCl 100 mM (pH 7.0 up to 10.0). Activity was expressed in relative percent activity being the highest value of absorbance at 470 nm considered as 100%.

Effect of metal ions and organic inhibitors on umbu-POX activity

The effects of the metal ions Mn^{2+}, Mg^{2+} and Ca^{2+} as well as of the organic inhibitors sodium azide, EDTA and tropolone were analysed at assay concentrations of 0.01, 0.1, 1 and 10 mM. Activity was expressed in relative percent activity in relation to the control, where no additions were made to the umbu-POX sample.

Km, Vmax and Vmax/Km determination

Km and Vmax were calculated from the data obtained with the substrates guaiacol and methylcatechol at increasing concentrations of 0.1, 0.5, 1.0, 2.0, 4.0, 8.0 and 16 mM for the former and 1, 2, 5, 10, 15, 20 and 50 mM increasing concentrations for the latter. Thereafter, the data was determined with Lineweaver-Burk plot.

POX activity staining in xylopodium tissue

Xilopodium of *S. tuberosa* plants of six months old were cut off, and sections of 4 to 5 mm were obtained. The sections were immersed in 100 mM phosphate buffer pH 7.0, 5 mM guaiacol, 20 mM hydrogen peroxide, for 5 min. Control experiment was performed with sections immersed only in phosphate buffer. After treatment, the slices were photographed (Cesarino et al., 2013).

RESULTS AND DISCUSSION

A single umbu peroxidase polypeptide was present in xilopodium exsudate (Figure 1). A native gel electrophoresis condition was adopted, differing from previous work protocols (Retamal et al., 1999; Scheer and Ryan, 2001), to protect the enzyme against eventual lost of activity. The purified enzyme showed a molecular mass of ca. 66 kDa (Figure 2). Peroxidase purification by conventional methods using salt precipitation and chromatographic steps have low yields index (yield of 2.67% for a lettuce stem peroxidase) (Estèban-Carrasco et al., 2002); yeld of 3.3% for *Aloe barbadensis* cationic peroxidase (Hu et al., 2012); 9.7% for a Turkish black radish peroxidase (Şişecioğlu et al., 2010). Purification yield by protocol used was as high as 74.75% (Table 1). This high yield was due to the adoption of a unique purification step such as to the one reported by similar protein purification methodology (Retamal et al., 1999; Scheer and Ryan, 2001). Umbu POX showed a range between 6.0 and 7.0 for its optimum activity. The pH curve showed a bell-shape distribution suggesting an involvement of two amino acid residues in catalysis. A broad range of pH was observed for umbu POX, the activity was maintained at a 70% level, in a range from 4.5 up to 7.5 (Figure 3). A similar pH curve profile was reported for a POX from tomato juice (Vernwal et al., 2006). However, an acid optimum pH (5.5) was observed in contrast to umbu POX that has optimum pH near to

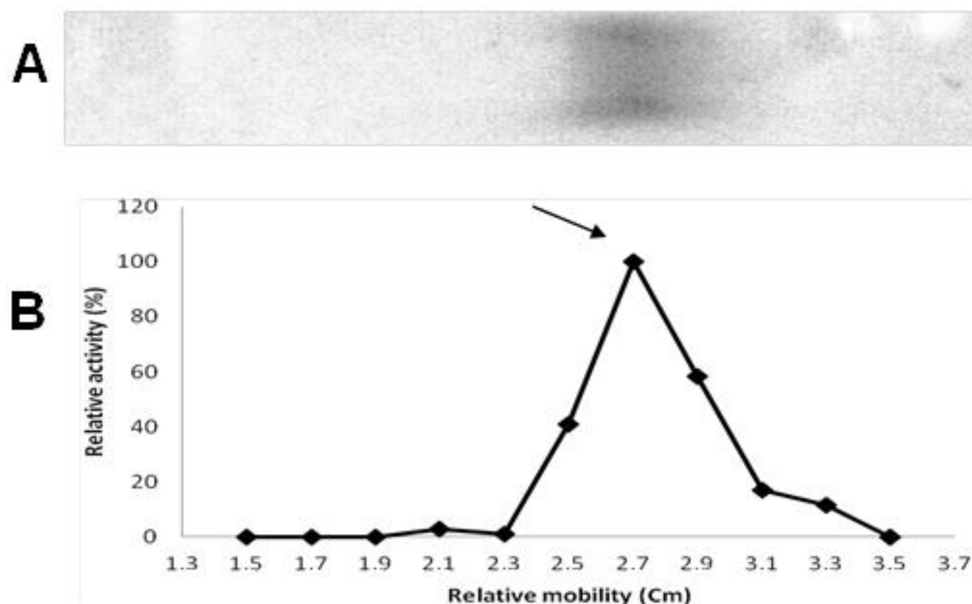

Figure 1. (A) POX activity measurement of different fractions extracted from gel electrophoresis strip. **(B)** Relative mobilities, expressed in centimeters of band migration (Cm). The arrow indicates the most active fraction with relative mobility of 2.7 Cm.

Figure 2. Activity staining of peroxidase with guaiacol after SDS-PAGE, showing total xylopodium extracts (TE) and of 2.5, f 2.7 and f 2.9 isolated fractions from previous native gel (Figure 1).

Table 1. Purification of umbu peroxidase by direct extraction from electrophoresis gel.

Purification steps	Total volume (mL)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Yield (%)	Purification fold
Xilopodium exsudate	6	0.93	891.79	958.91	100	1
Isolated umbu POX fraction	2.5	0.024	666.67	27777.92	74.75	28.97

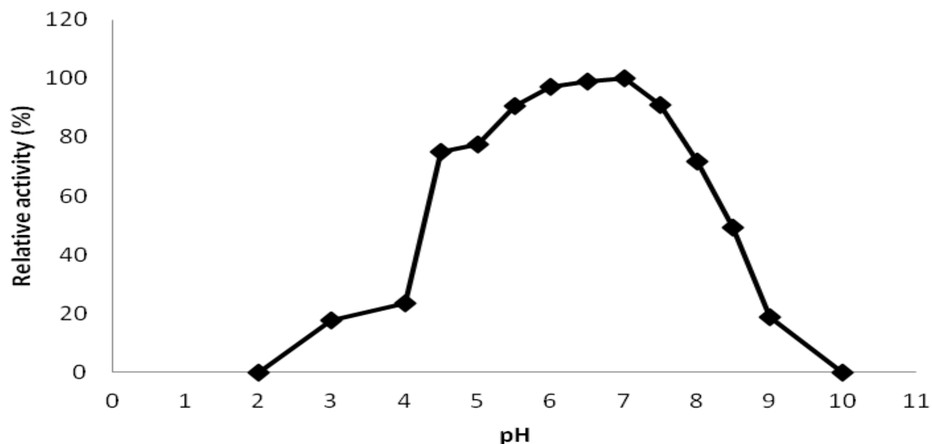

Figure 3. Effect of pH on umbu POX activity using guaiacol as substrate.

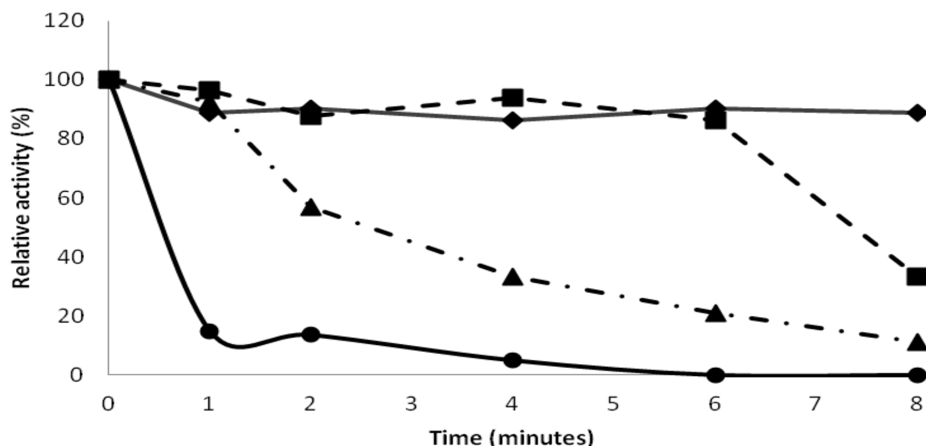

Figure 4. Determination of thermal stability of umbu-POX at different temperature: 60 (♦), 70 (■), 80 (▲) and 90°C (●).

neutral. Peroxidases from vegetal sources have a great variation of optimum pH for activity. For example, optimum pH for peroxidase isoforms isolated from apple fruit ranges from 5.0 up to 7.0 (Ros-Barceló and Pomar, 2002). Most peroxidases of this family have optimum pH in the acidic range possible due to the pH present in vacuole and intercellular way (Ros-Barceló and Pomar, 2002). Umbu POX, differently, has shown a high level of activity in pH close to neutrality. The pH of xylopodium exsudate was determined here as being 6.12, which was suggested that umbu POX is able to reach maximum activity in natural medium. Umbu POX seen to maintain 95% of activity at 70°C for 8 min (Figure 4), showing a higher thermal stability. Plant POXs have a variable thermal stability, for example: acidic broccoli POX loses 50% activity after 17 min at 65°C (Thongsook et al., 2005). Carrot peroxidase are low stable to high temperature, loses 70% of activity after 10 min at 60°C, while POX of solenaceae species such as tomato, potato and eggfruit are termal stable (Suha et al., 2013).

Most plant peroxidases are glycosylated and may have among 0 to 25% of glycan moieties. This feature is one of the main factors associated to the thermal resistance of these proteins (Ros-Barceló and Pomar, 2002). Sugar is able to protect several macromolecules from denaturation (Neves and Silva, 2007). Purified umbu POX is less thermally stable in its isolated form than as present in crude umbu exsudate (Figure 5). Under a temperature condition of 80°C, the activity of umbu POX is four times higher in crude exsudate then in the purified fraction. Since, xilopodium exsudates are rich in soluble sugars (Silva et al., 2009), is possible that these substances may be acting as a natural thermal protector for umbu POX. Other possibilities must be considered since for

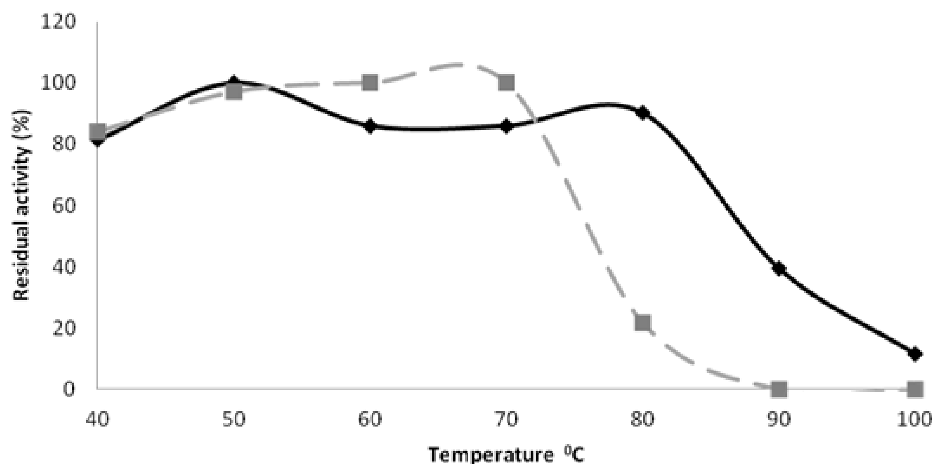

Figure 5. Comparative effect of heating (5 min) on POX activity from purified fraction (■) and from crude xylopodium exsudate extract (♦).

Table 2. Activity of umbu POX under the influence of several metallic ions and organic inhibitors.

Metallic ion and organic inhibitor	Percent activity (%)			
	0.01 mM	0.1 mM	1 mM	10 mM
Mn^{+2}	95	77	77	59
Ca^{+2}	100	111	117	134
Mg^{+2}	100	100	115	115
Azide	100	100	64	3
EDTA	100	100	100	100
Tropolone	100	100	100	100

horseradish peroxidase isoenzyme C (HRP), which has eight N-linked glycans, it was found that, despite glycosylation has significantly enhanced the kinetic stability, it did not influence the thermodynamic stability of this enzyme (Mathé et al., 2010).

A stimulatory effect of Ca^{2+} on umbu POX activity was observed, at concentrations from 0.1 mM onwards. Mg^{2+} had similar effect but at a lower proportion, at concentrations from 1 mM onwards while Mn^{2+} displayed inhibitory effect from concentrations of 0.01 mM onwards (Table 2). The effect of metal ions on peroxidase activity is highly variable over enzymes from different sources, yet the ion Ca^{2+} has important effect on molecular folding and usually shows stimulatory effects on plant class III peroxidases (Ros-Barceló and Pomar, 2002). The effect of Mg^{2+} on plant peroxidases may be inhibitory, as shown for peroxidase from *Jatropha curcas* (Cai et al., 2011) or stimulatory as for *Carica papaya* peroxidase (Pandey et al., 2012). Mn^{2+} ions as well as heme and Ca^{2+} are prosthetic groups for specific peroxidases known for their particular catalytic activity (Mathé et al., 2010). Class III peroxidase are importantes detoxicant enzymes agaist intoxication due to high maganese concentration. Mn^{2+} may be oxidade and insolubilited by guaiacol peroxidases (Christoffers et al., 2007). Umbu peroxidase was poorly inhibited by Mn^{2+} indicating a no specific inhibition or a substrate competition with guaiacol in reaction medium.

Inhibitory effect was not noticed in the presence of EDTA or tropolone, while azide was an effective inhibitor for umbu POX activity, reducing it to 3% when added at a 10 mM concentration (Table 2). EDTA is a metal chelator with affinity to Fe^{3+} and due to this property affects the activity of peroxidase from *Moringa oleifera* and many others peroxidases (Khatun et al., 2012). The absence of inhibition of umbu POX by EDTA indicates that this chelator was unable to fully combine with Fe^{3+} atom found in the enzyme active center, under the adopted experimental conditions. Azide as well as cyanide both strongly binds to iron from heme groups. Azide, cyanide and fluoride are the most thoroughly characterized inorganic inhibitors of peroxidases (Battistuzzi et al., 2004). Tropolone is an effective inhibitor for PPO (Saniewski et al., 2014). Since peroxidases and polyphenoloxidases share common features in their phenolic compounds oxidation mechanisms, mistakes may be committed when discriminating these enzymes.

Table 3. Kinetic parameters (Km, Vmax and Vmax/Km) of umbu POX towards guaiacol and methylcatechol.

Substrate	Km	Vmax	Vmax/Km
Guaiacol	6.83	4.16	0.61
Methylcatechol	22.25	9.77	0.44

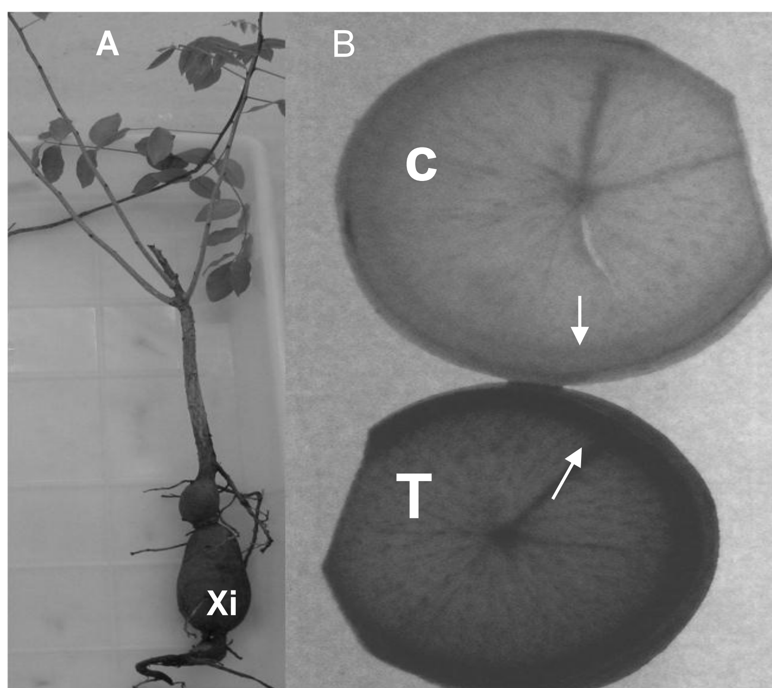

Figure 6. A) Six months old plant of *S. tuberosa*, a xylopodium fullly formed (Xi). **B)** Transversionally sectioned xylopodium immersed in phosphate buffer (Control – **C**), or phosphate buffer plus guaiacol (Test – **T**). The arrow indicate a dark region labeled for POX activity.

The absence of inhibition of umbu POX by tropolone reinforces the idea of its peroxidase nature.

Umbu POX showed a lower Km towards guaiacol than methylcatechol (6.83 and 22.25, respectively). The higher affinity as well as catalytic efficiency towards guaiacol suggests that the enzyme may be a guaiacol peroxidase (Table 3). Peroxidases oxidize several different substrates under the presence of hydrogen peroxide. Although, guaiacol could be a general substrate for all peroxidases, it was better oxidized than methylcatechol by the umbu POX. Peroxidase activity in xilopodium was more concentrated in the outermost layers of tissue, such as the endoderm (Figure 6B). Analyses of peroxidase in sugarcane stems have indicated a similar high level of activity in outer layer of cells in oldest plants, what has been associated to lignification processes in this species (Şişecioğlu et al., 2010). Peroxidases are molecular tools for biotechnology. Despite being one of the most extensively studied enzymes, the search for new peroxidases displaying particular features is yet a relevant scientific goal. In this work, umbu POX, the first protein characterized from *S. tuberosa*, was proven to be a thermal stable enzyme with the desirable ability to function under a wide range of pHs. These features may be valuable for the use of umbu POX as a molecular tool in the future.

Conclusions

In this work, the peroxidase from *S. tuberosa* present at high concentration in exsudate from xilopodium roots, was purified and characterizated. The characteristics of umbu peroxidase enzyme was able to show the activity in a large range of pH and temperature; although many guaiacol peroxidases have similar property, umbu POX

are different among them, and, it is not inhibited by EDTA, and these can be employed in biotechnological engineering. The high level of specific activity of POX peroxidase in umbu xylopodium exsudate, suggests the important role of physilogical condition to *S. tuberosa*, such as it was seen when the level of activity is higher in outer areas of xylopodium tissue.

Conflict of interests

The authors did not declare any conflict of interest.

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