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A novel bi-enzyme electrochemical biosensor for selective and sensitive determination of methyl salicylate

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ABSTRACT

An amperometric sensor based on a bi-enzyme modified electrode was fabricated to detect methyl salicylate, a volatile organic compound released by pathogen-infected plants via systemic response. The detection is based on cascadic conversion reactions that result in an amperometric electrochemical signal. The bi-enzyme electrode is made of alcohol oxidase and horseradish peroxidase enzymes immobilized on to a carbon nanotube matrix through a molecular tethering method. Methyl salicylate undergoes hydrolysis to form methanol, which is consumed by alcohol oxidase to form formaldehyde while simultaneously reducing oxygen to hydrogen peroxide. The hydrogen peroxide will be further reduced to water by horseradish peroxidase, which results in an amperometric signal via direct electron transfer. The bi-enzyme biosensor was evaluated by cyclic voltammetry and constant potential amperometry using hydrolyzed methyl salicylate as the analyte. The sensitivity of the bi-enzyme biosensor as determined by cyclic voltammetry and constant potential amperometry were 112.37 and 282.82 μA cm^{-2} mM^{-1} respectively, and the corresponding limits of detection were 22.95 and 0.98 μM respectively. Constant potential amperometry was also used to evaluate durability, repeatability and interference from other compounds. Wintergreen oil was used for real sample study to establish the application of the bi-enzyme sensor for selective determination of plant pathogen infections.

1. Introduction

Agricultural economic losses due to pest and pathogen infections amount to $40 billion annually in the U.S. alone (Roberts et al., 2006). Minimizing crop damages through proper disease management practices demand new technologies for detecting infections at very early stages. Currently there is no rapid detection method available to detect the existence of plant diseases on the field. Among the various off-field methods explored for the detection of pathogenic diseases in crops, profiling of voltaic organic compounds (VOCs) released by plants has been identified as an useful route to non-destructively analyze the sample. The chemical signature of infected plants contains unique information related to the nature of the infection and hence can be used as a reliable marker to identify pathogenic infections in crops. An infected plant would produce different amount of VOCs as opposed to a healthy plant (Fang et al., 2014). The VOCs in infected plants are produced through various biosynthetic pathways, including the octadecanoid pathway leading to fatty-acid derived green leaf volatiles (GLVs), monoterpenes, diterpenes, sesquiterpenes, isothiocyanates and a large diversity of aromatic metabolites (Schoonhoven et al., 2005). Among the various compounds in the volatile signature of plants, methyl salicylate (MeSA) is released in large quantities during pathogenic infections and infestation and therefore is a suitable target compound (marker) for detecting biotic stresses of plants. Plants produce MeSA through Shikimate biosynthesis pathway, during a biotic stress event such as the pathogenic infection and herbivorous infestation. For instance, the production of MeSA was observed from *Tetranychus urticae* infested lima beans (De Boer and Dicke, 2004; De Boer et al., 2004; James and Price, 2004; Pickett et al., 2006). MeSA production was also reported from maize and pepper infected by *Fusarium* and *Phytophthora capsici* respectively (Buttery et al., 1969; Piesik et al., 2011). MeSA is an allelochemical that is released not just at the site of pathogen infection but throughout the plant through a systematic response (Dudareva et al., 2006; Pichersky et al., 2006). It is one of the key markers for volatile based detection of fungal diseases such as fruit blight, leaf blight, leather rot etc., which

Abbreviations: AOD, alcohol oxidase; CNT, carbon nanotube; CV, cyclic voltammetry; DMF, dimethylformamide; GC–MS, gas chromatography-mass spectrometry; GC, glassy-carbon; GLV, green leaf volatile; HRP, horseradish peroxidase; LOD, limit of detection; LOQ, limit of quantification; MeOH, methanol; MeSA, methyl salicylate; PB, phosphate buffer; PBSE, pyrenebutanoic acid succinimidyl ester; RDE, rotating disc electrode; RSD, relative standard deviation; SA, salicylate; VOC, volatile organic compound

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primarily affect the cucurbit crops. Therefore it is a suitable target analyte (marker) for development of biosensors to detect pathogenic diseases in crops. Moreover, the hydrolyzed form of MeSA (consisting methanol) can be amperometrically detected on an enzymatic bioelectrode, providing the possibility of highly selective quantitative detection based on bio-electrochemical redox reactions. Although gas chromatography-mass spectrometry (GC–MS) is the most commonly used technique to study the volatile signature of infected plants, this technique requires the sample to be collected in the field and analyzed elsewhere in a laboratory (Ewen et al., 2004; Lui et al., 2005; Prithiviraj et al., 2004; Vikram et al., 2006). Moreover the high cost of the instrumentation and complexity of the analysis prohibit it from being used in the field for real time monitoring of plant diseases (Fang and Ramasamy, 2015). On the other hand amperometric electrochemical sensors such as the one described in this work, offer many unique advantages for this application due to the following characteristics: accuracy, rapid detection, robustness, non-invasive detection, selective and ultra-low detection limits (Bakker, 2004). They cost less and require no skilled analysts to infer the results.

In this article, we report the successful development of a bi-enzyme based amperometic biosensor for selective determination of methyl salicylate. The biosensor platform is a glassy-carbon electrode, the surface of which is modified with multiwalled carbon nanotubes (CNTs). The CNT acts as both immobilization support for the enzymes and as amperometric transducer. The bio-recognition element consists of two enzymes namely alcohol oxidase (AOD) and horseradish peroxidase (HRP) immobilized on a matrix of multiwalled CNTs. The immobilization was achieved through an established molecular tethering approach developed and extensively demonstrated by us for other bio-electrochemical systems (Calkins et al., 2013; Lau et al., 2012; Ramasamy et al., 2010; Umasankar et al., 2014; Umasankar and Ramasamy, 2013). As illustrated in Fig. 1, the detection is carried out in three steps. First, MeSA is hydrolyzed in potassium hydroxide (KOH) to form potassium salicylate (SA) and methanol (MeOH). The pH was adjusted to 7.6 by adding phosphoric acid. Next the enzyme alcohol oxidase (AOD) converts methanol into formaldehyde via its native biochemical reaction, during which a simultaneous reduction of O_2 to H_2O_2 takes place (Ozimek et al., 2005; Patel et al., 1981). The third step involves the bio-electrochemical reduction of H_2O_2 to water using the second enzyme horseradish peroxidase (HRP), which results in an amperometric signal for detection on the electrode (Akkara et al., 1991; Ghindilis et al., 1997; Veitch, 2004). The amperometric signal is proportional to the concentration of the hydrolyzed MeSA in the electrolyte. The setup offers a simple bi-enzyme biosensor for the detection of an important plant allelochemical namely methyl salicylate.

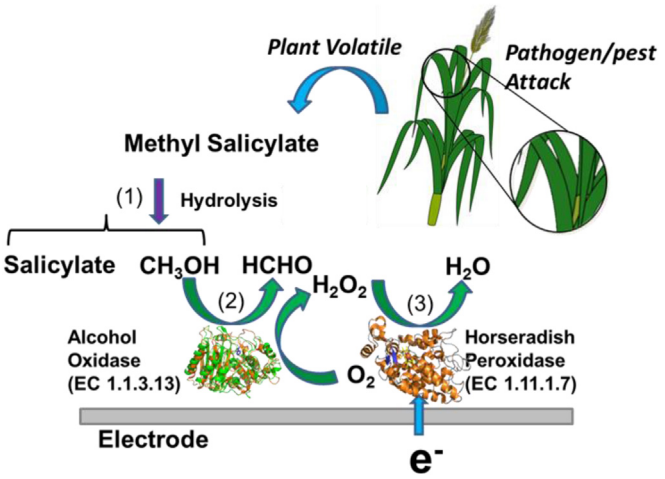

Fig. 1. Schematic illustration of methyl salicylate detection by bi-enzyme modified electrode. The process begins with the hydrolysis of methyl salicylate to form salicylate and methanol (1), oxidation of methanol and production of hydrogen peroxide (2) and direct electron transfer from electrode to hydrogen peroxide by horseradish peroxidase (3). Not drawn to scale.

2. Materials and methods

2.1. Materials

Alcohol oxidase (EC 1.1.3.13) from *Pichia pastoris* was purchased from Sigma-Aldrich and used as received. Horseradish peroxidase (specific activity 281 U/mg) was purchased from Calbiochem Inc. Multiwalled carbon nanotube (CNT) was obtained from DropSens Inc. Pyrenebutanoic acid succinimidyl ester (PBSE) was purchased from AnaSpec Inc. (Fremont CA). Dimethylformamide (DMF) was purchased from Acros Organics Inc. Chemicals for the interference study such as *cis*-3-hexenol, hexyl acetate and *cis*-hexenyl acetate were obtained from TCI America (Portland, OR) and used as received. Wintergreen oil purchased from Piping Rock Health Products was used as obtained for the real sample study. Methyl salicylate (MeSA) was used as received from Sigma-Aldrich Inc. All other chemicals used in the work were of the analytical grade. 100 mM phosphate buffer (PB) (pH 7.6) was used as the electrolyte for all experiments. All the aqueous solutions were prepared using 18.2 MΩ nano pure de-ionized (DI) water. Electrolyte (buffer) solutions were oxygenated by purging with pure oxygen for 15 min before each experiment.

2.2. Apparatus

Cyclic voltammetry (CV) and constant potential amperometry were performed using CHI 920 c potentiostat. A conventional three-electrode system consisting a Pt wire as the counter electrode and 3 M Ag/AgCl as the reference electrode was used for electrochemical measurements. The working electrodes were glassy carbon (GC) electrode, modified multiwalled CNT or a glassy carbon rotating disk electrode (RDE) from Pine Instrument Inc. All experiments were carried out at 25 ± 2 °C.

2.3. Electrode preparation and electrochemical measurement

GC and RDE electrodes were surface cleaned by polishing on a polishing pad with 0.05 μm alumina polishing powder before each experiment. The electrodes were then cleaned using ultrasonic cleaner and rinsed by DI water to remove the fine alumina powder adhered to the electrode surface. The CNT suspension was prepared by ultrasonication of 1 mg of multiwalled CNTs in 1 mL DMF for 1 h. The CNT modified electrodes were prepared by drop casting 8 μL (in 8 steps of 1 μL) for GC electrode and 12 μL (in 3 steps of 4 μL) for RDE followed by drying at 70 °C. CNT modified electrodes were placed on the ice and allowed to cool down before 2 and 4 μL of 10 mM PBSE in DMF were added on GC and RDE respectively. The electrodes were incubated for 15 min to allow the formation of non-covalent linkage between CNT and PBSE. Then the electrodes were rinsed by DMF and 100 mM PB (pH 7.6) sequentially to remove excessive PBSE (Ramasamy et al., 2010). HRP solution was prepared by dissolving 5 mg HRP in 1 mL 20 mM PB (pH 7.6). The bi-enzyme solution was simply prepared by mixing 5 μL of alcohol oxidase solution and 5 μL HRP solution. 10 μL of bi-enzyme solution was drop casted on the electrodes and incubated on ice for 30 min for enzyme immobilization. The electrodes were rinsed with 100 mM PB (pH 7.6) to remove any unimmobilized enzyme. For CV measurements, the potential range used was from 0.2 V to 0.7 V at a scan rate of 20 mV s^{-1} with a

sample interval of 0.001 V. The initial potential for constant potential amperometry with RDE was 0.45 V with 0.1 s interval for data collection.

2.4. Hydrolysis of methyl salicylate

The MeSA was mixed with 0.19 M KOH in 15 mL falcon tube to balance the ionic strength of the buffer for hydrolysis. The falcon tube was sealed and placed in boiling water bath for 30-min hydrolysis. Then the falcon tube was cooled down to room temperature before adding phosphoric acid to adjust the pH to 7.6 († Supplementary data Fig. S1).

3. Results and discussion

3.1. Cyclic voltammetry on bi-enzyme modified electrode

As methanol is the product of the hydrolysis step and the first reactant in the enzymatic conversion reactions, the bi-enzyme system was evaluated first for methanol detection using 3 mM of methanol in the electrolyte. For comparison purposes, a CNT modified GC electrode functionalized with only one enzyme (HRP or AOD) as bio-recognition element was also evaluated as two different controls. As shown by the cyclic voltammograms in Fig. 2 (a), the amperometric signal indicated by the reduction wave originating at 0.6 V (with a peak at 0.45 V) was generated only

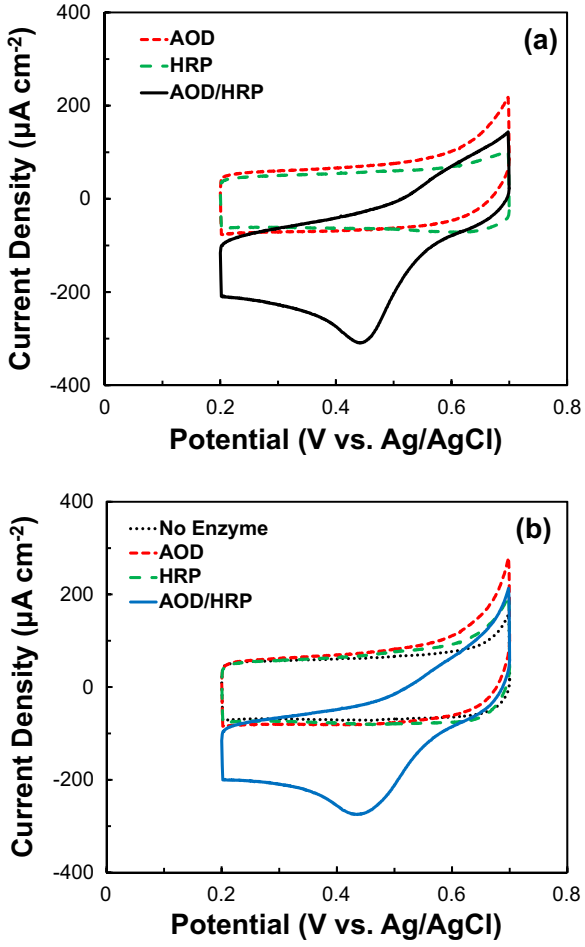

Fig. 2. CV responses of 3 mM methanol on bi-enzyme modified, AOD modified and HRP modified electrodes (a) and 1.88 mM hydrolyzed methyl salicylate on bi-enzyme modified, AOD modified, HRP modified and no enzyme modified electrodes (b).

when both enzymes are present on the electrode. The cathodic wave corresponds to the electrochemical reduction of hydrogen peroxide to water, which is the last step of the cascade reactions as described in the Fig. 1. No such reduction was observed in Fig. 2(a), when any one of the two enzymes was absent on the electrode. The results indicate that the cascade reactions do not proceed if one of the enzymes was absent and that the hydrogen peroxide is formed as a result of the cascade reactions. Moreover there was no peak attributable to direct methanol oxidation at the electrode was observed in any of the voltammograms in Fig. 2(a), suggesting that the amperometric signal could be solely attributed to the hydrogen peroxide reduction reaction.

The next step in the analysis is to evaluate if the intermediate compounds in the cascade reactions namely potassium salt of salicylate (SA) and formaldehyde would contribute to the amperometric signal in the region of interest (0.6 V and below along the cathodic wave in Fig. 2(a)). For this, the HRP and AOD immobilized electrodes were tested using CV in the same potential window using reagent solutions of salicylate (SA) and formaldehyde solutions respectively and the corresponding voltammograms are given in the Supplementary data† Fig. S2. The results in Fig. S2(a) suggest that formaldehyde did not contribute to the reduction current in the potential range of interest. The SA however, exhibits a small redox peak around 0.5 V, but the signal is weak and much smaller compared to the electrochemical signal for hydrogen peroxide reduction († Supplementary data Fig. S2) (Zhang et al., 2010). There was also a large oxidation peak for salicylate oxidation above 0.65 V noticed in the voltammograms in Fig. S2. However this oxidation current does not interfere with the reduction of hydrogen peroxide, which happens only below 0.6 V. It can also be observed that SA does not interfere the current signal collected from methanol on bi-enzyme electrode († supplementary data Fig. S2(b)). The results shown in Fig. 1(a) and Fig. S2 demonstrate that the methanol (from hydrolyzed MeSA) is the only compound responsible for the amperometric signal observed in the voltammograms. This serves as a positive control for our actual metahyl salicylate detection described further below.

The next step in the analysis is to determine if MeSA can be detected amperometrically through the cascade reactions with no initial methanol present in the system. For this purpose, 1.88 mM hydrolyzed MeSA was used as the analyte in the electrochemical cell and CV was performed in the same potential window. As shown in Fig. 2(b), the voltammograms showed a similar electrochemical response for the bi-enzyme electrode for the hydrolyzed MeSA analyte as it was for methanol, with the hydrogen peroxide reduction peak occurring at 0.45 V. The electrodes modified with only one of the two enzymes as well as the electrode with no enzyme modification exhibited no redox response suggesting that the amperometric signal is generated only when both enzymes are present, to carry out all reactions in the cascade. The results also confirm that the electrochemical reduction of hydrogen peroxide requires HRP, as there was no distinct signal observed in the voltammograms for direct reduction of H_2O_2 in the absence of HRP. Similarly no electrochemical signal was generated for direct oxidation of either methanol or SA in the absence of enzymes. The results in Fig. 2(b) demonstrate that only the bi-enzyme modified electrode can detect the MeSA in the analyte. The optimal loading of enzymes on the electrode was optimized by a series trial experiments with different mass ratios of both enzymes (2.8 U AOD/14.0 U HRP, 5.5 U AOD/7.0 U HRP and 11.0 U AOD/3.5 U HRP), the results of which are given in supplementary data† Fig. S3. The optimal mass ratio was determined to be 5.5 U of AOD and 7.0 U of HRP (1:1 mass ratio of AOD: HRP) on the CNT modified electrode.

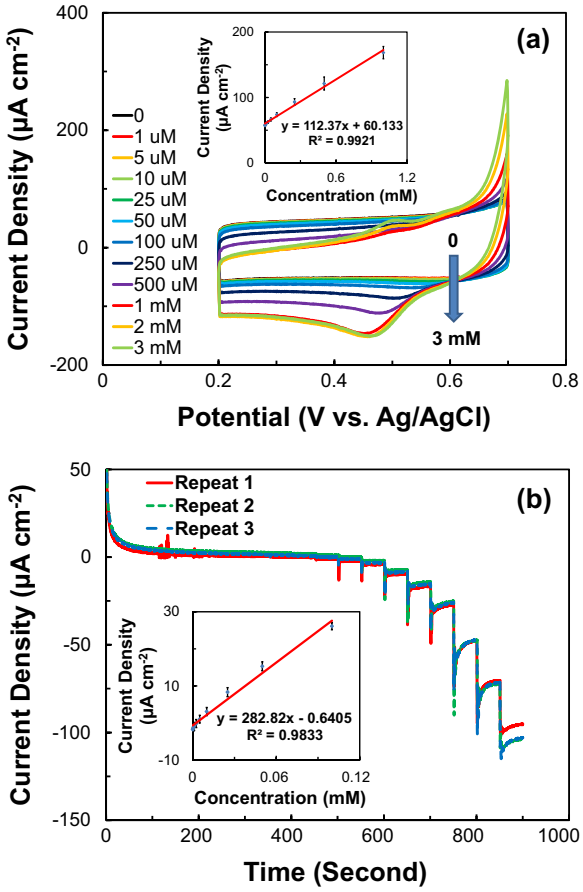

Fig. 3. CV responses of hydrolyzed methyl salicylate from 0, 1 μM to 3 mM on bi-enzyme modified GC electrode (a). Inset in (a) shows current density versus concentration. Constant potential amperometry of hydrolyzed methyl salicylate from 0, 0.1 to 1 mM on bi-enzyme modified RDE (b). Inset in (b) shows current density versus concentration.

3.2. Electrochemical response of the bi-enzyme sensor

The amperometric response of the bi-enzyme modified electrode at different concentrations of the analyte was analyzed using both CV and constant potential amperometry. The 11 different concentrations of the hydrolyzed MeSA ranged from 1 μM to 3 mM and were studied by stepwise addition. The system was equilibrated for 10 seconds after the stepwise analyte addition at each concentration before a measurement was made. The above concentration range was chosen based on a series of experiments, where the lowest limit was determined based on the noticeable increase in reduction current upon an incremental addition of hydrolyzed MeSA into the electrolyte. Similarly, the upper concentration limit was chosen based on the rate of decrease in oxidation current during subsequent additions of hydrolyzed MeSA. Voltammograms shown in Fig. 3(a) indicate that before the addition of hydrolyzed MeSA no redox peak was observed. When the concentration of hydrolyzed MeSA in the electrolyte was increased by stepwise additions to 10 μM and beyond, noticeable H_2O_2 reduction wave was observed below 0.6 V with a peak around 0.45 V. The peak current increased with increasing concentrations of hydrolyzed MeSA in the electrolyte. When the concentration of hydrolyzed MeSA exceeds 1 mM an oxidation peak was also observed at 0.5 V, which could be attributed to the oxidation of SA present in the hydrolyzed MeSA. Amperometric sensor parameters such as sensitivity, limit of detection (LOD), and limit of quantification (LOQ) can be obtained by the following equations.

$$\text{Sensitivity} = \frac{\text{Slope of calibritaion curve } (AM^{-1})}{\text{Area of electrode } (cm^2)}$$

$$\text{LOD} = 3.3 \times \frac{\text{SD of peak current in the absence of analyte (A)}}{\text{Slope of linear calibration curve } (AM^{-1})}$$

$$\text{LOQ} = 10 \times \frac{\text{SD of peak current in the absence of analyte (A)}}{\text{Slope of linear calibration curve } (AM^{-1})}$$

The data and values for the above parameters as well as the linear range of reliable detection and initial threshold concentration for amperometric response were tabulated in the Supplementary data† Table S1.

Since CV response includes non-faradaic contributions, it does not provide a steady state response of the amperometric sensor. Therefore in order to establish the steady state response of the biosensor, constant potential amperometry (chronoamperometry) was also used to determine the amperometric response. For this a RDE electrode modified with the bi-enzyme/CNT matrix was used as the working electrode and the measurements were made at a rotational speed of 1200 rpm to eliminate mass transfer limitations. The bi-enzyme modified RDE was stabilized for 300 s before the addition of hydrolyzed MeSA from 0.1 μM to 1 mM in stepwise additions after each 50 s. The first observable stepwise increase appears at the concentration of 0.5 μM as it shown in Fig. 3(b). The relevant electrochemical parameters obtained from the amperometry data is also reported in Supplementary data† Table S1. The results suggest a higher sensitivity for constant potential amperometry (282.82 μA cm^{-2} mM^{-1}) as opposed to that for cyclic voltammetry (112.37 μA cm^{-2} mM^{-1}). Furthermore, the limit of detection for constant potential amperometry can be lowered down to 0.98 μM as opposed to cyclic voltammetry (22.95 μM), which is reported in Supplementary data† Fig. S4. To put this LOD into perspective, a typical MeSA release rate by a plant is 283 ng hr^{-1} $plant^{-1}$ (Shulaev et al., 1997). At this typical rate, one could accumulate enough sample from the plant for reliable detection within 1.05 h during which about 0.98 μM of MeSA can be accumulated in a 2 mL electrochemical cell (supplementary data† Table S1). Though the linear range for constant potential amperometry is narrower than CV, it is a preferable amperometric technique over CV based on other estimated parameters.

3.3. Interference studies

MeSA is not the only VOC produced by plants during biotic stress events. Other VOCs such as green leaf volatiles (GLVs) that are non-specific to the pathogen infections are also often released at high concentrations, which could cause interference during MeSA measurement. Therefore a representative set of such GLV compounds namely, *cis*-3-hexenol, hexyl acetate and *cis*-3-hexenyl acetate were used to determine the effect of interference on the biosensor response using constant potential amperometry. The bi-enzyme modified RDE was preconditioned until 300 s and hydrolyzed MeSA was added to maintain 50 μM of working concentration, which is in the middle of linear range. The RDE was further stabilized for 500 s before adding different concentrations of hydrolyzed GLVs from 10 μM to 1 mM (final concentration in the electrolyte) at 50-second intervals. The experiment was conducted for all three GLVs as well as the same volume of 100 mM phosphate buffer (pH 7.6) as the control. The raw interference data of current vs. time is reported in Supplementary data† Fig. S5. While the typical GLV concentration is about 10 μM for *cis*-3-hexenol, 20 μM for *cis*-hexenyl acetate and 1 μM for hexyl acetate (Umasankar et al., 2012), a wider concentration range (0 to 1 mM) of these interfering compounds were tested to simulate extreme

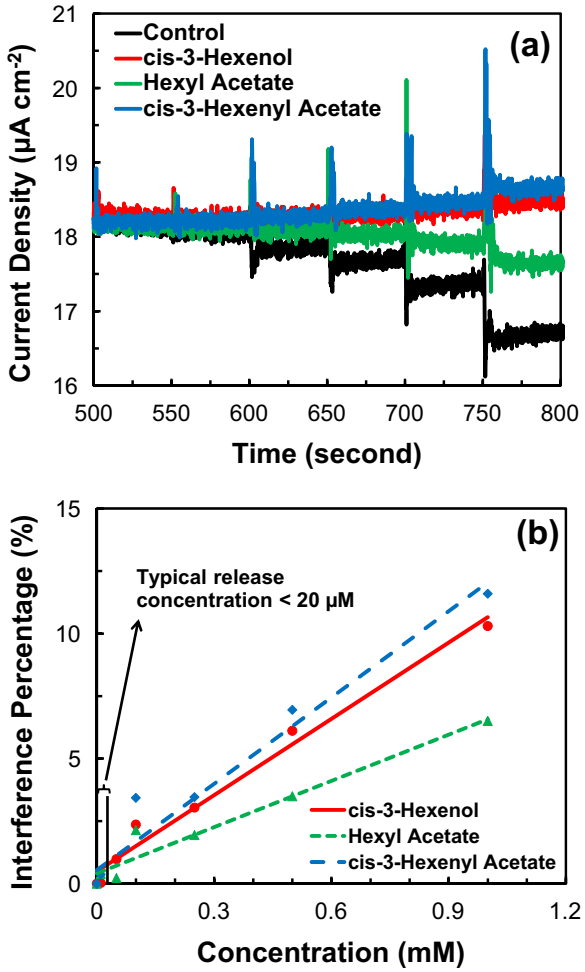

Fig. 4. Interference of *cis*-3-hexenol, hexyl acetate and *cis*-3-hexenyl acetate on the amperometric measurements. Phosphate buffer is used as the control interfering compound (a). Percentage of interference caused by each compound at different concentrations (b).

scenarios (Danner et al., 2011).

Fig. 4(a) displays the results from the interference studies, where the variation in the amperometric signal of the sensor was plotted against time during stepwise additions of each interfering compound, namely *cis*-3-hexenol, hexyl acetate and *cis*-3-hexenyl acetate. To account for the effects that arise due to the volume change of the electrolyte, a control interference study was performed, where phosphate buffer (PB) was added stepwise to mimic the volume change in the electrolytes. The decrease in the current values during stepwise addition of PB in control experiment could be attributed to the dilution effects arising from the stepwise addition, as the buffer itself does not contribute to interference. On the other hand, amperometric signal in the presence of GLV were slightly higher compared to the control. Fig. 4 (b) shows the percentage of interference at different concentrations of the interfering compounds, which ranges from 0% (for 0 μM of GLVs) to 2.36%, 2.14% and 3.43% respectively, (for 100 μM of *cis*-3-hexenol, hexyl acetate and *cis*-3-hexenyl acetate) as reported in Supplementary data† Table S2. Since 100 μM of GLVs represent is about two orders of magnitude higher than typical concentrations of GLVs, we could conclude that bi-enzyme sensor for MeSA detection suffers minimally from the interference of GLVs at their typical concentrations in plant volatile signature.

In addition to the above-mentioned compounds, methanol, which produced by most of C3 plants, could also potentially interfere with MeSA signal, since the detection of MeSA is based on methanol that was formed after hydrolysis (Fall and Benson, 1996). Therefore, the sensor is primarily meant for use with C4 plants such as corn. Previous research has indicated that significant amount of MeSA was released by juvenile corn (*Zea mays*) while methanol production was not reported (Bernasconi et al., 1998; MacDonald and Fall, 1993). The existing biosensor could be applied for crops like tobacco, as the quantity of methanol released by the tobacco crop is negligible compared to the quantity of MeSA (Bernasconi et al., 1998; Shulaev et al., 1997). Moreover pretreatment of the volatile sample to remove methanol vapors could also be applied to avoid false positive signals from methanol vapors (Premkumar and Krishnamohan, 2010).

3.4. Durability and repeatability

Durability of the bi-enzyme biosensor was evaluated for 10 days and the original data is reported († Supplementary data Table S3). The duration of the durability study was decided based on the duration it takes to notice a significant decrease in the amperometric signal. For this, constant potential amperometry was used to detect 50 μM hydrolyzed MeSA using the bi-enzyme modified RDE, where the net current density was measured before and after addition of the hydrolyzed MeSA. The RDE was then stored in 100 mM PB (pH 7.6) at 4 °C. The experiments were performed on day 0, 1, 2, 4, 7 and 10 by using the same procedure. The result of the durability shows that the current density for H_2O_2 reduction decreased gradually over the 7 days (Supplementary data† Fig. S6). The day when initial measurements were made was considered as day 0. On day 1, the current dropped from 100% to 95%. However, between day 1 and 4, the electrode behaved relatively stable with very little variations. The amperometric signal current was above 90% of the initial value even after 7 days. However, on day 10, the current sharply decreased to 79%, which could be attributed to the loss of enzyme activity (Supplementary data† Table S3). Since the sensor electrodes are meant for single-use testing, the durability data here shows satisfactory retention of signal durability by the bi-enzyme sensor for 7 days.

Tests were also conducted to evaluate the repeatability of the bi-enzyme biosensor performance. The experiments were conducted on the bi-enzyme modified RDE using identical concentrations of 50 μM hydrolyzed MeSA (Supplementary data† Fig. S7). The current density differed slightly between each measurement, which could be attributed to the slight change in enzyme orientation on the surface of the electrode. Upon the immobilization, the enzyme could orient itself on the surface in multiple ways, and therefore the active site of the enzyme could be either exposed to the electrolyte side or facing the electrode. The variation in the orientation could impact substrate binding and reaction thereby causing small variations in the electrochemical parameters measured in each experiment. Difference in the currents before and after adding hydrolyzed MeSA was used for relative standard deviation (RSD) calculation. The RSD for the repeatability experiments was 6.6% († Supplementary data Table S4), which is quite acceptable based on for enzyme-based sensors.

3.5. Real sample study

Real sample study was conducted in order to evaluate the bi-enzyme biosensor on a native analyte that contain methyl salicylate. Wintergreen oil essentially consists 98% of MeSA, which is produced by the enzymes of the wintergreen when it is under stress. Wintergreen oil was hydrolyzed as stated before and used as the analyte for real-sample studies. However, MeSA, which is the main compound in wintergreen oil, is not present in the plant initially. It is only produced enzymatically from a glucoside within the leaves when they are macerated in warm water. In this

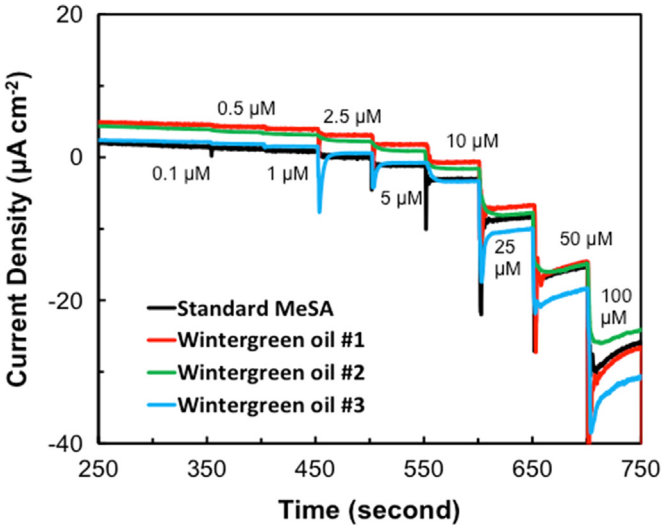

Fig. 5. Constant potential amperometry of the electrode upon the stepwise additions of wintergreen oil (3 replicates) resulting in concentrations of 0.1, 0.5, 1, 2.5, 5, 10, 25, 50, 100 μM (98% MeSA) wintergreen oil. A control experiment using standard methyl salicylate at identical concentrations (black curve) is also shown for comparison.

Table 1
Standard concentration, calculated concentration, recovery, SD and RSD of hydrolyzed wintergreen oil for real sample study.

Std. conc. (μM)	Cal. conc. (μM)	Recovery (%)	SD (μM)	RSD (%)
2.45	2.24	91	0.28	12.38
4.90	5.78	118	0.28	4.90
9.80	12.64	129	0.29	2.29
24.5	31.38	128	0.96	3.06
49.0	59.81	122	2.71	4.53

experiment, wintergreen oil was introduced to simulate the situation when plant produces MeSA due to plant infection. Different quantities of hydrolyzed wintergreen oil containing 98% MeSA was added stepwise to result in concentrations of 0.1, 0.5, 1, 2.5, 5, 10, 25, 50 and 100 μM were added into electrochemical cell and the amperometric signal was measured and compared against the amperometric signal of pure MeSA standards, in order to determine the estimated concentration of MeSA in the wintergreen oil. Recovery was calculated from the estimated concentration obtained from the working curve of standard MeSA sample and the known standard concentration.

As shown in Fig. 5, three replicates of measurements were obtained using hydrolyzed wintergreen oil and the trend was found to be the same as that of pure MeSA. As seen in Table 1, the RSD values ranged from 91% to 122%. The bias could arise due to the presence of other electrochemical active compounds in wintergreen oil such as α-pinene, myrcene, δ-3-carene, limonene, 3,7-guaiadiene and δ-cadinene reacted directly on electrode (Gurung, 2007). From the RSD values in Table 1, it can be concluded that the real sample experiments were highly repeatable when the concentration is close to the middle point of the linear range (*e.g.* 49 μM). On the other hand, the repeatability decreased when concentrations were at the lower limit (2.45 μM) and upper limit (98 μM) of the linear range.

4. Conclusion

A bi-enzyme based amperometric electrochemical biosensor has been developed using an established immobilization method. The biosensor was successfully tested for the detection of MeSA, a common VOC released by plants during pathogen infections. The sensor was characterized using CV and constant potential amperometry. Constant potential amperometry demonstrates higher sensitivity and lower limit of detection, which allows for faster sample collection for MeSA detection. The interference study with three GLVs indicates that the biosensor signal is not significantly affected by GLVs. The biosensor also displays satisfactory durability and acceptable repeatability. Wintergreen oil was used for real sample study, for demonstrating the on-field applicability of the sensor. Future work would focus on optimizing the sensor performance in terms of sensitivity, durability and repeatability by adopting more specific enzyme like salicylate hydroxylase and other enzyme stabilization methods. The fabricated biosensor will be used to test the head-space gas produced by the plants in glass bell jar in the prototype demonstrations and would eventually be used in the greenhouse. The work demonstrates an application of an electrochemical sensor with bio-recognition element for selective determination of plant pathogen infections based on methyl salicylate production.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2016.01.095.

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