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ARTICLE

Electrochemical ecology: VIMP monitoring of plant defense against external stressors

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Abstract: The use of the voltammetry of immobilized particles (VIMP) approach, by means of the voltammetric response of microparticulate films from ethanolic leaf extracts in contact with aqueous electrolytes and direct contact probe leaf voltammetry, to monitor the defense of plants against external stressors is described. This approach is applied to study the chemical communication between plants of *Peperomia obtusifolia* A. Dietr. submitted to herbivory by the beetle *Monoplatus* sp.

Keywords: chemical ecology, chemical signaling, electrochemistry, metabolomics**,** voltammetry of immobilized particles.

Introduction

Many plants respond to external stress such as mechanical damage or attack by phytophagous insects producing defensive substances.[1] In most cases, plant defensive strategies involve the release of chemical volatile organic compounds (VOCs) repelling the ovipositing insects,[2] or attracting parasitoids and predators of the same.[3] Interestingly, plants appear to distinguish between mechanical wounding and the attack of hervibores.[4]

From the chemical point of view, studies on chemical plant defense against external stressors requires the analysis of the involved molecular recognition events and the identification of the secondary metabolites acting as defensive compounds and/or chemical signals. In the first group, alkaloids, terpenoids, cyanogenic glucosides and phenolic compounds, among others, have toxic, anti-digestive or repellant properties on phytophagous insects.[2,5]

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In this context, there is evidence that VOCs emitted from damaged plants can induce defense responses in intact neighbor plants, which was demonstrated by changes in transcription of defense-related genes.[6] The notion of chemical communication between plants and between plants and other organisms is currently an accepted ecological phenomenon.[7] In addition to the well-known communication of plants with mutualists, such as pollinators and fruit dispersers, through both chemical and visual cues, communicate with themselves, with each other, with herbivores and with predators of those herbivores.

The VOCs acting as chemical signals comprise indole and methyl salicylate, terpenoids (cyclic and acyclic), oximes and nitriles.[4,8] The identification of such compounds is preferentially made by using chromatographic methods (HPLC-MS, GC-MS) and multinuclear magnetic resonance techniques.

Here, we describe the application of another technique: the voltammetry of immobilized particles (VIMP) for monitoring the chemical signatures associated to the defense of plants against external stressors. This technique is applied to detect the communication between plants of *Peperomia obtusifolia* A. Dietr. (baby rubber plant or pepper face**)** a widely distributed American ornamental plant, submitted to herbivory by the beetle *Monoplatus* sp., studied in parallel with conventional chromatographic and multinuclear resonance spectroscopy techniques.[9] The VIMP methodology, developed by Scholz et al.,[10] enables to determine the voltammetric response of sparingly soluble solid materials in contact with suitable electrolytes.[11]

The proposed method exploits the electrochemical activity of much natural compounds entering into the composition of the plant

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leaves. Two sensitive ways to detect the electrochemical response of these compounds is reported here: a) forming microparticulate films on electrodes from the leaf extracts with organic solvents, and b) direct contact probe voltammetry of leaf fragments immobilized onto carbon paste electrodes, in both cases in contact with aqueous electrolytes. As a result, variety-characteristic profiles are obtained from vegetal samples.[12] This electrochemical response can be attributed mainly to flavonoids and other polyphenolics of well-known electrochemistry in solution[13] and solid state using the VIMP approach[14] also used for monitoring the antioxidant properties of vegetables.[15] The specificity of the electrochemical response of the plants and the accessibility of the technique, which includes the possibility of using portable equipments for field analysis, makes it potentially interesting for chemoecological studies. It is pertinent to note that there is a variety of biotic and abiotic factors influencing the expression of molecules with functions in plant immune systems as well as the composition of secondary metabolites so that electrochemical data have to be necessarily complemented with other relevant biochemical tools.

Materials and methods

Plant material. A series of undamaged *P. obtusifoila* adult plants were transferred from the University of Sao Paulo and planted individually in plastic pots and irrigated and incubated under controlled conditions as reported elsewhere (see also Supporting information).[9] The plants were divided into three subgroups of ten individuals confined in plastic boxes permeable to chemicals but not to insects. The first group (I: 'herbivory group') was submitted to herbivory by 25 beetles (*Monoplatus* sp.); the second group, labeled as 'internal control' (II), was allowed to communicate with the herbivory group by placing the specimens in the same incubator. The third group, termed 'external control' (III), was isolated in a separate incubator under identical conditions (temperature, illumination, humidity) than the herbivory and internal control groups.

Electrochemical methods. Electrochemical experiments were performed at 298±1 K in a conventional three-electrode cell with CH 660I equipment. A BAS MF2012 glassy carbon working electrode (GCE) (geometrical area 0.071 cm^2), a platinum wire auxiliary electrode and an Ag/AgCl (3M NaCl) reference electrode were used. Ethanolic extract of leaves of plants of the different groups were obtained prior to electrochemical runs by macerating ca. 0.5 g of sample with 1 mL of the solvent in an agate mortar and pestle. Films of the extracts on glassy carbon electrode were prepared by pippeting a drop (50 μL) of the extracts on the surface of glassy carbon electrode (GCE) and allowing the solvent to evaporate. The voltammetric response of the resulting microparticulate films was studied upon immersion into 0.10 M aqueous phosphate buffer saline (PBS) at pH 7.4. Contact probe experiments were performed upon pressing a Pt microelectrode (20 μm diameter) onto a leaf fragment adhered to a carbon paste electrode. The same arrangement but separating the Pt microelectrode from the substrate was used for SECM experiments, here using $K_4Fe(CN)_6$ as a redox probe. In order to test the possibility of field studies, no electrolyte deaeration was performed.

Complementary techniques. High performance liquid chromatography with diode array detection (HPLC-DAD) was applied to plant extracts using a Phenomenex Luna column (C18, 5 μm, 250 × 4.6 μm) and acetonitrile:water with 1% formic acid as a mobile phase. ATR-FTIR spectra of leaves of the plants were performed upon pressing different regions of the leaf specimen on the diamond window of the spectrometer. A Vertex 70 Fourier transform infrared spectrometer with a FR-DTGS (fast recovery deuterated triglycine sulphate) temperature-stabilized coated detector. Additional experimental details are provided as Supplementary information.

Results and discussion

Experiments using headspace gas chromatography-mass spectrometry (HS-GC/MS), high-performance liquid chromatography with diode array detection (HPLC-DAD) and multinuclear magnetic resonance were consistent, after multivariate analysis of data, with the hypothesis that chemical communication between plants of the herbivory group and the internal control group occurred.[9] Figure 1 compares the cyclic voltammograms of microparticulate deposits of leaf extracts from the different groups of plants after 10 days of herbivory by *Monoplatus* sp., in contact with air-saturated PBS solution at biological pH.

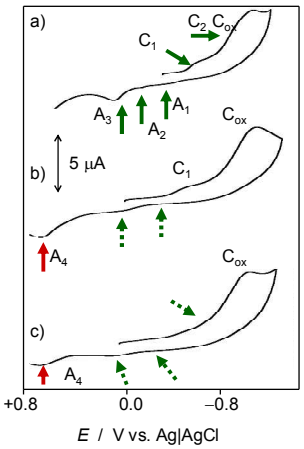

Figure 1. Cyclic voltammograms of microparticulate deposits of leaf extracts from: a) external control; b) herbivory group; c) internal control of *P. obtusifolia* plants after 10 days of herbivory by *Monoplatus* sp., in contact with air-saturated 0.10 M PBS solution at pH 7.4. Potential scan rate 50 mV s^{-1}.

Upon scanning the potential in the negative direction, the external control group of plants displays a cathodic shoulder at -0.40 V (C_1) followed by two prominent overlapping cathodic peaks at –0.85 (C_2) and –1.02 V vs. Ag/AgCl. In the subsequent anodic scan, oxidation waves at –0.10 (A_1), +0.20 (A_2) and +0.56 V (A_3) appear. This pattern is substantially modified in the extracts of the herbivory plants and those presumably communicated with the above (internal control group) whose the voltammograms exhibit an essentially identical pattern. Here, the peaks C_1, C_2 and A_2 vanish whereas the peak A_1 decreases significantly and appears to be followed by weak oxidation signals in the positive region of potentials where a new prominent oxidation peak is recorded at +0.82 V (A_4) which is accompanied by weak signals in the region of potentials between –0.25 and +0.75 V.

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This voltammetry can be interpreted in terms of the superposition of the signals for the electrochemical oxidation (A-peaks) and reduction (C-peaks) -the later superimposed to the ubiquitous signal for the reduction of dissolved oxygen, C_{ox},- of unidentified compounds present in the leaf extract. The anodic signals are particularly sensitive to changes in the composition of the extract. Apart from polyphenolic compounds (typically flavonoids, flavones, etc., relatively abundant in plant leaves)[22,23] there is possibility of attributing some of these oxidation processes to sesquiterpene compounds such as α-eudesmol, guaiol and δ-cadinene, particularly abundant in the essential oils from *P. obtusifolia*.[16] The first compounds could be electrochemically oxidized mimicking their chemical oxidation to dihydroxyeudesmane[17] and decalone,[18] respectively. In turn, δ-cadinene could be biochemically oxidized to 8-hydroxy-δ-cadinene further yielding gossypol (see Figure 2).[19] This compound, having o-catechol units, can experience an electrochemical oxidation (analogously to flavonoids having this unit)[13] to the corresponding o-quinone(s) resulting in anodic signals differing from those of the parent sesquiterpene.

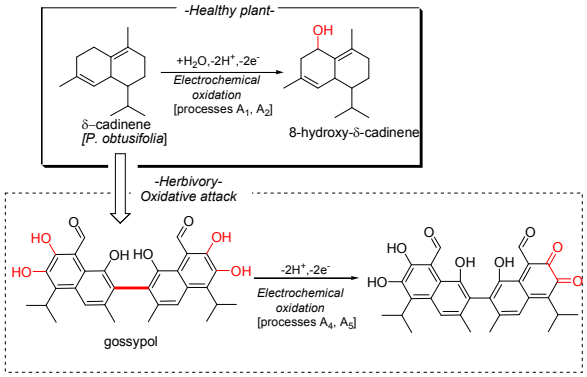

Figure 2. Scheme of possible electrochemical and chemical oxidation processes involved in δ-cadinene signaling.

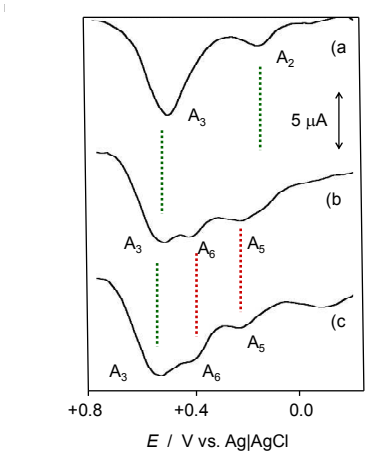

Figure 3. Square wave voltammograms, after semi-derivative convolution, of microparticulate deposits of leaf extracts from: a) external control; b) herbivory group; c) internal control of *P. obtusifolia* plants after 30 days of predation by *Monoplatus* sp., in contact with air-saturated 0.10 M PBS solution at pH 7.4. Potential scan initiated at –0.85 V in the positive direction; potential step increment 4 mV; square wave amplitude 25 mV; frequency 5 Hz.

A more detailed testing of the defensive response of plants against external stress was obtained using square wave voltammetry, an electrochemical technique which minimizes capacitive effects. Figure 3 shows the square wave voltammograms of microparticulate deposits of leaf extracts from *P. obtusifolia* plants of the groups III (external control, unpredated plants), I (submitted to herbivory); and II (internal control), all collected after 30 days of herbivory by *Monoplatus sp*. of the plants of the group I. Here, semi-derivative convolution was performed to increase peak resolution. Upon scanning the potential in the positive direction, the external control (unpredated) plants (Fig. 3a) show anodic peaks at +0.20 (A_1) and +0.56 V (A_3). Such peaks appear at freshly extract-modified electrodes upon scanning the potential from –0.10 V in the positive direction, thus denoting that the species responsible for peaks A_1-A_3 were in principle different from those yielding the signals C_1 and C_2. In agreement with cyclic voltammetric data, the extracts of herbivory samples were remarkably different and similar to those of the plants of the internal control group. In such voltammograms (Figs. 3b,c), the peak A_2 vanishes whereas the peak A_3 becomes diminished and overlapped with peaks at +0.23 (A_5) and +0.41 V (A_6). Essentially identical voltammetric features were obtained for 10-, 20- and 30-day plant samples.

The second approach consisted of recording the direct voltammetric response of leaf fragments in contact with aqueous electrolytes. Now, the voltammetric response would be possibly limited to nanocurrents, possibly associated to the reductive/oxidative dissolution of some vegetal compounds. The possibility of an effective contact probe electrochemistry was assessed upon recording the scanning electrochemical microscopy (SECM) images of such leaf fragments using $Fe(CN)_6^{4-}$ as a redox probe. Figure 4 depicts the corresponding images of the borderline of a leaf fragment of *P. obtusifolia* immersed into 2.0 mM $K_4Fe(CN)_6$ solution in phosphate buffer. Upon applying to the tip of a potential E_T sufficiently positive to promote the diffusion-controlled oxidation of $Fe(CN)_6^{4-}$, the tip current reflects the variations in the topography and conductivity of the immobilized substrate. Then, the leaf appears as a negative feedback featured region surrounded by the conducting carbon paste when no potential inputs are applied to the substrate electrode (Fig. 4a). When, following the redox competition strategy,[22] it is applied to the substrate a potential E_S positive enough to promote the oxidation of polyphenolic compounds (Fig. 4b), the profile of the boundary region between the leaf, the base carbon paste and the electrolyte becomes slightly modified, in agreement with the hypothesis that some oxidation process yielding water-soluble compounds occurs, similarly to that occurring in the voltammetry of microparticulate deposits of ion-insertion solids[20] and as observed for organic compounds experiencing solid-sate proton-assisted redox processes.[21]

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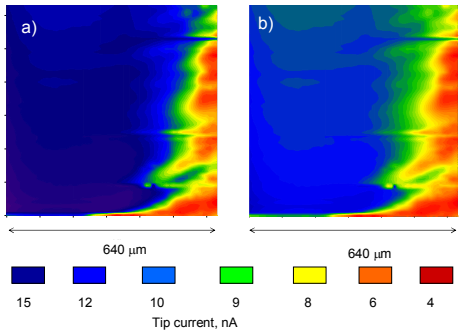

Figure 4. SECM map colors of the borderline of a leaf of *P. obtusifolia* immobilized onto carbon paste in contact with 2.0 mM $K_4Fe(CN)_6$ plus 0.10 M PBS, pH 7.4. E_T = +0.30 V; a) E_S = 0.00 V; b) E_S = +0.75 V.

Consistently with the SECM results, contact probe experiments performed upon pressing a with a Pt microelectrode onto leaf fragments immobilized onto a conventional carbon paste electrode and placed in contact aqueous phosphate buffer displayed oxidative nanocurrents in the region of potentials where polyphenolic compounds are oxidized. Again, differences between external control (group III), herbivory group (I) and internal control (group II) specimens of *P. obtusifolia* plants were obtained. This can be seen in Figure 5, where the 'direct' square wave voltammograms of leaves in contact with PBS are depicted. Under these conditions, electroactive compounds different from those extracted with ethanol are responsible of the observed voltammetry. Then the voltammogram in Fig. 5a, consists of a unique anodic peak at +0.35 V (A_7), differs from that in Fig. 3a. Remarkably, however, and in agreement with previous data on microparticulate deposits of ethanolic extracts, the voltammograms of the herbivory and internal control specimens was essentially identical and differed from that of the external control by the weakening of the signal A_7 and the appearance of a new, prominent anodic peak at +0.22 V (A_8). The disappearance or weakening of the signals C_1, C_2 and A_1-A_3 appearing in the voltammogram of external control plants and the appearance of new signals (A_5, A_6) appearing in extracts from both herbivory plants and those of the internal control, can be considered as consistent with the hypothesis of the existence of a chemical communication between *P. obtusifolia* plants associated to their predation by *Monoplatus* sp. Apparently, chemical defense to the external stress results in the loss of the compounds responsible for the signals C_1, C_2, and A_1-A_3 and the formation of new compounds responsible for signals A_4-A_6.

These features were consistent with HPLC-DAD and ATR-FTIR data (see Supplementary information). Figure 6 compares the fingerprint region of the infrared spectra of leaves of the groups I, II and III. As in the case of the voltammetric responses: the spectra of the plants undergoing herbivory (group I) was essentially identical to that of plants protected from herbivory but allowed to communicate with predated plants (group II), but differed from the spectra of isolated plants (III). It should be emphasized that taking into account the variety and complexity of the factors determining the composition of secondary metabolites and molecules involved in the plant immune system, a detailed scenario of the plant-predator interaction and plant-plant communication can only be obtained from other relevant biochemical techniques. Additionally, it is pertinent to note that plant defense can be activated by a variety of factors (in our case associated to herbivory) including wound damage, beetle-associated microorganisms inducing plant stress and immune response. In this context, the electrochemical methodology described here can be considered as a complementary tool potentially interesting for chemoecological studies aimed to study plant defense against external stress. Figure 7 shows a schematic representation of the relationship between the above voltammetric data and the phenomena of external stress, defense and signaling/communication referred to the voltammetric features recorded for ethanolic extracts of leaves of *P. obtusifolia* plants.

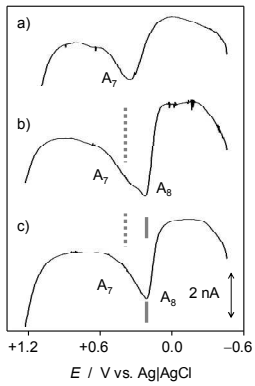

Figure 5. Contact probe square wave voltammograms at Pt microelectrode of leaf fragments of: a) external control; b) herbivory group; c) internal control of *P. obtusifolia* leaves after 30 days of predation by *Monoplatus* sp., in contact with air-saturated 0.10 M PBS solution at pH 7.4. Potential scan initiated at −0.45 V in the positive direction; potential step increment 4 mV; square wave amplitude 25 mV; frequency 5 Hz.

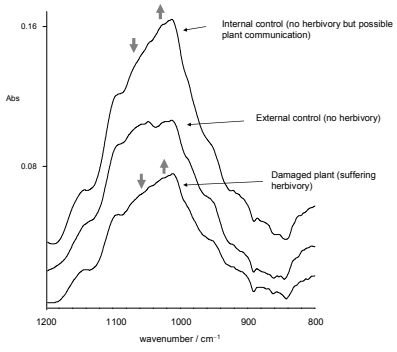

Figure 6. Detail of the ATR-FTIR spectra of leaves of *P. obtusifolia* specimens submitted to herbivory (group I), isolated from predators but with allowed communication (group II) and entirely isolated (group III) from herbivory and possible communication. The arrows mark the regions where the spectra of the damaged by beetle and 'communicated' plant differed

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from the spectrum of the external (non-damaged, non-communicated) control plant.

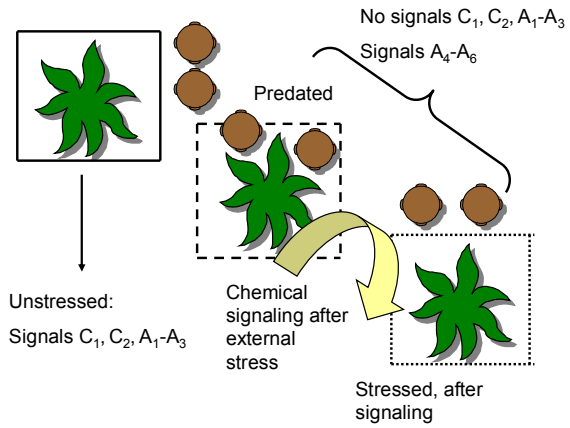

Figure 6. Schematic representation of the relationship between plant external stress, defense and signaling/communication phenomena and voltammetric data.

Conclusions

Using contact probe methodology and voltammetric experiments from microparticulate deposits of ethanolic leaf extracts it is possible to monitor the chemical plant defense against external stressors. This methodology is applied to study the response of *P. obtusifolia* submitted to predation by *Monoplatus* sp. beetles. The voltammetric response of plants undergoing herbivory was essentially identical to that of plants protected from *Monoplatus* but allowed to communicate with predated plants. These features would be consistent with the hypothesis of a chemical communication between plants associated to their defense against external stress. The sensitivity of the involved electrochemical methodology is just determined by the confluence of two favoring factors: i) the existence of only a limited number of compounds electrochemically active avoid the appearance of multiple signals which made difficult to discern between different plants (requiring multiparametric chemometric techniques) and the significant variations in the composition of such compounds associated to the process of external stress. These characteristics make voltammetric techniques a potentially useful technique for chemoecologic studies.

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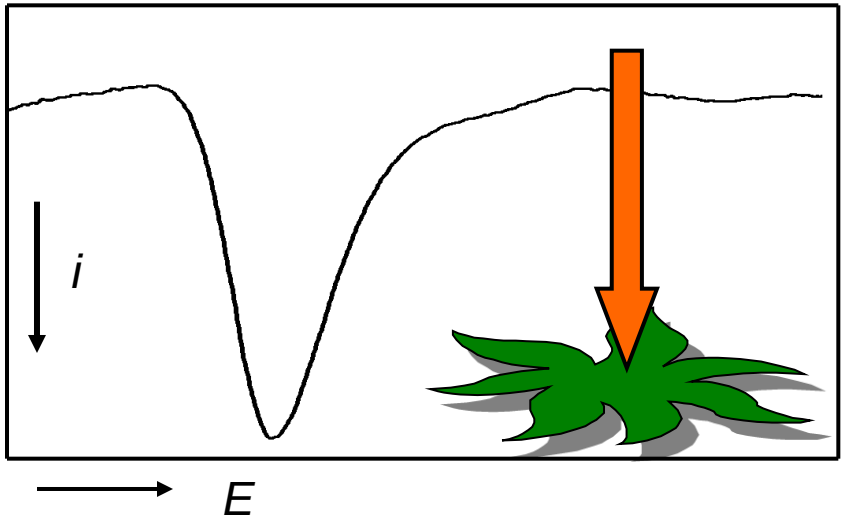

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