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The Use of Gas-Sensor Arrays in the Detection of Bole and Root Decays in Living Trees: Development of a New Non-invasive Method of Sampling and Analysis

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Abstract: Wood rot is a serious fungal disease of trees. Wood decay fungi penetrate and gain entry into trees through pruning cuts or open wounds using extracellular digestive enzymes to attack all components of the cell wall, leading to the destruction of sapwood which compromises wood strength and stability. On living trees, it is often difficult to diagnose wood rot disease, particularly during extreme weather conditions when trees can fail, causing tree parts to fall onto people and property. Today, tree stability evaluation and inner decay detection are performed visually and by the use of commercial instruments and methods that are often invasive, time-consuming and sometimes inadequate for use within the urban environment. Moreover, most conventional instruments do not provide an adequate evaluation of decay that occurs in the root system. A long-term research project, initiated in 2004, was aimed at developing a novel approach for diagnosing inner tree decays by detecting differences in volatile organic compounds (VOCs) released by wood decay fungi and wood from healthy and decayed trees. Different commercial electronic noses (ENs) were tested under laboratory conditions and directly in the field, on healthy and artificially-inoculated stem wood chips, and root fragments. The first stage of the research was focused on testing different commercially available electronic noses (e-noses) for the capabilities of discriminating between different strains and species of wood decay fungi as well as sapwood belonging to different tree species. In the second stage, sapwood of different tree species was artificially inoculated with decay fungi to test the diagnostic ability of the e-noses to detect differences in aroma bouquets emitted by healthy and inoculated woods. Root fragments were then inoculated with specific root decaying fungi and incubated under different types of soils to assess whether soil odors could influence the ability of the e-nose to discriminate between non-inoculated and diseased root fragments. For the final stage, soil air was evaluated for the presence of VOCs released by root-decaying fungi on diseased standing trees cultivated in the urban environment. *Copyright © 2015 IFSA Publishing, S. L.*

Keywords: Electronic nose, Decay detection, Urban forestry, VOCs, Tree.

1. Introduction

Trees in urban environments are cultivated under conditions that are extremely hostile (causing many stresses and negative effects) and consequently inadequate to sustain healthy plant life. Frequently, trees must face high levels of air, soil and water pollution [1]. Root development is often limited by permanent water stress and very small soil volume for root expansion, providing inadequate support of above-ground plant parts [2-3]. Moreover, road works, hasty or poor pruning methods, and vandalism increase tree stress in the urban environment. These adverse environmental factors dramatically increase physiological stresses that decrease tree fitness and increase susceptibility to attack by pathogenic agents [4].

Wood decay fungi are some of the worst microbial pathogens because they can take advantage of tree physiologic stresses by attacking and destroying all woody components, reducing tree structural stability, leading to failure (breaks) especially during severe weather events [5-6]. Root rots (decays) are even more dangerous and severe, due to difficult detection and the possibility of causing wind throw to the ground (complete tree loss). Trunk and root rot diagnoses in standing trees currently are performed primarily by electrical conductivity meters, constant feed drills, single pulse sonic and ultrasonic techniques, core samples, computerized tomography, and molecular methods for identification of decay fungi [7]. These tools and methods are pensive, invasive, require very skilled personnel, and do not provide systemic information. For these reasons, a multi-year study aimed at developing a novel approach for diagnosing inner tree decays using several gas sensor arrays or ENs was tested.

2. VOCs Emitted by Wood Decaying Fungi

Live standing trees containing decayed wood release a particular mixture of volatile organic compounds (VOCs) consisting of fungal metabolites, tree metabolites, and fungus-induced tree antimicrobial defense compounds (e.g. phenolic metabolites, terpenoids, isoprenoids, and phytoalexins). The composition of metabolites released by individual fungi is controlled largely by the types and combinations of metabolic pathways specific to microbial species, which are regulated by genetic, substrate and environmental factors [8]. Korpi, *et al.* [9] found microbes that released pinenes, acrolein, ketones and acetylenes that were irritants to mice.

Other investigations have focused on the identification of VOCs released by food spoilage fungi [10-11]. The compound 1-octen-3-ol was detected in damp houses containing various mold fungi [12]. Numerous other chemical species have been reported as fungal metabolites, including complex acids, sesquiterpenes, methyl ketones and alcohols [13].

Relatively few recent studies have reported on the release of VOCs by healthy and decayed trees. An analysis of healthy *Populus* spp. and *Pinus* spp. indicated the presence of mainly monoterpenes, acetone and small amounts of isoprene [14]. Other studies have indicated increases in toluene and α-pinene emissions associated with *P. sylvestris* under pathogen attack [15], and a decrease in isoprene emissions from diseased *Quercus fusiformis* L. and *Q.virginiana* L. [16].

The bacteriostatic role of plant VOCs was studied by Gao, *et al.* [17] who found emissions of terpenoids, alcohols, aldehydes, organic acids, and esters released by five healthy coniferous species in which α-pinene, β-pinene, 2,(10)-pinene, myrcene and d-limonene represented more than 95 % of total VOC emissions. Increased levels of α-pinene, limonene, nonaldehyde and benzaldehyde also were found in artificially-inoculated wood shaves in the same study

3. Electronic Noses

The EN is an instrument that mimics the human olfactory apparatus to detect VOCs through a series of sensors (sensor array) that provide digital signatures (sensor patterns) of all volatile chemicals present in the aroma bouquet of the sample analyte. It typically consists of a multisensor array, an information-processing unit such as an artificial neural network (ANN), software with digital pattern-recognition algorithms, and reference-library databases. The cross-reactive sensor array is composed of incrementally-different sensors chosen to respond to a wide range of chemical classes and discriminate diverse mixtures of possible analytes. The output from individual sensors are collectively assembled and integrated to produce a distinct digital response pattern. Identification and classification of an analyte mixture is accomplished through recognition of this unique aroma signature of collective sensor responses. A reference library of digital aroma signature patterns for known samples is constructed prior to analysis of unknowns. The ANN is configured through a learning process (neural net training) using pattern recognition algorithms that look for differences between the patterns of all analyte types included in the reference library. This process continues until a previously selected level of discrimination is met. The results are validated and assembled into the reference library to which unknown samples can be compared. Identification of unknowns is based on the distribution of aroma attributes or elements that the analyte pattern has in common with patterns present in databases of the reference library.

In this experiment, we employed three different commercially available ENs.

3.1. AromaScan A32S Electronic Nose

The AromaScan 32S (Osmetech Inc., Wobum, MA, USA) is a conducting polymer (CP) EN that contains an organic matrix-coated polymer-type 32-sensor array designed for general use applications with 15 V across sensor paths. The sensor array response to different VOCs was tested previously [8].

Sensors responses are measured as a percentage of electrical resistance changes to current flow in the sensors relative to baseline resistance (%ΔR/Rbase). The sorption of headspace volatiles, composed of specific VOC mixtures, to the CP sensor surfaces induces a change in the electrical resistance to current flow, which is detected and measured to produce the sensor array output. Sensor responses varied with the type of plastic polymer used in the sensor matrix coating, produced by electropolymerization of either polypyrrole, polyaniline or polythiophene derivatives, which have been modified with ring substitutions of different functional groups and with the addition of different types of metal ions to the polymer matrix in order to improve and modulate sensor response.

All measurements were statistically compared using normalized sensor outputs from the sensor array. Conducting polymer analysis methods used with this instrument employ application-specific reference libraries for aroma pattern recognition and neural-net training algorithms.

3.2. Lybranose 2.1 Electronic Nose

Operation of this EN is based on the quartz crystal microbalance (QCM) technology, which can be described as an ultrasensitive sensor capable of measuring small changes in mass on a quartz crystal recorded in real-time. The heart of the QCM is the piezoelectric AT-cut quartz crystal sandwiched between a pair of electrodes. The electrodes are attached to an oscillator. When an AC voltage is applied over the electrodes, the quartz crystal starts to oscillate at its resonance frequency due to the piezoelectric effect. If sample volatiles are evenly deposited onto one or both of the electrodes, the resonant frequency will decrease proportionally to the mass of the adsorbed layer according to the Sauerbrey equation [18].

The LibraNose 2.1 (Technobiochip, Pozzuoli, NA, Italy) sensor array consists of eight 20 MHz AT-cut QCM sensors with a gold surface (Gambetti Kenologia, Binasco, PV, Italy) coated with either metalloporphyrines, deposited by solvent casting, or by polypyrrole polymer) films (Technobiochip patent. No. 04425560.2-2102) deposited by means of Langmuir-Blodgett technology using a KSV 5000 film-deposition device (KSV Instruments, Helsinki, Finland). This process utilizes 0.3 mg/mL polymers dissolved in chloroform and ultrapure, distilled water as a subphase.

3.3. PEN3 Electronic Nose

The PEN3 EN (Airsense Analytics GmbH, Schwerin, Germany) is a very compact instrument (255 × 190 × 92 mm), light-weight (2.1 kg) and portable olfactory system. It consists of an array of 10 different doped metal-oxide semi-conductive (MOS) gas sensors positioned into a very small chamber with a volume of only 1.8 ml.

The instrument operates with filtered, ambient air as a carrier-gas at a flow rate of 10-400 ml min^{-1}, sample-chamber temperature of 0-45°C, and sensor array operating temperature of 200-500°C. The sensing reaction is based on an oxygen exchange between the volatile gas molecules and the metal coating material. Electrons are attracted to the loaded oxygen and result in decreases in sensor conductivity. Instrument sensitivity to various gas analytes ranges from 0.1-5.0 ppm.

4. Main Goals

This research project, a pioneer in the field of plant pathology and urban forestry, is based on following steps starting from basic research to be applied to finalized research solution. In every phase we have formulated a hypothesis derived from a question, with the aim to verify if the instrument could give positive answers.

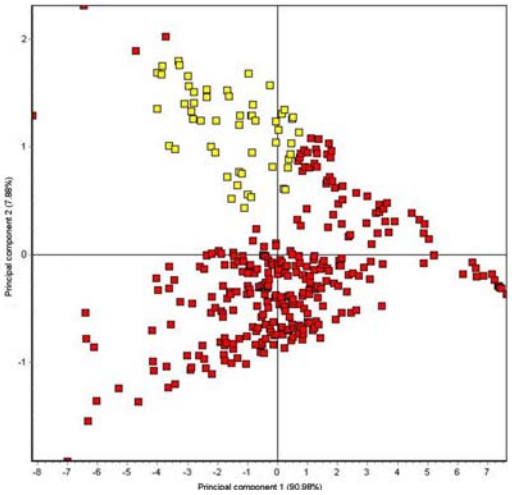
4.1. Can the EN Discriminate Between Healthy and Inoculated Wood Samples?

The first step of the research was aimed at determining if wood decay fungi emit certain combinations of VOCs that can be detected and recognizable by ENs [19]. 11 wood decay fungi (WDF) strains were selected, cultivated and inoculated on wood chips samples (sapwood) taken from 19 tree species: *Fraxinus pennsylvanica* Marsh., *Liquidambar styraciflua* L., *Pinus taeda* L., *Platanus occidentalis* L., *Populus deltoids* Bartr. ex Marshall, *Quercus nuttallii* Palm., *Quercus lyrata* Walt., *Thuia occidentalis* L., *Taxodium distichum* L. *Acer negundo* L., *A. saccharinum* L., *Aesculus hippocastanum* L., *Castanea sativa* Mill., *Cedrus deodara* (D. Don) G. Don fil., *Celtis australis* L., *Platanus x acerifolia* Brot., Quercus rubra L., *Robinia pseduoacacia* L., and *Tilia* spp.

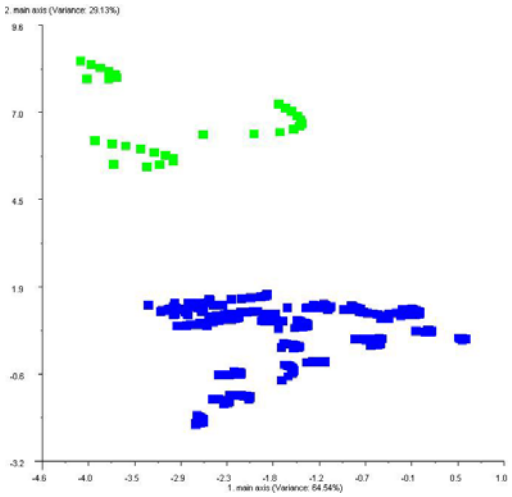
Species were selected from among the hardwood and conifer species most common in the lower Mississippi Delta and Northern Italy urban and forest environment, where the experiments were conducted.

After 6, 12 and 24 months of incubation under standard conditions we evaluated the discrimination ability of all three selected ENs. Fig. 1 – Fig. 3 show some results of this step.

Fig. 1(a) reports about the ability of Lybranose 2.1 in discrimining healthy and inoculated wood samples of all tree species with all fungal species.



(a)



(b)

Fig. 1. Discrimination of volatiles from healthy and decayed wood block using PCA. Labels are as follows: yellow and green labels indicate volatiles from healthy controls and red and blue labels indicate volatiles from decayed samples.

Although some zones of the graph show some overlaps between the two types, it is possible to assert that WDF emit volatiles which are clearly discriminable for the instrument. PEN3, differently, but clearly discriminated healthy and inoculated wood samples (Fig. 1(b)).

Running Principal Component Analysis (PCA) on samples belonging to one single tree species inoculated with different fungal strains, it is clear as the EN (Fig. 2) can easily discriminate the different WDF species.

4.2. Can the EN Discriminate Between Healthy and Inoculated Root Samples Under Soil Conditions?

The root system is the most important organ for initiating plant growth as it is dedicated to the uptake of water and minerals. In trees, structural roots give mechanical support to the heavy woody structure. The root system is by nature the least known of all tree organs as it is not assessable other than by destructing methods. As far as root decay diagnoses are concerned, there are not commercially available tools capable of assessing and diagnosing decays in the root system.

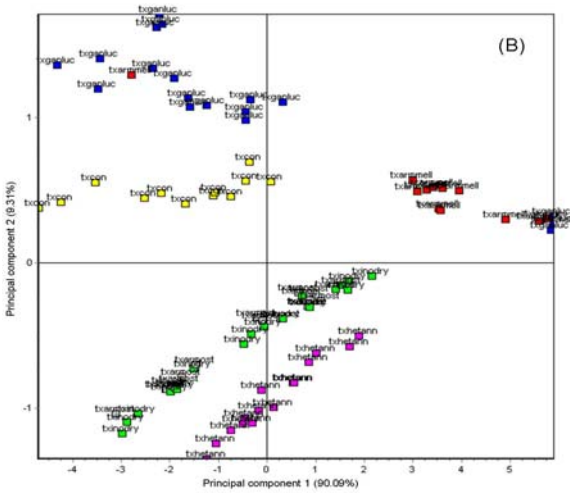


Fig. 2. Discrimination of volatiles from artificially-inoculated decayed wood samples of *Tilia* spp. by PCA. Different color labels indicate different wood decay fungi responsible for decay. Undecayed (control) wood block are labeled in yellow.

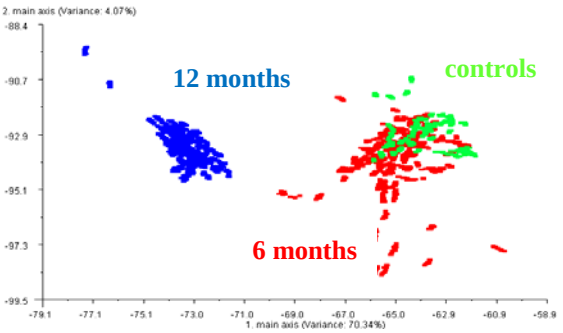


Fig. 3. Discrimination of VOCs from healthy controls (green labels) and artificially-inoculated root chips after 6 months (red labels) and 12 months (blue labels) from inoculation using PCA.

This phase of the research, aimed at determining if the presence of VOCs emitted by wood decaying fungi or decayed living wood can be detected even under-soil conditions, utilized root tissue form four species of shade trees [7]. Parts of 1 cm healthy roots were sampled from each tree in which roots were prepared and inoculated with four different WDF strains (two strains of *Armillaria mellea*, one of *Ganoderma lucidum* and one of *Heterobasidion annosum*). Inoculated root chips were then incubated under two different kinds of soils (very poor urban soil and professional soil for horticulture) for 12 months at standard laboratory conditions.

Our results show that:

1) PEN 3 EN could easily discriminate between inoculated and non-inoculated root chips after 12 months of incubation;

2) PEN 3 EN could not discriminate the inoculated samples from the healthy ones after only 6 months from the inoculation. This means that under soil conditions, wood decay fungi take a little more time to develop enough VOCs to be detected from the EN (Fig. 3);

3) Soil type does have an influence on the discrimination capability of the instrument. This is probably due to the fact that professional soil type, which is still rich in microorganisms, emits a strong aroma bouquet.

4.3. Can the EN Detect the Presence of a Decay in the Root System of Standing Trees Directly in the Field ?

All previous steps of this long research were aimed at evaluating the diagnostic feasibility of EN under the stable and standardized conditions of the laboratory environment. In this stage, the EN was employed directly in the field to detect the presence of VOCs emitted by wood decay fungi attacking the root system.

A very important postulate of this research is that the diagnostic system (tool as well as sampling method) should be totally non-invasive for the plant. Wounds caused by sampling, diagnosis or analysis method could eventually be preferred entry points for further pathogenic attack. According to this, a revolutionary sampling method was tested based on detecting decay fungi that emit VOCs which diffuse in soil air macropores. To sample and analyze soil air, a pump system was designed and built as seen in Fig. 4, in which soil air is sucked in by the pump and directly carried to Nalophan bags for e-nose analysis.

Fig. 4. The automatic pump employed in the field to put directly soil air in Nalophan bags.

For this final stage of the research, more than 60 trees planted in the city of Milano, Italy, belonging to five different species [*Acer negundo* L., *A. negundo* 'Variegatum', *A. pseudoplatanus* L., *Aesculus hippocastanum* L., and *Platanus x acerifolia* (Aiton) Willd were sampled]. All of these trees were previously assessed via conventional methods for the presence of stem and root decays. Soil air was sampled six times over a period of two years.

Our preliminary results, shown in Fig. 5 – Fig. 7, demonstrate that WDF VOCs can be found in soil macropores, and that their concentration in the zone of the root system is high enough to be detected by EN sensors. Fig. 5 show a linear discriminant analysis (LDA) performed on soil air samples taken about 30 cm from the bole of healthy and decayed trees. The etiologic agent causing decays in sample trees, previously recognized via traditional methods, was also recognized by the EN. Fig. 6 shows the diagnostic feasibility of the PEN 3 EN in discriminating between different WDF species.

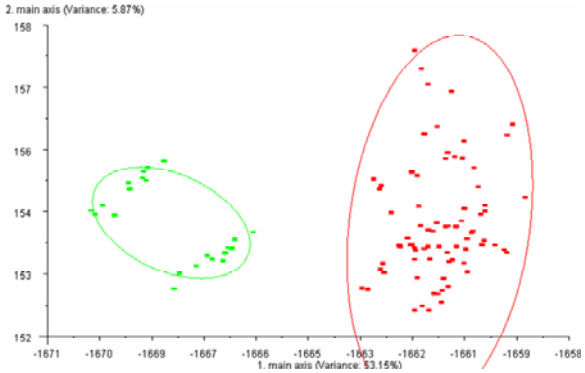


Fig. 5. Linear Discriminant Analysis performed on volatiles in soil air samples taken 30 cm from the bole of healthy (red labels) and decayed (red labels) standing trees.

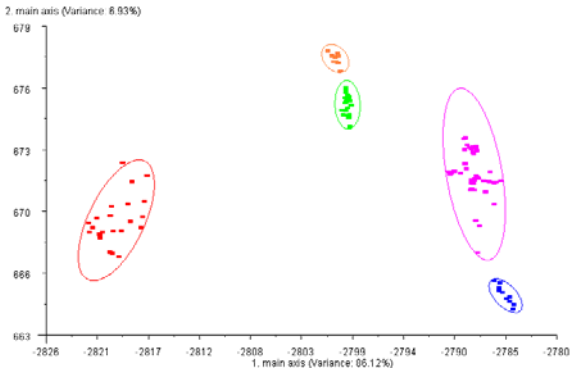


Fig. 6. PCA performed on volatiles in soil air samples taken 30 cm from the bole of healthy (green labels) and decayed standing trees. Different colors correspond to main etiologic decay agent: *Armillaria* spp. (red labels), *Meripilus giganteus* (orange), *Ganoderma* spp. (pink) and *Perenniporia* ssp. (blue labels).

Soil air or healthy control trees also were used to check if healthy root systems release the same VOCs

as those released by trunk sapwood; and if ENs can discriminate between species on the basis of those VOCs. Fig. 7 shows the discrimination feasibility of the PEN 3 EN between different healthy tree species on the basis of VOC analysis in the aroma bouquet released by root systems sampled at about 30 cm from the bole by the use of an air pump.

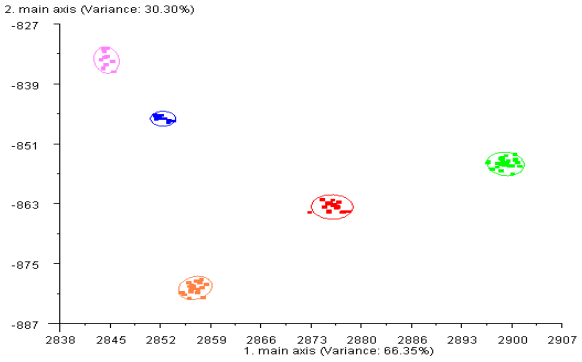


Fig. 7. PCA performed on volatiles in soil air samples taken 30 cm from the bole of healthy standing trees. Different colors correspond to different species as following: *Metasequoia glyptostroboides* (pink labels), *Fagus sylvatica* (blue), *F. sylvatica* 'Pendula' (red), *Aesculus hippocastanum* (green) and *Quercus rubra* (orange).

7. Conclusions

Tree cultivation in the urban environment requires some agronomic works, which are particularly important and expensive for Public Administrators. Among these, pruning and maintaining tree stability most influence the annual budget of ordinary management. Tree stability assessment, a fundamental duty to prevent sudden tree failures so ensure citizen's safety, is performed by very skilled personnel who employ commercial instruments and tools which are, in most of cases, invasive and very expensive. Decay assessment of the root system is not currently performed, as there are no commercially available instruments besides ENs capable of these assessments.

Our experimental research started about 10 years ago, was aimed at developing a sampling and analysis methodology to determine the presence of active wood decay and root rots on standing trees, in a rapid and non-invasive way, applicable in all situations and usable by non-skilled operators. Through multiple stages of research, we have demonstrated that three different commercial ENs can discriminate: between different tree species and WDF species by analyzing the VOCs in the aroma bouquet released by healthy (non-inoculated) and inoculated trunk wood chips; between healthy (non-inoculated) and inoculated root chips incubated under two different kinds of soils; between healthy and decayed living and standing trees, between different species of healthy standing trees, and between etiologic agents of diseased standing trees on the basis of the analysis of the aroma bouquet present in the soil air (macropores) sampled near the tree bole.

The EN system is not fully ready to be employed daily in the field yet, as it is necessary to build application-specific aroma signature databases of healthy tree species at different stages of growth, development and phonologic phase, as well as diseased tree species, decayed by different wood decay fungi species, in order to train ENs to yield immediate answers directly in the field.

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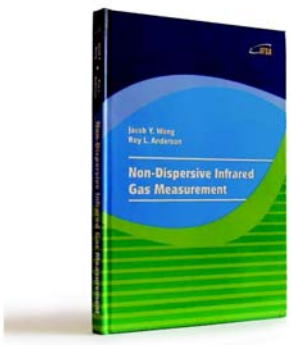

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Jacob Y. Wong, Roy L. Anderson

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