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Differential Changes in Some Antioxidant Enzymes and Biomolecules in Leaves of Tomato Infected by *Phytophthora infestans* (Mont.) de Bary

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

In Cameroon, tomato yields remain low due to attack by pathogens and insects. *Phytophtora infestans* (PI) is a phytopathogenic fungus responsible for the downy mildew of tomatoes, a fungal disease responsible for enormous economic losses. To contribute to the search for means of control, the stimulatory effect of the tomato defence system by extracts of some plants was evaluated. Tomato plants were treated with the aqueous macerates of *Callistemon citrinus* (*C.*

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citrinus) and *Cymbopogon citratus* (*C. citratus*) at 10% (W/V). After 4 days of spraying with the macerates, plants were inoculated with a virulent strain of PI. Tomato leaves collected at 2-day intervals for 10 days were used to determine the contents of chlorophyll a, proteins, carbohydrates, lipids, amino acids and proline. The activities of the main antioxidant enzymes were also assessed: Ascorbate Peroxidase (APX), Phenylalanine Ammonia Lyase (PAL), Peroxidase (POX), Superoxide Dismutase (SOD). The results showed that treatment of tomato plants with aqueous macerates of *C. citrinus* and *C. citratus* and their inoculation with PI induced an increase in chlorophyll a, protein, carbohydrate, lipid, amino acid and proline contents in tomato leaves; an increase in APX, PAL, POX, and SOD activities. Our results suggested that increased metabolite synthesis on the one hand, and increased enzymatic activities, on the other hand, could mitigate the oxidative damage that occured during pathogen expansion. Aqueous macerates of *C. citrinus* and *C. citratus* could be used as natural products to stimulate the defence system in tomato against *Phytophtora infestans*.

Keywords: Tomato; fungal diseases; plant extracts; antifungal; induced resistance.

1. INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) is a vegetable cultivated in almost all countries of the world. Tomato has a high biological value due to its content in carotenes, vitamins C and E and lycopene and a balanced content of mineral elements [1]. Its global production in 2020 was estimated to 186 million tons with an average yield of 197 tons/ha. In Cameroon, tomato remains the most important vegetable in terms of production. In 2020, its production was estimated at 1.246 million tons with a yield of 15.14 tons/ha [2]. This yield is far from the world yield, this gap is mainly justified by many constraints faced by tomato cultivation. Among these, the most striking in Cameroon are adverse climatic conditions and pests, susceptibility to diseases (alternariosis, fusariosis, mildew...) [3]. Indeed, tomatoes are the target of many bacterial and fungal diseases. Phytosanitary analysis of crops and sampling carried out in tomato growing sites in Cameroon in the Centre(Yaounde) and Western (Dschang) regions revealed a recurrence of fungal diseases caused by several fungi including *Fusarium oxysporum* f.sp*. lycopersici* that causes leaf yellowing, wilting and damping-off. This plant pathogenic fungus causes field losses ranging from 46 to 100% [4].

Nowadays, the control of most biotic plant diseases is mainly based on the very intensive use of synthetic chemical bactericides and fungicides [5]. However, despite the acceptable results, chemical control has several limitations, namely environmental pollution, high cost of synthetic chemicals, the risk of pathogen resistance to chemical substances, and human intoxication, especially since analyses of vegetables and fruits in Cameroon have shown pesticide residues. These limitations motivate and justify the search for effective and eco-friendly alternatives to control pathogenic microorganisms and crop pests. Thus, the use of natural substances (essential oils and solvent extracts) as plant disease control agents is receiving increasing attention. In Cameroon, local plant extracts have been reported as having properties in vitro and in vivo against various pathogens [3]. Botanicals with antifungal compounds have been identified and can be exploited for the management of plant diseases because they have low mammalian toxicity, target specificity, biodegradability and contain many active ingredients [6]. *Callistemon citrinus*, *Cymbopogon citratus* and *Oxalis barelierri* because of their antifungal activity notably against *Phytophtora infestans* and *Fusarium oxysporum* of tomato are used as biocontrol agents [4]. Plant extracts contain compounds that not only have a direct antimicrobial effect on the pathogen, but also stimulate the plant's natural defences, making them one of the most promising alternatives among crop protection strategies [7]. Studies have shown that spraying rice plants with *Datura metel* leaf extracts can induce systemic resistance of this plant against *Rhizoctonia solani* and *Xanthomonas oryzea* pv *oryzea* through the accumulation of pathogenicity-related proteins [8]. Treatment of plants with aqueous extracts has enabled plants to resist pathogenic microorganisms and control several fungal diseases through metabolic changes (accumulation of phenolic compounds and antioxidant defence enzymes) [9].

The objective of this work was to evaluate the stimulating effect of the defence system of aqueous macerates of *Callistemon citrinus* and *Cymbopogon citratus* in tomato (*Lycopersicon esculentum* Mill.) infected by *Phytophtora infestans* (Mont.) de Bary.

2. MATERIALS AND METHODS

2.1 Plant and Soil Materials

Seeds of the Rio Grande variety of tomato were purchased at the Mfoundi market (Yaounde, Cameroon).

The soil used was obtained from the soil of Nkolbisson (Yaounde, Cameroon).

The leaves and stems of *Callistemon citrinus* and *Cymbopogon citratus* (DC) STAFW were harvested in Mbandjock (Yaoundé,Cameroon).

Salicylic acid, a natural stimulator of the plant defence system, was used as a positive control.

2.2 Fungal Materials

A strain of *Phytophtora infestans* (Mont.) de Bary was isolated from tomato fruits and leaves harvested in the field and showing downy mildew symptoms. Its isolation and identification were done in the Phytopathology laboratories of the *Institut de Recherche Agricole pour le Développement (IRAD)* of Nkolbisson (Yaounde,Cameroon) and the Institut du Développement Rural (IDR) of the *Université Polytechnique de Bobo-Dioulasso* (Burkina Faso). Identification was done according to Agarwal et al., [10] and Mathur and Kongsdal [11] and confirmed by the Phytopathology Laboratory of the Danish Seed Health Center (DSHC), Copenhagen, Denmark. The pathogenicity of these strains was proven on 20-day-old tomato plants from which the same pathogens were re-isolated. The strain of *Phytophtora infestans* (Mont.) de Bary was maintained on V8 agar medium in 90 mm diameter Petri dishes at 20 ± 2 °C in the dark. The strain was cultured for 7-10 days to allow sporulation of the fungi, subcultured from time to time to keep the growth active and kept at 5°C in a V8 agar medium. Fourteen-day cultures were used for the inoculation of tomato plants.

2.3 Assessment of the Stimulatory Potential of the Defence System of Plant Macerates (*C. citrinus* and *C. citratus*) and Salicylic Acid in the Tomato/PI Interaction

2.3.1 Preparation of aqueous macerates and salicylic acid solutions

The plant material (*Callistemon citrinus* and *Cymbopogon citratus*) was carefully harvested and shade-dried in the greenhouse for 14 days and then crushed with a mill. A 10% (W/V) macerate was prepared. The mixture was kept for 48 hours in the greenhouse and at room temperature in the laboratory. The aqueous macerates were obtained by pressing and filtering through a sieve and a fine cloth. The extracts obtained were used for spraying tomato plants.

Salicylic acid was used as a positive control and prepared at 0.01% (W/V) as described by Mandal et al., [12].

2.3.2 Obtaining tomato plants

The seeds were weighed and sterilized with 1% (v/v) sodium hypochlorite solution. Then they were rinsed twice with sterile distilled water for 02 minutes while shaking vigorously each time.

The seeds obtained were sown in a nursery tray containing sterile soil consisting of a sand/soil mixture in the proportions 1/3 (v/v). The plants were left to grow with regular watering for 14 days and then transplanted into 10 kg pots (1 plant per pot) in a greenhouse. 10 days after transplanting, the tomato plants were divided into 4 lots:

- The first batch was made up of control plants sprayed with distilled water (S);
- The 2nd batch consisted of plants treated with 10% macerate *C. citratus* (Tr Cymb);
- The 3rd batch consisted of plants treated with 10% macerated *C. citrinus* (Tr Call);
- The 4th batch consisted of plants treated with salicylic acid (Tr AS).

After 3 days of treatment (spraying), each batch was divided into 2 subgroups: subgroup 1 consisting of tomato plants of each of the above treatments but not inoculated: S, Tr Cymb, Tr Call, Tr AS; subgroup 2 consisting of tomato plants of each of the above treatments inoculated with PI: I (S having been inoculated), (Tr Cymb + I), (Tr Call + I), (Tr AS +I).

For each treatment, five replicates were conducted independently and the pots were arranged in the greenhouse in a completely randomised design.

The experiment was conducted in a completely randomised block design.

2.3.3 Pathogen inoculation

From a 14-day PI culture on agar V8 medium, the fungal suspension was prepared and

adjusted to 5×105 conidia/ml. Two (2) ml of the conidia suspension was homogenized and inoculated onto the tomato leaves. The inoculated plants (subgroup 2) were covered with black polyethylene bags for 48 h to create adequate moisture for fungal growth and development. Non-inoculated tomato plants (subgroup 1) were used as controls. For biochemical assays, leaves were collected on the day of inoculation and at 2-day intervals during 10 days. These samples were washed with tap water, drained, weighed and frozen for further analysis.

2.3.4 Determination of chlorophyll a

The extraction of chlorophyll was done in 80% acetone at 4°C. For this purpose, 5 g of leaves were cut into slices of 2 cm diameter and put into test tubes containing 3 ml of 80% acetone. The tubes were incubated in the dark for 24 hours to allow solubilisation of the photosynthetic pigments. The determination of chlorophyll a was done by determining the optical densities at 663 and 646 and the calculation was done according to the formula of Lichtenthaler and Welburn [13].

Chlorophyll a (mg.g-¹FW) = 12.21 OD663 - 2.81 OD646

2.3.5 Extraction and determination of total soluble proteins

The extraction of the total soluble proteins was carried out according to the method described by Mbouobda et al., (2007). Two (2) grams of leaves were ground cold (4° C) in 4 ml of TAMET buffer (Tris 0.5 M; 0.3M ascorbic acid; 2% permethylethanol; EDTA 0.01 M; Triton x100, 2%, pH 7) supplemented with the addition of polyvinyl pyrrolidone (PVP) and sterile fine sand. The grind was incubated for 30 min at 4° C and then centrifuged at 10,000 g for 20 min. The recovered supernatant constituting the total soluble protein extract was stored at -20° C. for subsequent analysis. The amount of protein in the crude extract was determined using the Bradford method [14] with bovine serum albumin (BSA) as standard. The protein content was expressed in $mg.g^{-1}$ of fresh matter.

2.3.6 Determination of carbohydrates

The plant samples were boiled in 80% ethanol for extraction. Test tubes containing 1 ml of ethanolic extract each were placed into the water bath for evaporation. The residue and 1 ml of distilled water were incubated at 49° C. for 30 min. The solution was then neutralised with 1N NaOH using methyl red as a colour indicator. 1 ml of Nelson's reagent was added to each tube. The tubes were heated for 20 min in a boiling water bath, were cooled and 1 ml of the arsenomolybdate was added. The solution was mixed well and diluted to obtain 25 ml and the OD was measured at 495 nm using a spectrophotometer. The concentration of reducing sugars was calculated from the glucose standard and expressed in $\mu g.g^{-1}$ of fresh matter [15].

2.3.7 Determination of total lipids

The tube containing 350 µl of supernatant (with 80% ethanol extract) was evaporated to complete desiccation in the heating blocks at 95° C. 37.5 µl of 96% sulphuric acid were added and the mixture was heated for five minutes. After cooling, 1000 µl of Vanillin was added and then after 10 to 15 min to read the OD at 525 nm after development of a characteristic red coloration. A calibration curve obtained from commercial vegetable oil was used to calculate the lipid concentration [16]. The lipid content was expressed in $\mu g.g^{-1}$ of fresh material.

2.3.8 Determination of total free amino acids

In test tubes, 1 ml of ethanol extract from the leaves was taken up in 25 ml of distilled water and neutralised with 0.1N NaOH using methyl red as a colour indicator [17]. 1 ml of ninhydrin was added and the mixture was heated in a boiling water bath for 20 min, then 5 ml of a dilute solution (distilled water/n-propanol in equal volume) was added. This was followed by cooling and dilution to 25 ml. The absorbance was measured at 570 nm with a spectrophotometer. The standard line uses a leucine solution. The total amino acid content was expressed in $mg.g^{-1}$ of fresh matter.

2.3.9 Determination of proline

The Proline Assay was carried out on a sample of 100 mg of fresh material taken and placed in a test tube to which 2 ml of 40% methanol was added. The sample was heated for 1 hour in a water bath at 85°C. After cooling, 1 ml of the extraction solution was added to 1 ml of the mixture (distilled water/acetic acid/ortho-phosphoric acid) (6/15/4) and 2 ml of ninhydrin. The mixture was brought to a boil at 100°C for 30 min in a water bath [18]. After cooling, 5 ml of

toluene was added after vortexing. The OD reading was taken at 528 nm. The proline content was calculated from the proline standard and expressed as $\mu g.g^{-1}$ of fresh material.

2.3.10 Assessment of Ascorbate Peroxidase (APX) activity

APX activity (EC 1.11.1) was assessed according to the method of Nakano and Asada [19]. Enzyme activity was determined by following the decrease in absorbance of ascorbate at 290 nm. The reaction medium consisted of: 50 mM phosphate buffer, pH 7; 0.5 mM ascorbic acid; H_2O_2 0.1 mM (5 / 2 /1) (V/V/V) in a final volume of 5 ml. To each test tube containing 5 ml of the reaction medium, the enzyme extract (10µl) was added. The optical density was read at 290 nm against a blank in which the enzyme extract is replaced by the extraction buffer. The molar extinction coefficient 2.8 $mM^{-1}.cm^{-1}$ was used to calculate the ascorbate peroxidase activity expressed as moles of H_2O_2 reduced. $min^{-1}.g^{-1}$ of fresh material. One Unit of Ascorbate Activity is required for the reduction of 1 mole of H_2O_2 per minute at 25°C.

2.3.11 Evaluation of Phenylalanine Ammonia Lyase (PAL) activity

PAL activity (EC.4.3.1.5) was assessed based on the rate of cinnamic acid production as described by Saunders and McClure [20]. Thus, 1 ml of 50 mM Tris-HCl buffer pH 8.8 containing β-mercaptoethanol, 0.5 ml of 10 mM L-phenylalanine, 0.4 ml of distilled water and 0.1 ml of enzyme extract were incubated at 37° C for 1 h. The reaction was stopped by adding 0.5 ml of 6M HCl and the product was extracted with 15 ml of ethyl acetate followed by evaporation of the extraction solvent using a rotary evaporator. The solid residue was taken up in 3 ml of 0.05 M NaOH and the concentration of cinnamic acid was quantified by reading the optical density at 290 nm. One PAL activity Unit is equal to 1 µmol of cinnamic acid produced per minute per g of fresh plant material.

2.3.12 Evaluation of peroxidase activity (POX)

POX activity (EC.1.11.1.7) was assessed by the method described by Hammerschmidt et al., [21]. The reaction medium (3 ml) consisted of 10 mM sodium phosphate buffer pH 6 containing 10 mM H_2O_2 at 0.25% (V/V). A volume of 100 µl of enzyme extract was added to initiate the reaction. The enzyme activity was determined by calculating the ratio of optical Density at 470 nm per minute per 0.01 which was defined as the Activity Unit of peroxidase activity expressed in terms of Unit per g of fresh plant material.

2.3.13 Assessment of superoxide dismutase (SOD) activity

SOD activity (EC.1.15.1.1) was assessed using the method described by Beauchamp and Fridovich [22]. It measures the ability of the enzyme to inhibit the photochemical reduction of nitro blue tetrazolium chloride (NBT). The reaction medium (3 ml) consists of: 50 mM phosphate buffer pH 7.8; 13mM L-methionine; NBT 75µM; EDTA 1 mM; 0.05M sodium carbonate; 20 µl of enzyme extract. Riboflavin 2µM was added after all reagents and the reaction was initiated by placing the test tube 30 cm below a 15W ampoule for 10 min at 25°C. The tube was then transferred into the dark for 10 min and the optical density read at 560 nm. One SOD Activity Unit was defined as the amount of enzyme required to inhibit the reduction of NBT by 50%. SOD activity was expressed as Units per minute per g of fresh plant material.

2.4 Statistical Analysis

The results obtained were subjected to statistical analysis for the calculation of means, standard deviations and the search for significant differences, using the SPSS 22.1 software. The one-factor ANOVA test coupled with the Student-Newman-Keuls test was used to evaluate the Smallest Significant Difference (PPDS) at $P < 0.05$.

3. RESULTS

3.1 Effect of Plant Extracts on Chlorophyll a Content

In healthy plants, the Chla content on day 0 was 12.50 ± 0.63 $mg.g^{-1}$ and did not vary significantly with time until day 8. When the plants were inoculated (I), there was a significant decrease in Chla on day 2 and on day 10, the percentage was 37%.In treated plants (Tr), the Chla content increased significantly ($P < 0.05$) for all treatments. On day 10, the content increased by 178.03% and 186.58% with *C. citrinus* and *C. citratus* respectively. In treated and inoculated plants (Tr + I), Chla content decreased significantly on days 2 and 4 in *C. citratus* treated plants and on days 2, 4 and 6 in *C. citrinus*

treated plants. A gradual and significant increase was then noted for all treatments. On day 10, the increase was 173.26% and 271.13% for *C. citrinus* and *C. citratus* respectively. The Chla content increased significantly from day 2 for all treatments (Table 1).

3.2 Effect of Plant Extracts on Protein Content

In healthy plants, the protein content was 38.77 ± 0.76 mg.g^{-1}FW on day 0. It increased significantly ($P < 0.05$) from day 2 and reached the peak on day 6. When the plants are inoculated (I), the protein content of 40.43 ± 1.04 mg.g^{-1} FW at day 0 decreased at day 4 and then there was a progressive increase until day 10. In plants treated with plant extracts, the protein content increased progressively and significantly over time by more than 100% at day 10 for all treatments. Inoculation after treatment with plant extracts and salicylic acid (Tr + I) resulted in a significant and progressive increase in protein content over time. Protein contents were 2 to 5 times higher on day 10 for all treatments (Fig. 1).

3.3 Effect of Plant Extracts on Carbohydrates Content

In healthy plants (S), the carbohydrate content was 77.33 ± 1.53 mg.g^{-1} FP at day 0, which increased significantly from day 2 to day 8. In the inoculated plants (I), the carbohydrate content increased gradually and significantly ($P < 0.05$) with a peak on day 6, an increase of 183.46%. After treatment with plant extracts, this content increased by more than 100% from day 2 for all treatments and reached a maximum value on day 6. In the inoculated plants after treatment (Tr + I), there was a significant ($P < 0.05$) increase in carbohydrate content from day 2. The percentage increase was 205.12% and 223.50% with *C. citrinus* and *C. citratus* respectively. This content increased gradually over time. In general, the increase of this content is higher than in the treated plants (Tr) (Fig. 2).

3.4 Effect of Aqueous Plant Macerates on Lipid Content

In healthy plants (S), the lipid content on day 0 was 27.5 ±2.00 mg g^{-1} FW. When the plants were inoculated (I), there was a significant decrease in lipid content ($P < 0.05$) on days 2 and 4. The lipid content then increases on day 8 and decreases on day 10. The decrease in lipid content on day 2 was 50.19%. Treatment of the plants with plant extracts resulted in a gradual and significant increase from day 2 with a peak on day 8. The increase at day 8 was more than 100% for all treatments. In the post-treatment inoculated plants (Tr + I), lipid content increased gradually and significantly ($P < 0.05$) by more than 100% from day 2 and peaked on days 10 (*C. citrinus*) and 8 (*C. citratus*). In general, the increase in lipid content was greater in treated and inoculated plants (Tr + I) than in treated plants (Tr) over time (Fig. 3).

3.5 Effect of Plant Extracts on Amino Acids Content

In healthy plants (S), the amino acid content was 8.57± 0.20 mg.g^{-1} FW on day 0. When the plants were inoculated (I), the amino acid content increased gradually and significantly ($P < 0.05$) with a maximum content at day 10 with increase of 54.18%. After treatment with plant extracts, there was a significant and gradual increase in the amino acid content. From day 2, the percentage increase was 71.63% and 625.00% respectively with *C. citrinus* and *C. citratus* and it continued until day 10. In plants inoculated after treatment (Tr + I), there was a significant ($P < 0.05$) and gradual increase in amino acid content from day 2. The percentages of increase on day 2 were 71.81% and 55.46% respectively with *C. citrinus* and *C. citratus* treatments. In general, the amino acid content in treated and inoculated plants (Tr + I) was higher than that of treated plants (Tr) (Fig. 4).

3.6 Effect of Plant Extracts on Proline Content

In healthy plants (S), the proline content on day 0 was 1.60 ± 0.10 µg.g^{-1} FW. When the plants were inoculated (I), there was a significant increase in proline content ($P < 0.05$) from day 2 of 145.62%. The treatment of the plants with the plant extracts resulted in a gradual and significant increase in proline content over time with a maximum value at day 10. In the inoculated plants after treatment (Tr + I), the proline content increased gradually and significantly ($P < 0.05$) in the same way as in the treated plants (Tr). In general, the proline content was higher in treated and inoculated (Tr + I) plants than in treated (Tr) plants over time (Fig. 5).

Table 1. Chlorophylle content (expressed in mg.g^{-1}FW) in tomato leaves on a time course after treatment with plants extracts and inoculation with PI and in the control

DAI	*C. citrinus*				*C. citratus*		Salicylic acid	
	S	I	Tr	Tr+I	Tr	Tr+I	Tr	Tr+I
0	12.50 ^{b} ± 0.63	12.10 ^{b} ± 0.99	11.70 ^{a} ± 0.08	11.56 ^{b} ± 0.21	11.70 ^{a} ± 0.18	11.57 ^{b} ± 0.21	11.70 ^{a} ± 0.18	11.57 ^{a} ± 0.21
2	11.70 ^{ab} ± 0.30	7.16 ^{a} ± 0.20	12.50^{b} ± 0.52	6.50 ^{a} ± 0.10	13.10 ^{ab} ± 0.20	5.10 ^{a} ± 0.12	44.43 ^{b} ± 1,97	22.80 ^{c}± 1.50
4	11.67 ^{ab} ± 0.40	6.83 ^{a} ± 0.53	13.50 ^{c}± 0.30	4.33 ^{a} ± 0.15	15.50 ^{bc}± 0.30	4.83 ^{a} ± 0.13	44.10 ^{b} ± 1.20	19.90 ^{b} ± 1.20
6	11.83 ^{ab} ± 0.15	5.16 ^{a} ± 0.13	13.16 ^{c}± 0.15	5.00 ^{a} ± 0.10	16.58^{c}± 0.30	20.66^{c}± 1.53	56.74^{c} ± 1.97	22.83^{c}± 1.20
8	11.40 ^{ab} ± 0.20	5.16 ^{a} ± 0.12	20.83^{d}± 1.53	21.80^{c}± 1.10	22.00^{d}± 2.00	27.00^{d}± 2.00	66.10^{d}± 5.20	87.40^{d}± 5.20
10	11.33 ^{a} ± 0.45	4.50 ^{a} ± 0.20	20.83 ^{d}± 1.25	20.03^{c}± 1.53	21.83^{d}± 1.15	31.37^{e}± 1.53	69.67^{e}± 1.89	101.00^{e}± 2.10

Data are means ± SD of five replications. Different letters after the numbers in each column indicate a significant difference ($p<0.05$) between values, Duncan test. DAI: day after inoculation; S: control plants; I: plants inoculated with PI; Tr: plants treated with extract or salicylic acid; Tr + I: plants treated and inoculated with PI

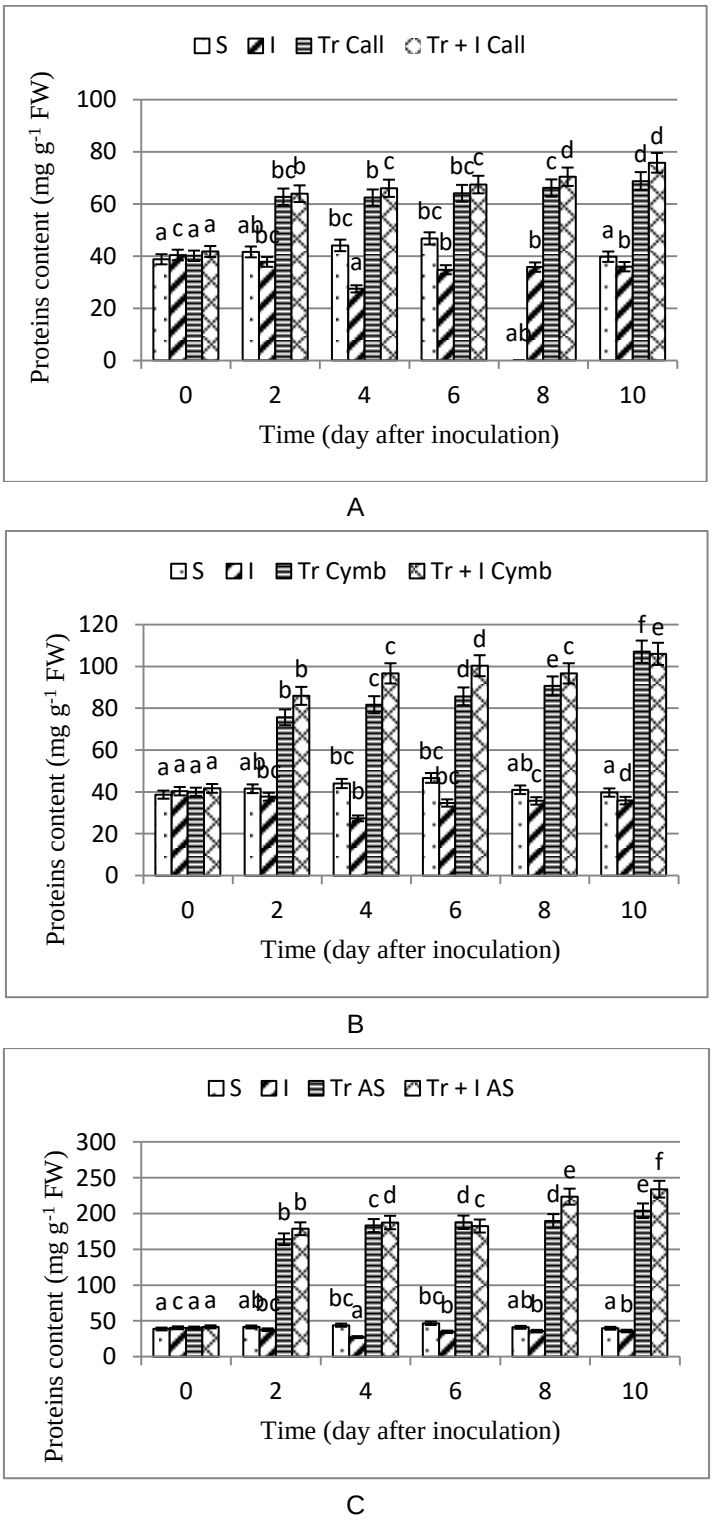
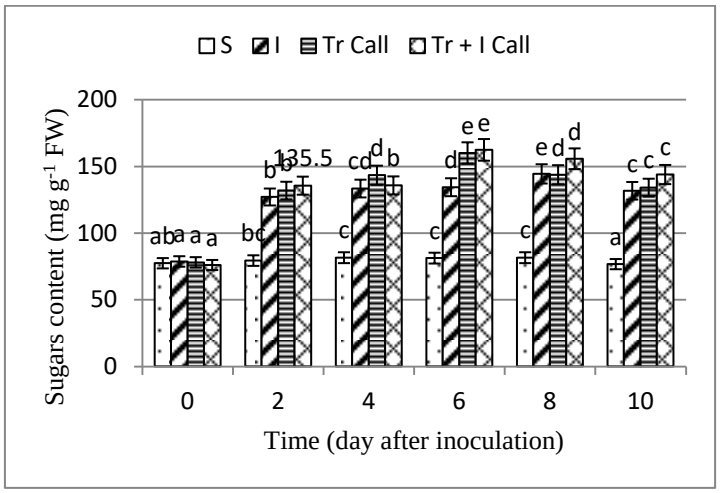


Fig. 1. Proteins content (expressed in mg.g^{-1} FW) in leaves of tomato plants on a time course after treatment with plants extracts and inoculation with PI and in the control

Data presented are the means ± SD of five replicates. Different letters indicate a significant difference ($p<0.05$) between values, Duncan test. A: C. citrinus; B: C.citratus; C: Salicylic acid. S: control plants; I: plants inoculated with PI; Tr C. citrinus, Tr C. citratus, Tr Salicylic acid: plants treated respectively with Callistemon citrinus, Cymbopogon citratus and salicylic acid. Tr + I C. citrinus, Tr + I C. citratus, Tr + I salicylic acid: Plants treated with Callistemon citrinus, Cymbopogon citratus and salicylic acid and inoculated with PI, respectively



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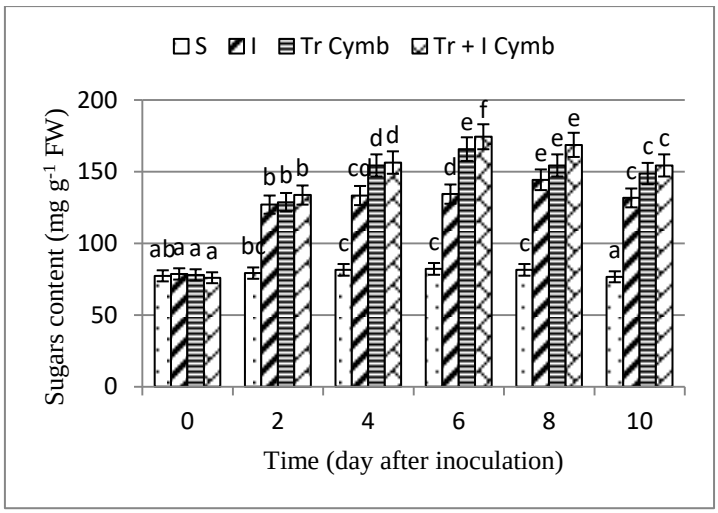

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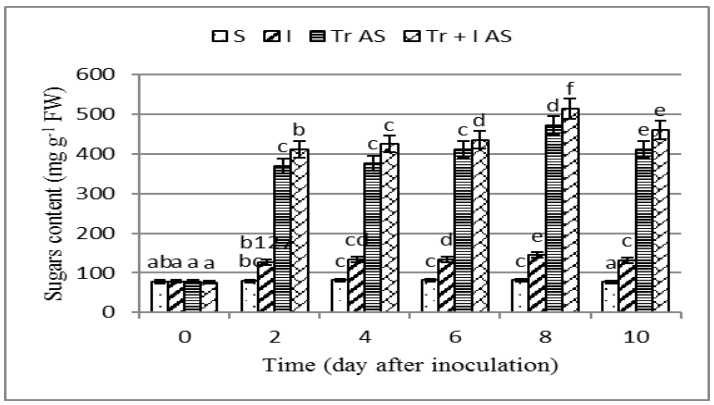
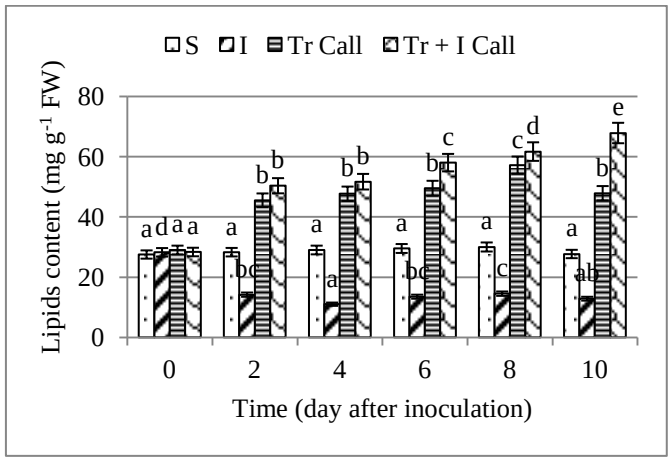


C

Fig. 2. Carbohydrates content (expressed in mg.g^{-1} FW) in leaves of tomato plants on a time course after treatment with plants extracts and inoculation with PI and in the control

Data presented are the means ± SD of five replicates. Different letters indicate a significant difference ($p<0.05$) between values, Duncan test. A: C. citrinus; B: C.citratus; C: Salicylic acid. S: control plants; I: plants inoculated with PI; Tr C. citrinus, Tr C. citratus, Tr Salicylic acid: plants treated respectively with Callistemon citrinus, Cymbopogon citratus and salicylic acid. Tr + I C. citrinus, Tr + I C. citratus, Tr + I salicylic acid: Plants treated with Callistemon citrinus, Cymbopogon citratus and salicylic acid and inoculated with PI, respectively.



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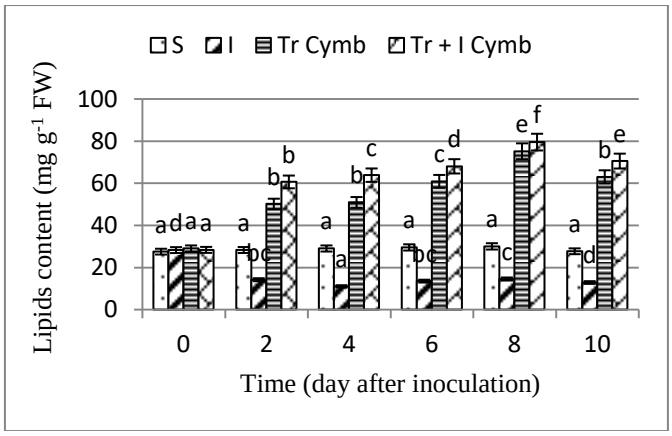

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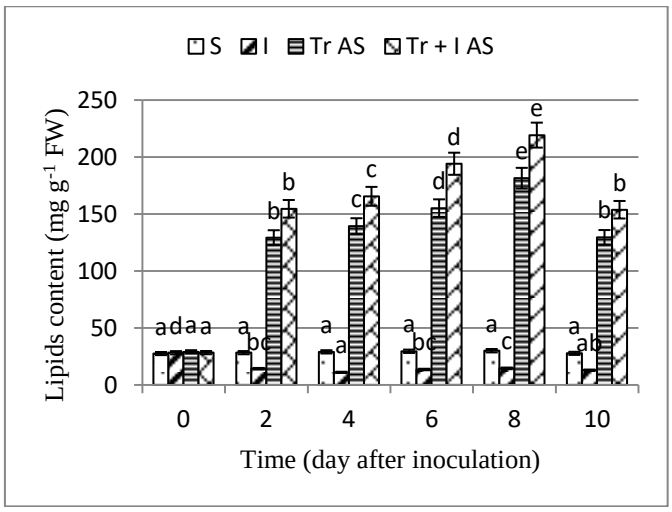
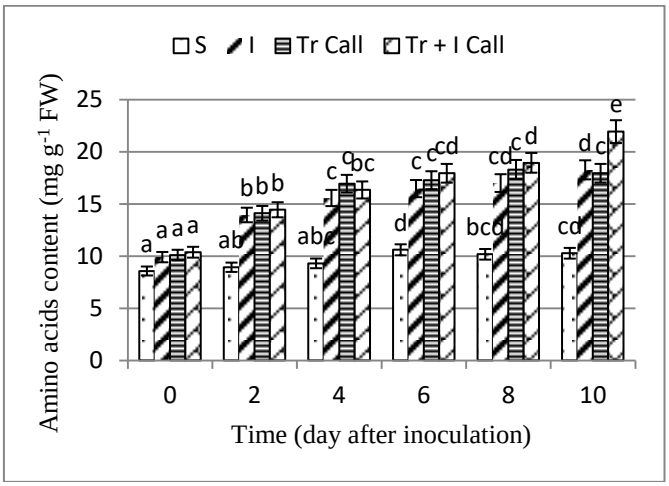


C

Fig. 3. Lipids content (expressed in mg.g^{-1} FW) in leaves of tomato plants on a time course after treatment with plants extracts and inoculation with PI and in the control

Data presented are the means ± SD of five replicates. Different letters indicate a significant difference ($p<0.05$) between values, Duncan test. A: C. citrinus; B: C.citratus; C: Salicylic acid. S: control plants; I: plants inoculated with PI; Tr C. citrinus, Tr C. citratus, Tr Salicylic acid: plants treated respectively with Callistemon citrinus, Cymbopogon citratus and salicylic acid. Tr + I C. citrinus, Tr + I C. citratus, Tr + I salicylic acid: Plants treated with Callistemon citrinus, Cymbopogon citratus and salicylic acid and inoculated with PI, respectively



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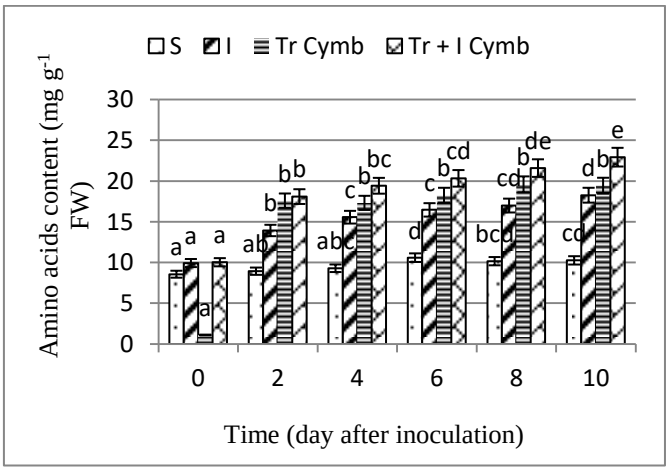

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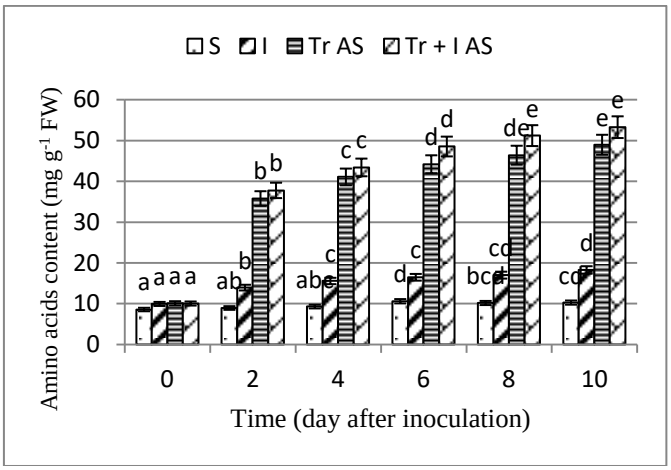

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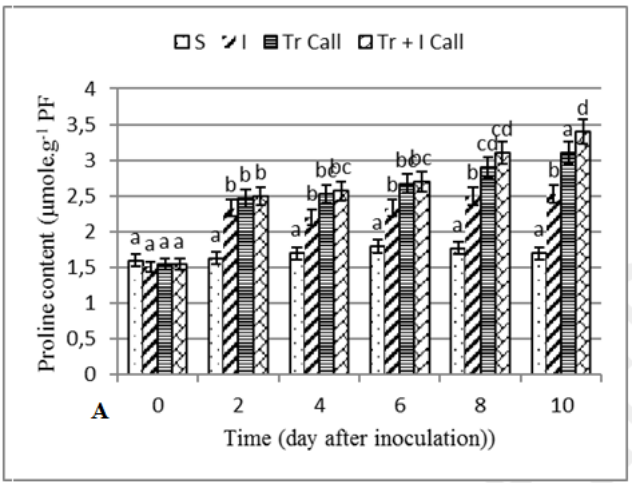
Fig. 4. Amino acids content (expressed in mg.g^{-1} FW) in leaves of tomato plants on a time course after treatment with plants extracts and inoculation with PI and in the control

*Data presented are the means ± SD of five replicates. Different letters indicate a significant difference ($p<0.05$) between values, Duncan test. **A**: C. citrinus; **B**: C.citratus; **C**: Salicylic acid. S: control plants; I: plants inoculated with PI; Tr C. citrinus, Tr C. citratus, Tr Salicylic acid: plants treated respectively with Callistemon citrinus, Cymbopogon citratus and salicylic acid. Tr + I C. citrinus, Tr + I C. citratus, Tr + I salicylic acid: Plants treated with Callistemon citrinus, Cymbopogon citratus and salicylic acid and inoculated with PI, respectively*



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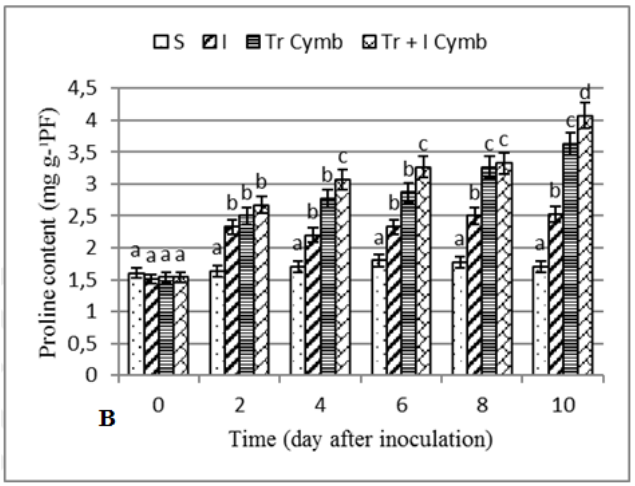

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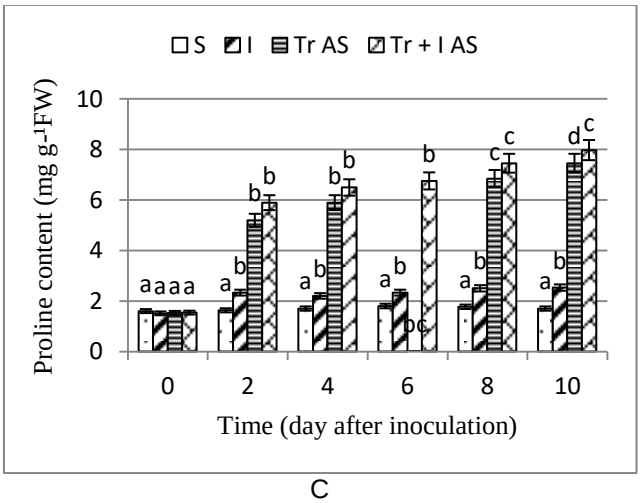

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Fig. 5. Proline content (expressed in µg.g^{-1} FW) in leaves of tomato plants on a time course after treatment with plants extracts and inoculation with PI and in the control

Data presented are the means ± SD of five replicates. Different letters indicate a significant difference (p<0.05) between values, Duncan test. A: C. citrinus; B: C.citratus; C: Salicylic acid. S: control plants; I: plants inoculated with PI; Tr C. citrinus, Tr C. citratus, Tr Salicylic acid: plants treated respectively with Callistemon citrinus, Cymbopogon citratus and salicylic acid. Tr + I C. citrinus, Tr + I C. citratus, Tr + I salicylic acid: Plants treated with Callistemon citrinus, Cymbopogon citratus and salicylic acid and inoculated with PI, respectively

3.7 Effect of Plant Extracts on the Variation of Antioxidant Enzymes Activities in *L. esculentum* Leaves

3.7.1 Ascorbate Peroxidase (APX) activity

In healthy plants (S), ascorbate peroxidase activity was 20.7 ± 1.00 $U.g^{-1}$FW from day 0 and did not vary significantly until day 10. When plants were inoculated (I), APX activity increased significantly ($P < 0.05$) with a peak of activity obtained at day 2. APX activity then progressively decreased until day 10 with values that remain higher than in healthy plants. Treatment of the plants with the plant extracts resulted in a significant ($P < 0.05$) increase in APX activity. With values of 25.00 ± 2.00 $U.g^{-1}$ FW (*C. citrinus*) and 19.9 ± 2.00 $U.g^{-1}$ FW (*C. citratus*) at day 0, it increased with a peak at day 2 with 3 and 8 times higher values. It then decreased progressively until day 10 but with higher values than healthy (S) and inoculated (I) plants. When the plants were inoculated after the treatment with the plant extracts (Tr + I), the APX activity increased in the same way as in the treated plants but with higher values (Fig. 6).

3.7.2 Phenylalanine Ammonia Lyase (PAL) activity

Phenylalanine Ammonia Lyase (PAL) activity varied from 68.67± 1.53 $\mu mol.min^{-1}.g^{-1}$ FW to 73.67 ± 1.53 $\mu mol.min^{-1}.g^{-1}$ FW at day 0 as a function of treatment in healthy plants. When plants were inoculated (I), PAL activity increased significantly ($P < 0.05$) from day 2 to 71.95%. After treatment of the plants (Tr), there was a significant ($P < 0.05$) increase in PAL activity from day 2 of 62.00%, and 61.80% respectively in *C. citrinus* and *C. citratus*. It then increased gradually and peaks in activity are obtained on day 6. When plants are inoculated after treatment (Tr + I), PAL activity increased in the same way as in treated plants but with higher values. Peaks in activity were obtained at day 6 and this activity remains high and higher than in healthy (S), inoculated (I) and treated (Tr) plants (Fig. 7).

3.7.3 Peroxidase (POX) activity

In the leaves of healthy plants (S), the peroxidase activity (POX) varied from 2.87 ± 0.25 $U.g^{-1}$ FW to 4.33 ± 0.15 $U.g^{-1}$ FW. When the plants were inoculated (I), it increased on day 2 and reached a peak on day 4 (9.00 ± 1.88 $U.g^{-1}$ FW). After the treatment of the plants, there was a significant increase ($P < 0.05$) in POX activity. From a value of 2.93 ± 0.21 $U.g^{-1}$ FP on day 0, it was 2 to 7 times higher from day 2. PAL activity then increased gradually with a peak in activity on day 4. When plants are inoculated after treatment (Tr + I), POX activity increases in the same way as in treated plants but with higher values. A peak in activity is obtained on day 4 with the different treatments (Fig. 8).

3.7.4 Superoxide dismutase (SOD) activity

In the leaves of healthy plants (S), the SOD activity of 40.10 ± 0.20 $U.min^{-1}.g^{-1}$ FW at day 0 did not vary significantly until day 10. When plants were inoculated (I), SOD activity increased significantly ($P < 0.05$) over time with a maximum value at day 4. After treatment of the plants (Tr), there was a significant ($P < 0.05$) increase in activity with 2-3 times higher values on day 2 depending on the treatment. When plants are inoculated after treatment (Tr + I), SOD activity increased in the same way as in treated plants but with higher values. A peak in activity is obtained on day 4 with the different treatments (Fig. 9).

4. DISCUSSION

The effect of plant extracts on the tomato defence system against *Phytophthora infestans* (PI) was evaluated through the determination of the content of biomolecules (chlorophyll a, proteins, carbohydrates, lipids, amino acids, and proline) in tomato leaves and the evaluation of the activities of antioxidant enzymes (ascorbate peroxidase (APX), Phenylalanine Ammonia Lyase (PAL), peroxidase (POX), Superoxide dismutase (SOD)).

Our study revealed that when tomato plants were inoculated with PI, the chlorophyll a content decreased. This decrease was believed to be a consequence of downy mildew, the pathogen released toxins that triggered the release of ROS. It has been proven by that the toxins produced by PI inhibit photosynthesis. The decrease in Chlorophyll a content was also believed to be due to the high level of lipid peroxidation causing cell death in tomato leaf tissues [23]. When plants were treated with plant extracts, an increase in Chl a content was noted. This increase could be justified by the stimulating effect of the extracts on the enzymatic activity of ribulose 1,5 bisphosphate carboxylase, a key a key enzyme of photosynthesis [24].

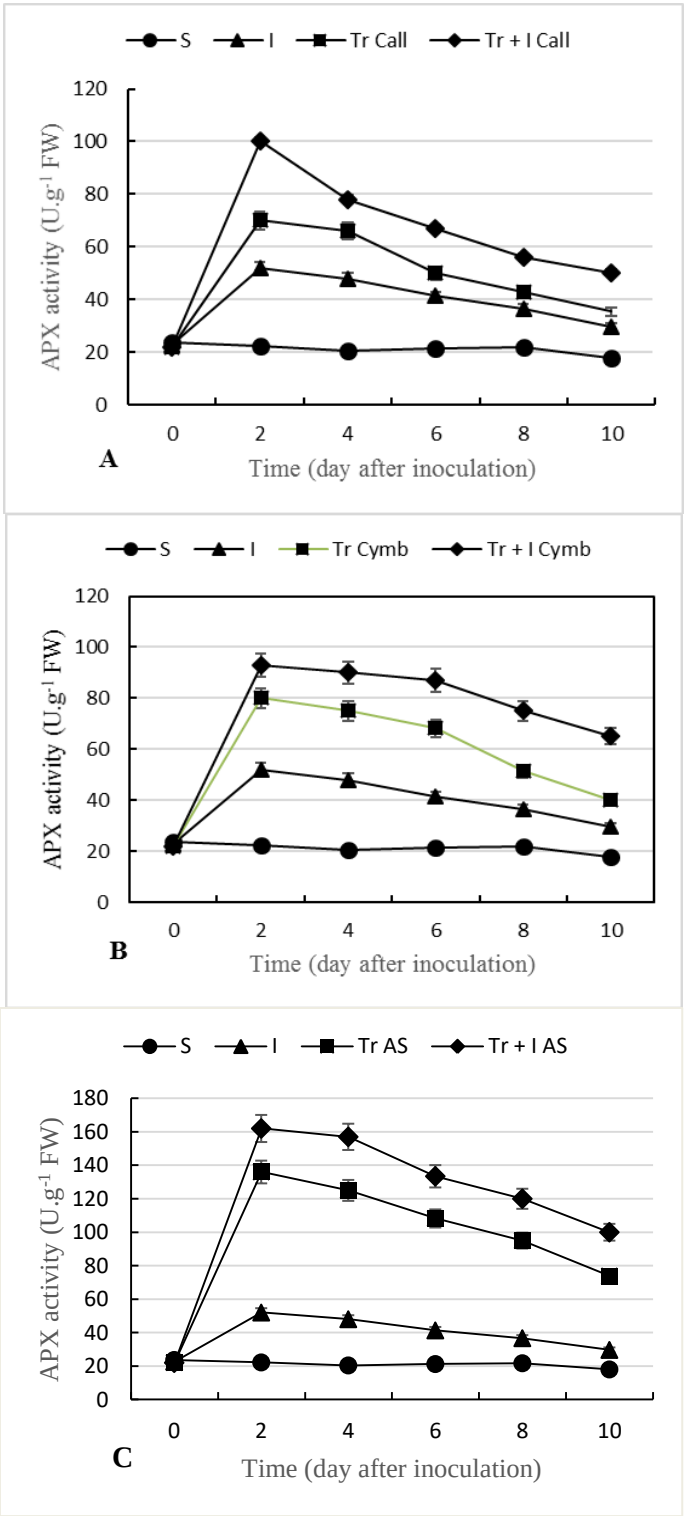

Fig. 6. APX activity (expressed in U.g^{-1} FW) in leaves of tomato plants on a time course after treatment with plants extracts and inoculation with PI and in the control

Data presented are the means ± SD of five replicates. Different letters indicate a significant difference ($p<0.05$) between values, Duncan test. A: C. citrinus; B: C.citratus; C: Salicylic acid. S: control plants; I: plants inoculated with PI; Tr C. citrinus, Tr C. citratus, Tr Salicylic acid: plants treated respectively with Callistemon citrinus, Cymbopogon citratus and salicylic acid. Tr + I C. citrinus, Tr + I C. citratus, Tr + I salicylic acid: Plants treated with Callistemon citrinus, Cymbopogon citratus and salicylic acid and inoculated with PI, respectively

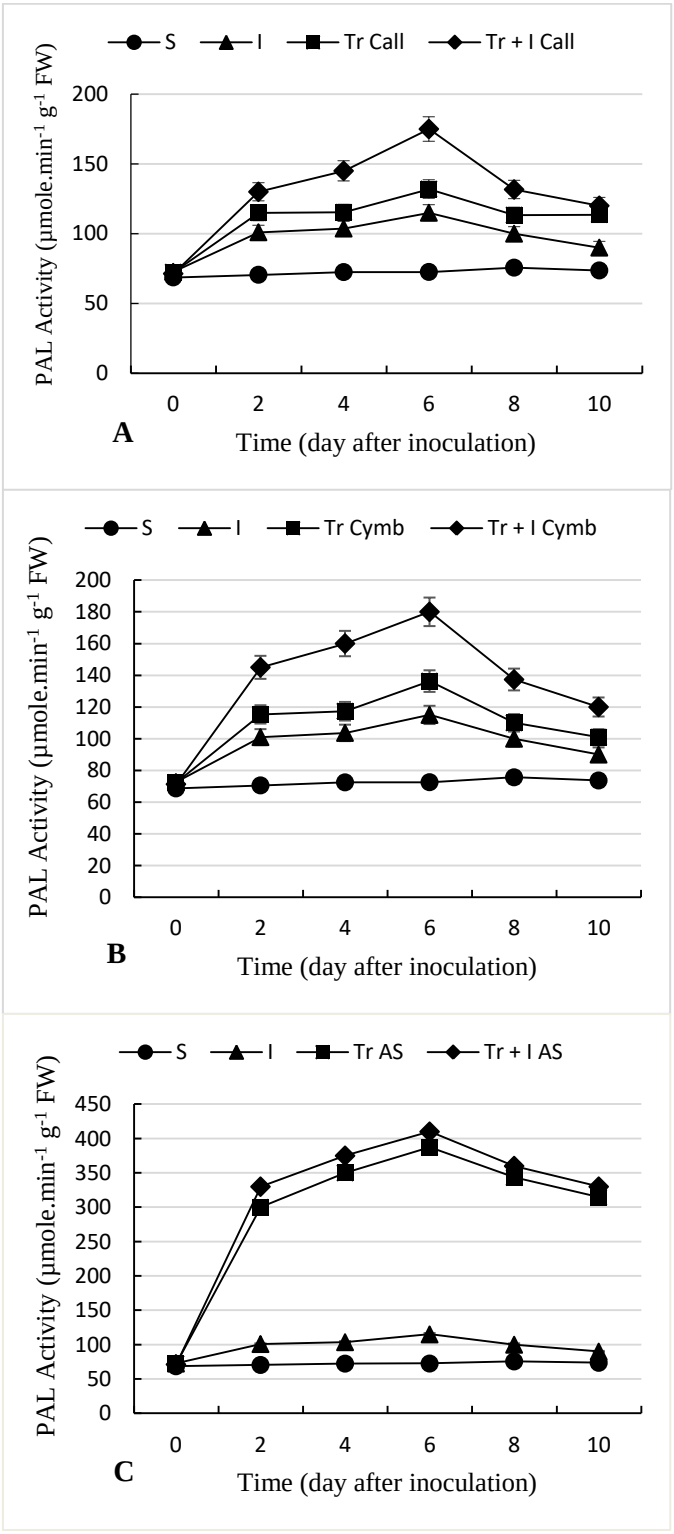

Fig. 7. PAL activity (expressed in U.g^{-1} FW) in leaves of tomato plants on a time course after treatment with plants extracts and inoculation with PI and in the control

Data presented are the means ± SD of five replicates. Different letters indicate a significant difference ($p<0.05$) between values, Duncan test. A: C. citrinus; B: C.citratus; C: Salicylic acid. S: control plants; I: plants inoculated with PI; Tr C. citrinus, Tr C. citratus, Tr Salicylic acid: plants treated respectively with Callistemon citrinus, Cymbopogon citratus and salicylic acid. Tr + I C. citrinus, Tr + I C. citratus, Tr + I salicylic acid: Plants treated with Callistemon citrinus, Cymbopogon citratus and salicylic acid and inoculated with PI, respectively

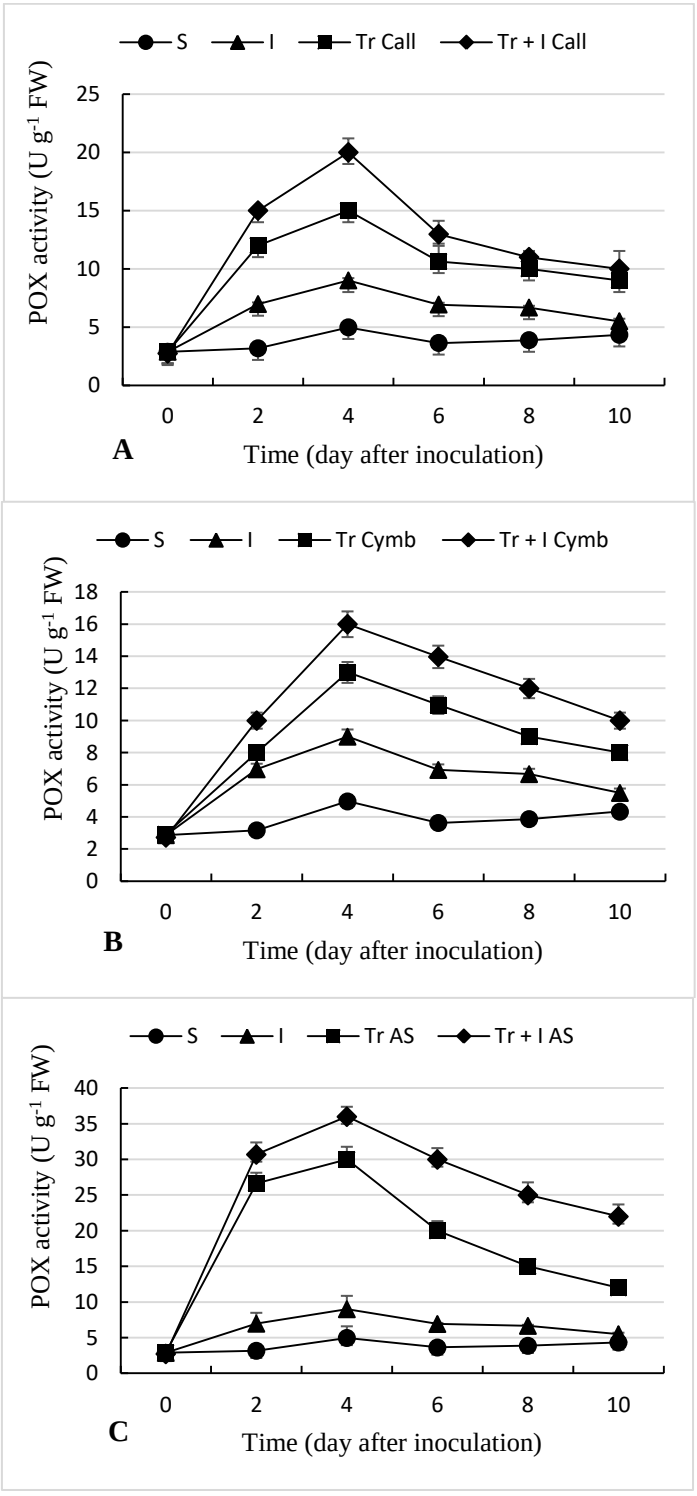

Fig. 8. POX activity (expressed in U.g^{-1} FW) in leaves of tomato plants on a time course after treatment with plants extracts and inoculation with PI and in the control

Data presented are the means ± SD of five replicates. Different letters indicate a significant difference ($p<0.05$) between values, Duncan test. A: C. citrinus; B: C.citratus; C: Salicylic acid. S: control plants; I: plants inoculated with PI; Tr C. citrinus, Tr C. citratus, Tr Salicylic acid: plants treated respectively with Callistemon citrinus, Cymbopogon citratus and salicylic acid. Tr + I C. citrinus, Tr + I C. citratus, Tr + I salicylic acid: Plants treated with Callistemon citrinus, Cymbopogon citratus and salicylic acid and inoculated with PI, respectively

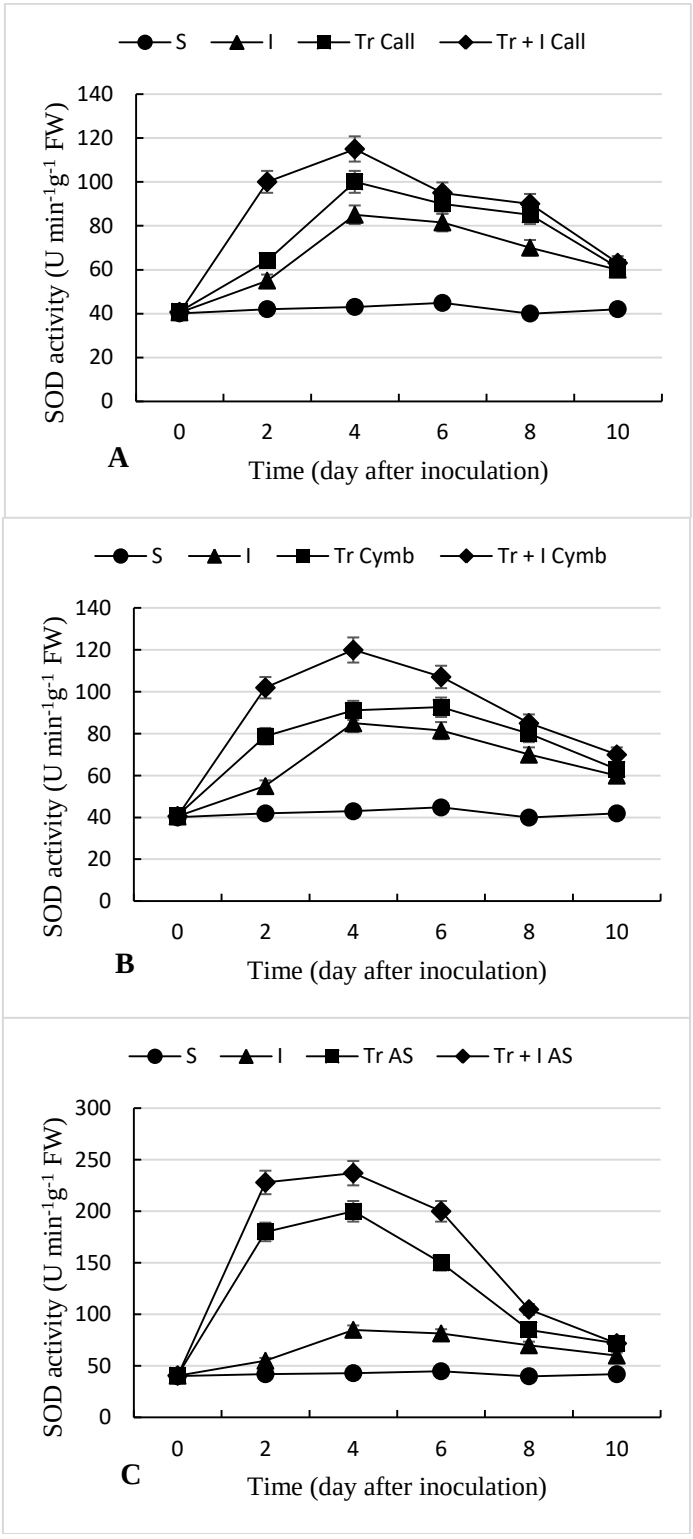

Fig. 9. SOD activity (expressed in U min^{-1} g^{-1} FW) in leaves of tomato plants on a time course after treatment with plants extracts and inoculation with PI and in the control

Data presented are the means ± SD of five replicates. Different letters indicate a significant difference ($p<0.05$) between values, Duncan test. A: C. citrinus; B: C.citratus; C: Salicylic acid. S: control plants; I: plants inoculated with PI; Tr C. citrinus, Tr C. citratus, Tr Salicylic acid: plants treated respectively with Callistemon citrinus, Cymbopogon citratus and salicylic acid. Tr + I C. citrinus, Tr + I C. citratus, Tr + I salicylic acid: Plants treated with Callistemon citrinus, Cymbopogon citratus and salicylic acid and inoculated with PI, respectively

Protein content decreased in leaves under inoculated conditions, but when tomato plants were treated with plant extracts, there was an increase in protein content. This result was in agreement with that obtained by Samir et al., (2019) who showed that tomato plants inoculated with *Fusarium oxysporum* accumulate proteins. According to this author, the increase in protein content could be explained by an increased synthesis of the enzymes involved in detoxification reactions.

When tomato plants were inoculated or treated with plant extracts, there was an increase in total free sugar content. Indeed, plant extracts are at the origin of the physiological notification of tomatoes due to photosynthesis. The accelerating effect of the extracts on photosynthesis will allow an increased synthesis of carbohydrates. Furthermore, it has been shown that cell wall glycoproteins rich in glycine, hydroxyproline, and proline are involved in the response to plant stress [25]. Couée et al., [26] have shown that in some plant species, increased synthesis of sugar such as mannitol is linked to increased resistance to oxidative stress.

The different actions carried out on tomatoes, namely PI inoculation, the treatment with plant extracts and the treatment followed by inoculation, resulted in a significant increase in the MDA content, which is a product of the membrane lipids peroxidation. It is a marker of oxidative damage and its concentration indicates that induction of oxidative stress was effective [27]. The increase in MDA concentrations in plants could be explained by a higher peroxidation of lipids in root cells.

When tomato plants were inoculated or treated with plant extracts, there was an increase in the content of total amino acids and proline particularly. This result is in agreement with the one obtained by Omokolo and Boudjeko [28] in the *Xanthosoma sagittifolium/Pythium myriotylum* interaction. Indeed, an increase in amino acids content after treatment with plant extracts could serve in the synthesis of molecules such as proteins, phenolic compounds and phytoalexins which are molecules involved in defence mechanisms [29]. The particular increase in proline content suggested the preferential orientation of the metabolic pathways of amino acid synthesis pathways towards proline synthesis. Akladious et al., [30] showed that the treatment of tomato plants with salicylic acid under oxidative stress conditions was responsible for the accumulation of proline. Several other authors have shown that plants under various stresses accumulate proline and this accumulation is positively correlated with the tolerance of oxidative stress [31,32].

When plants were inoculated with PI, APX activity increased. Treatment of plants with extracts resulted in a significant increase in APX activity. When plants were inoculated after treatment with plant extracts, APX activity increased in the same way as in treated plants but with higher values. This result is in agreement with the one obtained by Mbouobda [33] in the roots of *Xanthosoma sagittifolium* treated with Benzo-(1,2,3)-thiadiazole-7-carbothionic acid-S-méthyl ester acid (BTH) and chitosan (CHT) and inoculated with *Xanthosoma sagittifolium*. Localized in peroxisomes and chloroplasts in plants, APX uses ascorbate as an electron donor and prevents H_2O_2 from leaving peroxisomes. In addition to being involved in various physiological processes such as photosynthesis, differentiation and growth, it is thought to be involved in stabilising plants under various biotic or abiotic stresses.

After inoculation of the tomato plants, the significant increase in PAL activity in the leaves indicates the activation of this crossroads enzyme which would trigger the different defence mechanisms in the plant. When plants were treated with plant extracts and then inoculated with PI a significant increase in PAL activity is noted. Shadle et al., [34] have shown that PAL activity in tobacco increases after infection, which reduce the incidence of disease by stimulating defence reactions. This confirms the key role of PAL in the synthesis of phenylpropanoids which are molecules involved in the synthesis of secondary metabolites, mainly phytoalexins and salicylic acid which act in reducing the incidence of plant diseases through direct antifungal activity and stimulation of plant defence mechanisms [35].

POX activity was higher in plants treated with extracts than those not treated. This would be related to oxidative stress with the involvement of peroxidase activity as an antioxidant response against H_2O_2. For Samir et al., (2019), this stress response involves a homeostatic response and control of the degradation process to maintain the integrity of vital cell functions. Mandal and Mitra, [36] have shown that treatment of *Lycopersicon esculentum* with chitosan induces a significant increase in POX activity, and an

increase in the content of phenolic compounds, which protects them against parasitic attacks.

Effective destruction of H_2O_2 requires SOD action. Our results indicate an increase in SOD activity in the leaves of tomato plants treated with plant extracts, treated and inoculated. This increase in activity maintains a positive ROS balance in favour of H_2O_2 accumulation. This result is in agreement with the one obtained by Li et al., [37] suggesting an involvement of SOD in the apple defence mechanism at all maturity stages.

5. CONCLUSION

The treatment of tomato with the aqueous extracts of *Callistemon citrinus* L., *Cymbopogon citratus* (DC) STAFW and *Oxalis barrelieri* L. stimulates its antioxidant defence system against *Phytophthora infestans*. This stimulation was manifested in tomato leaves by an increase in the content of chlorophyll a, proteins, carbohydrates, lipids, amino acids and proline; an increase in ascorbate peroxidase, phenylalanine ammonia lyase, peroxidase and superoxide dismutase activities. The increase in metabolite synthesis on the one hand, and the increase in enzyme activities on the other hand could mitigate the oxidative damage that takes place during the expansion of the pathogen. Aqueous macerates of *C. citrinus* and *C. citratus* could be used as natural products to stimulate the tomato defence system against *Phytophthora infestans*.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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