INDUCTION OF SYSTEMIC RESISTANCE IN *ENSETE VENTRICOSUM* CLONES BY THE LEAF EXTRACT OF *AGARISTA SALICIFOLIA* AGAINST *XANTHOMONAS CAMPESTRIS* pv. *MUSACEARUM*

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SUMMARY

The potential role of *Agarista salicifolia* leaf extract to trigger induced resistance, biochemical changes in *Ensete ventricosum* and the subsequent suppression of Enset bacterial wilt (EBW) was evaluated under field condition. Resistant (*Genticha*) and susceptible (*Midasho*) Enset clones were grown in the field and treated with *A. salicifolia* extract and *Xanthomonas campestris* pv. *musacearum* (Xcm). The results revealed that treated plants of both clones had an increased activity of phenylalanine ammonia lyase (PAL), peroxidase, polyphenol oxidase and greater amount of total phenolics content (TPC) and total soluble sugars (TSS). The activity of tested enzymes and amounts of TPC and TSS were significantly increased in resistant plants treated with leaf extract and Xcm as compared to susceptible ones. The activity of PAL progressively increased in resistant plants treated with the leaf extract. The biochemical changes that occur after the application of treatments imply the onset of induced systemic resistance. Extract induced resistance brought a 33.33% and 3.1% disease incidence reduction in susceptible and resistant plants, respectively, compared to infected controls. The result suggests that induced resistance could be used as a feasible approach to control EBW. However, the need for further studies on other application methods that augment efficacy and scaling up is required.

Keywords: *Enset* bacterial wilt, inducible responses, phenylpropanoid pathway, phenolic compounds.

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INTRODUCTION

Enset bacterial wilt (EBW) is a devastating disease of Enset and banana threatening the lives of millions of people in eastern and central Africa (Addis *et al*., 2010). It is caused by the bacterium *Xanthomonas campestris* pv. *musacearum* (Xcm). The disease was first reported in 1968 in Ethiopia on Enset and the first natural epidemic of EBW occurred in 1974 on banana in Keffa-Sheka zone, south-western Ethiopia (Yirgou and Bradbury, 1968, 1974). Since 1980s, the disease is becoming a serious problem for Enset and banana production and now it is recognized as a national problem with an increased severity and spread into most Enset and banana growing agro-ecological zones of the country. Furthermore, the disease is widespread in East and central African regions, where banana forms a large proportion of the diet for about 25 million people, posing a serious threat to household food security and income (Smith *et al*., 2008).

EBW is the major constraint of *E. ventricosum* (Welw.) Cheesman, cultivation; a multipurpose crop widely grown in the south and southwestern parts of Ethiopia (Wolde-Michael *et al*., 2008). Unlike other diseases, EBW is both extreme and rapid causing gradual increasing losses over years. It has been reported to have a considerable impact on Enset cultivation. Surveys conducted in the early 1980's and 1990's in the major Enset growing zones revealed the occurrence of EBW disease in all zones with differing degrees of severity (Ashagari, 1985). The economic impact of EBW is so severe that it can kill the whole plant (mother plant) that would otherwise contribute to the continuation of Enset production cycles. The bacterium survives well on infected plant material and moves easily in soil water, making the disease very difficult to manage (Smith *et al*., 2008).

Under most agro-ecological conditions in Ethiopia, the main means of EBW transmission are infected planting materials (i.e. suckers) and contaminated farming tools. The spread of the disease is commonly prevented by cultural management practices such as burying infected plants, restricting movement of infected plant materials, sterilizing farming tools, and raising awareness about the disease (Tripathi *et al*., 2009; Addis *et al*., 2010). However, these methods are not effective due to the fact that farmers

are inconsistent and reluctant to employ labor-intensive disease control measures (Tripathi *et al*., 2009). Moreover, there is no commercial bactericide available to date that effectively controls the infection (Smith *et al*., 2008; Seleim *et al*., 2011). Considering all these, there is an imperative need for additional approaches of controlling the disease, and thus, induced resistance delivers the promise of durable, broad-spectrum disease control using the plant's own natural immune system.

In addition to pre-existing defense barriers to pathogens, plants possess an inducible immune system that controls the activation of defence mechanisms after recognition of a pathogen. Induced resistance is a physiological "state of enhanced defensive capacity" elicited by specific environmental stimuli, whereby the plant's innate defenses are potentiated against subsequent biotic challenges (Vallad and Goodman, 2004). It is a novel technology in the management of plant diseases and in practicing it, plant defenses are preconditioned by prior treatment that results in resistance against subsequent challenge by a pathogen (Vallad and Goodman, 2004). Induced resistance offers the prospect of durable, broad-spectrum, eco-friendly and cost-effective disease controlling approach using the plant's own resistance (Abd El-Rahman *et al*., 2012; Jadesha *et al*., 2012). It also increases the ability of susceptible plants to withstand pathogens in a non-genetic way (Ma and Cui, 2002).

Induced resistance can be provoked with the aid of biotic and abiotic agents. These agents are capable of mimicking the perception of a pathogen by a plant, thereby triggering induction of a sophisticated defense response in plants (Mandal, 2010). They bind to specific receptors of the plant and trigger a signaling cascade that eventually results in biochemical and mechanical defenses (Mandal, 2010). One often used method is the application of plant extracts as natural plant protection products. Plant extracts act directly on the pathogen and/or indirectly induce the host plant natural immune system (Ahmad *et al*., 2010). Therefore, the aim of this study was to activate the natural immune system of *E. ventricosum* using crude leaf extract, investigate the biochemical changes in the plant and evaluate EBW disease suppressive capacity of induced resistance under field condition.

MATERIALS AND METHODS

Enset clones. The Enset clones used in this study were selected based on research reports about their sensitivity to Xcm and the preference of Enset growing farmers in terms of quantity and quality of products such as *Bulla*, *Kocho* and *Amicho*. Accordingly, *Genticha* is known as resistant/tolerant and *Midasho* as susceptible clones (Wolde-Michael *et al*., 2008). One Xcm strain was used throughout the experiment. The strain was isolated from infected Enset in Sidama zone where the study was conducted. This zone was selected as a source of Enset clones and Xcm strain because of the presence of high natural epidemic of Enset bacterial wilt and of more virulent Xcm isolates, as reported by Handoro and Wolde-Michael (2007).

Growing conditions. The field experiment was conducted on an area of 1224 m^2, partitioned into three blocks, each consisting of six, 6 m×6 m (36 m^2) plots. There were 2 m and 3 m spacing between blocks and plots, respectively. One year old and approximately equal sized Enset suckers of the clones under study (*Ganticha* and *Midasho*) were bought from farmers and transplanted at the beginning of the rainy season. Each Enset clone was randomly assigned to each plot in a completely randomized block design in three replications. Nine individual Enset suckers were transplanted in each plot. After two weeks, equal amount of undecayed cattle manure was added around each Enset sucker. Enset management techniques such as tilling and weeding commonly practiced by the Enset farmers were carried out as needed.

Leaf extract preparation. Fresh leaves of *Agarista salicifolia* (Com.ex Lamb.) G. Don (Ericaceae) was collected from Amaro district, southern Ethiopia. The species was selected on the basis of follow-up of antimicrobial activity reports and traditional use of the plant against diseases (Fabricant and Farnsworth, 2001). It is a tree that grows on steep, rocky slopes in *Juniperus-Erica* scrub, open grassland with *Combretum*, *Protea*, *Erica* and *Helichrysum*, bamboo forest, evergreen forest relict in valleys at altitude ranging 2050-2900 m above sea level (a.s.l.). It is found widespread in Africa (Hedberg and Hedberg, 2003). The leaf samples were collected from a tree grown at 2131 m a.s.l. The leaf of this species is traditionally used to treat gastrointestinal diseases such as diarrhea in humans (Shangal *et al*., 2008). The bark and the root are used to treat sores (external boil) and toothache, respectively (Shangal *et al*., 2008). The shoot is also used to treat cattle disease such as Babesiosis (Andarge *et al*., 2015). Specimen of the species was freshly pressed, mounted and identified by experts in the National Herbarium, Department of Plant Biology and Biodiversity Management, Addis Ababa University. A voucher specimen of the species has been made available in the National Herbarium.

The leaves were dried under shade at room temperature and ground to fine powder using mechanical grinder. Extraction was carried out using maceration method. Leaf powder and the solvent (methanol) were added into conical flasks in the ratio of 1 : 10 (w/v). The flasks were tightly closed and shaken for 72 h using orbital shaker at a speed of 250 rpm under room temperature. The extract was filtered first by four layer of cheese cloth and cotton followed by Whatman's No. 1 filter paper. The extract was dried and concentrated by evaporating methanol using rotary evaporator. The antibacterial activity the leaf extract was tested using disc diffusion method and minimum

inhibitory concentration (MIC) of the extract was determined using agar dilution method. The leaf extract of *A. salicifolia* exhibited high potency and low MIC value. Thus, the MIC value (12.5 mg/ml) was used to induce defense responses in *E. ventricosum* in the field experiment (data not shown). Methanol was used as a solvent.

Xcm isolation and identification. The Xcm strain was isolated from infected Enset pseudostem samples obtained from the study area. The samples were cut into smaller pieces using sterile scalpel. The pieces were surface disinfected by dipping in 5% sodium hypochlorite solution for one min and immediately immersed in distilled water three times to remove the disinfectant. Then after, the cut pieces were placed in a test tube containing 5 ml of distilled water and allowed to stand for 5 min until the bacterial population diffuses out of the cut tissue into the distilled water. Serial dilutions of the bacterial suspension were prepared and loopful of the dilutions (10^{-2} and 10^{-3}) were streaked to sterilized semi-selective growth medium composed of yeast extract (10 g l^{-1}), peptone (10 g l^{-1}), sucrose (10 g l^{-1}), agar (15 g l^{-1}), cephalexin (50 mg l^{-1}) and amphotericin (150 mg l^{-1}) (Tripathi *et al*., 2007). The streaked Petri dishes were incubated in an inverted position at 28°C for 72 h. Sub-culturing was carried out by taking loopful of the bacterium directly from separate and uniform colonies in growth plates. Identification was made based on colony characteristics. Accordingly, yellowish, mucoid and circular colonies were identified as Xcm. The identity of the pathogen was confirmed by pathogenicity test. Culture collection of the Xcm strain is available at Ethiopian Institute of Biodiversity.

Pathogenicity test. Plastic buckets filled with soil, sand and manure in the ratio of 2 : 1 : 1 were prepared in the glasshouse and suckers of a susceptible Enset clone were transplanted. After establishment, individual Enset plants were inoculated with 10 ml of Xcm suspension adjusted to 1.5×10^8 CFU/ml (0.5 McFarland standard) at the base of midrib in three replications. The negative control was inoculated with the same amount of distilled water using syringes with metal needle. A week after inoculation, symptom development was monitored for every other day. Yellowing of the inoculated leaf was seen after three weeks. Pseudostem of the infected plants was taken and the bacterium was re-isolated using the standard isolation procedure.

Xcm inoculum preparation. Xcm inoculum was prepared from 72 h old bacteria grown in yeast extract (1%), peptone (1%), sucrose (1%) and agar (1.5%) medium (YPSA). The upper surfaces of several isolated (pure) colonies were swabbed with cotton swab and dissolved in distilled water in a test tube. The content of the test tube was thoroughly shaken until a homogenous suspension was formed. The absorbance of the bacterial suspension was measured with a spectrophotometer (NV202, Sunny) at 600 nm and adjusted to 0.132 which is equivalent to the density of 0.5 McFarland standards (Sutton, 2011). The bacterial population at 0.5 McFarland standards is approximately equal to 1.5×10^8 CFU/ml. Xcm was preserved in Petri dishes for short term (one month) storage and in slant cultures for relatively long period (two months) at 4°C. In both cases, Xcm was grown on YPSA growth medium.

Application of treatments. The treatments were applied after one year from transplanting. In each block, there were six plots; three for each Enset clone. The treatments were assigned randomly in plots. All plants grown in a plot received the respective treatments. The three treatments were: 1) Enset plants inoculated with 10 ml of Xcm suspension adjusted to 1.5×10^8 CFU/ml. Xcm inoculated plants were taken as infected controls (positive controls); 2) Enset plants treated with 10 ml of 12.5 mg/ml *A. salicifolia* crude leaf extract dissolved in methanol. The value 12.5 mg/ml was the minimum inhibitory concentration of the leaf extract. Plants pretreated with the leaf extract were inoculated with Xcm suspension after 30 days when symptoms of infected control plants fully develop. Both inoculants were thoroughly shaken before inoculation; 3) Enset plants treated with 10 ml of methanol, the solvent of the leaf extract, were taken as the controls (negative controls) of the experiment. All treatments were applied at the base of the newly expanding youngest leaf using mid vein injection method. One leaf/plant was treated with the corresponding treatments throughout the experiment. Separate 5 ml capacity hypodermic syringes with metal needles were used for the application of the three treatments. To protect transmission of the pathogen, separate management tools were used for each treatment after inoculation.

Leaf sample collection. Leaf samples were collected from plants grown under each treatment to study the induction of secondary metabolism after the application of treatments. Leaf discs approximately equal to 10 cm×10 cm in size were collected from the third leaf, different from treated leaf, from the top at 0, 24, 48, 72, 96 and 120 h after treatment application. The midrib was not included during sampling. The leaf discs were taken from three to five randomly selected plants in each plot. The leaf samples were stored in liquid nitrogen and transported to Addis Ababa University. The leaf discs of each clone were sorted by blocks and treatments, and further cut into small pieces. The leaf pieces of each treatment were homogenized block wise and used for biochemical analysis separately. All assays were performed independently a minimum of two times for each Enset clone, replication (block), treatment and sample collection period. Similar trends were observed between replicate samples under trail times during each bioassay.

Assay for the detection of phenylalanine ammonia lyase (PAL). The activity of PAL was estimated according to Dickerson *et al.* (1984) as described in Eisa *et al.* (2013). One gram (1 g) of leaf sample from each treatment was crushed and homogenized in 10 ml of borate buffer (pH 8.8) using pre-chilled mortar and pestle. This was carried out in the presence of 0.4 ml of 5 mM mercaptoethanol l^{-1}. The homogenate was centrifuged at 12,000 rpm at 4°C for 10 min and the supernatant was collected and used as an enzyme extract.

An aliquot (0.2 ml) of enzyme extract was transferred into a separate tube containing 2.5 ml of borate buffer and 1 ml of 0.1 mM L-phenylalanine (pH 8.8). This was done for each treatment and clone for every sampling time. The mixture was incubated for 30 min at 32 ± 2°C. Enzyme reaction was stopped by the addition of 0.5 ml of 2 N HCl. Enzyme extract (0.2 ml) along with 0.5 ml of borate buffer, 1.3 ml of distilled water, 0.5 ml of 2 N HCl and 1 ml of L-phenylalanine were added into a test tube in sequence and used as a blank. The PAL activity was determined from the production of cinnamate (Qin and Tian, 2005) and absorbance was measured at 290 nm with a spectrophotometer. The amount of cinnamic acid produced was determined from cinnamic acid calibration curve and PAL activity was expressed as µmoles of cinnamic acid produced g^{-1} of fresh weight.

Peroxidase assay. In the presence of hydrogen donor, pyrogallol peroxidase converts H_2O_2 to H_2O and O_2. The oxidation of pyrogallol to a coloured product called purpurogalli can be followed spectrophotometrically at 470 nm. One gram of Enset leaf sample was weighed and crushed in pre-chilled (cooled) mortar and pestle in the presence of 0.1 M sodium phosphate buffer (pH 7) in the ratio of 1 : 4 (w/v). The extract was allowed to pass through a funnel and the filtrate was centrifuged at 4000 rpm for 20 min. The supernatant was used for estimation of peroxidase activity.

Two sets of tests were run representing the experimental test and the control. To all the test tubes of the two sets, 2.5 ml of sodium phosphate buffer (pH 7) and 0.2 ml of the enzyme extract were added. To the experimental set, 0.2 ml of 0.05 M pyrogallol solution was added and mixed. Then 0.1 ml of 1% H_2O_2 was added to all the test tubes. Contents were mixed thoroughly and left at room temperature for 20 min. A solution consisted of 0.2 ml of distilled water, 2.5 ml of phosphate buffer (pH 7) and 0.1 ml of 1% H_2O_2 was used as a blank.

Absorbance of the experimental and control tests was measured at 470 nm using a spectrophotometer (NV202, Sunny). Each measurement was replicated three times to ensure maximum conformity of results. Optical density (OD) of all the tests was taken against the blank. Peroxidase activity was estimated according to Anjum *et al.* (2012) using the formula as follows:

$$\text{Units mg}^{-1}\text{ fresh wt.} = \frac{\text{O.D of test} - \text{O.D of control}}{\text{O.D of control} \cdot \text{mg of plant sample}}$$

Peroxidase activity was expressed in terms of absorbance changes min^{-1} g^{-1} of leaf sample.

Polyphenol oxidase assay. Polyphenol oxidase activity was determined according to the method described by Esterbauer *et al.* (1977). Using a pre-chilled mortar and pestle, 0.5 gram of leaf sample was homogenized in 2 ml of the extracting solvent containing 50 mM Tris-HCl (pH 7.2), 0.4 M sorbitol and 10 mM NaCl in the ratio of 3 : 1 : 1. The homogenate was centrifuged at 2000 rpm for 10 min at 4°C and the supernatant was used for the assay.

An aliquot of 2.5 ml of phosphate buffer and 0.3 ml of catechol solution and 0.2 ml of enzyme extract were added in a cuvette and the absorbance of the mixture was measured at 495 nm using a spectrophotometer. Change in absorbance was recorded every 30 seconds interval for 3 min. However, absorbance was nearly stable approximately after 2 min. One unit of polyphenol oxidase is defined as the amount of enzyme that transforms one µmole of dihydrophenol to one µmole of quinone per min. The activity of polyphenol oxidase was calculated using the formula:

$$\text{Enzyme unit in the sample} = K \times (\Delta A / \text{min})$$

where, K for catechol oxidase (polyphenol oxidase) = 0.272.

Estimation of total phenolics content. Enset leaf sample (1 g) from each treatment was homogenized in a mortar and pestle in 10 ml of 80% methanol (w/v). The homogenate was filtered in a funnel and centrifuged at 10,000 rpm for 10 min. The supernatant was used for total phenolics content determination. A 0.2 ml aliquot of the supernatant was taken and diluted to 3 ml with distilled water. Consecutively, 0.25 ml of Folin Ciocalteau reagent was added. After 3 min, 1 ml of 20% (w/v) sodium carbonate was added and thoroughly mixed. The tubes were placed in boiling water for 1 min and cooled.

A blue color was developed in each test tube because the phenols undergo a complex redox reaction with phosphomolibdic acid in Folin Ciocalteau reagent in alkaline medium. This blue colored complex was molybdenum blue. The absorbance was measured at 650 nm against a reagent blank using a spectrophotometer (NV202 Spectrophotometer, Sunny). The blank was composed of 3 ml of distilled water, 0.25 ml of Folin Ciocalteau and 1 ml of 20% sodium carbonate. The absorbance of the blank was subtracted from each reading. Catechol was used to prepare the standard calibration curve from which the amount of total phenols in the samples was calculated. The amount of total phenols was expressed in mg catechol equivalent of phenol/g of leaf sample (Zieslin and Ben-Zaken, 1993).

Estimation of total soluble sugars. The amount of total soluble sugars was estimated using phenol-sulfuric acid method as mentioned in Nielsen (2010). Freeze dried Enset leaf sample (0.5 g) was homogenized with 10 ml of 80% ethanol as described in Salt *et al.* (1988). The solution was poured in a funnel and the filtrate was centrifuged at 2000 rpm for 20 min. The supernatant was collected separately for each clone, treatment and sampling time. In a test tube, 0.1 ml alcoholic leaf extract and 1 ml of 2% phenol solution were added. This concentration of phenol solution was chosen due to the smallest difference in the absorbance readings among the three sugars (glucose, fructose and galactose). Moreover, measuring absorbance at 490 nm with the use of 2% phenol in the sugar assay gives the closest match to the absorption coefficients of the three basic monomers without compromising the sensitivity of the assay (Chow and Landhausser, 2004). To the extract-phenol solution, 5 ml of concentrated sulphuric acid was added. Each tube was gently agitated during the addition of the acid and allowed to stand in a water bath adjusted to 30°C for 20 min.

The absorbance of the yellow orange color thus developed was measured at 490 nm in a spectrophotometer. The blank was composed of all the constituents of the test without the enzyme extract. The amount of total soluble sugars was determined from a standard curve prepared by using known concentration of glucose followed by the addition of equal amounts of phenol solution and sulfuric acid. The amount of sugar was expressed as mg/g fresh weight of leaf tissue.

Disease incidence. Disease incidence (DI) was determined 4 months after treatment application. Nine plants were grown in each plot and each received the corresponding treatment. The numbers of dead plants in each plot and treatment were counted and recorded. DI was calculated separately for each treatment and clone under each plot and the average values were taken**.**

Percentage of disease incidence was calculated according to Haggag and El-Gamal (2012) with little modification using the formula as follows:

$$\text{Disease incidence (\%)} = \frac{\text{number of dead plants} \times 100}{\text{total number of plants inoculated}}$$

Statistical analysis. All data collected were subjected to analysis of variance using SPSS (Statistical Package for Social Sciences, version 20). This was done after carrying out test of homogeneity and normal distribution for each measured parameter. Means were compared by Tukey's Honestly Significant Difference (HSD) test at 95% confidence interval.

RESULTS

PAL activity. Analysis of variance revealed that the activity of PAL was quickly elevated in leaf extract induced and infected control plants (Fig. 1A, 1B). The enzyme activity measured at various time points after treatment application was significantly higher ($P < 0.01$) in treated plants than untreated (0 h) ones (Fig. 1A, 1B). The difference between treatments in each clone was also statistically significant ($P < 0.05$). In the resistant clone, induced plants showed an 8, 4.6, 3, 13 and 4.6 fold increments in PAL activity compared to controls at 24, 48, 72, 96 and 120 h post treatment, respectively. On the other hand, infected control plants showed a 7, 3.6, 2.3, 10.6 and 2.4 fold increments compared to controls. In this study, the resistant clone treated with *A. salicifolia* leaf extract showed the highest PAL activity 120 h followed by 96 and 72 h post treatment (Fig. 1A). Similarly, induced plants of the susceptible clone exhibited 1.5, 1.7, 1.4, 4.2 and 4.4 fold increments in PAL activity compared to controls at 24, 48, 72, 96 and 120 h post treatment, respectively, while infected control plants showed a 0, 1.3, 1.4, 3 and 2 fold increments in PAL activity compared to controls at similar

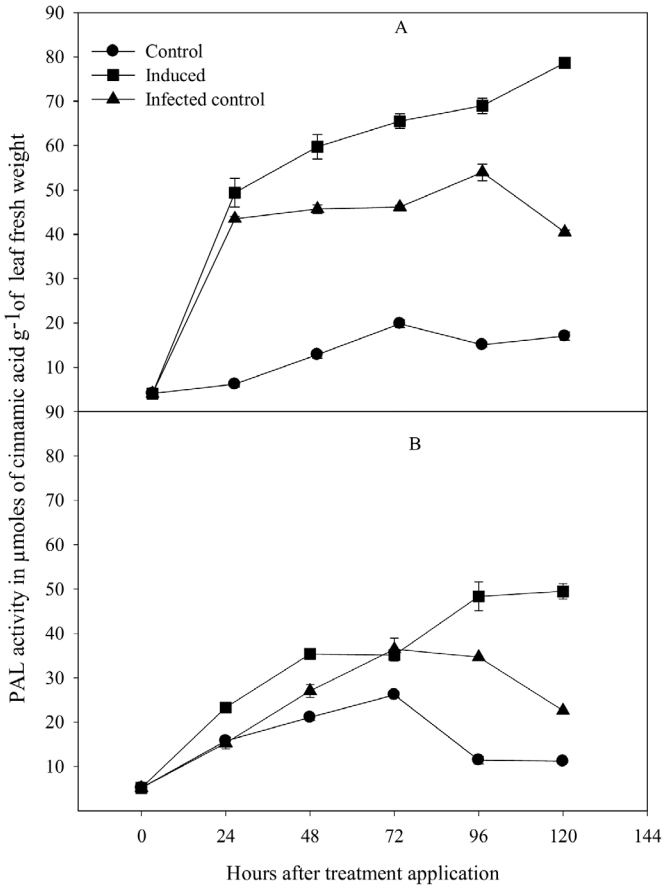

Fig. 1. Time course activity of PAL in leaves of resistant (A) and susceptible (B) Enset clones inoculated with *X. campestris* pv. *musacearum* and treated with *A. salicifolia* leaf extract (12.5 mg/ml) and methanol one year after transplanting. Values represent means of six replicates. Bars indicate mean ± SE.

sampling time. In this clone, the highest PAL activity was observed in leaf extract treated plants 120 and 96 h after treatment application. The activity of PAL declined after 96 h from treatment application in infected control plants of both clones (Fig. 1A, 1B). Higher PAL activity was recorded in induced plants of both clones compared to other treatments. Furthermore, PAL activity was increased progressively up to 120 h post treatment (Fig. 1A, 1B). Induced and infected plants of the resistant clone had significantly ($P < 0.01$) higher PAL activity than the corresponding plants of the susceptible clone (Fig. 1A, 1B).

Peroxidase activity. The Post Hoc test for multiple comparisons of treatments revealed that the activity of peroxidase in treated resistant/tolerant and susceptible plants was significantly ($P < 0.01$) increased with respect to untreated plants (Fig. 2A, 2B). In the resistant clone, it showed a significant variation between treatments (Fig. 2A). In control plants, peroxidase activity was boosted up 24 h and increased linearly till 120 h post treatment, but this activity of the enzyme was significantly lower than values of others treatments (Fig. 2A). Infected control plants of the resistant clone attained the highest peroxidase activity 72 and 48 h post treatment followed by induced plants 96 and 120 h after treatment application, respectively (Fig. 2A). The differences between the aforementioned values were significant ($P < 0.01$). Peroxidase activity measurements at the remaining sampling periods showed statistically significant variation between treatments (Fig. 2A). In the susceptible clone, the activity of peroxidase in infected control plants was the highest at 48 and 72 h post treatment and these values were significantly ($P < 0.01$) different from the other measurements (Fig. 2B). Besides, induced plants had also higher enzyme activity at 96 and 120 h post treatment as compared to others treatments (Fig. 2B).

Moreover, there were no significant differences between untreated plants of the resistant and susceptible Enset clones (Fig. 2A, 2B). When treated, the clones showed a significant variation in peroxidase activity between treatments. Under the control treatment, the resistant clone had significantly higher peroxidase activity than the susceptible one at 120 h post treatment. In addition, induced plants of the resistant clone attained significantly higher peroxidase activity than the susceptible ones at 48 and 72 h post treatment. Conversely, induced plants of the susceptible clone had significantly ($P < 0.01$) higher activity than the resistant clone at 96 and 120 h after treatment application (Fig. 2A, 2B). In both Enset clones, peroxidase activity was elevated earlier and dropped lately in infected control plants than induced ones (Fig. 2A, 2B).

Polyphenol oxidase activity. The studied resistant and susceptible Enset clones showed an increased polyphenol oxidase activity post treatment with leaf extract, Xcm suspension and methanol (Fig. 3). The activity of the enzyme was significantly ($P < 0.05$) higher in treated plants of the resistant clone 24, 48, 72, 96 and 120 h after treatment application compared to untreated (0 h) plants (Fig. 3A). The highest polyphenol oxidase activity was recorded 72 and 120 h post treatment in induced and infected control plants of the resistant clone, respectively (Fig. 3A). In the susceptible clone, the activity of the enzyme in induced plants was higher than untreated (0 h) ones at 24, 48 and 96 h post treatment (Fig. 3B). However, significant differences were observed between induced and untreated plants 96 h after treatment application. The activity of the enzyme drastically declined 96 h post treatment in infected control plants of the susceptible clone (Fig. 3B). The activity of polyphenol oxidase in induced plants of the resistant and susceptible clones showed an increasing trend up to 72 and 96 h post treatment, respectively (Fig. 3A, 3B). The activity then after declined. On the other hand, the activity of polyphenol oxidase under untreated condition was insignificantly different between the two Enset clones. Nonetheless, clear and significant ($P < 0.05$) differences were observed between the two clones after the application of treatments.

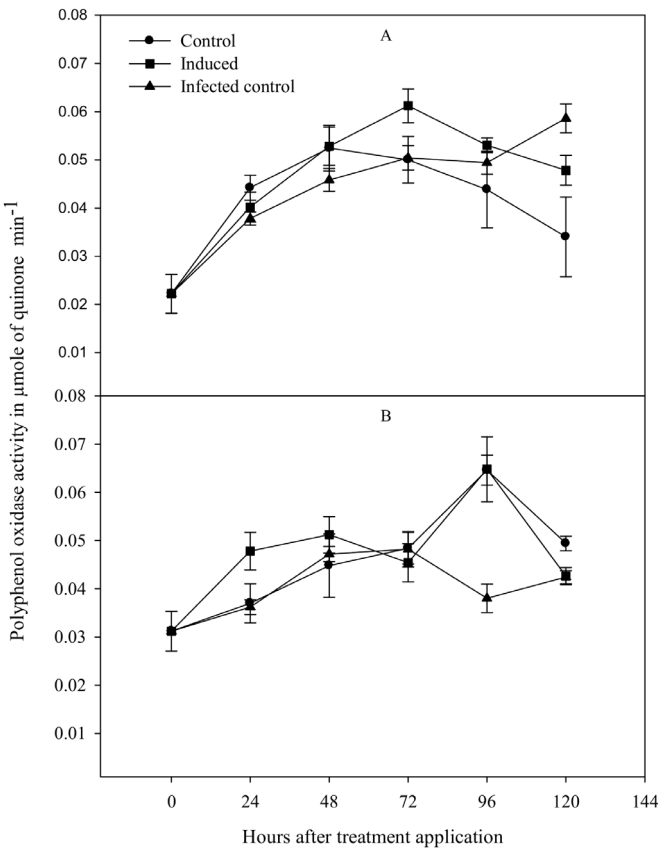

Fig. 2. The activity of peroxidase enzyme in leaves of resistant (A) and susceptible (B) Enset clones treated with *X. campestris* pv. *musacearum*, *A. salicifolia* leaf extract (12.5 mg/ml) and methanol one year after transplanting. Values represent means of six replicates. Bars indicate mean ± SE.

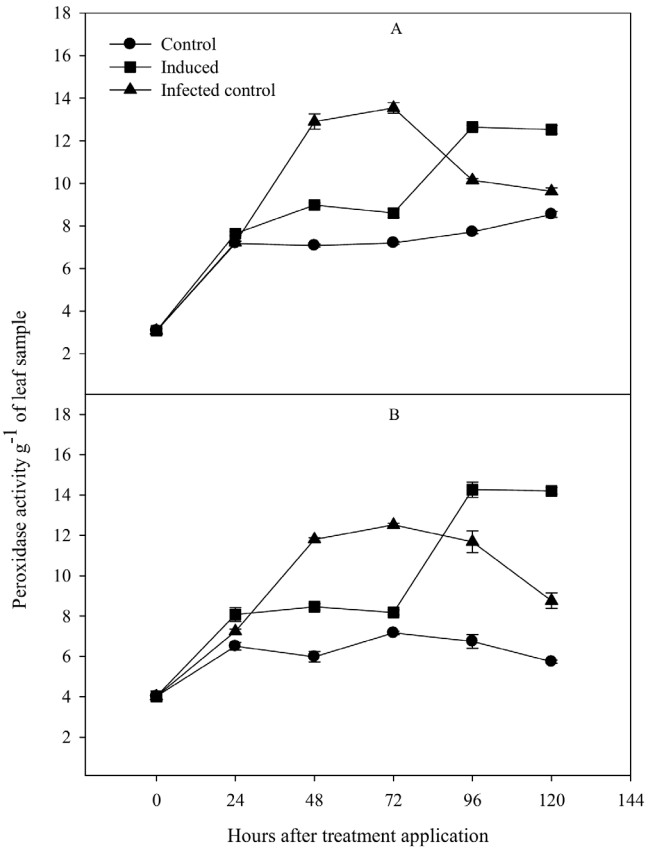

Fig. 3. Induction of polyphenol oxidase activity in leaves of resistant (A) and susceptible (B) Enset clones treated with *X. campestris* pv. *musacearum*, *A. salicifolia* leaf extract (12.5 mg/ml) and methanol one year after transplanting. Values represent means of six replicates. Bars indicate mean ± SE.

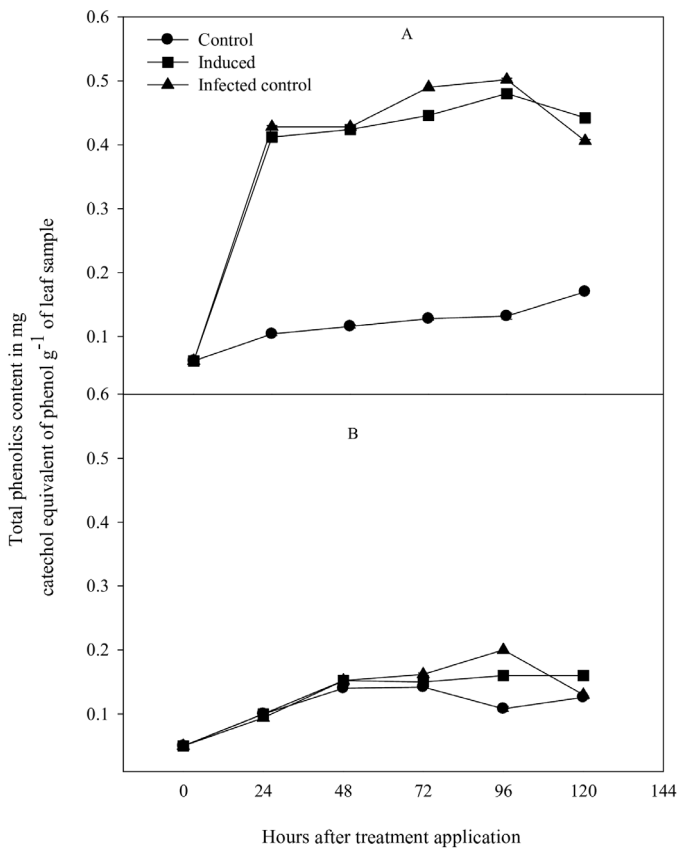

Fig. 4. Changes in TPC in leaves of resistant (A) and susceptible (B) Enset clones treated with *X. campestris* pv. *musacearum*, *A. salicifolia* leaf extract (12.5 mg/ml) and methanol one year after transplanting. Values represent means of six replicates. Bars indicate mean ± SE.

Total phenolics content. Fig. 4 depicts that total phenolics content (TPC) of the study clones was significantly ($P < 0.05$) higher in treated plants than untreated ones. Statistically significant differences were recorded in the amount of TPC between the different sampling periods within treatments in both clones. TPC showed an increasing trend in time (Fig. 4A, 4B). The highest TPC of the control treatments was recorded in plants of the resistant clone at 120 h post treatment. From all the treatments, plants of the resistant clone inoculated with Xcm suspension produced the highest TPC and the maximum amount was recorded at 96 and 72 h followed by induced plants 96 h post treatment (Fig. 4A). These values of TPC were significantly higher than others treatments (Fig. 4A, 4B). Similarly, higher amount of TPC was produced by infected control plants of the susceptible clone as compared to other treatments within the clone (Fig. 4B).

At 0 h (untreated), the TPC difference between the two clones was not significant (Fig. 4A, 4B). Under the control treatment, plants of the susceptible clone had higher TPC than the resistant at 48 and 72 h after treatment application (Fig. 4A, 4B). On the other hand, the TPC of infected control and induced plants of the resistant clone were significantly higher than the susceptible clone. The amount of TPC in the resistant clone was twofold greater than the susceptible (Fig. 4A, 4B). TPC content was drastically boosted up 24 h post treatment and then increased slowly in infected control and induced plants of the resistant clone (Fig. 4A).

Total soluble sugars. Unlike other measured parameters, the amount of total soluble sugar (TSS) showed a significant ($P < 0.01$) difference between the two clones under untreated condition (Fig. 5A, 5B). The resistant clone had higher TSS than the susceptible clone at most sampling periods (Fig. 5A, 5B). Besides, resistant plants in the control treatment had significantly higher TSS in comparison to the susceptible and these plants achieved the successive maximum TSS values at 72, 96, 48 and 120 h post treatment, respectively (Fig. 5A, 5B). Infected control plants of the resistant clone produced lower TSS than the other treatments 72, 96 and 120 h after treatment application (Fig. 5A). On the other hand, induced plants of the susceptible clone attained significantly higher TSS at 72, 96 and 120 h post treatment than others treatments (Fig. 5A, 5B). Additionally, the chemical analysis result showed that the concentration of free proline under the various treatments in both Enset clones at all sampling periods was negligible

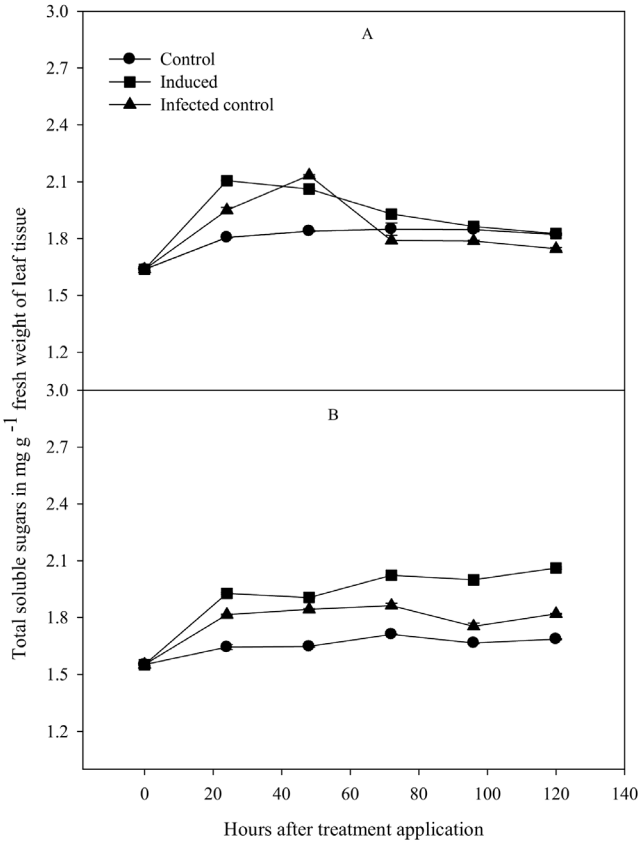

Fig. 5. Total soluble sugar content in *X. campestris* pv. *musacearum*, *A. salicifolia* leaf extract (12.5 mg/ml) and methanol treated leaves of resistant (A) and susceptible (B) Enset clones. Values represent means of six replicates. Bars indicate mean ± standard error.

(data not shown). Overall, the performance of the resistant clone was better than that of the susceptible clone under most treatments.

The Pearson correlation analysis showed that a positive correlation exists between measured parameters (Table 1). In the control plants of the resistant clone, the amount of TPC showed a significant positive correlation with PAL, peroxidase and polyphenol oxidase activities, and TSS (Table 1). In induced resistant plants, PAL activity had a significant positive correlation with peroxidase, TPC and polyphenol oxidase. The TPC of infected control plants had a significant positive correlation with PAL, peroxidase and polyphenol oxidase activities. Similarly, the amount of TSS of control plants of the susceptible clone had positive correlation with PAL, peroxidase, polyphenol oxidase and TPC (Table 1). PAL activity revealed a significant positive correlation with peroxidase activity, TPC and TSS in induced plants, whereas in infected control plants it had a significant positive correlation with peroxidase activities and TSS (Table 1).

Disease incidence. The disease incidence assessment showed variation between treatments and clones (Fig. 6). Infected control plants of the susceptible clone showed

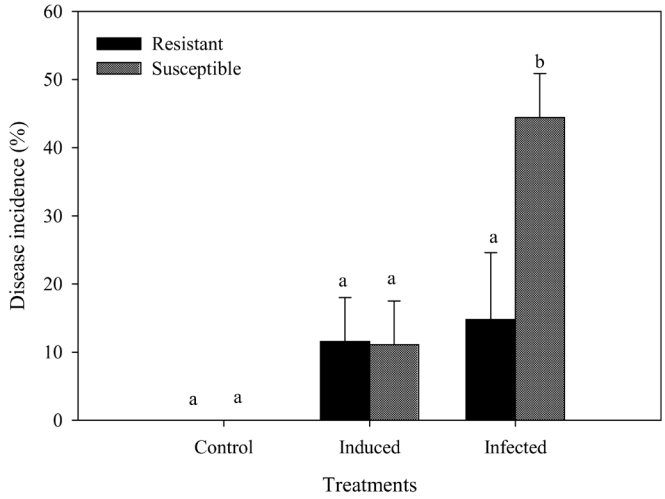

Fig. 6. Disease incidence of resistant and susceptible Enset clones four months after the application of treatments (*X. campestris* pv. *musacearum*, leaf extract and methanol). Values represent means of three replicates. Bars indicate mean ± SE.

the highest disease incidence, while induced susceptible plants showed a 33.33% disease incidence reduction as compared to infected control plants (Fig. 6). All Xcm inoculated plants developed symptoms. However, most plants of the resistant clone endured the pathogen and continued to survive. In the resistant clone, induction prior to Xcm inoculation minimized disease incidence only by 3.2%.

DISCUSSION

The results of our study demonstrate that the activity of defense related enzymes, PAL, peroxidase, polyphenol oxidase and compounds such as TPC and TSS, increased in *Ensete ventricosum* clones upon inoculation with Xcm and treatment with the leaf extract and methanol. The level of induction significantly varied between treatments and clones. Similar results have been reported in *Arachis hypogaea* tissues infected with stem rot (Reddy and Sireesha, 2013), *Gossypium hirsutum* infected by cotton leaf curl Burewala virus (Siddique *et al*., 2014) and *Vicia faba* treated with plant growth promoting rhizobia inoculants and *Salix alba* extracts as foliar and seed applications (Sofy *et al*., 2014). It is a well established fact that induced enzymes such as PAL, peroxidase, polyphenol oxidase and compounds like total phenolics play an important role in crop disease resistance (Ngadze *et al*., 2012; Reddy and Sireesha, 2013). In the present study, the magnitude of induction is evaluated in terms of increased activity of PAL, peroxidase and polyphenol oxidase, and elevated amount of TPC and TSS.

The activity of PAL increased in both clones in the control, leaf extract induced and infected control treatments as compared to the non-inoculated ones. It increased in time in the two Enset clones. However, the increment in

Table 1. The bivariate relationship between biochemical responses of the resistant and susceptible Enset clones treated with methanol, *A. salicifolia* leaf extract and Xcm suspension under field condition.

Biochemical responses	Resistant clone														
	Control					Induced					Infected control				
	PAL	POX	PPO	TPC	TSS	PAL	POX	PPO	TPC	TSS	PAL	POX	PPO	TPC	TSS
PAL	1	.713	.551	.845*	.752	1	.936**	.915*	.954**	.485	1	.804	.835*	.991**	.536
POX	.713	1	.580	.905*	.927**	.936**	1	.758	.862*	.281	.804	1	.803	.830*	.534
PPO	.551	.580	1	.367	.829*	.915*	.758	1	.915*	.525	.835*	.803	1	.849*	.340
TPC	.845*	.905*	.367	1	.772	.954**	.862*	.915*	1	.673	.991**	.830*	.849*	1	.492
TSS	.752	.927**	.829*	.772	1	.485	.281	.525	.673	1	.536	.534	.340	.492	1
	Susceptible clone														
PAL	1	.758	.242	.670	.787	1	.939**	.629	.899*	.962**	1	.972**	.776	.693	.957**
POX	.758	1	.724	.857*	.754	.939**	1	.542	.792	.811	.972**	1	.879*	.757	.930**
PPO	.242	.724	1	.800	.584	.629	.542	1	.691	.682	.776	.879*	1	.866*	.648
TPC	.670	.857*	.800	1	.904*	.899*	.792	.691	1	.911*	.693	.757	.866*	1	.594
TSS	.787	.754	.584	.904*	1	.962**	.811	.682	.911*	1	.957**	.930**	.648	.594	1

*Correlation is significant at 0.05 level; **Correlation is significant at 0.01 level. PAL = Phenylalanine ammonia lyase; POX = Peroxidase; PPO = Polyphenol oxidase; TPC = Total phenolics content; TSS = Total soluble sugars.

the resistant clone was greater and progressive than in the susceptible clone. PAL activity was significantly higher in induced and infected control plants of the resistant clone at all time courses compared to control, indicating a possible means to develop disease resistance. In this respect, the works of Purwar *et al.* (2012) and Punithavalli *et al.* (2013) are congruent to the results of the present study. According to Purwar *et al.* (2012), the activity of PAL in resistant genotypes of wheat increased 2-3 folds higher than susceptible genotypes when infected with Karnal Bunt disease. Similarly, the activity of PAL has been increased significantly in the resistant and wild genotypes of rice infested with *Cnaphalocrocis medinalis* as compared to susceptible ones (Punithavalli *et al.*, 2013). Moreover, application of leaf extracts of *Adathoda vasica*, *Cymbopogen citrus* and *Ocimium sanctum* as seed treatment to control sheath blight disease of rice increased the activity of PAL (Govindappa *et al.*, 2011; Pal *et al.*, 2011). Several researches demonstrate that challenge treatments of plants with biotic and abiotic agents raise the activity of PAL earlier than other metaboilic changes (Kagale *et al.*, 2004; Guleria and Kumar, 2006). The increase in PAL activity indicates the activation of phenylpropanoid pathway. PAL is the enzyme in the pathway that catalyzes deamination of *L*-phenylalanine into trans-cinnamic acid (Kagale *et al.*, 2004), which serves as a precursor of various secondary metabolites that might result in higher salicylic acid synthesis leading to induction of resistance (Guleria and Kumar, 2006; Nafie and Mazen, 2008).

The activity of peroxidase increased almost equally in the resistant and susceptible clones under untreated conditions. Nonetheless, the activity of the enzyme significantly increased under induced and infected control plants of both clones. Similarly, Arun *et al.* (2010) reported increased peroxidase activity in pearl millet infected with *Sclerospora graminicola.* Cocoa plants induced by acibenzolar- *S*-methyl (ASM) against *Crinipellis perniciosa* and *Verticillium dahlia* showed a significant increase in peroxidase activity (Resende *et al.*, 2002). *Azadirachta indica* leaf extract treated sesame plants against *Alternaria sesami* (Guleria and Kumar, 2006) and rice leaves treated with *Datura metel* leaf extract and inoculated with *Rhizoctonia solani* or *Xanthomonas oryzae* pv. oryzae (Kagale *et al.*, 2004) showed a remarkable increase in peroxidase activity. According to the research reports mentioned above and others, increased expression of peroxidase is involved in the biochemical reaction necessary for lignification (Punithavalli *et al.*, 2013).

On the other hand, the response of polyphenol oxidase activity in the two *E. ventricosum* clones was linear under the various treatments and over time. Besides, the difference across treatments and between clones was insignificant. This result is against the works of Govindappa *et al.* (2011), Ngadze *et al.* (2012) and Jadesha *et al.* (2012) who observed discernible increase of polyphenol oxidase in rice induced with *Adathoda vasica* leaf extract, in potato tubers inoculated with various pathogen suspensions, and in banana peel and pulp dipped in leaf extract, respectively. Although there are no clear and definite ways about the potential anti-pathogen effects of polyphenol oxidase, Li and Steffens (2002) suggested several possibilities, including general toxicity of polyphenol oxidase-generated quinones to pathogens and plant cells accelerating cell death, alkylation and reduced bioavailability of cellular proteins

to the pathogen and cross-linking of quinones with protein or other phenolics, forming a physical barrier to pathogens in the cell wall.

Associated with augmented activity of PAL and peroxidase, the amount of TPC was significantly increased upon treatment of *E. ventricosum* clones with methanol, leaf extract and Xcm during the different leaf sampling periods. In addition, TPC of the resistant clone was significantly higher than the susceptible one. Likewise, Arun *et al.* (2010), Ngadze *et al.* (2012) and Punithavalli *et al.* (2013) reported similar TPC responses of resistant and susceptible pearl millet, potato and rice cultivars, respectively. In this respect, Guleria and Kumar (2006) reported that sesame plants treated with *Azadirachta indica* leaf extract produce significantly high content of phenolic compounds. Abd El-Rahman *et al.* (2012) also reported greater accumulation of phenolic compounds in infected and induced-infected *Lupinus albus* seedlings compared to healthy plants. Moreover, Kagale *et al.* (2004) reported increase in level of phenols in rice leaves treated with *Datura metel* leaf extract and inoculated with *Rhizoctonia solani* or *Xanthomonas oryzae* pv. *oryzae*. These evidences might help us to infer that TPC has a key role in disease resistance development. Thus, accumulation of phenolic compounds after challenge inoculation has been correlated with the restriction of pathogen development, since such compounds are toxic to pathogens (Punithavalli *et al.*, 2013; Ojha and Chatterjee, 2012). Also, phenolic compounds may impede pathogen infection by increasing the mechanical strength of the host cell wall (Ojha and Chatterjee, 2012). These results agree with the general speculation that when plant cells are under infection, there is a switch from the normal primary metabolism to a multitude of the secondary defense pathway, and activation of novel defense enzymes and genes (Tan *et al.*, 2004).

Total soluble sugar responses of *E. ventricosum* clones exhibited variations between treatments. In both clones, significantly higher amounts of total soluble sugars were produced by leaves of induced plants compared to other treatments. This corroborates with the results of Khallal (2007) and Hao *et al.* (2013) on glucohexose treated tomato and arbuscular mycorrhizal fungi and/or jasmonic acid and salicylic acid treated cucumber plants, respectively. Both researchers reported increased production of TSS in plants treated with elicitors than plants infected with the respective pathogens. On the contrary, greater rates of TSS decrease were noticed in cucumber and barley infected leaves than in healthy ones (Abood and Losel, 2003; Singh *et al.*, 2009). In this study, infected control plants of the resistant clone generated relatively higher amount of TSS than susceptible plants. Similarly, Misra *et al.* (2008) disclosed comparable results between resistant and susceptible taro genotypes. This shows that total soluble sugar responses of plants seem to be dependent on genotypes. The accumulation of total soluble sugars in bioagent or chemical induced and infected plants signifies the relationship between sugar regulation and activation of systemic resistance (Khallal, 2007). It is evident from several research reports that soluble sugars are involved in the responses of plants to abiotic and biotic stresses, and act as nutrient and metabolite signaling molecules (Couee *et al.*, 2006).

There was a significant difference in disease incidence between the clones in infected controls. Disease incidence was significantly higher in the susceptible infected control plants as compared to other treatments. However, in plants pre-treated with leaf extract and consequently inoculated by Xcm after 30 days, there were 33.3% and 3.2% disease incidence reduction in the susceptible and resistant clones, respectively. This shows that inducing the natural resistance increases the ability of susceptible plants to withstand pathogens in a non-genetic way (Ma and Cui, 2002). The result of the present study is in agreement with Resende *et al.* (2002) who reported 5-84% reduction in disease incidence of witches' broom on cocoa seedlings sprayed with acibenzolar- *S*-methyl (ASM) 30 days before inoculation. Moreover, chemical inducing agents can cause disease incidence reduction ranging between 20–80% (Walters and Fountaine, 2009). Arbuscular mycorrhizal fungi plus jasmonic acid treatments can reduce disease incidence by 92% (Khallal, 2007). The resistant clone showed the lowest disease incidence both in the induced and infected control plants. This might be related to the biochemical responses of the clone after treatment. The biochemical responses of the susceptible clone revealed induction of resistance against Xcm which was not as significant as the resistant clone. This is reflected in the reduction of disease incidence in induced and infected control plants of the clone. Therefore, the present study demonstrates that the biochemical changes occurring after application of the leaf extract and the pathogen can act as markers for systemic acquired resistance, triggering the crops' natural immunity. Exploiting systemic acquired resistance is a feasible disease controlling alternative strategy that diminishes the use of toxic chemicals.

ACKNOWLEDGEMENTS

The authors appreciate Addis Ababa University, Office of Vice President for Research and Technology Transfer for its financial support to conduct this research. Moreover, others who have contributed to the success of the study are duly acknowledged.

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Received December 20, 2015
Accepted June 17, 2016