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Research Article

COMPARATIVE STUDY OF TREATMENT METHOD FOR *TECTONA GRANDIS* L. PLANTS WITH SALICYLIC ACID AND CHITOSAN ON ACCUMULATION OF PHENOLIC COMPOUNDS

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A B S T R A C T

Increasingly, cultivation of teak is experiencing problems of pressure of pathogens in Côte d'Ivoire; This will affect quality of wood and disappearance of certain plantations in future. Objective of present work is to study one of natural defenses of plants, synthesis of phenolic compounds. Leaves of teak plants were treated with salicylic acid and chitosan separately and simultaneously; and amount of phenols was evaluated. Results showed that these elicitors amplify synthesis and accumulation of phenols. In treated leaves, high values were recorded with salicylic acid (81.07 mg tyrosin / gFM), followed by salicylic acid + chitosan treatment. But accumulation was earlier with chitosan, 12 hours after treatment. Basal and apex leaves also responded to treatment with phenol synthesis. Phenol content of leaves after treatment was more dependent on elicitors than phyllotaxy. However, cumulative action of salicylic acid and chitosan on synthesis and accumulation of phenols follows effect of chitosan.

INTRODUCTION

Phenolic compounds are secondary metabolites having in common presence of one or more benzene rings bearing one or more hydroxyl functions (Azar, 2007). These molecules are found in all parts of higher plants. But most of them are found in plant cells vacuoles (Boizot and Charpentier, 2006). In higher plants, phenols are involved in such varied roles as defense responses against aggressors, cell growth, organogenesis, bud dormancy, flowering, tuberization, or induction of somatic embryogenesis. (Boizot and Charpentier 2006, Kouakou 2010). Role played by these compounds against aggression has been demonstrated by Franke *et al*. (2002). Indeed, when plant is subjected to mechanical injuries or infections, phenols are synthesized and characteristic peroxidase activity of lignifying tissues is stimulated. These reactions result in formation at wound level of scar tissue that is resistant to infection. Likewise, thanks to their antimicrobial properties, oxidized phenolic compounds limit proliferation of infection and alteration of plant tissues (Zawistowski *et al*., 1991). These protective barriers have been observed in *Ipomoea batatas*, *Daucus carota* and *Solanum tuberosum* (Cheriot 2007).

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According to this author, ability of a plant species to resist attack of insects and microorganisms is often correlated with phenolic compounds content. Among these phenolic compounds, salicylic acid plays a key role in plant resistance mechanisms against pathogens. It is involved in both establishment of local and generalized resistance in plants (Grant and Lamb, 2006). Tagging experiments of this acid showed that more than 60% of this compound, measured in uninfected zone of plant, came from its transport from infected areas (Shulaev *et al*., 1995). The work done by Nawrath and Métraux (1999) mentioned that *Arabidopsis sp* mutants, unable to produce this acid, are susceptible to pathogenic microorganisms *Pseudomonas syringae* and *Peronospora parasitica*. According to the work of Gogbeu *et al* (2015) in *Manihot, esculenta*, resistance can be restored in these plants after an exogenous application of salicylic acid. Chitosan, a polysaccharide derived from chitin (Tolaimate *et al*., 2003), stimulates germination (Xue *et al*., 2002) and plant production (Salama *et al*., 2015). But, chitosan has been used more successfully in plant protection against abiotic (Pongprayoon *et al*., 2013) and biotic stress (Falcón-Rodriguez *et al*., 2014). These results clearly show that chitosan is used as both a biostimulant and a biopesticide. Based on these observations, salicylic acid and chitosan are referred to as elicitors because they stimuled defense responses in plants. First events that occur after recognition of elicitor by plant, are disturbances in cell membranes. As a result of these disturbances, ion (Ca^{2+},

K^+, and Cl^-) transfers occur between various cell compartments and outside cell (Heath, 2000). These ion flows have main effect of causing an increase calcium content in cytoplasm of stressed cells. Ca^{2+} ions activate protein kinases (Ruiz *et al*., 2003), which in turn cause phosphorylation reactions responsible for triggering defense processes (Jourdan *et al*., 2008). This transduction contributes to general resistance establishment in uninfected tissues of host plant. Purpose of this study is to evaluate amount of phenolic compounds synthesized and accumulated in *Tectona grandis* leaves under influence of salicylic acid and chitosan elicitors.

METHODOLOGY

Experimental apparatus

110-day old *Tectona grandis* plants were used for study. Seeding was done according to Gogbeu *et al*. (2018) method from cuttings. Each cutting was placed vertically in a perforated polyethylene bag filled with 5 kg of sterilized compost (a mixture of dead leaf powder and undergrowth soil). Crop was placed in a greenhouse lit by natural light and cuttings were watered regularly. At 110 days after germination of cuttings, plants obtained were divided into four lots according to treatment method: control plants, plants treated with salicylic acid, plants treated with chitosan and plants treated simultaneously with salicylic acid and chitosan.

Leaf treatment with salicylic acid and chitosan

Contact time of salicylic acid and / or chitosan on synthesis and accumulation of phenolic compounds was evaluated in two ways, in local and systemic reactions. In local reaction, evaluation was made from 0 to 96 hours. From 0 to 12 hours, 2nd row (F2) open leaves from apex were harvested every 4 hours and beyond at 24, 48, 72 and 96 hours. On this sheet, treatment was made on underside by a deposit of 40 µl of salicylic acid 5 mM and / or chitosan 100 mg/L on a wound previously made by pressure with aid of abrasive paper of diameter 1 mm. In systemic reaction, three leaf levels were considered. These are opposite leaf (F2opp) to treated leaf, one of immediate leaves located above (F1) of treated leaf and one of immediate leaves below (F3) of treated leaf. In control plants, same leaf levels were chosen. A total of 48 plants were used, ie 24 plants per type of experiment. Harvested leaves were immediately immersed in bath at 2°C for subsequent phenol extraction.

Extraction and dosage of phenolic compounds from leaves

Extraction of phenolic compounds was done according to Gogbeu *et al*. (2012) method. 1 g of fresh limb was milled in 3 mL of alcohol (80%, v / v). Ground material was centrifuged at 5000 rpm for 10 min at 4 °C. Supernatant obtained constituted phenolic extract. Amount of total phenols was determined according to Swain and Hillis (1959) method. Presence of phenols was revealed in extract by addition of 0.5 mL of Folin-Ciocalteau (0.5 N) reagent followed by 1.5 mL of sodium carbonate (17%, w/v). After 45 min incubation in dark, absorbance was determined at 725 nm on spectrophotometer. Amount of phenols was determined using a standard curve made with different tyrosin concentrations. Phenol content of leaves was calculated in milligram equivalent tyrosin per milligram of fresh material (mg tyrosin / mg FM).

Statistical analysis of the data

All experiments conducted in this work were repeated three times. SPSS software version 11.5 was used for comparison of averages. Analysis of variance at a classification criterion was made at 5% threshold. If $p \leq 0.05$, homogeneous groups were determined by the Duncan method.

RESULTS

Quantity of phenolic compounds in leaves according to phyllotaxy Content of leaves in phenolic compounds varied according to phyllotaxy (Table 1).

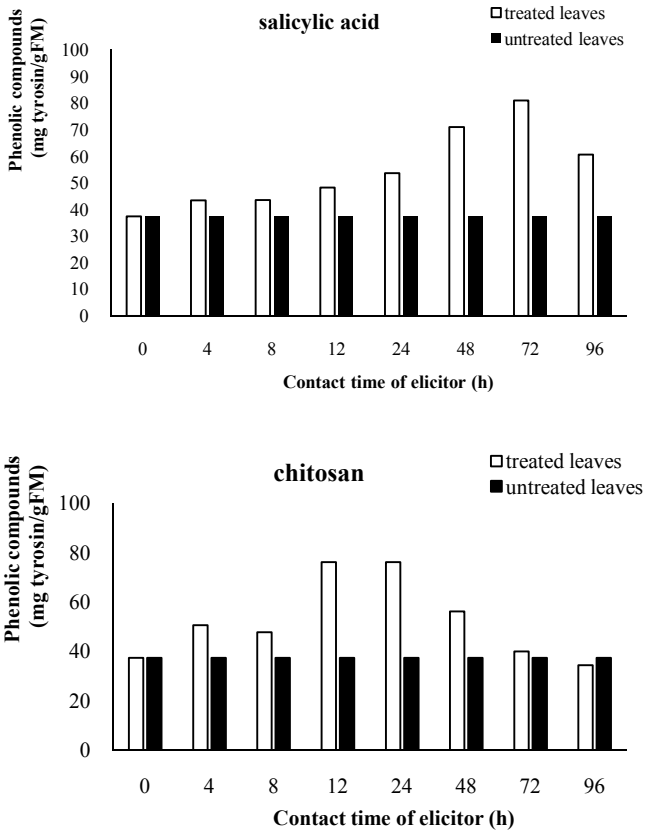
Table 1 Content of leaves in phenolic compounds

Phenolic compounds	Phyllotaxy				*Statistical test*	
	F_1	F_2	F_2opp.	F_3	*F*	*p*
(mg tyrosin/gFM)	**35,90[a]**	**37,48[a]**	**37,51[a]**	**37,56[a]**	***2,06***	***0,18***
Standard deviation	± 0,95	± 1,14	± 0,83	± 1,06		
Proportion (%)	95,77	100	100,05	100,22		

F1: untreated leaves of rank 1, F2: treated leaves of rank 2, F2opp.: untreated leaves of rank 2, F3 : untreated leaves of rank 3

From an average value of 35.90 mg tyrosin / gFM at first leaf level (F1), it reached value of 37.56 mg tyrosin / gFM at third level (F3) from apex to base. Leaves of second level (F2 and F2opp) had substantially same content of phenolic compounds. By taking phenol content of F2 sheets as 100%, only phenol value of F1 leaves (95.77%) remained lower. However, statistical test performed shows no significant difference between phenolic compounds content of leaves ($F = 2,06$; $P > 0.05$).

Effects of contact time of salicylic acid or chitosan on amount of phenolic compounds synthesized in F2 leaves Figure 1 shows an increase in amount of phenolic compounds in leaves after treatment of them with salicylic acid or chitosan.



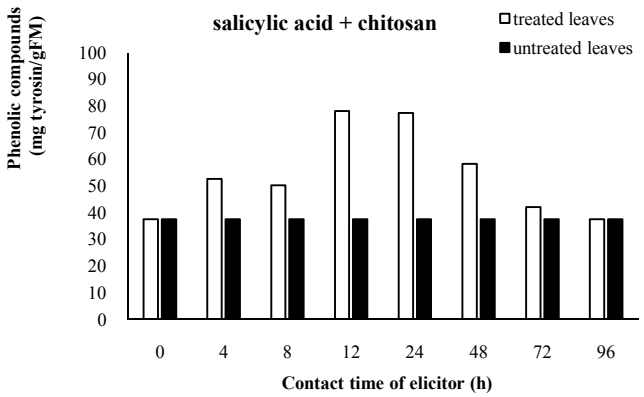
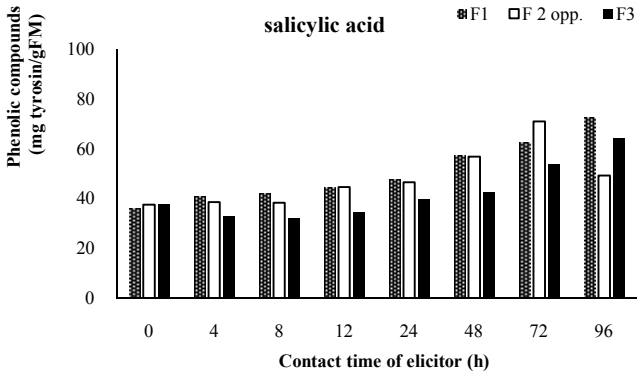
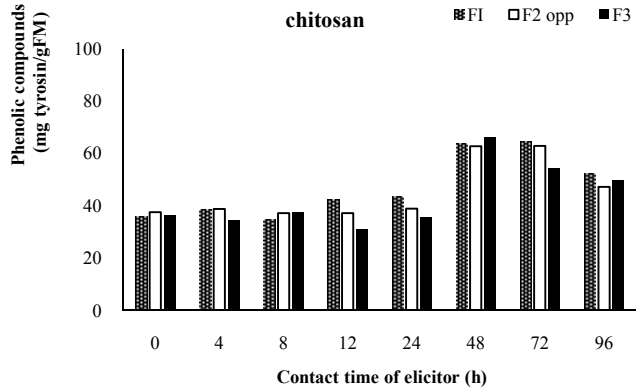


Figure 1 Effects of contact time of salicylic acid and chitosan on synthesis and accumulation of phenolic compounds in teak leaves.

In untreated leaves, value remained around 37.48 mg tyrosin / gFM. In fact, in presence of salicylic acid, maximum content of phenolic compounds was around 81.66 mg tyrosin / gFM after 72 hours of treatment. From an initial average value of 37.48 mg tyrosin / gFM in treated leaves (F2), it increased as contact time increased. After this time, it decreased but still remained high compared to control at end of experiment (96 hours: 60.66 mg tyrosin / gFM). Statistical test performed to compare phenol content of leaves during experiment revealed a significant difference (*F =4,09; p < 0,05).* When chitosan was applied to the leaves (Figure 2), synthesis and accumulation of phenolic compounds was early compared to salicylic acid.





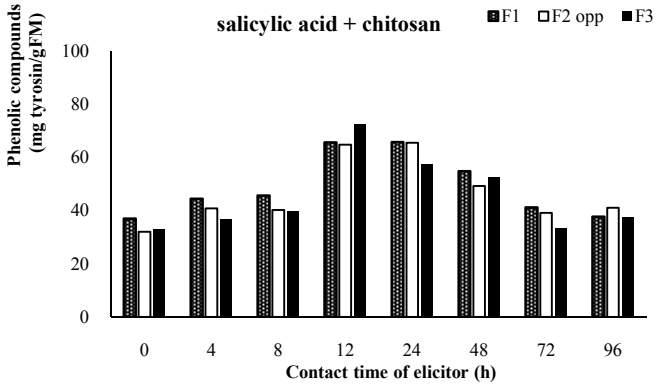


Figure 2 Action of salicylic acid and chitosan on synthesis and accumulation of phenolic compounds in untreated teak leaves. *F1:untreated leaves of rank 1, F2opp .: untreated leaves of rank 2, F3: untreated leaves of rank 3*

Maximum values were recorded at 12 (76.14 mg tyrosin / gFM) and 24 hours (76.10 mg tyrosin / gFM) after treatment after a slight increase recorded at 4 hours (50.58 mg tyrosingFM). Maximum content of phenolic compounds in leaves was substantially same in presence of salicylic acid (72 hours: 81.07 mg tyrosin / gFM) and chitosan (24 hours: 76.14 mg tyrosin / gFM). In presence of salicylic acid and chitosan applied to same leaves F2, synthesis and accumulation of phenols were stimulated rapidly. From 38.52 mg tyrosin / gFM, content reached 52.66 mg tyrosin / gFM after 4 hours of treatment. It decreased to 8 hours before recovering to 12 hours when it reached value of 78.21 mg tyrosin / gFM. Up to 24 hours of treatment, accumulation remained maximal. After this period, amount of phenolic compounds decreased gradually in leaves as contact time increased. At different sampling times, amount of phenols was not same (*F = 4,27; p < 0,05*).

Effects of contact time of salicylic acid or chitosan on synthesis and accumulation of phenolic compounds in F1, F2opp and F3 leaves during systemic reaction Amount of phenolic compounds in different leaves of untreated teck plants (F1, F2opp and F3) with salicylic acid and chitosan varies with phyllotaxy (Figure 2). In contrast to treated leaf (F2opp), maximum value of phenols was 70.97 mg tyrosin / gFM. It was also recorded after 72 hours of treatment. On other hand, in leaves below (F3) and above (F1) treated leaves, maximum phenol content was obtained only after 96 hours of treatment. It was respectively 64.03 and 72.38 mg tyrosin / gFM. At each hour of sampling, statistical analysis showed a significant difference between quantities of phenols accumulated in different leaves (*F = 34,08 ; p <0,05*). When plants were treated with chitosan, amount of phenols in leaves opposite treated leaves (F2opp) and that of leaves below (F3) and above (F1) also varied with contact time. In these untreated leaves (F1, F2opp and F3), highest valueswere recorded from 48 hours of treatment. They were 62.79, 64.59 and 66.34 mg tyrosin / gFM respectively for F2opp, F1 and F3 leaves. By comparing influence of these two elicitors on accumulation of phenolic compounds leaves, it appears that at each sampling time, distribution of amount of phenols is not a function of elicitor, of phyllotaxy position of leaf (*F = 8,57; p < 0,05*). When salicylic acid and chitosan were applied to same leaves, phenol accumulation was rapid in untreated leaves. In leaves at apex (F1), amount of phenols increased steadily to 65.66 mg tyrosin / gFM at 12 hours of treatment. It remained high until 24 hours (65.79 mg tyrosin / gFM) before decreasing. In leaves opposite treated leaves, amount of phenols also increased gradually, but remained below level recorded in F1 leaves except value obtained at 96 hours (41.10 mg tyrosin / gFM). In basal leaves, at 12 hours of treatment, amount of phenols was 72.55 mgtyrosin / gFM. It is in these leaves that greatest value in phenolic compounds of leaves has been obtained. After 12 hours of treatment, phenol content decreased as contact time increased.

Comparison of effects of salicylic acid and chitosan on synthesis and accumulation of phenolic compounds according to phyllotaxy Analysis in Table 2 indicates that according to phyllotaxy, distribution of amount of phenolic compounds in leaves was substantially same (*F = 2.06, p >0.05*).

Table 2 Comparative action of salicylic acid and chitosan on synthesis and accumulation of phenolic compounds in teak leaves.

Phyllotaxy	Elicitors	Contact time of elicitors (h)							
		0	4	8	12	24	48	72	96
Untreated Leaves rank1	SA	35,90^{a} ± 0,95	40,72^{b} ±1,04	42,03^{bc} ±1,23	44,59^{a} ±1,13	47,86^{a} ±3,07	57,24^{b} ±4,30	62,66^{a} ±1,02	72,38^{b} ±2,17
	CH	35,90^{a} ±2,94	38,38^{a} ±6,31	34,90^{a} ±3,57	42,38^{a} ±0,97	43,62^{a} ±2,88	63,61^{a} ±6,17	64,59a ±4,53	52,41^{a} ±5,67
	SA+CH	36,93^{a} ±2,95	44,44^{c} ±0,98	45,69^{c} ±2,89	65,66^{b} ±6,21	65,79^{b} ±5,11	54,83^{b} ±5,92	41,14^{b} ±5,13	37,66^{c} ±4,51
Treated leaves rank 2	SA	37,48^{a} ±1,14	43,45^{a} ±2,83	43,66^{a} ±2,24	48,24^{a} ±1,15	53,66^{a} ±2,17	71,03^{a} ±1,28	81,07^{a} ±1,63	60,66^{a} ±3,17
	CH	37,48^{a} ±5,66	50,59^{b} ±2,37	47,79^{b} ±4,50	76,14^{b} ±3,26	76,10^{b} ±4,26	56,24^{b} ±4,96	40,07^{b} ±5,00	34,48^{b} ±4,16
	SA+CH	38,52^{a} ±7,67	52,66^{b} ±2,38	50,34^{c} ±5,01	78,21^{b} ±3,27	77,48^{b} ±4,30	58,31^{b} ±4,97	42,14^{b} ±5,00	37,59^{b} ±4,51
Untreated leaves, rank 2	SA	37,51^{a} ±0,83	38,62^{a} ±2,48	38,28^{a} ±1,96	44,62^{b} ±2,60	46,52^{b} ±3,58	56,79^{b} ±2,56	70,97^{b} ±2,33	49,28^{b} ±3,95
	CH	37,51^{a} ±0,79	38,76^{a} ±2,29	37,14^{a} ±5,87	37,10^{a} ±5,12	38,90^{a} ±5,22	62,76^{c} ±1,62	62,79^{b} ±1,89	47,24^{b} ±4,32
	SA+CH	32,07^{a} ±1,88	40,77^{b} ±2,53	40,24^{b} ±6,33	64,83^{c} ±1,62	65,55^{c} ±1,88	49,31^{a} ±4,32	39,17^{a} ±5,12	41,10^{a} ±5,33
Untreated Leaves rank 3	SA	37,56^{a} ±1,06	32,76^{a} ±2,25	32,03^{a} ±1,82	34,41^{a} ±4,26	39,62^{b} ±3,59	42,52^{a} ±2,43	53,83^{b} ±4,78	64,03^{c} ±4,19
	CH	37,56^{a} ±2,00	34,55^{bc} ±4,98	37,48^{b} ±5,40	31,07^{a} ±1,45	35,69^{a} ±7,44	66,34^{c} ±4,78	54,34^{b} ±3,79	49,66^{b} ±6,09
	SA+CH	32,69^{a} ±9,89	36,62^{c} ±4,99	39,55^{b} ±5,41	72,55^{b} ±10,19	57,45^{c} ±5,48	52,41^{b} ±6,80	33,14^{a} ±1,45	37,41^{a} ±6,97

For each group of sheets, values followed by the same alphabetical letter are not significantly different, SA: Salicylic acid, CH :chitosan

In the lot of treated leaves of rank 2, high values were recorded in salicylic acid treated leaves (81.07 mg tyrosin / gFM). Then, leaves of salicylic acid + chitosan treatment batch gave a large amount of phenols; and finally leaves treated with chitosan. In latter, maximum amount hovered around 76 mg tyrosin / gFM. However, accumulation was early in presence of chitosan (12 hours after treatment) and combined action of salicylic acid and chitosan. On the other hand, with salicylic acid, maximum value was recorded at 72 hours of treatment, ie 48 hours after that of chitosan. In lot of leaves opposite treated leaves (opposite leaves rank 2), highest values of phenolic compounds were obtained with salicylic acid, followed by chitosan. Response of leaves to treatments was fast with salicylic acid + chitosan. It takes place 12 hours after treatment. With chitosan, optimum was obtained at 48 hours and with salicylic acid at 72 hours. In extreme leaves (F1 and F3), with salicylic acid, significant amounts of phenols were obtained at 96 hours after treatment (F1: 72.38 mg tyrosin / gFM, F3: 64.03 mg tyrosin / gFM). Chitosan was recorded at 72 hours (64.59 mg tyrosin / gFM) for leaves of rank 1, and 48 hours (66.34 mg tyrosin / gFM) for leaves of rank 3. With salicylic acid + chitosan, synthesis was maximal at 12 h (F1: 65.66 mg tyrosin / gFM, F3: 72.55 mg tyrosin /gFM).

DISCUSSION

This study showed that teak leaves were rich in phenolic compounds. According to Lattanzio*et al*. (2008), presence of phenols in plants would be greatly influenced by environment and genetic control. In natural selection during plant evolution, phenols would therefore be characteristics of adaptation (Treutter, 2006, Russel *et al*., 2009). Inoculation of leaves with salicylic acid and chitosan, however, triggered synthesis and accumulation of phenolic compounds in them. Amount of phenol synthesized, however, varied with sampling time and phyllotaxis. These results corroborate those obtained in other plants by Loakeand Grant (2007), Dogbo *et al*. (2008), Vlot *et al*. (2009) and Liu *et al*. (2012) who used salicylic acid and chitosan as elicitor. These authors reported that salicylic acid and chitosan induced activity of stress enzymes. Regarding cassava, Dogbo *et al*. (2008) obtained rapid activation of polyphenoloxidases following inoculation of plant leaves with salicylic acid. In inoculated leaves, they noted maximal enzymatic activity between 1 and 3 hours after treatment. Similarly, work done by Gogbeu *et al* (2015) has shown induction of new forms of polyphenoloxidases after treatment or inoculation of cassava plants with phosphorous acid or with *Colletotrichum sp*.

With regard to chitosan, work done by Liu *et al*. (2012) showed that this compound induces production of PR-protein. Among them, chitinases and β-1.3 glucanases that would be markers of plant defense system. Salicylic acid and chitosan, however, acted differently in teak. In local reaction, maximum content of phenolic compounds (81.06 mg tyrosin / gFM) was recorded in presence of salicylic acid elicitor, 72 hours after treatment. On the other hand, it was earlier with chitosan, 12 hours after treatment. When both compounds were applied simultaneously, maximum amount of phenols was also recorded at 12 hours after treatment. These data on synthesis and accumulation of these compounds indicate their involvement in establishment of defense system in the plant (Li and Zhu, 2013, Xing *et al*., 2015) and that salicylic acid and chitosan would also be elicitors. Phenolic compounds would have antimicrobial properties (Martini *et al*., 2009). Ma *et al*. (2013) and Xing *et al*. (2015) noted that exogenous application of chitosan induces plant resistance to several pathogens by increasing phenylalanine ammonia liases activities. However, important activity of this enzyme would make available toplant cinnamic acid which is precursor of most secondary compounds, namely phenols. This rapid accumulation of phenols would stop progression of pathogen (May*et al*., 1997). In a systemic reaction, distribution of accumulated phenols varied according to phyllotaxy. With salicylic acid, reaction was early in leaves above treated leaves; but maximum accumulation of phenols was recorded in leaves opposite treated leaves. With chitosan, it is leaves opposite treated leaves and leaves above that have reacted quickly. But, maximum accumulation was observed in leaves below. With salicylic acid and chitosan mixture, three leaf levels reacted quickly. However, content of accumulated phenols was significant in leaves below treated leaves. These observations clearly show that applied compounds take phloem pathway. Similar results have been reported by Dogbo *et al*. (2008) in cassava. Leaves of this plant, located on both sides of treated leaf reacted to salicylic acid treatment by synthesis and accumulation of phenolic compounds. In general, increase in phenol level is observed ininfected tissues compared to control (Mikulič-Petkovšek*et al*., 2011). On apple bark, high level of hydroxycinnamic acid around *Venturiaina equalis* infection site is related to presence of this pathogen. This testifies to role of phenols in setting up the defense mechanism of plants. These same observations have been reported by Mikulič-Petkovšek*et al*. (2009) and Leser and Treutter (2005) as responses to stress conditions. These plants reacted to the artificial inoculation responses of the pathogen *Colletotrichum lindemuthium*. Thus, phenols restrict viability of pathogen and are

often deposited in intracellular space to provide first line of defense against entry of pathogen or its infection (Schwalb and Freucht, 1999). This study in teak has shown that phenolic compounds can be compounds involved in defense mechanisms of plant.

CONCLUSION

This study showed that teak plants react with salicylic acid and chitosan by synthesis of phenolic compounds. In unstressed plants, amount of accumulated phenols does not vary with position of the leaf. When plants are stressed, phenol content changes from one leaf to another. However, reaction time is early in treated leaves followed by leaves opposite treated leaves. Cumulative action of salicylic acid and chitosan elicitors on synthesis and accumulation of phenols follows effect of chitosan.

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