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Research article

Effects of chitosan on vegetative organs growth and peroxidases activities in cassava (*Manihot esculenta* Crantz) cultivars YACE, 9620A, TMS4(2)1425 and TMS30572

Kouakou Dappah KRA[1]*, Seu Jonathan GOGBEU[2], Kafana SORO[3], Kouassi Joseph KOUAKOU[4], Koffi II Nazaire KOUASSI[5] and Denezon Odette DOGBO[1]

[1]Laboratory of Biology and Improvement of Plant Production, University Nangui Abrogoua, Côte d'Ivoire
[2]Laboratory of Plant Physiology and Pathology, University Jean Lorougnon Guédé, Côte d'Ivoire
[3]Laboratory of Floristic Biodiversity, Ecology Research Center, University Nangui Abrogoua, Côte d'Ivoire
[4]Laboratory of Biology and Improvement of Plant Production, University Nangui Abrogoua, Côte d'Ivoire
[5]Laboratory of Plant Physiology, university Félix Houphouet-Boigny, Côte d'Ivoire

*Corresponding Author: dappahk@yahoo.fr
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Abstract: In this work, the influence of chitosan on vegetative organs growth and peroxidases activities in cassava (*Manihot esculenta*, Euphorbiaceae) plants grown in hydroponic was studied. The experiment was carried out under a greenhouse lit by natural light at the Ecology Research Center (Côte d'Ivoire). Six concentrations of chitosan (0, 25, 50, 75, 100 and 125 mg L^{-1}) added to the culture media were tested on four cassava cultivars (Yace, 9620A, TMS4(2)1425 and TMS30572). These media are renewed every 15 days and growth of the plants lasted two months. The results showed that chitosan stimulated the growth of 1.1 to 1.4 according to cultivars and organs. Peroxidases activities were 1.5 to 2.6 times higher in leaves and roots in presence of 75 or 100 mg L^{-1} of chitosan depending on the cultivars. This activity is multiplied by 2.3 to 2.6 respectively in leaves and roots of *Yacé*, 2.6 and 1.5 of TMS30572, 1.9 and 2.2 of TMS4(2)1425. In 9620A, this factor was less than 2. These results show that chitosan could be a biostimulator in cassava.

Keywords: Peroxidases activities - Chitosan concentration - Growth - Cassava cultivar - Vegetative organs.

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INTRODUCTION

Chitosan is a natural copolymers of N-acetyl-D-glucosamine and D-glucosamine in variable proportion (Katiyar *et al.* 2014). It is rare in nature but could be found in cell walls of certain fungi such as zygomycetes (Tayel *et al*. 2010). In addition to these organisms, chitosan is obtained after deacetylation of chitin, which is the second most abundant natural biopolymer in nature after cellulose (Shahidi *et al.* 1999). This is explained by fact that chitin is one of the protective cuticle constituents of certain invertebrates teguments, such as insects, molluscs and crustaceans as well as cell walls of fungi (Rinaudo 2006, Tayel *et al*. 2010).

Chitosan is a biodegradable and non-polluting polymer for the environment. It has used in many fields, including agriculture (Dzung 2005, Dzung 2007). Several authors have reported that this molecule influences positively plants agronomic parameters. Indeed, chitosan stimulates germination (Xue *et al*. 2002), growth (Mondal *et al*. 2012, Piyavadee 2013, Mondal *et al*. 2016) and plants production (Salachna & Zawadzińska 2014, Salama *et al*. 2015). It has been used successfully in plants protection against abiotic (Lizarraga-Pauli *et al*. 2011, Jabeen & Ahmad 2013, Pongprayoon *et al*. 2013) and biotic stress (Coqueiro & Piero 2011, Falcón-Rodriguez *et al*. 2014).

These different actions of chitosan on plants would be done by metabolism modification. Indeed, works

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carried out by some authors have shown that this compound stimulates synthesis of several enzymes including hydrolases, polyphenol oxidases, catalases and peroxidases (Falcón-Rodríguez *et al*. 2009, Ricardo *et al*. 2013, Ben-Shalom *et al*. 2013).

The Objective of this work was to evaluate chitosan effects on the vegetative organs growth and peroxidases activities in cassava cultivars (*Manihot esculenta* Crantz) Yacé, 9620A, TMS4(2)1425 and TM30572 cultivated in hydroponic.

MATERIAL AND METHODS

Plant material

Stems of cassava cultivars Yace, 9620A, TMS4(2)1425 and TM30572 were obtained at National Center for Agronomic Research (CNRA) and multiplied on an experimental plot. This plot is located at Azaguié, about 45 km from Abidjan. Among these cultivars, *Yace* is a local cultivar, *9620A* is a cultivar improved by CNRA whereas TMS4(2)1425 and TM30572 were cultivars proved from International Institute of Tropical Agriculture (IITA).

Obtaining plants and treatments

Stems of these different cultivars were sterilized with sodium hypochlorite 3.6% (w/v) and alcohol 70% (v/v). After three rinses with sterile distilled water, stems were cut into cuttings at least 20 cm long and placed in liquid nutrient medium according to Gogbeu *et al.* (2012) method. In this medium, different amounts of chitosan (SIGMA) were added so as to obtain final concentrations of 0; 25; 50; 75; 100 and 125 mg L^{-1}. Seedlings from cuttings were kept in culture for 2 months under a greenhouse illuminated by natural light. All treatments of a cultivar were randomly arranged. Each treatment was replicated three times.

Determination of agronomic parameters

Agronomic parameters measured on these 2-month-old seedlings were leaf surface, length of shoot and root. Measurements were taken on all plants of each treatment. Length of a plant was determined from the insertion point of shoot on cutting to seedling apex. Length of root was determined from its point of insertion on the cutting to root apex. These measurements were made using a tape measure. Leaf surface was determined by the method described by Gogbeu *et al.* (2015). It consisted in spreading sheet on paper of known dimensions. Initial mass (M) and surface (S) of paper were determined. Cassava leaf is then spread on this paper and outline of it was delimited with a pencil. The bounded surface was cut with a blade and sheet of paper was weighed again and final mass (M') was obtained. Leaf surface (LS) was determined according to the following formula:

$$LS = S - \left(\frac{S \times M'}{M}\right)$$

Average leaf surface (ALS) for each treatment was determined according to the following formula:

$$ALS = \Sigma LS/N, \text{ with N = total number of leaves.}$$

Estimation of peroxidases activities

After determining agronomic parameters, leaves and roots of these seedlings were used for determination of peroxidases activities. Leaves taken are those of ranks 3, 4, 5 and 6 denoted f3, f4, f5 and f6, numbered from apex cauline to base. One (1) g of limb or root was crushed in 5 mL of sodium phosphate buffer (0.2 M, pH 6.8), 0.1 g of polyvinylpyrrolidone and 50 µL of Triton X-100 (1%, v/v). The ground material was centrifuged at 8000 rpm for 10 min at 4°C. Resulting supernatant was incubated with 3% (w/v) Dowex 50 (SIGMA) for 30 min at 4°C under shaking. The mixture was centrifuged as before. This last supernatant constituted enzymatic extract used for peroxidases assay. Dosage of peroxidases was done by Criquet *et al.* (2001). Reaction medium is composed of 0.1 ml of enzymatic extract, 1 ml of H_2O_2 (0.01 M) and 1 ml of guaiacol (0.01 M). Control consists of 0.1 mL of enzymatic extract and extraction buffer solution. The volume of each medium was supplemented to 3 mL with extraction buffer solution. Tubes containing reaction media were stirred rapidly and then incubated for 5 min in dark at 25ºC. At the end of incubation time, tubes were homogenized and absorbance was determined spectrophotometrically at 470 nm. Enzymatic activity was expressed as absorbance per minute and per milligram of protein (ΔDO min^{-1} mg^{-1}prot.).

Determination of proteins

Extracts used for enzyme assay are those used for determination of proteins. The protein assay was performed according to Bradford (1976) colorimetric method. Amount of protein was determined using a standard curve made from different concentrations (0 to 100 µg mL^{-1}) of Serum Albumin Bovine solution.

Statistical analyzes

All experiments conducted in this work were repeated three times. Analysis of variance (ANOVA) was done at 5% threshold with STATISTICA software 7.1. When $p \leq 0.05$, the difference is said to be significant. Homogeneous groups were determined by test of Duncan.

RESULTS

Agronomic parameters

The analysis in table 1 showed that agronomic parameters evaluated were variously influenced by chitosan according to cultivars. In most cultivars, 25 and 50 mg L^{-1} of chitosan did not affect organs growth except TMS4(2)1425 and 9620A leaves whose presented slight stimulation. For the latter, statistical treatment performed indicated a significant difference at 5% level. Concentrations 75 and 100 mg L^{-1} of chitosan induced growth of all evaluated organs. However, highest stimulation was achieved by 100 mg L^{-1} treatment except TM30572 leaves, which slightly increased in presence of 75 mg L^{-1} of chitosan. Organ growth was inhibited with an increase in chitosan concentration to 125 mg L^{-1}. Statistical analysis showed a significant difference apart from Yace leaves.

Table1. Effect of chitosan concentration on growth of stems and roots, and leaf surface of cassava aged 2 months.

Chitosan Concent. (mg L^{-1})	Cassava											
	Yace			9620A			TMS4(2)1425			TMS30572		
	Stem (cm)	Root (cm)	Leaf (cm^2)	Stem (cm)	Root (cm)	Leaf (cm^2)	Stem (cm)	Root (cm)	Leaf (cm^2)	Stem (cm)	Root (cm)	Leaf (cm^2)
0	14.22 ±0.27b	9.15 ±0.13c	39.62 ±0.65a	13.52 ±0.72c	11.05 ±0.37b	36.72 ±0.64e	13.7 ±0.50c	12.02 ±0.53b	38.67 ±0.64^{e}	13.97 ±0.31^{c}	9.07 ±0.39^{d}	40.12 ±0.60^{bc}
25	13.85 ±0.10b	9.27 ±0.19c	39.67 ±0.70a	13.6 ±0.58c	10.95 ±022b	37.02 ±0.65de	13.27 ±0.4c	11.75 ±0.52b	39.05 ±0.82^{de}	14.5 ±0.30^{c}	9.9 ±0.15^{c}	39.07 ±0.41^{c}
50	14.35 ±0.56b	9.55 ±0.30c	40.02 ±0.34a	13.65 ±0.54c	11.47 ±0.34b	37.8 ±0.58d	15.2 ±0.34b	12.55 ±0.34ab	40.3 ±0.98^{d}	14.57 ±0.28^{c}	9.97 ±0.33^{c}	39.8 ±0.54^{c}
75	14.87 ±0.76b	11.07 ±0.15b	41.22 ±0.61a	16.6 ±0.50b	12.65 ±0.29a	40.35 ±1.46b	15.82 ±0.38ab	13.22 ±0.23a	42.62 ±1.31^{b}	15.55 ±0.31^{b}	11.05 ±0.67^{b}	42.8 ±1.87^{a}
100	16.72 ±0.93a	13.05 ±0.27a	40.82 ±0.97a	18.02 ±0.83a	13.05 ±0.27a	42.7 ±2.12a	16.65 ±0.31a	13.3 ±0.219a	44.5 ±1.94^{a}	16.8 ±0.36^{a}	12.32 ±0.58^{a}	40.87 ±0.57^{b}
125	13.17 ±0.39b	11.17 ±0.76b	39.32 ±0.69a	13.92 ±0.27c	11.45 ±0.23b	39.5 ±1.27c	15 ±0.33b	12.05 ±0.31b	41.8 ±1.30^{c}	13.82 ±0.48^{c}	9 ±0.37d	39.32 ±0.40^{c}

Note: In each column, the averages followed by the same alphabetical letter are not statistically different at the 5% threshold (Duncan test).

Peroxidases activities in cassava leaves

Analysis of table 2 showed a variation of peroxidases activities in different leaves of cassava [Yace, 9620A, TMS4(2)1425 and TMS30572], treated with chitosan. In general, these activities increased with leaf age and chitosan concentration in all cultivars. In control leaves, peroxidases activities ranged from 0.12 to 0.23 ΔDO min^{-1} mg^{-1}prot. for f3 and from 0.35 to 0.41 ΔDO min^{-1} mg^{-1}prot. for f4 according to cultivars. In leaves 5 and 6, enzyme activity oscillated from 0.25 to 0.32 ΔDO min^{-1} mg^{-1}prot. apart from 9620A, for which peroxidases activities have increased to 0.45 ΔDO min^{-1} mg^{-1}prot.

In treated leaves, peroxidases activities increased significantly with concentrations in 4^{th} and 5^{th} leaves where it was maximum at 75 or 100 mg L^{-1} of chitosan according to cultivars (Table 2). It went from 0.31 to 0.87 ΔDO min^{-1} mg^{-1} prot., overall, 175 to 260% stimulation at optimal concentration. In leaf 6, increase in activity was 188 to 244% depending on cultivars for same concentrations. Statistical analysis revealed a significant difference at only 5% between leaf levels and concentrations. Lowest enzymatic activity was detected in leaves of rows 3 regardless of concentration used.

Peroxidases activities in roots of cassava

Peroxidases activities evaluated in plants roots of different cassava cultivars presented in table 3 shows that chitosan stimulated activities of these enzymes. Their activities have increased with the concentration of chitosan and reached optimum at 75 or 100 mg L^{-1} of chitosan in treatment medium. In *Yace* cultivar, there was a marked increase in peroxidase activity from 0.67 to 1.45 ΔDO min^{-1} mg^{-1} prot. 50 to 75 mg L^{-1}, respectively. This latter concentration was most inducing of peroxidases activities (1.45 ΔDO min^{-1} mg^{-1} prot). From this maximum value, peroxidases activities decreased to 1.37 ΔDO min^{-1} mg^{-1}prot. for 125 mg L^{-1} of chitosan.

For cultivar TMS30572, the increase in peroxidases activities was gradual until it reached its optimum (0.93 ΔDO min^{-1} mg^{-1} prot) at 100 mg L^{-1} chitosan. Statistical treatment did not show a significant difference at 5%

threshold for treatments of 100 and 125 mg L^{-1} of chitosan. Peroxidases activities were maximal (1.29 ΔDO min^{-1} mg^{-1} prot) in 9620A for 100 mg L^{-1} of chitosan. However, it decreased slightly to 125 mg L^{-1}, namely 1.25 ΔDO min^{-1} mg^{-1} prot. As for cultivar TMS4(2)1425, the increase in peroxidases activities were consistently 0 to 75 mg L^{-1} of chitosan. From 75 mg L^{-1}, an increase in activity was observed from 0.78 to its optimum at 1.33 ΔDO min^{-1} mg^{-1} prot. for concentration of 100 mg L^{-1} of chitosan. The activity of enzyme decreased slightly (1.27 ΔDO min^{-1} mg^{-1} prot.) with increasing chitosan concentration.

Table 2. Effect of different concentrations of chitosan on peroxidases activities (ΔDO min^{-1} mg^{-1} prot.) in cassava leaves.

Casava		Concentrations of chitosan (mg L^{-1})					
		0	25	50	75	100	125
Yace	F3	0.24±0.02ab	0.25±0.01a	0.26±0.01a	0.27±0.01a	0.27±0.05a	0.27±0.01a
	F4	0.37±0.02d	0.40±0.01d	0.45±0.01c	0.62±0.08b	0.87±0.02a	0.83±0.01a
	F5	0.30±0.01c	0.35±0.01d	0.40±0.01c	0.56±0.02b	0.76±0.01a	0.72±0.02a
	F6	0.25±0.01d	0.30±0.02c	0.35±0.01b	0.47±0.01a	0.41±0.01a	0.43±0.01a
9620A	F3	0.18±0.01d	0.20±0.01d	0.20±0.04d	0.28±0.01c	0.36±0.02b	0.54±0.02a
	F4	0.35±0.04c	0.37±0.01c	0.53±0.01b	0.63±0.01a	0.67±0.01a	0.66±0.01a
	F5	0.45±0.02d	0.46±0.01d	0.58±0.01c	0.71±0.02b	0.79±0.02a	0.72±0.02b
	F6	0.30±0.02de	0.35±0.01d	0.40±0.01c	0.56±0.01b	0.61±0.01a	0.55±0.01b
TMS4(2)1425	F3	0.20±0.02c	0.26±0.01ab	0.28±0.01ab	0.33±0.01a	0.28±0.01ab	0.29±0.01a
	F4	0.41±0.01d	0.43±0.01d	0.58±0.02c	0.72±0.02b	0.80±0.01a	0.76±0.02b
	F5	0.32±0.02d	0.36±0.02d	0.54±0.01c	0.64±0.01b	0.70±0.02a	0.64±0.02b
	F6	0.29±0.01c	0.29±0.01c	0.40±0.01b	0.62±0.01a	0.61±0.01a	0.59±0.01a
TMS30572	F3	0.12±0.10c	0.19±0.01b	0.21±0.02b	0.23±0.02ab	0.28±0.01a	0.28±0.01a
	F4	0.37±0.02d	0.38±0.08d	0.50±0.02c	0.65±0.01b	0.75±0.01a	0.71±0.03a
	F5	0.30±0.00d	0.31±0.02d	0.56±0.01c	0.79±0.01a	0.75±0.01ab	0.74±0.01ab
	F6	0.25±0.02c	0.28±0.01c	0.39±0.01b	0.61±0.01a	0.59±0.01a	0.57±0.02a

Note: F3, Leaves 3; F4, Leaves 4; F5, Leaves 5; F6, Leaves 6.
Number in line marked with the same letters do not differ significantly at P≤ 0.05 (Duncan test).

From the analysis of this table 3, in TMS30572, TMS4(2)1472 and 9620A cultivars, concentration of 100 mg L^{-1} of chitosan was most stimulating of peroxidases activities. In cultivar *Yace*, concentration of 75 mg L^{-1} of chitosan was more inducing to activity of enzyme. This activity was much higher than that evaluated in the other three cultivars.

Table 3. Effect of chitosan concentrations on peroxidases activities (ΔDO min^{-1} mg^{-1}prot.) of cassava plant roots.

Cultivars	Chitosan (mg L^{-1})					
	0	25	50	75	100	125
Yacé	0.55 ±0.01a	0.64±0.02b	0.67±0.01b	1.45±0.01d	1.41±0.05d	1.37±0.03c
9620A	0.73±0.04a	0.77±0.02a	0.97±0.01b	1.20±0.01c	1.29±0.01d	1.25±0.01c
TMS4(2)1425	0.57±0.01a	0.63±0.02b	0.74±0.01c	0.78±0.02c	1.30±0.03d	1.30±0.02d
TMS30572	0.61±0.01a	0.65±0.01a	0.72±0.02b	0.82±0.01c	0.93±0.03d	0.92±0.01d

Note: Number in line marked with the same letters do not differ significantly at P≤ 0.05 (Duncan test)

DISCUSSION

Results of this study indicate that chitosan promoted growth of stem, leaf and root organs. Elongation of stems and roots, and increase of leaf surface would result from cells division and/or their extension. Indeed, organs growth is done by cells mitoses of meristematic zones which, after cell turgor increase of dimensions. Whole of merèse and auxèse phenomena is at the origin of organs growth of plants. Work done by some authors (Dixon *et al*. 1994, Thain *et al*. 1995, Amborabe *et al*. 2004) have shown that chitosan acts on depolarization of plasma membrane that is manifested by the influx of Ca^{2+} and H^{+}, and efflux of Cl^{-} and K^{+}. Activation of ion channels causes changes in intra and extracellular media. A decrease of calcium in pectocellulosic walls increases their extensibility; which leads to cell growth or elongation. Growth induction of various plants by chitosan has been reported by several authors (Asghari-Zakaria *et al*. 2009, Algam *et al*. 2010, Saad 2011, Mondal *et al*. 2012, Mondal *et al*. 2013, Ramkissoon *et al*. 2016). As obtained in this study, their work showed that 75 mg L^{-1} and 100 mg L^{-1} chitosan were the most growth-inducing factors in spinach, okra, beans, chickpeas and chickens. On the other hand, shoots and roots growth of green anise (*Pimpinella anisum* L.) was stimulated after seed treatment by 50, 100 and 200 mg L^{-1} of chitosan (Batool 2016). Regardless of inducing concentration, growth obtained is also due in part to activation of nitrogen metabolism (Ke *et al*. 2001). This mineral is essential for the synthesis of molecules necessary for the vital functions of plant cells such as proteins, nucleic acids and chlorophylls.

Peroxidases activities were detected in leaves and roots of all plants examined. Presence of these enzymes in various organs demonstrates that they exercise physiological functions therein. Indeed, bibliographical synthesis carried out on these enzymes by Delannoy *et al.* (2004), indicated that they are involved in several metabolic pathways, among others, regulation of certain hormones, synthesis of lignins and defense of plants against abiotic and biotic stress. Low peroxidases activities in young leaves, increased in mature leaves and then decreased in older leaves. This variation is related to cell differentiation and more or less important synthesis of auxin and lignin. After germination of plants in chitosan solution, peroxidases activities were increased and multiplied by 1.7, 1.9, 2.3 and 2.6 at maximum activity respectively in 9620A, TMS4(2)1425, Yace and TMS30572 leaves. High peroxidases activities in leaves of rows 4 and 5 could be interpreted by their high metabolic activity. Indeed, in these leaves, chitosan activates photosynthesis (Mondal *et al.* 2012), which produced hydrogen peroxide. However, this compound is a substrate for peroxidases. In addition, work by some authors has reported that chitosan stimulates key enzymes in the biosynthetic pathway of phenolic compounds such as phenylalanine ammonia-lyase and tyrosine ammonia-lyase (Khan *et al.* 2003) and those involved in plant defense such as peroxidases (Coqueiro & Piero 2011). In total, the presence of hydrogen peroxide and precursors of lignin biosynthesis would have promoted activation of peroxidases.

Peroxidases activities evaluated in roots of controls was generally higher than that detected in leaves. This increase was 1.5 to 2.6 depending on cultivars. This result could be interpreted by the fact that germination was done in an aqueous medium which caused more marked water stress in the roots; these being in direct contact with the water. In the presence of chitosan, the enzyme activity was similar in the leaves and roots of the same cultivar except in TMS30572 for which peroxidase activity was 1.7 times stronger in the leaves. Water stress and chitosan concomitantly have stimulated the activity of peroxidases. These results confirmed those obtained by Lizarraga-Pauli *et al.* (2011) and Pongprayoon *et al.* (2013), on the induction of plant resistance by chitosan against abiotic stress. In addition, these results are in the same direction as those of El-Hassni *et al.* (2004) and Falcón-Rodríguez *et al.* (2014) whose have obtained the activation of peroxidases after application of this product. However, this peroxidase activity was a function of the concentration used but could also depend on the degree of acetylation of chitosan (Coqueiro & Piero 2011).

CONCLUSION

This study showed that chitosan promoted growth of all organs studied and stimulated peroxidases activities in cassava. Depending on the cultivar, its action was maximum at 75 or 100 mg L^{-1}.

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