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Study of some characteristics of soluble polyphenol oxidases from six cultivars callus of cassava (*Manihot esculenta* Crantz).

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Key words:
Cassava, callus, polyphenol oxidases, substrates, physicochemical parameters

1 SUMMARY

The objective was to study the influence of some physicochemical parameters on polyphenol oxidases (PPO) activities from callus of cassava (*Manihot esculenta* Crantz). The enzyme was extracted at different pH values, ranging from 2.0 to 11.0, and then treated with anion Dowex ®3% (w/v). The PPO activities were optimally active at pH range 4.5 to 8.0 and temperatures varying from 30 to 55 °C. The enzyme activity remained stable during the thermal treatment at 50 °C for 30 min but, at 100 °C, 60% of PPO activities were lost in most cultivars after 20 min. Catalytic efficiency (v_{max}/k_M) calculated starting with 4 substrates (L-tyrosin, dopamine, pyrocatechol and pyrogallol) showed that dopamine (cultivars *yacé, bonoua2* and *I88/00158*) and pyrocatechol (cultivars *TMS4 (2)1425, TMS30572* and *9620A*), were the best substrates of PPO. For these two diphenolic compounds, PPO activities were 1.2 to 1.8 times stimulated in the presence of 5 to 10 mM of the ions Ca^{2+} and Cu^{2+}. At the same concentrations, Mg^{2+} and Zn^{2+} had almost no effect on the enzymes.

2 INTRODUCTION

Polyphenol oxidases (PPO, EC. 1.14.18.1 and EC. 1.10.3.2) are oxydoreductases with two copper atoms (Krebs *et al.*, 2004) which catalyse the oxidation of phenols to *o*-quinones in the presence of molecular oxygen (Thipyapong *et al.*, 2007). In plants, PPO intervenes in several biological phenomena which proceed during the life of the plant (Kuwabara & katoh, 1999; Doğan *et al.*, 2005). PPO intervenes also in the defence of the plants (Doğan *et al.*, 2005). When a resistant plant was attacked by a pathogenic agent, the synthesis of the PPO was stimulated. In this mechanism , PPO oxide the endogenous phenolic compounds in quinones of which some are toxic for the pathogenic

agents (Weir *et al*., 2004; Felton, 2005; Dogbo *et al*., 2007).

Work carried out on these enzymes showed that several factors influence PPO catalytic activity, namely the pH and the temperature of the reaction mixture, availability and nature of substrate, some organic and inorganic compounds and as well as the origin of an enzymatic extract. Indeed, Chevallier *et al.* (1999) showed that the pH value leading to maximum PPO activities in *Prunus armeniaca* (apricot) was 4.4 when the 4-methylcatechol is used as the substrate. But under similar conditions, optimal pH for chlorogenic acid oxidation was 6.6 (Dijkstra & Walker, 1991). In *Olea europaea* (olives), the oxidation of the caffeic acid was accelerated in the presence of cobalt (Co^{2+}) and manganese (Mn^{2+}) ions (García *et al*., 1996). Work carried out by Barthet (1997) on cassava tubers (*Manihot esculenta*) revealed the presence of three isozymes which were expressed differently according to the mode of extraction. The influence of all these factors on PPO synthesis and activities raised up many studies in various plants: *Nicotiana tabacum* (tobacco) (Shi *et al*., 2001), *Origanum vulgare* (oregano) (Doğan *et al*., 2005), *Trifolium pratense* (red clover) (Schmitz *et al*., 2008).

In this work, the objective was to determine the optima conditions of PPO activities extracted from six cultivars callus of *Manihot esculenta*: two (2) local cultivars (*yacé* and *bonoua2*) and four (4) improved cultivars (*TMS30572, TMS4(2)1425, I88/00158* and *9620A*). The evaluated parameters were the pH, the temperature, phenolic substrates (L-tyrosin, dopamine, pyrocatechol and pyrogallol) and some divalent ions (Ca^{2+}, Cu^{2+}, Mg^{2+} and Zn^{2+}).

3 MATERIAL AND METHODS

3.1 Biological materiel: The cultivars used came from the collection of the National Centre for Agricultural Research (CNRA, Côte d'Ivoire). Callus were obtained by *in vitro* culture of cassava cultivars (*yacé, bonoua2, TMS30572, TMS4 (2)1425, I88/00158 and 9620A*). Petioles of immature sheets resulting from germination under greenhouse of stem fragments were successively cleaned with alcohol 70% (v/v) for 1 min, sodium (hypochlorite) 3.6% (v/v) for 7 to 8 min and rinsed three times with sterile distilled water. After sterilization, these petioles were divided into 0.5 cm long and then cultured on Murashige & Skoog (1962) medium enriched with vitamins B5 (0.46%, w/v) (Gamborg *et al*., 1968), glucose (3%, w/v) and solidified with agar (0.8%, w/v). The medium was supplemented with 2, 4-dichloro-phenoxyacetate (0.2 mg/L), 6-benzylaminopurine (0.5 mg/L) and picloram (0.2 mg/L) as growth regulators. This medium was autoclaved for 30 min at 121 °C, and the callus obtained after six weeks of culture was used.

3.2 Extraction of PPO: Polyphenol oxidases extraction was modified according to the method of Dogbo *et al.* (2007). The extraction buffer of enzymes varied according to the cultivar and the substrates used. One (1) g of fresh callus was crushed in 10 mL of 0.2 M Tris-HCl buffer of pH 8 for cultivars *yacé and bonoua2*. For cultivars *TMS4 (2)1425* and *I88/00158*, 0.2 M sodium phosphate and 0.1 M citrate for buffer pH 4.5 were used. Also 0.2 M sodium phosphate buffer pH 6 for cultivars *TMS30572* and *9620A* was used. After centrifugation at 16000 x g for 30 min at 4 °C (centrifuge, SELECTA), the supernatant recovered constituted the crude extract. This extract was treated with anion Dowex® 3% (w/v) was shaken for 30 min at 4 °C before being centrifuged under the same conditions. The supernatant resulting from this second centrifugation constituted the partially purified enzymatic extract.

3.3 Measurement of PPO activities: PPO activities were determined by the modified method of Dogbo *et al.* (2007). The enzyme activity was assayed in 3 mL of reaction mixture, consisting of 50 µL of partially purified enzymatic extract and 50 mM [PPO extracted from *yacé* (*y*PPO)] or 100 mM [PPO extracted from *bonoua2* (*b*PPO) and *I88/00158* (*i*PPO)] of dopamine. For PPO extracted from cultivar *9620A* (*a*PPO), and cultivars *TMS4 (2)1425* (t_4PPO) and *TMS30572* (t_{30}PPO), 90

mM and 125 mM of pyrocatechol were used, respectively. The reaction mixture was adjusted to 3 ml with extraction buffer. After 5 min incubation at 30 °C (t_{30} PPO), 35 °C (*y*PPO), 40 °C (t_4PPO and *i*PPO), and 55 °C (*b*PPO and *a*PPO), the reaction mixtures were cooled in a controlled bath at 4 °C. PPO activities were determined by measuring the absorbance (spectrophotometer, Milton Roy) at 470 nm (*b*PPO, *i*PPO and *y*PPO) or 420 nm (t_{30}PPO, t_4PPO and *a*PPO). The enzymatic activity was expressed in UI/mg protein (µmole of quinones/ min/ mg protein) or expressed as a percentage of activity.

3.4 Protein determination: The protein concentration of the partially purified extract was assayed according to the method of Bradford (1976) by using bovine serum albumin as the standard.

3.5 Influence of pH on PPO activities: The PPO activities were measured at different pH values, ranging from 2.0 to 11.0. Three buffers were used for this experiment: 0.2 M sodium phosphate – 0.1 M citrate buffer for pH values, ranging from 2.0 to 5.0; 0.2 M sodium phosphate buffer for pH between 5.0 and 7.5 and 0.2 M Tris-HCl buffer for pH values ranging from 7.5 to 11.0. The substrates L-3-[4-hydroxyphenyl] alanine (L-tyrosin, MERCK), 3, 4-dihydroxyphenethylamine (dopamine, SIGMA), 1, 2-benzenediol (pyrocatechol, SIGMA) and 1, 2, 3-trihydroxybenzene (pyrogallol, LABOSI) were prepared in buffers at different pH. The reaction mixture adjusted to 3 mL with extraction buffer contained 50 µL of partially purified enzymatic extract, and 100 mM of substrate. The PPO activities were determined at 30 °C under the standard test conditions and three replicates were carried out in each case. *O*-quinones content starting from the dopamine and L-tyrosin was estimated respectively at 470 and 472 nm (Wititsuwannakul *et al.*, 2002) and those resulting from pyrocatechol and pyrogallol at 420 nm (Duangmal & Owusu-Apenten, 1999).

3.6 Effect of temperature on PPO activities: The influence of temperature on PPO activities was investigated by equilibrating the reaction mixture (buffer plus substrate) in the water bath (10-80 °C) for 10 min before introducing the enzymatic extract. PPO activities were measured at different temperatures, in range 10-80 °C (Doğan *et al.*, 2005). The study of thermal inactivation was carried out at 50 and 100 °C with dopamine and pyrocatechol. For each cultivar, tubes containing 1 mL of partially purified extract were preincubated at these temperatures for up to 30 min. Aliquots were withdrawn at intervals (5 min) and immediately cooled at 4 °C. Residual activities, determined under the standard test conditions, were expressed as percentage of zero-time control of untreated enzymes.

3.7 Influence of substrate concentration on PPO activities: Various concentrations of substrate (2.5, 5, 10, 15 and 20 mM) were used. Michealis constant (K_M) and the maximum velocity (V_{max}) were calculated from a plot of 1/activity versus 1/substrate concentration by the method of Lineweaver & Burk (1934). Thus, the criterion for the best substrate was the catalytic efficiency (v_{max}/k_M) (Duangmal & Owusu-Apenten, 1999).

3.8 Influence of metal ions Ca^{2+}, Cu^{2+}, Mg^{2+} and Zn^{2+} on PPO activities: The influence of metal ions on PPO activities was studied according to the method of Montero *et al.* (2001). The effectors Ca^{2+}, Cu^{2+}, Mg^{2+} and Zn^{2+} were introduced into the reaction mixture using respectively $CaCl_2$, $CuCl_2$, $MgCl_2$ and $ZnCl_2$. Concentrations were 1, 5, 10, 15, 20, 25 and 30 mM. PPO activities were started by addition of dopamine (cultivars *yacé, bonoua2 and I88/00158*) or pyrocatechol (cultivars *TMS4 (2)1425, TMS30572* and *9620A*). The relative activity was calculated by the expression $T = (A^* / A) \times 100$, where A and A * represent the absorbance in absence and in presence of the effectors.

3.9 Data analysis: Data shown are means of triplicates for each treatment. Statistical analyse was performed with the analysis of variance ANOVA with the software SPSS version 11.5. Means were compared with Duncan multiple range comparison test ($\alpha = 0.05$).

4 RESULTS AND DISCUSSION

4.1 Optimal pH of PPO activities: The effect of pH on PPO activities of cassava callus was examined at different pH values, ranging from 2.0 to 11.0 (Figure 1).

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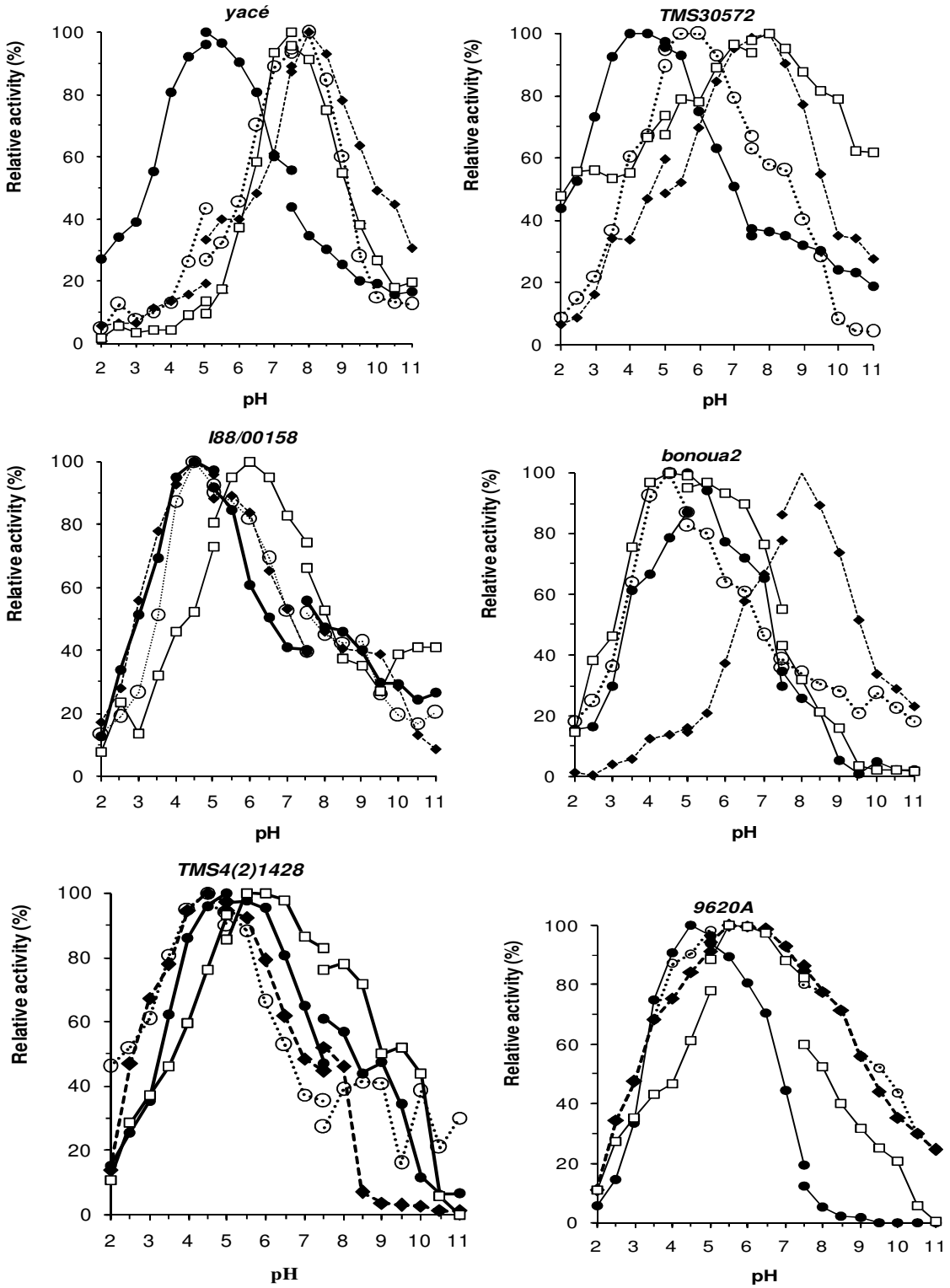

Figure 1 : pH-activity profile from cassava callus PPO in 0.2 M sodium phosphate – 0.1 M acetate buffer (pH 2.0 - 5), 0.2 M sodium phosphate buffer (pH 5.0 -7.0) and 0.2 M Tris-HCl buffer (pH 7.0 - 11.0). Substrates: L-tyrosin (●), dopamine (◆), pyrocatechol (○), pyrogallol (□)

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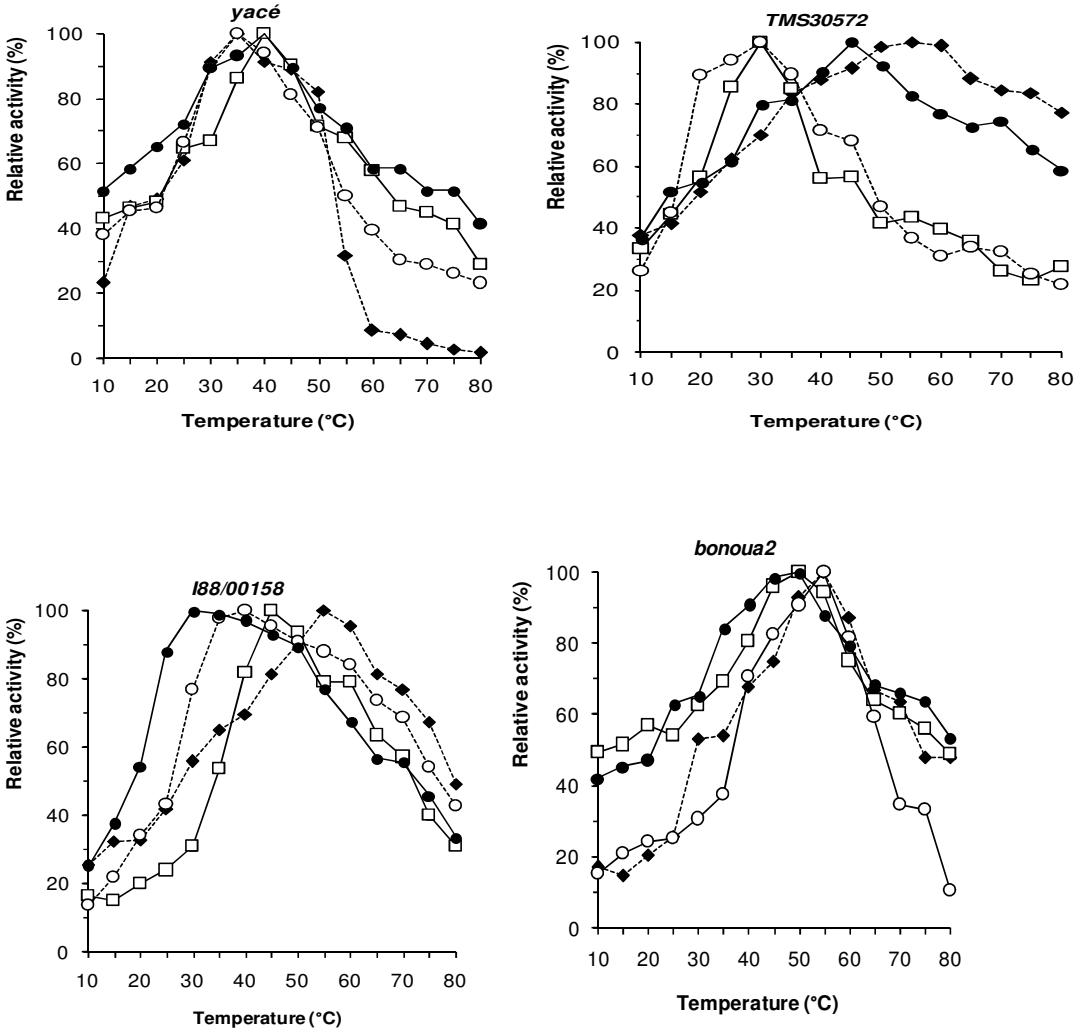
In all cultivars, the optimum pH of PPO activities varied from 4.5 to 8.0 according to the substrates tested. This study showed that PPO activities were optima in pH (4.5 and 5.0) with L-tyrosin and, between 4.5 and 8.0 with di- and triphenolic substrates. Similar results on pH profile in various plants were obtained by several authors (Barthet, 1997; Kavrayan & Aydemir, 2001; Montero et al., 2001). Indeed, the work carried out by Barthet (1997) on isozymes isolated from tuber of Manihot esculenta indicated that optima pH were 6.5, 6.8 and 7.5 with dopamine and pyrocatechol. Those carried out by Montero et al. (2001) on Panaeus japonicas (prawns) indicated optima pH at 5.0 and 8.0 when dopamine was used. In the same way, PPO extracted from roots and buds of O. vulgare had a maximum activity at pH 8.0 with pyrocatechol (Doğan et al., 2005). According to Alyward & Haisman (1965) cited by Aydemir & Akkanl (2006), the multiplicity of optima pH of PPO activities in plants could be explained by method of extraction, substrates used and localization of the enzyme in the vegetable cell.

4.2 Optimal temperature of PPO activities: Figure 2 presents the optima temperatures for PPO activities extracted from Manihot esculenta. These temperatures varied from 30 to 55 °C according to cultivars and substrates.



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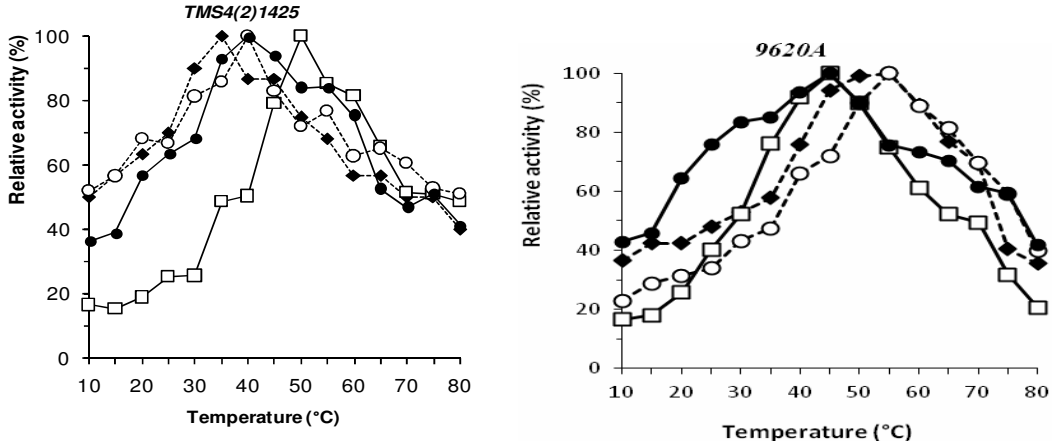
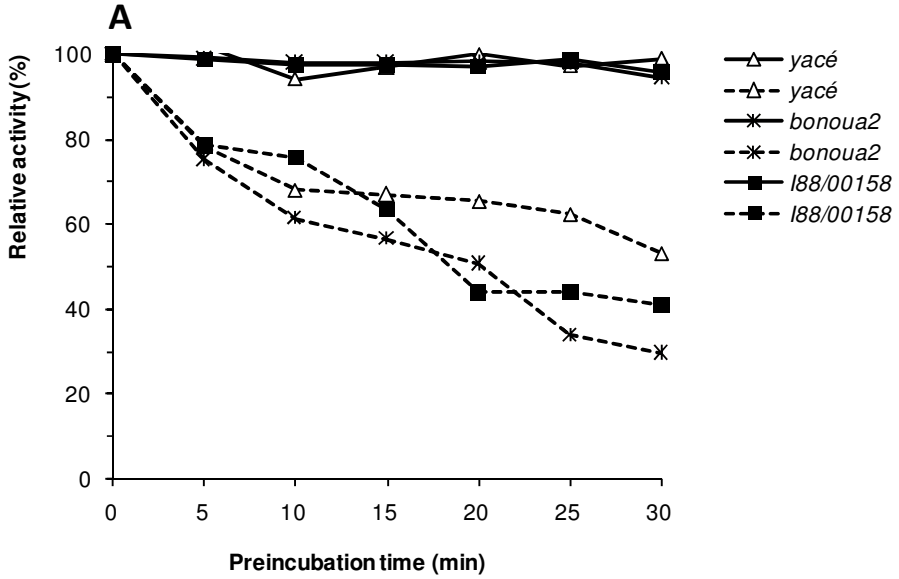


Figure 2: Temperature activity profiles from cassava callus PPO in appropriate pH for each substrate. Substrates: L-tyrosin (●), dopamine (◆), pyrocatechol ( ), pyrogallol (□)

This study results are in agreement with those of Dincer *et al.* (2002). Indeed, in *Mespilus germanica* (medlar), these authors recorded an optimal temperature of PPO activity at 55 °C with catechol and L-dopamine, whereas those of *Mentha piperita* (peppermint) were located at 30, 50 and 55 °C respectively with catechol, D-L dopa and L-dopa (Kavrayan & Aydemir, 2001).

Concerning the study of thermal stability (Figure 3), at 50 °C, PPO preserved approximately 95% of their catalytic activity after 30 min preincubation. At 100 °C, 20% loss in PPO activities were observed during the first 5 min in all cultivars, however, at the end of 30 min more than 60% (*I88/00158* and *TMS30572*) to 70% (*bonoua2* and *TMS4(2)1425*) activity was lost. Mazzafera & Robinson (2000) had the same results after exposing PPO extracted from sheets of coffee-tree (*Coffea arabica L.*) at 50 and 90 °C for 30 min. These authors revealed that at 50 °C, PPO conserved 100% of their catalytic activity whereas at 90 °C, 90% of the initial activity was almost lost.



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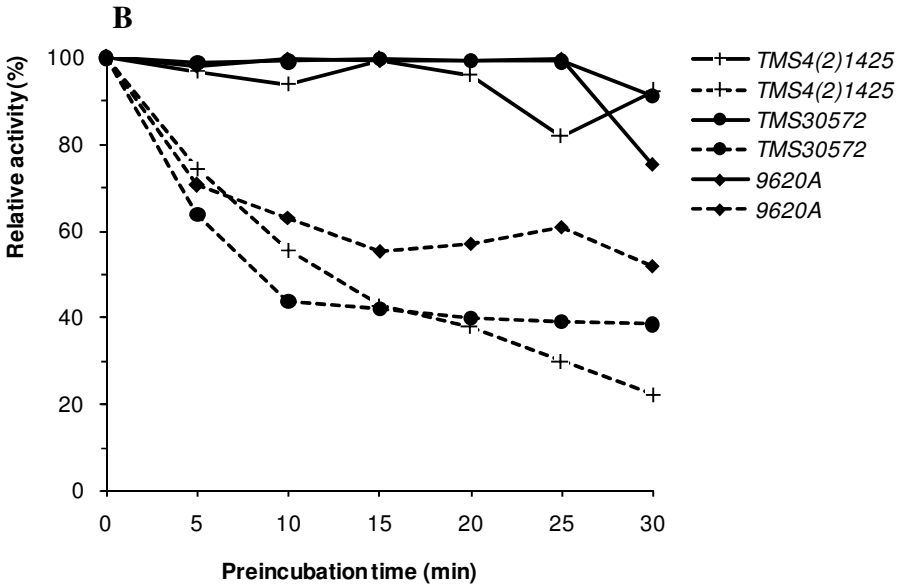


Figure 3: Thermal stability profiles of cassava callus PPO. The enzyme solution was preincubated for various time intervals (5-30 min) at the specified temperature (50 and 100°C) and rapidly cooled. The activity measured with non preincubated extract was taken as 100%.
A: dopamine substrate, **B**: pyrocatechol substrate, 50 °C (—), 100 °C (- - -).

43 Enzyme kinetics: The effect of substrates concentrations ranging from 2.5 to 20 mM on *Manihot esculenta* PPO activities was investigated. The K_M, V_{max} and V_{max}/K_M values obtained for *y*PPO, *b*PPO, t_{30}PPO, t_4PPO, *t*PPO and *a*PPO starting with 4 substrates (L-tyrosin, dopamine, pyrocatechol and pyrogallol) are summarized in Table 1.

Table 1: Kinetic parameters for the oxidation of phenolic substrates (L-tyrosin*, dopamine**, pyrocatéchol** and pyrogallol***) by cassava callus PPO

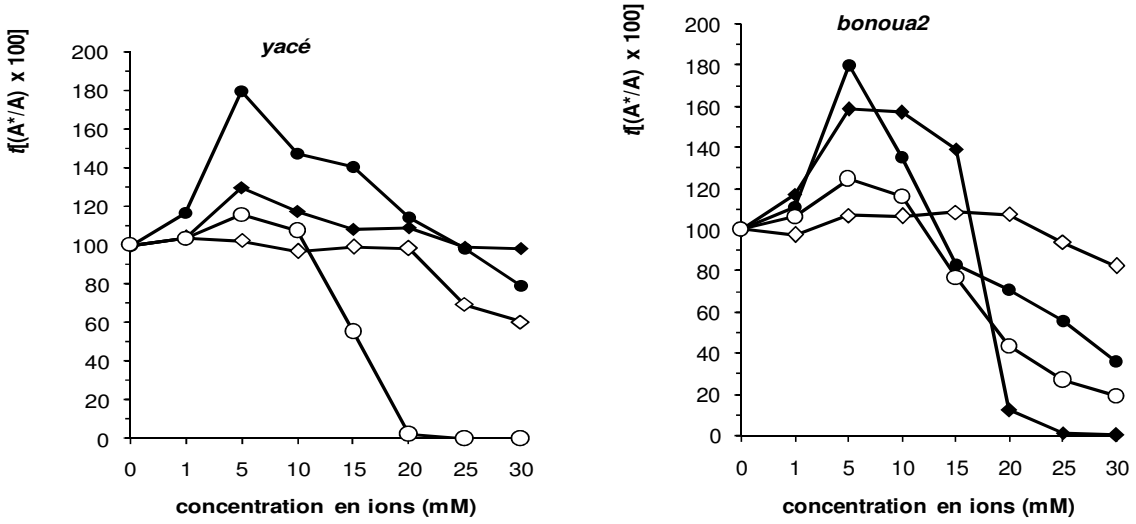
Cassava cultivars	Phenolic substrates	Kinetic parameters		
		Vmax (UI/mg protein)	KM (mM)	Vmax/KM
	L-tyrosin	1.93	20.05	0.09
	dopamine	27.78	26.31	1.06
yacé	pyrocatechol	28.57	55.17	0.52
	pyrogallol	3.55	8.11	0.44
	L-tyrosin	0.46	14.57	0.03
	dopamine	4.00	1.08	3.70
bonoua2	pyrocatechol	3.70	9.79	0.38
	pyrogallol	2.15	11.21	0.19
	L-tyrosin	2.63	18.89	0.13
	dopamine	13.70	21.71	0.63
TMS30572	pyrocatechol	14.93	6.60	2.26
	pyrogallol	6.71	3.99	1.68
	L-tyrosin	1.41	9.59	0.14
	dopamine	15.38	28.88	0.53
TMS4(2)14	pyrocatechol	22.22	33.33	0.63

25				
	pyrogallol	6.41	17.51	0.37
	L-tyrosin	4.49	9.99	0.44
	dopamine	45.45	18.09	2.51
I88/00158	pyrocatechol	76.92	26.85	2.87
	pyrogallol	37.04	26.89	1.38
	L-tyrosin	10.41	95.18	0.01
	dopamine	9.35	30.52	0.31
9620A	pyrocatechol	12.99	14.06	0.92
	pyrogallol	6.54	32.11	0.20

*monophenolic substrate; **diphenolic substrate; ***triphenolic substrate

For t_{30}PPO, t_4PPO, *i*PPO and *a*PPO, the catalytic efficiency were high with pyrocatechol followed by dopamine except for t_{30}PPO where dopamine was replaced by pyrogallol. Pyrocatechol thus was regarded as a better substrate of PPO extracted from these cultivars. As concern *i*PPO, statistical analysis carried out indicated that there is no significant difference (p = 0.257) between V_{max}/K_M ratio obtained with dopamine (2.51) and pyrocatechol (2.87). Consequently, *i*PPO had high affinity for dopamine (lowest K_M value). For *y*PPO and *b*PPO, dopamine was the best substrate followed by pyrocatechol. All in all, PPO extracted from *Manihot esculenta* presented a low affinity for L-tyrosin (monophenol) and a relatively average activity for pyrogallol (triphenol). In contrast, the *o*-diphenols used (dopamine and pyrocatechol) were the best substrates for these enzymes. Similar results were obtained by several authors (Mazzafera & Robinson, 2000; Dincer *et al.*, 2002) where *o*-diphenols were the best substrates compared to mono- and triphenols. This low affinity for monophenol (L-tyrosin) substrate could be explained by the fact that, in higher plants, the monophenol oxidase / diphenol oxidase ratio generally low (1/10) climb down even to 1/40 (Nicolas *et al.*, 1994; Sanchez-Ferrer *et al.*, 1995). Moreover, for the monophenol oxidasic activity, PPO must be in the form "Oxy {Cu^{II} - O_2^2 - Cu^{II}}"; this form was only able to bind the monophenol (L-tyrosin) to the enzyme. However, according to Eicken *et al.* (1999), the form "Oxy" of the catalytic mechanism of PPO seems much more unstable than the form "Met {Cu^{II} - Cu^{II}}", which strongly reduces their affinity for monophenols.

4.4 Influence of metal ions on PPO activities: The ions Cu^{2+} and Ca^{2+} displayed activity on PPO at low concentrations; but this stimulation varies with cultivars (Figure 4).



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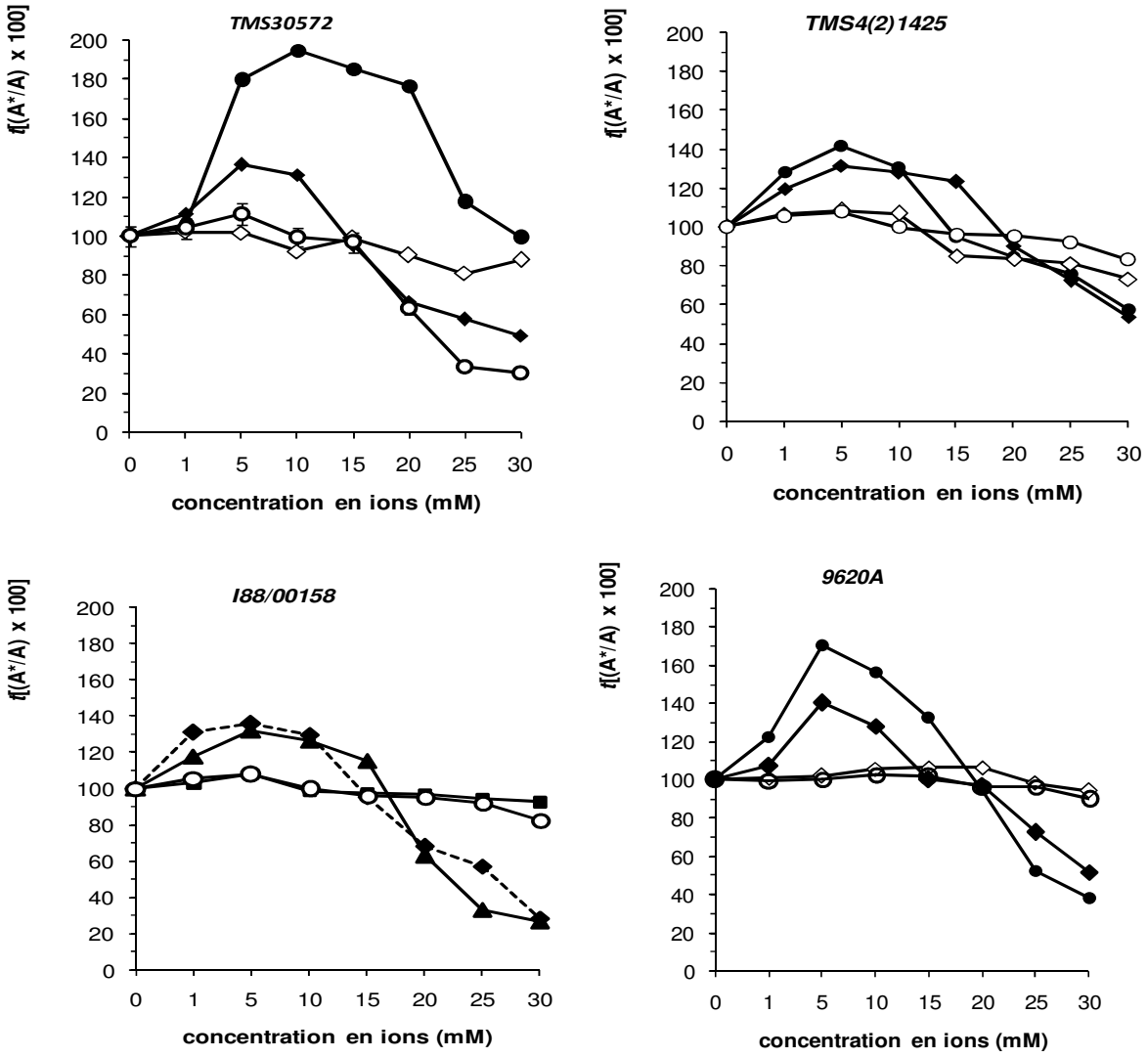

Figure 4: Effect of ions (Cu^{2+}, Mg^{2+}, Ca^{2+} and Zn^{2+}) concentration on cassava callus PPO activities. Ions were introduced in the form of $CaCl_2$, $MgCl_2$, $CaCl_2$ and $ZnCl_2$, respectively. $CuCl_2$ (●), MgCl2 (◇), $CaCl_2$ (○), ZnCl2 (◆).

PPO from *Manihot esculenta* was more stimulated when these ions were at 5 mM for all cultivars except *TMS30572* which was highly enhanced in the presence of 10 mM Cu^{2+}. The activation rate varied from 40 to 95% with the strongest value for PPO extracted from cultivar *TMS30572* (95%). In the presence of Ca^{2+}, the maximum values of activation of the enzyme border 35% for all PPO except *b*PPO (61%) and *i*PPO (15%). As regards cations Zn^{2+} and Mg^{2+}, they do not exert any effect on the activity of enzymes at low concentration (5 and 10 mM). In addition, high concentrations (25 to 30 mM) generally inhibited PPO activities ($T = 0$). The statistical test indicated a significant difference ($p = 0$) of salt influence on PPO activities. The stimulation of *Manihot esculenta* PPO by Cu^{2+} is tempting to speculate that these enzymes require the presence of copper atoms at their active site to

perform catalysis. However, the results from this study differed from those of Aydemir & Akkanl (2006) which indicated that Cu^{2+} ($CuSO_4$) did not have any influence on PPO activities, whereas Ca^{2+} ($CaCl_2$) strongly inhibited *Apium graveolens* (celery) PPO.

5 CONCLUSION

All studied enzymatic parameters (pH, temperature, nature and concentration of the substrates, metal cations) differently influenced the activity of PPO according to cultivars. These results could testify the presence of several isozymes of PPO in *Manihot esculenta*. Thus, it would be interesting to carry out purification and electrophoresis of these enzymes in order to confirm the presence or not of these various forms.

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