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COMPARATIVE ACTIVITIES OF PHENYLALANINE AMMONIA-LYASE AND TYROSINE AMMONIA-LYASE AND PHENOLIC COMPOUNDS ACCUMULATED IN CASSAVA ELICITED CELL

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ABSTRACT

Plants respond to attack by pathogens by initiating a change in cellular metabolism, leading to synthesis of antifungal proteins, production of phytoalexins and/or accumulation of phenolic compounds, namely lignins and salicylic. Lignins reinforce pectocellulosic cell walls and limit the invasion of plant tissues by pathogens; while salicylic acid plays a role in signals plant defense against pathogens. The objective of this study was to evaluate the activities of phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) and tyrosine ammonia-lyase (TAL, EC 4.3.1.5); and to determine the level of their involvement in the biosynthetic pathway of these phenylpropanoids in cells of cassava (*Manihot esculenta* Crantz, cv *Yacé*) elicited with salicylic acid (SA). PAL and TAL activities were demonstrated in crude extract enzyme. PAL activity was 9.8 times greater than that of TAL in the pellet obtained with 20% (w/v) ammonium sulphate. In the extract treated with Dowex 2 (cationic), TAL activity was 36.7 times greater than that of PAL. pH and temperature optima of PAL (8; 40 °C) differed from those of TAL (8.5; 30 °C). In the presence of SA, PAL and TAL activities were respectively maximum 24 and 72 hr after inoculation. TAL activity and induced phenols were much higher than PAL. PAL and TAL activities were optimised respectively, by 75 and 100 µM of SA. The synthesis of phenolic compounds was concomitant with enzymes stimulation. These results show that PAL is different from TAL and the two enzymes are involved in the biosynthetic pathway of phenylpropanoids in cassava.

Key Words: *Manihot esculenta*, phenylalanine ammonia-lyase, salicylic acid

RÉSUMÉ

Les plantes répondent à l'attaque des pathogènes par l'initiation d'un changement du métabolisme cellulaire, conduisant à la synthèse des protéines antifongiques, la production des phytoalexines et/ou l'accumulation des composés phénoliques appelés lignines et salicyliques. Les lignines renforcent les membranes cellulaires péctocellulosiques et limitent l'invasion des tissues des plantes par les pathogènes, alors que l'acide salicylique joue un rôle dans les signaux de défense des plantes contre les pathogènes. Cette étude avait pour objectif d'évaluer les activités du phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) et du tyrosine ammonia-lyase (TAL, EC 4.3.1.5) ainsi que de déterminer le niveau de leur implication dans la voie biosynthétique de ces phénylpropanoides dans les cellules de manioc (*Manihot esculenta* Crantz, cv *Yacé*) dues à l'acide salicylic. Les activités PAL et TAL étaient démontrées dans un extract de base d'enzyme. L'activité PAL était 9.8 fois plus élevée que celle de TAL dans la boulette obtenue du sulfate d'ammonium (w/v) 20%. Dans l'extract traité avec

Dowex 2 (cationique), l'activité TAL était 36.7 fois plus élevée que celle du PAL. Le pH et la température optima de PAL (8; 40 °C) différaient de ceux du TAL (8.5; 30 °C). En présence de l'acide salicylique, les activités PAL et TAL étaient respectivement maximum à 24 et 72 heures après inoculation. L'activité TAL ainsi que les phénols induits étaient plus élevés que PAL. Les activités PAL et TAL étaient plus optimisées respectivement par 75 et 100 µM de l'acide salicylique. La synthèse des composés phénoliques était concomitante avec la stimulation enzymatiques. Ces résultats montrent que PAL est différent de TAL et les deux enzymes sont impliqués dans la voie biosynthétique des phénylpropano¿des dans le manioc.

Mots Clés: *Manihot esculenta*, phénylalanine ammonia-lyase, acide salicylique

INTRODUCTION

Plants respond to pathogen attack through reactions triggered upon recognition of pathogens by plant (Pautot *et al*., 1999). The process is characterised by a change in cellular metabolism leading to the synthesis of antifungal proteins, production of phytoalexins and/or accumulation of phenolic compounds (Lange *et al*., 1995; Bacher *et al*., 2001). Among these phenylpropanoids synthesised are lignins and salicylic acid. Lignins reinforce pectocellulosic cell walls and limit the invasion of plant tissues by pathogens (Pautot *et al*., 1999; Lateur, 2002); while salicylic acid plays a fundamental role in signaling plant defense against pathogens (Martinez *et al*., 2000). Accumulation of this molecule in the plant or its application on plant tissues induces the synthesis of phenylalanine ammonia-lyase (PAL, EC 4.3.1.5), a key enzyme in the biosynthetic pathway of phenylpropanoids (Rösler *et al*., 1997; Wajahatullah *et al*., 2002; Berner *et al*., 2006).

However, the work of Dogbo *et al*. (2008) on seedlings of cassava, cv *Yacé*, elicited with salicylic acid showed that the amount of total phenolics ethano-soluble increased during the treatment, despite the drastic drop in PAL activity. This has led to a hypothesis of the existence of another pathway for the biosynthesis of these compounds: the path of tyrosine ammonia-lyase (TAL, EC 4.3.1.5) (Berner *et al*., 2006; Dogbo *et al*., 2007; Dogbo *et al*., 2008).

The objective of this study was to evaluate the activities of PAL and TAL, and their level of involvement in the biosynthetic pathway of phenolic compounds using cassava as the test crop.

MATERIALS AND METHODS

Plant material. The plant material used consisted of cells obtained from fragments of petioles culture of cultivar *Yacé*. Petioles of immature leaves of seedlings grown in a greenhouse were successively cleaned with 70% alcohol for 30 seconds, then with 2.4% sodium hypochlorite for 7-8 min and later rinsed four times with sterile distilled water. They were cut into fragments of 0.5 cm and placed in petri-dishes containing 20 ml of culture medium. This medium solidified by 2.5 g of Gelrite containing 0.46% Murashige and Skoog (1962) enriched with vitamins B_5 of Gamborg *et al*. (1968), 30% glucose, growth regulators such as 2,4-dichlorophenoxyacetic acid (0.2 mg L^{-1}) and 6-benzylaminopurine (0.5 mg L^{-1}). It was then autoclaved for 30 min at 121 °C and 1 bar. The callus obtained after 6 weeks of culture were used to prepare cell suspension.

Cell suspension and elicitation. Cell suspension was performed using the methods of Bui and Tran (2000). Friable callus from 6 weeks were separated and cells of same size were harvested after sorting through a sieve (diameter 2 mm). A sample of 1.5 g of cells was transferred into Erlenmeyer flasks each containing 30 ml of the same liquid culture medium. To condition the cells in hydroponics, the containers were placed for 3 days on an orbital shaker (Selecta) at 100 rpm in the culture room (temperature: 25 °C ± 2; luminous flux: 2000 lux, photoperiod: 16 hr). Cells were then elicited by salicylic acid (125 µM). For control cells, salicylic acid was replaced with sterile distilled water.

Extraction and assay of enzymes. Phenylalanine ammonia-lyase and tyrosine ammonia-lyase were

extracted using the method of Berner *et al*. (2006) with some modification. After the screening of pH, optimum pH was selected; it was 8 for PAL and 8.5 for TAL. One gramme of cells was ground in 10 ml of sodium borate buffer 50 mM, pH 8 and 8.5 for PAL and TAL, respectively. The homogenate was centrifuged at 5000 trs. min^{-1} for 15 min at 4 °C. The supernatant constituted the crude extract enzyme. The supernatant of PAL was precipitated with ammonium sulphate 20% (w/v); while that of TAL was treated with Dowex cation 0.3% (w/v). Partially purified extracts were used for assays of enzyme activities.

Assay of PAL and TAL was performed using the method of Berner *et al*. (2006), by using the optimal pH (8 for PAL and 8.5 for TAL) of these enzymes. The reaction mixture consisting of 0.5 ml of enzyme extract and 150 mM of L-phenylalanine or L-tyrosine was adjusted to 3 ml with the extraction buffer. Incubation was done at 30 °C for TAL and 40 °C for PAL for 30 min. PAL activity was determined at 290 nm, following the formation of *E*-cinnamic acid; and that of TAL at 310 nm by formation of *p*-coumaric acid. Specific activity of enzymes was expressed as millimoles *E*-cinnamic acid or *p*-coumaric acid formed per minute per milligramme of protein (mmol. min^{-1} mg prot).

Extraction and determination of phenolic compounds. Extraction of phenols was made by grinding 0.5 g of cells in 5 ml of ethanol 80% (v/v) containing 0.1 g of sodium dithionite (Dogbo *et al*., 2007). The homogenate was centrifuged at 5000 trs. min^{-1} for 10 min, at 15 °C. The supernatant obtained was used for the determination of phenols. Presence of phenols was revealed in 1 ml of this extract by adding 0.5 ml of Folin-Ciocalteau (0.5 N) and 1.5 ml of sodium carbonate (17% w/v) (Swain and Hillis, 1959). After 45 min. of the reaction, absorbance (A) was determined at 725 nm. Phenolics content were calculated according to a standard curve obtained from a Folin-Ciocalteu reaction with tyrosine, and expressed in microgramme tyrosine equivalent per gramme of fresh weight (µg TYR. g fresh wt^{-1}).

Determination of protein. Protein assay was performed according to the method of Bradford (1976). The bovine serum albumin was used as protein standard.

Optimisation of enzymes extraction. To optimise the extraction of enzymes, the crude extracts of PAL and TAL were subjected to either an ammonium sulphate precipitation (20 to 100% w/v) or a cationic Dowex 2X8 purification -100 or anionic 50 x 4-100. Ammonium sulphate precipitation was performed according to the nomogram of Jakoby *et al*. (1976). Ammonium sulphate was dissolved in the enzyme extract and the whole was mixed and incubated for 15 min at 4 °C. After centrifugation at 5000 tr. min^{-1} for 15 min at 4 °C, the precipitated proteins were recovered in the pellet and then homogenised in extraction buffer. The homogenate constituted the partially purified enzyme extract.

Dowex purification was made using the method of Regnier (1994) by utilising the best concentration of this resin. To 10 ml of crude extract, 0.1 g of Dowex (cationic or anionic) was added. The whole was stirred and incubated for 15 min at 4 °C. The homogenate was centrifuged as before. The supernatant collected was used as partially purified enzyme extract. The treatment that gave the highest activity was selected for further work.

pH and incubation temperature on the enzyme activities. The Influence of pH on enzyme activity was evaluated from pH 2 to 11. The extraction and assay of enzymes were performed at each pH at 30 °C. Three buffer solutions were used for this experiment: 0.1 M sodium phosphate - 0.1 M citrate buffer (pH 2-7), 0.2 M borate – 0.05 M sodium borate buffer (pH 7-9) and 0.2 M glycine – 0.2 M NaOH buffer (pH 9-11).

The optimum temperature for enzymes incubation was determined using the optimumal pH for each enzyme. Incubations were made at temperatures from 5 to 60 °C, at 5 °C intervals.

Contact time of salicylic acid on enzymes activity and phenolic compounds accumulation. To evaluate this parameter, the cells elicited with salicylic acid (SA) 125 µM were harvested at different times of contact with the inoculum (0.25, 0.5, 1, 4, 8, 12, 24, 48, 72, 96 and 120 hr). After

filtration through a web of 800 microns mesh, 0.5 and 1 g of cells of each treatment were taken for the respective extraction of phenolic compounds and enzymes. For each treatment time, 3 replicates were considered.

Salicylic acid concentration. The amount of salicylic acid (SA) required to induce maximal activity of the enzymes was evaluated at optimal contact time of PAL (24 hr) and TAL (72 hr). To do this, cells in the suspension were treated with different doses of SA (5, 25, 75, 100, 125, 250 and 500 µM).The cells, harvested after the respective times, were used to evaluated enzymes activities and quantification of phenolic compounds. For each concentration tested, three repetitions were performed.

Statistical analysis of data. The experiment was repeated three times and the data were subjected to analysis of variance of the Statistical Package for Social Scientists (SPSS) version 11.5. The differences between means at 95% confidence level were calculated using the Least Significant Difference (LSD) test.

RESULTS

Optimisation of enzymes extraction. The highest activity of PAL was detected in pellet obtained with 20% of ammonium sulphate (Table 1). For TAL, the highest activity occurred with Dowex cation. Subsequently, the partially purified enzyme extracts with ammonium sulphate (20%) and Dowex cation were used, respectively, for the assay of PAL and TAL.

pH and temperature on enzymes activities. The optimum pH for enzymes was located in the basic pH range; namely 8 to 8.5 for PAL and TAL (Fig. 1). PAL activity was at maximum at 40 °C and that of TAL climaxed at 30 °C (Fig. 2).

Contact time of salicylic acid, enzyme activity and phenolic compounds. Table 2 presents PAL and TAL activities. In the untreated cells, the activities remained almost constant; while in the elicited cells, an increase in activity of both enzymes was observed.

Enzyme activity increased at the beginning of treatment, but declined after 30 min. of

TABLE 1. Activities of PAL and TAL extracted from cells cassava cv *Yacé* treated with ammonium sulphate and Dowex

Treatment	Enzymes activities (10^{-8} mmol min^{-1} mg. prot.)		
	Nature	PAL	TAL
Witness	Crude extract	14.31 ± 0.55	13.01 ± 0.50
Dowex 2	2X8 - 100	0.54 ± 0.43	19.80 ± 0.17
Dowex 50	50X4 - 100	2.87 ± 0.19	11.00 ± 0.06
Ammonium sulphate(%)	20 (supn.)	12.00 ± 0.99	8.60 ± 0.57
	20 (culot)	21.61 ± 0.53	2.21 ± 0.11
	40 (supn.)	8.40 ± 0.96	7.11 ± 0.02
	40 (culot)	9.62 ± 0.02	4.30 ± 0.04
	60 (supn.)	6.70 ± 1.28	7.00 ± 0.03
	60 (culot)	2.71 ± 0.07	9.72 ± 0.04
	80 (supn.)	6.21 ± 0.43	5.41 ± 0.47
	80 (culot)	0.79 ± 0.04	8.60 ± 0.09
	100 (supn.)	5.02 ± 0.50	4.83 ± 0.11
	100 (culot)	0.32 ± 0.01	5.91 ± 0.17

Activity values are averages of three independent determinations ± standard deviation. Supn : supernatant

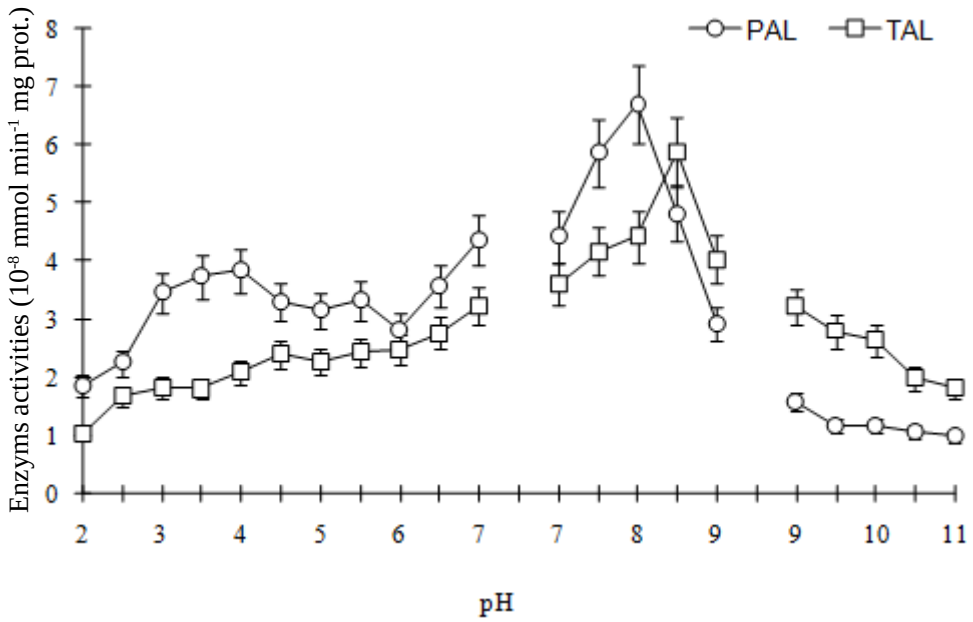

Figure 1. Determination of pH optimal of phenylalanine ammonia-lyase and tyrosine ammonia-lyase activities extracted from cells of cassava cv *Yacé*. 0.1 M sodium phosphate -0.1 M citrate buffer (pH 2-7), 0.2 M borate – 0.05 M sodium borate buffer (pH 7-9) and 0.2 M glycine – 0.2 M NaOH buffer (pH 9-11). Prot. = 0.05 mg ml^{-1}.

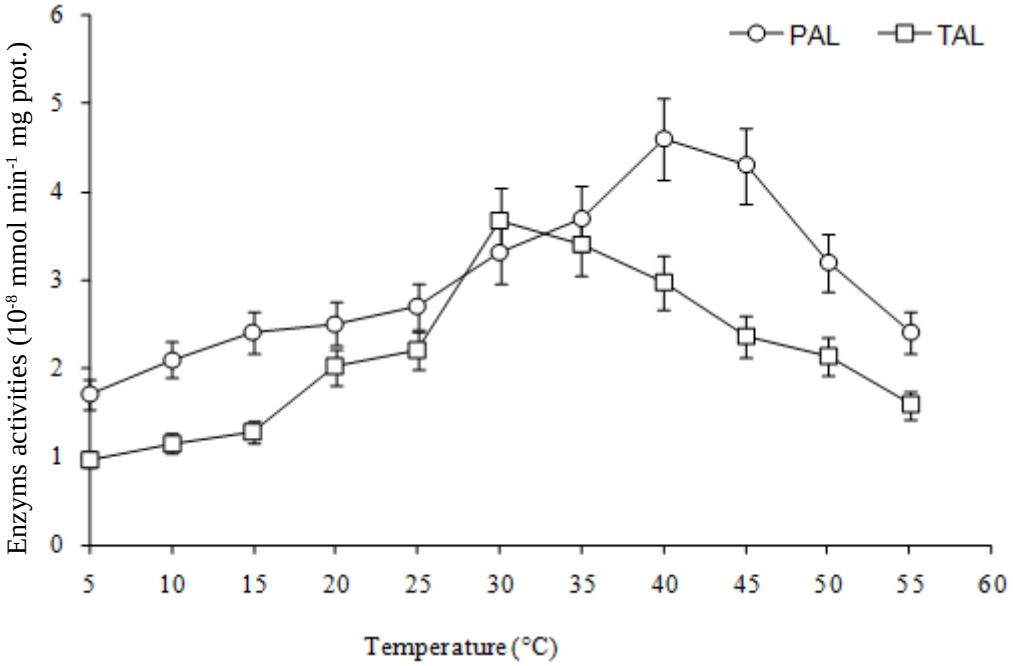

Figure 2. Influence of incubation temperature on activities of phenylalanine ammonia-lyase and tyrosine ammonia-lyase extracted from cells of cassava cv *Yacé*; Prot. = 0.05 mg ml^{-1}.

inoculation. PAL activity reached optimum 24 hr after inoculation and was 12 times higher than that of the control. On the other hand, the activity of TAL reached a maximum within 72 hr of the treatment and was 17 times higher than that of the control. Beyond the optimum point, enzyme activity decreased gradually (Table 2). For each treatment time, the activity of TAL was greater than that of PAL.

Accumulation of phenolic compounds in treated cells varied with time of contact (Fig. 3). After 25 min. of inoculation, the amount of phenolic compounds doubled. This was followed by a slight decrease during 1 to 4 hr, and thereafter,

TABLE 2. Correlation between contact time of salicylic acid (125 µM) and activities of PAL and TAL in cell suspension of cassava cv *Yacé*

Time post-elicitation (hr)	Enzymes activities (10^{-8} mmol min^{-1} mg prot.)			
	— — — PAL — — —		— — — TAL — — —	
0	2.07	± 0.00	2.06	± 0.01
0.25	2.74	± 0.05	6.46	± 0.02
0.5	5.80	± 0.17	7.06	± 0.06
1	14.97	± 0.21	22.10	± 0.27
4	14.17	± 0.11	21.07	± 0.04
8	17.00	± 0.11	28.90	± 0.53
12	22.31	± 0.47	32.36	± 0.20
24	40.13	± 0.67	52.22	± 0.19
48	38.28	± 0.14	53.44	± 0.03
72	31.92	± 0.22	57.06	± 1.47
96	24.87	± 0.17	35.10	± 0.12
120	18.68	± 0.33	25.77	± 0.70

Activity values are averages of three independent determinations ± standard deviation; Prot = 0.05 to 0.55 mg ml^{-1}

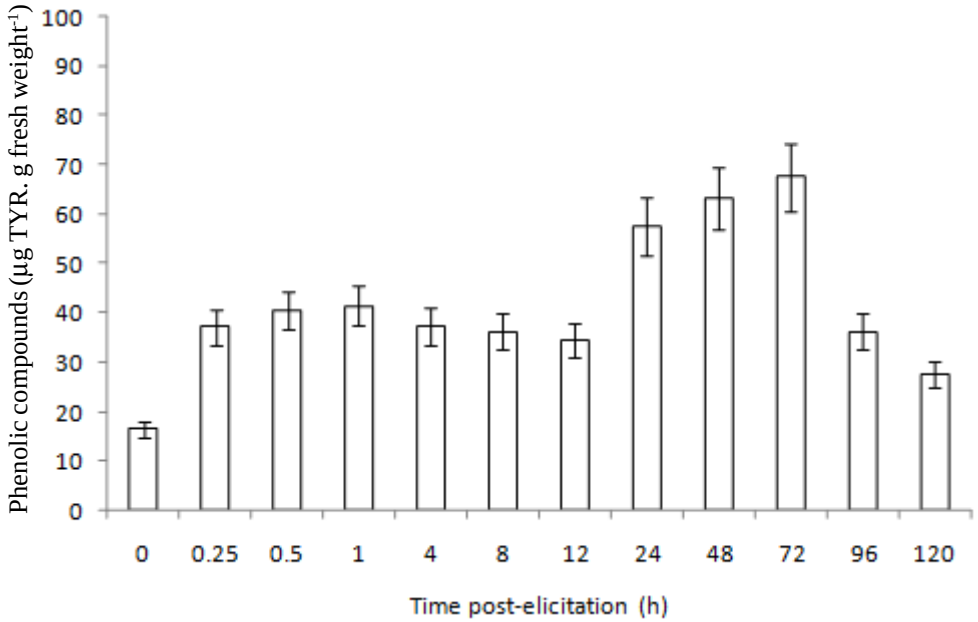

Figure 3. Changes in total phenolic ethano-soluble compounds content in cells of cassava cv *Yacé* during inoculation time with salicylic acid (125 µM). TYR = 0 to 100 µg ml^{-1}.

a sharp increase which reached maximum within 72 hr of inoculation. Subsequent to this optimum time, the amount of phenolic compounds decreased until the end of the experiment (Fig. 3).

Salicylic acid concentration. Table 3 shows that PAL and TAL activities were not influenced by Salicylic acid concentrations in the untreated cells (0 µM). In elicited the cells, enzyme activities increased with SA concentration. The activity of PAL (24 hr of treatment) reached a climax at 100 µM of SA. For TAL (72 hr of treatment), the induced activity reached a maximum with the 75 µM SA concentration. In fact, it was 13 times higher than that of the control (Table 3). For each concentration tested, activity of TAL was greater than PAL (P = 0.002). Phenolic content of untreated cells (0 µM) remained almost uninfluenced by SA concentrations.

In cells treated for 24 hr, phenolic compounds increased along with the concentration of SA. Its highest value on dry weight basis was obtained at the 100 µM of SA. For cells inoculated for 72 hr, the synthesis of phenolic compounds was

TABLE 3. Effect of variation salicylic acid concentration on activities of PAL (24 hr) and TAL (72 hr) extracted from cell suspension of cassava cv *Yacé* at the optimum time of the hypersensitivity reaction

Salicylic acid concentration (µM)	Enzymes activities (10^{-8} mmol min^{-1} mg prot.)			
	PAL		TAL	
0	2.00	± 0.01	2.01	± 0.00
5	6.36	± 0.03	11.68	± 0.06
25	7.49	± 0.16	11.67	± 0.10
75	9.47	± 0.32	25.51	± 0.20
100	11.38	± 0.59	25.95	± 0.17
125	9.77	± 0.33	26.73	± 0.28
250	9.43	± 0.08	25.73	± 0.27
500	8.66	± 0.12	24.20	± 0.13

Activities values are averages of three independent determinations ± standard deviation; Prot = 0.05 to 0.55 mg ml^{-1}

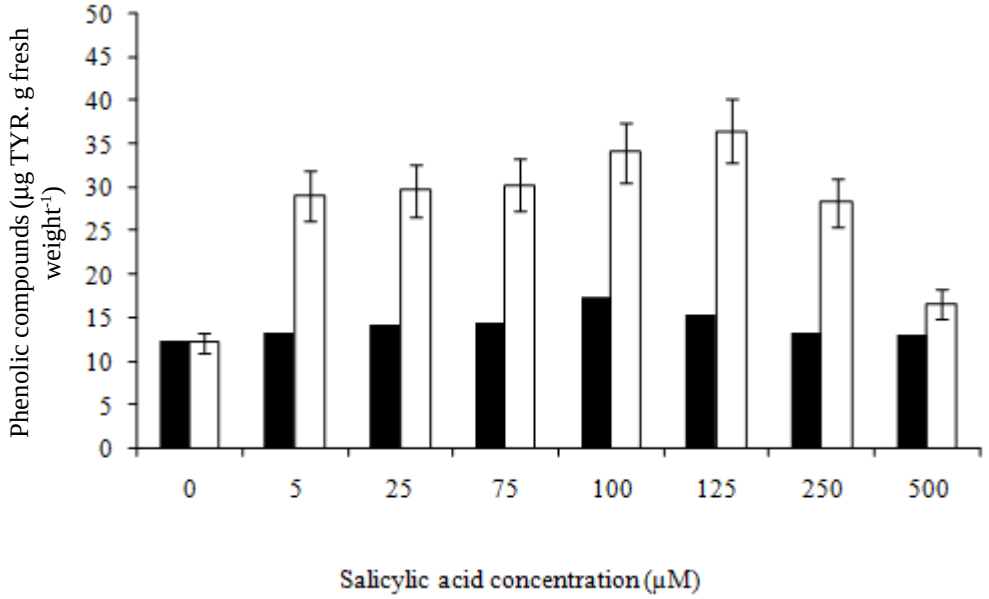

Figure 4. Total phenolics ethano-soluble accumulated in cell suspension of cassava cv *Yacé* under different concentrations of salicylic acid: treated 24 hr■ ; treated 72 hr□ ; TYR = 0 to 100 µg ml^{-1}.

elevated to a maximum at 125 µM and was 3 times higher than that of control cells. After the 125 µM concentration, the levels of the phenols suddenly decreased, but was maintained at a level higher than that of untreated cells (Fig. 4).

DISCUSSION

From this study, it is clear that the two enzymes used, phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) and tyrosine ammonia-lyase (TAL, EC 4.3.1.5), are different (Table 1, Figs. 1 and 2). Indeed, PAL activity was 9.8 times greater than that of TAL in the pellets obtained in 20% (w/v) saturation with ammonium sulphate. However, when the Dowex cation was used, the activity of TAL was 36.7 times higher than that of PAL. This difference could be due to the nature of the enzyme protein. Indeed, including PAL protein that precipitated at 20% (w/v) ammonium sulphate are lest soluble in the buffer solution used (Dawson *et al.*, 1986). In addition, the Dowex cationic complex anions inhibited PAL or complexed it; while TAL was stimulated it (Mihai *et al.*, 2004). Differences between pH and temperature optima of PAL and TAL are proxy evidence that the two enzymes differ in cassava.

Our results are corroborated by those of Dogbo *et al*. (2008) who worked on elicited seedlings of the same cassava cultivar. These authors found that the amount of phenols was maintained high despite stimulation of polyphenol oxidases (PPO) and the drastic drop in PAL activity. They suggested the existence and involvement of tyrosine ammonia-lyase (TAL) in the synthesis of these compounds. Our results confirm this hypothesis.

Lynda *et al*. (1985) and Raifa-Hassanein *et al*. (2005) also showed that TAL activity was distinct from that of PAL in the callus of *Nicotiana tabacum* and *Hibiscus sabdarifa*, respectively. PAL and/or TAL activation plays a fundamental role in plant adaptation to its environment. Indeed, cassava is grown in areas presenting different pedo-climatic characteristics. Also, the simultaneous activation of these enzymes, but staggered in time (Table 2), could then contribute. According to Rösler *et al*. (1997) and Wajahatullah *et al*. (2002), this activation depends on the species, genotype, environmental conditions and the availability of endogenous substrates. Simultaneous activation of these enzymes induced a strong synthesis of phenolic compounds. In addition, maintaining TAL activity after the dramatic fall in that of PAL was a real relay in the synthesis of these compounds (Table 2, Fig. 3).

It is clear from this study that TAL activity was consistently higher than that of of PAL (Tables 1 and 2). This could be explained by the fact that TAL activity was estimated by the formation of *p*-coumaric acid. Indeed, the *p*-coumaric acid may result from two biosynthetic pathways: the direct deamination of tyrosine and the transformation of *E*-cinnamic acid produced by the PAL, as suggested by Rösler *et al*. (1997). Consequently, *p*-coumaric acid remains a crossroad in the synthesis of most phenylpropanoids (Berner *et al*., 2006). TAL is, therefore, a shunt in this metabolic pathway (Rösler *et al*., 1997; Wajahatullah *et al.*, 2002). Stimulation of enzymes was early and concomitant with synthesis of phenolic compounds. TAL was more sensitive to elicitor and reached its optimum at 75 mM of salicylic acid in contrast to PAL (100 µM) (Table 3). The estimated amounts of phenolics increased regularly with the concentration of the elicitor to a limit value of 100 µM for 24 hr and 125 µM for 72 hr of exposure to inoculation. The decrease in the amount of phenolics could have resulted from their incorporation into ethanol-insoluble polymers or oxidation by polyphenol oxidases (Vanholme *et al*., 2010; De Leonardis and Macciola, 2011). Indeed, oxidised phenolics were not revealed by the assay methods employed. However, the cumulative effect of both enzymes was beneficial for the synthesis of phenols. This would favour the defense of cassava cultivar *Yacé* as suggested by some authors (Dogbo *et al*., 2007, 2008).

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