Talanta 129 (2014) 422–430

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Detection of potato brown rot and ring rot by electronic nose: From laboratory to real scale

E. Biondi*, S. Blasioli, A. Galeone, F. Spinelli, A. Cellini, C. Lucchese, I. Braschi

Department of Agricultural Sciences, University of Bologna, V.le Fanin, 44, 40127 Bologna, Italy

ARTICLE INFO

Article history:
Received 24 January 2014
Received in revised form
17 April 2014
Accepted 21 April 2014
Available online 2 May 2014

Keywords:
Volatile compounds
E-nose
Sensorial analysis
LDA statistical analysis
PCA statistical analysis

ABSTRACT

A commercial electronic nose (e-nose) equipped with a metal oxide sensor array was trained to recognize volatile compounds emitted by potatoes experimentally infected with *Ralstonia solanacearum* or *Clavibacter michiganensis* subsp. *sepedonicus*, which are bacterial agents of potato brown and ring rot, respectively. Two sampling procedures for volatile compounds were tested on pooled tubers sealed in 0.5–1 L jars at room temperature (laboratory conditions): an enrichment unit containing different adsorbent materials (namely, Tenax® TA, Carbotrap, Tenax® GR, and Carboxen 569) directly coupled with the e-nose (active sampling) and a Radiello™ cartridge (passive sampling) containing a generic Carbograph fiber. Tenax® TA resulted the most suitable adsorbent material for active sampling. Linear discriminant analysis (LDA) correctly classified 57.4 and 81.3% total samples as healthy or diseased, when using active and passive sampling, respectively. These results suggested the use of passive sampling to discriminate healthy from diseased tubers under intermediate and real scale conditions. 80 and 90% total samples were correctly classified by LDA under intermediate (100 tubers stored at 4 °C in net bag passively sampled) and real scale conditions (tubers stored at 4 °C in 1.25 t bags passively sampled). Principal component analysis (PCA) of sensorial analysis data under laboratory conditions highlighted a strict relationship between the disease severity and the responses of the e-nose sensors, whose sensitivity threshold was linked to the presence of at least one tuber per sample showing medium disease symptoms. At intermediate and real scale conditions, data distribution agreed with disease incidence (percentage of diseased tubers), owing to the low storage temperature and volatile compounds unconfinement conditions adopted.

1. Introduction

Electronic nose (e-nose) is a useful instrument for the detection and discrimination of odors that mimics the olfactory human system by means of a sensor array, which is able to detect mixtures of volatile compounds coming from complex matrices due to a broad overlap of selectivity of the various sensors in the array [1]. It is largely used in food to recognize the product freshness or to identify product adulterations [2], in the cosmetic and pharmaceutical industry for flavor analysis [3,4], as well as in environmental safety to detect explosive and flammable or toxic/hazardous gases [5,6].

In the nineties, e-nose had been applied in the clinical diagnosis for the identification of bacterial infections through the analysis of volatile compounds emitted from micro-organisms [7,8]. Recently, studies have been extended to plant pathogens to discriminate healthy from diseased plants on the basis of the changes in the volatile compound patterns caused by the biological pathogen activity [9,10] or among different pathogenic species [11].

Ralstonia solanacearum race 3 biovar 2 (Rs) and *Clavibacter michiganensis* subsp. *sepedonicus* (Cms) are the bacterial causal agents of potato brown and ring rot, respectively, and are considered the most damaging potato diseases worldwide; the former has been reported in more than 30 countries from sub-tropical to cold temperate areas, while the latter occurs in Northern America, North Eastern Europe and Asia. Both bacteria are included in the A2 list of quarantine pathogens in Europe and are subjected to EU directives (2006/63/EC for Rs and 2006/56/EC for Cms). Foliage symptoms include wilting of leaves and stems in both diseases (Figs. 1(a) and (c)). Tubers external symptoms are visible only at a late stage of infection, when the complete destruction of tubers occurs, opening the way for the attack of other secondary micro-organisms that can contribute to the degradation of potato tissues [12]. Internal symptoms are visible after potato cutting (Figs. 1(b) and (d)): a browning and possible necrosis of the vascular ring occurs in tubers infected by Rs while, in the presence of Cms pathogen, the vascular ring has a yellowish coloration and when

* Corresponding author. Tel.: +39 051 2096728; fax: +39 051 2096723.
E-mail addresses: enrico.biondi3@unibo.it (E. Biondi), sonia.blasioli@unibo.it (S. Blasioli), antogaleone@yahoo.it (A. Galeone), francesco.spinelli3@unibo.it (F. Spinelli), antonio.cellini2@unibo.it (A. Cellini), carla.lucchese@unibo.it (C. Lucchese), ilaria.braschi@unibo.it (I. Braschi).

http://dx.doi.org/10.1016/j.talanta.2014.04.057
0039-9140/

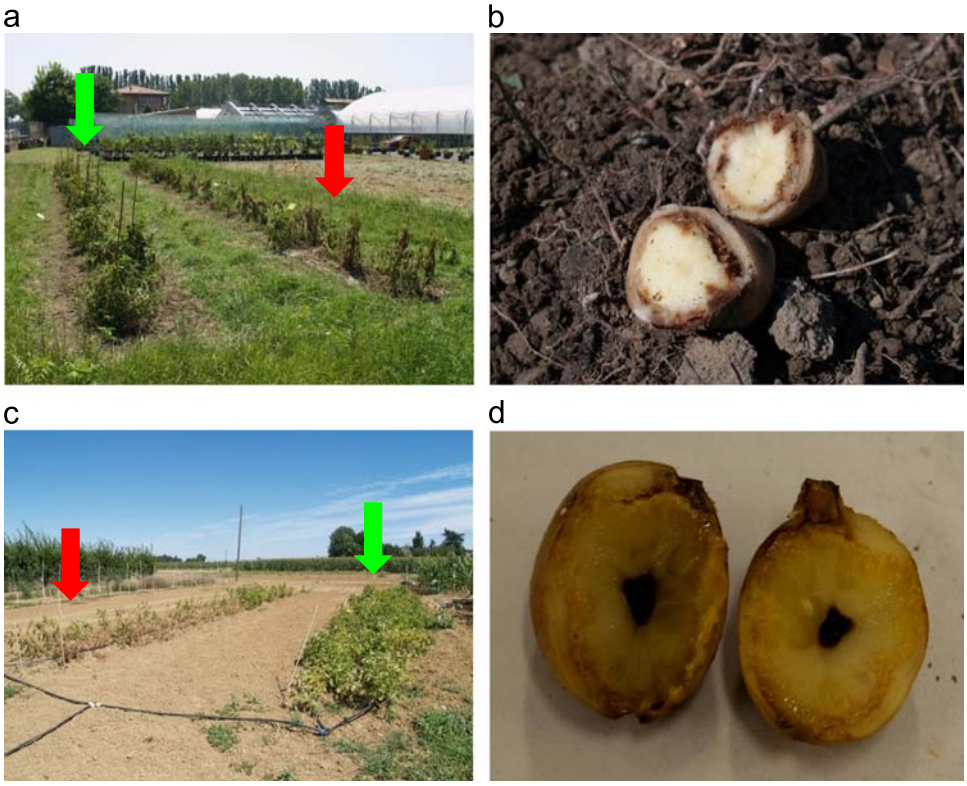

Fig. 1. Production of infected potatoes for laboratory and intermediate scale experiments: (a) field of potatoes inoculated with Rs and control (right and left arrow, respectively); (b) potato with brown rot symptoms; (c) field of potatoes inoculated with Cms and control (left and right arrow, respectively); and (d) potato with ring rot symptoms.

the tuber is gently squeezed, a cheese-like material emerges from vascular ring [13]. In case of latent infections, internal symptoms might be not visible even though the pathogens are present inside the tubers.

Potato flavor is composed by more than 100 volatile compounds including aldehydes, alcohols, ketones, acids, esters, hydrocarbons, amines, furans, and sulfur compounds. Micro-organisms such as fungi, bacteria, viruses, nematodes, and protozoa can modify the volatile pattern emitted from potatoes and produce markers of the pest. Acetone, ethanol, 2-butanone and 3-hydroxy-2-butanone were identified as markers of soft rot (caused by *Pectobacterium carotovorum* ssp. *carotovorum*) in stored potatoes [14,15]. Ethoxy-ethene, 2-methyl-l-butanol, 2-butanone, 2-methyl-2-butanamine, 2-2-propenyl-1,3-dioxolane, and 3,5-heptadiyn-2-one are fungal disease markers in stored potatoes [16,17]. Stinson et al. [18] found that potatoes at the final stage of Cms infection (when the whole tuber rots away) emit 3-methyl-2-pentanone as a marker of ring rot, whereas the variation of peak intensity of short-chain alcohols and ketones is indicative of brown rot disease. In a recent paper of Blasioli et al. [19], specific Rs and Cms markers in unwounded potatoes without external disease symptoms and with a low disease severity (to avoid cross-contaminations due to the presence of other secondary micro-organisms) have been identified: 1-hepten-3-ol, 3,6-dimethyl-3-octanone, 3-ethyl-3-methylpentane, 1-chloroctane and benzothiazole for potato brown rot, 2-propanol and toluene for potato ring rot.

Though the effect of potato diseases on volatile emission has been demonstrated, a few papers have investigated the use of e-nose for the detection of potato diseases. de Lacy Costello et al. [20] have developed an e-nose prototype which is able to detect, in a simulated storage box, one tuber with soft rot symptoms in 100 kg of healthy tubers and one infected tuber without visible signs of infection, within 10 kg of healthy tubers. In this study, potatoes were experimentally produced by inoculating a bacterial suspension into a wound made at the tubers surface [20]. In addition, Stinson et al. [18], have demonstrated the capability of a "home-made" e-nose to identify potatoes infected by Rs or Cms. In both studies, experiments have been conducted at laboratory scale or under simulated real conditions [20].

The present work is focused on the use of a commercial e-nose for the detection of Rs and Cms pathogens in potato tubers with the aim to develop a fast and reliable method—alternative to the standard methods based on molecular and microbiological analysis which are reported in EU Directives—for quarantine pathogen detection.

Here, the sampling and analysis protocol for brown and ring rot detection were developed from laboratory to real scale conditions. Two sampling methods of volatile compounds emitted from unwounded healthy and diseased potatoes (without external disease symptoms) were evaluated: (i) active sampling by means of an enrichment unit containing adsorbent materials directly coupled with the e-nose and (ii) passive sampling by means of a Radiello™ cartridge. Data collected with the sensorial analysis were statistically processed to build a database to be used for the offline discrimination between healthy and diseased samples.

2. Materials and methods

2.1. Production of infected potato tubers

2.1.1. Bacterial Strains

The virulent IPV-BO 5836 (Mazzucchi and Traversa, unpublished data) and IPV-BO 7695 (Mazzucchi and Mucini, unpublished data) strains, isolated from potato tubers, were used for this study.

Strains were routinely grown on tetrazolium (TZ) [21] and yeast dextrose calcium carbonate (YDC) [22] media at 27.0 ± 0.1 °C for 72–96 h, respectively.

2.1.2. Experimental inoculations

Infected potatoes were obtained by experimental inoculations with both bacterial pathogens in potato plants. Cultivars 'Spunta' and 'Kennebec' were used for the production of Rs and Cms infected potatoes, respectively (Fig. 1). 30 μL of a water suspension (ca. 10^9 CFU mL^{-1}) containing Rs or Cms, grown at 27.0 ± 0.1 °C for 48 h (growth exponential phase) on TZ and YDC, respectively, were injected at the stem (30–60% of the total stems). After the infiltration of the suspension, the wounds were closed with fluid paraffin. Sterile deionized water (SDW) was used as a negative control. The experimental inoculations of Cms were also made at seed stage: more details on experimental inoculations are reported in Blasioli at al. [19].

After approx. 4 months from the inoculation, the tubers of both experimental fields were harvested and stored in a refrigerated room at 4 ± 1 °C. The tubers infected with each pathogen and the corresponding negative control were harvested and stored separately to avoid contaminations.

2.2. Potato sample preparation for laboratory, intermediate, and real scale experiments

2.2.1. Laboratory scale experiment

Sensorial analyses were performed on unwounded pooled tubers, grouped as healthy (negative controls) or diseased samples (infected with Rs or Cms) placed into 0.5 L jars (1 L jars for volatiles passive sampling with Radiello™ in order to easily accommodate the cartridge into the jar, vide infra) up to fill the jar volume (approximately 250 and 400 g of potatoes per 0.5 and 1 L jar, respectively). Jars were tightly sealed with Teflon caps (Figs. 2(a) and (b)).

2.2.2. Intermediate scale experiment

A set of samples was prepared placing 100 healthy tubers (provided by Phytosanitary Inspection Service of Emilia Romagna Region, Italy) into net bags (Fig. 2(d)). This number is a half amount of the potato tubers which must be processed by phytosanitary inspectors to rule out the occurrence of quarantine pathogens, according to the EU directives. Some potato net bags were sealed in polyethylene bags, (from now on called "PE bags") to simulate the confined environment of closed jars (Fig. 2(c)). The response of PE bag to e-nose was assessed and did not affect those from both diseased and healthy potato emission. To validate e-nose detection method, net bags of 100 tubers were prepared at 50%, 75% and 100% of experimentally inoculated Cms potatoes.

2.2.3. Real scale experiment

Tubers stored in a refrigerating cell at 4 °C in 1.25 t polypropylene (PP) bags (Fig. 2(e)) were used as samples for the set of analyses in real conditions, directly performed in Ravenna Port (Italy). The potatoes were previously analyzed by the local Phytosanitary Inspection Service and were claimed healthy.

2.3. Volatile compound sampling methods

Before the sensorial analyses, volatile compounds were collected with two different sampling methods: (i) active sampling performed by a volatile compound trap, directly connected to e-nose, that concentrated volatiles from potato jars; and (ii) passive sampling performed by commercial Radiello™ cartridge (Supelco™, Sigma-Aldrich Co. LLC., USA) directly placed inside potato jars or bags. After sampling, volatile compounds were thermally desorbed (i) in the e-nose, in case of active sampling, and (ii) in an external apparatus in case of passive sampling.

2.3.1. Active sampling under laboratory conditions

The e-nose was coupled with an electronic desorption unit (EDU3, Airsense Analytics GmbH, Germany). The unit was provided with different vials embedding several adsorbent materials such as Tenax® TA, Carbotrap, Tenax® GR, and Carboxen 569. EDU3 unit firstly adsorbs volatile compounds which are subsequently released into the e-nose.

Four steps were involved in the sensorial analysis: (i) volatile compound adsorption into EDU3 unit by drawing the headspace of potato samples (previously incubated into jars at room temperature for 24 h) at 25 °C for 300 s (air flow rate = 100 mL min^{-1}); (ii) volatile compound desorption by heating the adsorption trap at 250 °C for 130 s (flow rate = 100 mL min^{-1}); (iii) injection of trapped flavors into the sensor array at 250 °C for 75 s (flow rate = 25 mL min^{-1}); (iv) cleaning of the adsorbent at 280 °C for 180 s, followed by a cooling step to get the instrument ready for the next analysis.

2.3.2. Passive sampling under laboratory conditions

A Radiello™ Carbograph cartridge was placed into 1 L jars filled with potato tubers (Fig. 2(b)) for 7 d at room temperature. Carbograph is a non porous, homogeneous and non specific adsorbent material, suitable for qualitative analysis of several volatile compounds. After adsorption, volatile compounds entrapped into the Radiello TM cartridge were thermally desorbed in an external homemade apparatus made of an aluminum tube placed for 10 min inside a tubular oven (Carbolite MTF 10/15/130, Carbolite, Hope, UK) kept at 380 °C under a chromatographic air flow (rate = 100 mL min^{-1}). The desorbed volatile compounds were collected into 1 L gas sampling bags (Supelco™, Sigma-Aldrich Co. LLC., USA). The subsequent e-nose analysis was performed by connecting each gas sampling bag kept at room temperature to the e-nose operating at 60 s injection time (air flow rate = 400 mL min^{-1}).

2.3.3. Passive sampling under intermediate conditions

A Radiello™ Carbograph cartridge was placed in 100 healthy tuber samples collected in PE and net bags at 4 °C and room temperature for 7 d (Figs. 2(c) and (d)). Radiello™ cartridges were also placed into net bags containing different percentages of Cms infected potatoes at 4 °C for 7 d (Fig. 2(d)).

After adsorption, volatile compounds were desorbed from Radiello™ and injected into e-nose as already described in the "Passive sampling under laboratory conditions" section.

2.3.4. Passive sampling under real conditions

A Radiello™ was placed for 7 d at 4 °C into 1.25 t PP bags containing healthy potatoes stored in the port of Ravenna (Fig. 2(e)). The procedures used to desorb and inject volatile compounds into e-nose were the same already described in the "Passive sampling under laboratory conditions" section.

2.4. E-nose analysis

The analyses were carried out with a commercially available portable e-nose (PEN3, Airsense Analytics GmbH, Germany). PEN3 consists of a sampling apparatus, a detector unit containing the sensor array, and a pattern recognition software (Win Muster v.1.6.2) for data recording. The sensor array contains 10 metal oxide semiconductor (MOS) sensors working in the 150–500 °C

Fig. 2. Sample preparation for e-nose analysis under laboratory, intermediate and real scale conditions: unwounded potato tubers infected with Rs or Cms in (a) 0.5 L and (b) 1 L jars sealed with Teflon caps (Radiello™ cartridge is visible in (b)); (c) net bag sealed in polyethylene (PE) bags; and (d) net bags; (e) 1.25 t polypropylene (PP) net bags.

range of temperature, which is not adjustable by the operator, to assure a correct classification and identification of volatile species. Each sensor is not specific and responds to a class of organic compounds as aromatics (MOS1 and MOS3), aromatics and aliphatics (MOS5), sulfur- and chloro-organic compounds (MOS7 and MOS9, respectively), methane and aliphatics (MOS10), broad range of alcohols and aliphatic substances (MOS2, MOS6 and MOS8). MOS4 is specific for hydrogen. The sensor response is expressed as resistivity (Ω).

The e-nose analyses were recorded in about 1 min (see injection time in "Volatile compound sampling methods" section) using an accumulation time of 1 s. The initial injection flow and the sensor chamber flow were set both at 25 mL min^{-1} using the enrichment unit, and at 400 mL min^{-1} without EDU3; the purge flow was 600 mL min^{-1} with dry air as a washing gas.

2.4.1. Data analysis

Principal Component Analysis (PCA) [23] and Linear Discriminant Analysis (LDA) [24,25] were used for the statistical analysis of data using a covariance matrix to build the PCA plot. The prediction capacity of the discriminant model was evaluated by the "leave-one-out" cross-validation to determine the stability of the model [26]. The software Minitab® 16 (MINITAB Inc., USA) was used for chemometrics analysis.

2.5. Phytopathometric analysis

After e-nose analysis, tubers were half cut to evaluate the disease severity. Symptoms on the vascular ring were visually analyzed, photographed and a five phytopathometric class ladder was built to help the correlation between the e-nose responses

and the disease severity (Table S1 in Supporting Information). The disease incidence (percentage of symptomatic tubers per sample) was also evaluated.

2.6. Bacterial pathogen reisolation and identification

A core was removed from each tuber at the heal-end and crushed in 2 mL of SDW. The isolation extract was left to settle and, after 15 min, 1.5 mL were processed for microbiological and molecular assays. The analyses for the detection and identification of both pathogens were carried out following the EU Directives. The protocols of Seal et al. [27] and Pastrik [28] were used for PCR assays to identify Rs and Cms, respectively. Details of procedure for the reisolation and identification of pathogens and for molecular assays are reported in Blasioli et al. [19].

3. Results and discussion

3.1. Yield of infected potato tubers

Diagnostic analyses, performed after sensorial analyses, on tubers produced in the field, highlighted a high percentage of disease incidences. After microbiological and molecular assays, 78% and 91% of tubers resulted infected by Rs and Cms, respectively. Among the infected tubers, 49 and 46% showed the typical internal symptoms of brown and ring rot, respectively. After phytopathometric evaluations, indeed, approximately half of the diseased tubers were latently infected (presence of the pathogen without symptoms), while the remaining tubers showed internal and/or external symptoms. Symptomatic tubers showed different degrees of disease severity and were assigned to the five phytopathometric classes of the built disease severity scale (Table S1), from asymptomatic/healthy tubers (class 0) to very high level of severity (class 5). In the fifth class, the degradation of potato tissues was attributable not only to Rs or Cms but also to other secondary micro-organisms contaminating the tuber after the primary infection.

The two Cms inoculation methods (namely, at the stem and seed) had a comparable effectiveness: 44% of tubers obtained from plants inoculated at the stem and 42% of those from plants inoculated at the seed stage were symptomatic. The symptomatic tubers produced by stem inoculation had a lower disease severity than those obtained with seed inoculation: indeed, ca. 40% and 50% symptomatic potatoes produced with the two inoculation methods, respectively, belonged to class 3–5. The inoculation at the seed stage with Rs was not applied because it would be more destructive, especially at the Italian latitude; to prevent the loss of daughter tubers, Rs was inoculated only at the stem. For this reason, the level of ring rot symptoms was higher compared to that of brown rot.

3.2. E-nose analysis

3.2.1. Active sampling of volatile compounds at laboratory scale

The enrichment unit, coupled with the commercial e-nose, can contain different adsorbent materials whose affinity for potato volatile compounds had to be carefully evaluated. For this reason, different adsorbent materials were tested to find out the best type of fiber able to discriminate healthy from diseased tubers. Tenax® TA, Tenax® GR, Carbotrap, and Carboxen 569 were screened for their adsorption capacity towards volatiles emitted from potatoes. These four materials are commercially available and usually employed in different fields. Tenax® TA is a porous polymer resin used for the trapping of volatiles and semi-volatiles.; Tenax® GR is composed by 70% Tenax® TA and 30% graphite and is especially useful for the analysis of volatile organic compounds in water. Carbotrap is a graphitized carbon black used for air monitoring applications. Carboxen 569 is a carbon molecular sieve adsorbent resin able to adsorb small organic molecules. All these resins have a low affinity for water. In terms of discrimination power percentage (severability percentage among different groups) between healthy and diseased tubers, resins were efficient in the order of Tenax > Carboxen 569 ≈ Carbotrap⩾Tenax GR for Rs infected tuber discrimination, and Tenax > Carboxen 569 > Carbotrap ⩾ Tenax GR for Cms (data not shown). On the basis of the screening results, Tenax TA resulted the best adsorbent material to trap volatiles emitted from healthy and diseased potatoes. Although Carboxen has been used by Blasioli et al. [19] to evaluate the volatile compound profile emitted by potato tubers infected with Rs or Cms by the GC–MS technique (in this work volatile compounds have been collected by solid phase microextraction equipped with Carboxen 569 fiber), this fiber was not suitable for e-nose analysis.

PCA plots of sensorial analysis data collected from healthy and Rs/Cms infected samples placed in 0.5 L jars (Fig. 2(a)) using Carboxen 569 and Tenax TA are reported in the Supporting information, (Fig. S1). Score plots were built using the first two principal components which explained 98.7% total data variance collected from healthy and Rs infected potatoes (Fig. S1(a)) and 99.9% from those Cms infected (Fig. S1(b)) using Carboxen 569 as an adsorbent material. 92.5% and 88.7% total data variance from healthy and diseased samples (Figs. S1(c) and (d) for Rs and Cms, respectively) were explained using Tenax TA. As it can be observed, Tenax TA was able to cluster the healthy samples in the plane whereas samples analyzed with Carboxen 569 resulted in a wider distribution. Therefore, the results highlighted Tenax TA as the resin more suitable to proceed with the work.

The Tenax TA behavior was confirmed by the score plot (Fig. 3(a)) built pooling the data reported in Figs. S1(c) and (d): the value of the first principal component was similar to that of the first principal component of the score plots built with data related to samples infected by each single pathogen, thus indicating the highest contribution of these data to the variance analysis since the healthy samples—though belonging to different cultivars—clustered in the same region of the plot. The loading plot in Fig. 3(b) showed the relationship between MOSn variables and their influence on the data analysis system through data vectors (lines focused in the origin). The MOSn variables were dominant in both principal components: namely, ten sensors contributed to the final results because their weight in the discrimination process was distributed in overall plane described by two principal components. The angles between two vectors were lower than 90°, indicating a high correlation between the variables.

The labels reported in the score plot in Fig. 3(a) described the percentage of disease incidence (percentage of symptomatic tubers per sample) and the phytopathometric class (only medium or high symptom level was indicated) of samples belonged to: it was evident that points outside the cluster of healthy samples contained at least one tuber with a disease severity ≥ 3. This value represents the threshold limit of our e-nose for the discrimination between healthy and infected potatoes: namely, our technique was not able to detect latent infections, very low and low level of internal symptoms. The e-nose discrimination power towards infected tubers was in agreement with GC–MS data reported in Blasioli et al. [19], where potato samples which gave differences in volatile compound profiles, in comparison with healthy samples, contained tubers with medium or high symptom level belonging to the class ≥ 3. As mentioned in [19], an increase of volatile concentration along with the emission of the disease markers was observed in tubers with higher disease severity.

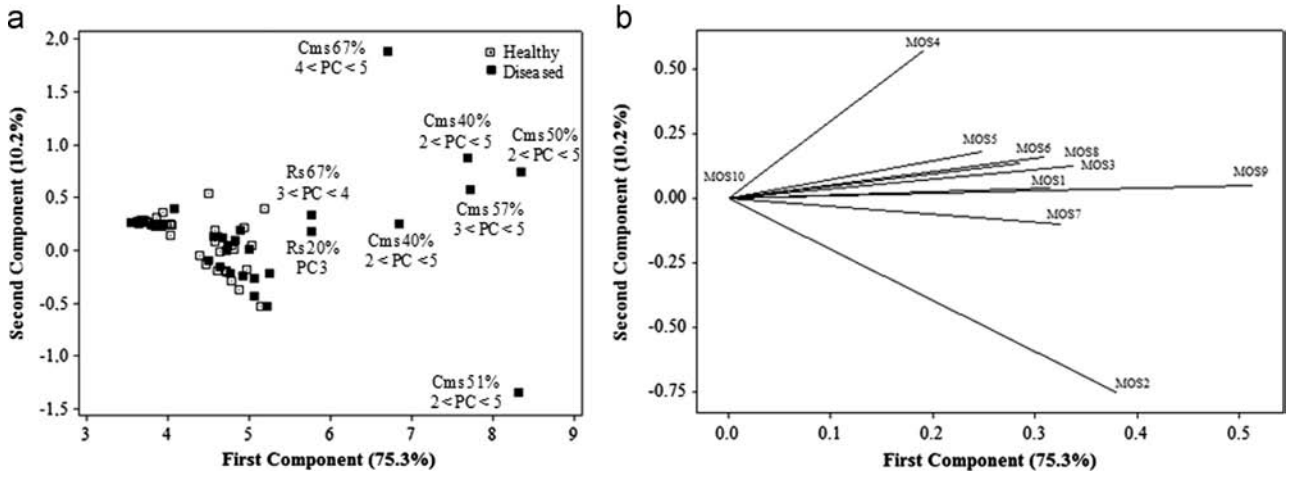

Fig. 3. (a) Score plot in the plane defined by the first two principal components of volatile compounds from potato samples placed in jars, analyzed by e-nose coupled with enrichment unit (active sampling at laboratory scale) using Tenax TA as an adsorbent material. For diseased samples discriminated from healthy ones, percentage of disease incidence and the higher phytopathometric class (PC), which the tubers composing the sample belongs to, are reported; (b) Loading plot of 10 MOS variables in the plane defined by the first two principal components.

Table 1
Classification of potato tubers samples kept in jars at room temperature using LDA with application of "leave-one-out" cross-validation method. Volatile compounds were collected by active sampling (Tenax TA was used as adsorbent material in the enrichment unit).

Put into group	True group	
	Healthy	*Diseased*
Diseased	14	21
Healthy	18	15
Total *N*	32	36
N correct	18	21
Recognition percentage (%)	56.3	58.3

Total number of samples = 68.
Total Recognition Percentage with cross-validation = 57.4%.

LDA was used to classify potato samples into "healthy" and "diseased" groups (Table 1). Classification method with "leave-one-out" cross-validation was able to correctly assign 57.4% samples to the related group: namely, little more than half of the 68 analyzed samples were correctly recognized from e-nose on the basis of the emitted volatile compounds. Due to the low value of the total recognition percentage, the active sampling was not applied to real conditions.

3.2.2. *Passive sampling of volatile compounds at laboratory scale*

The efficiency of Radiello™ to discriminate diseased potatoes from the healthy ones was preliminary evaluated on samples placed in 1 L jars (Fig. 2(b)). Radiello™ cartridge was exposed to a headspace of potato samples for 7 d at room temperature, to simulate the transit of potatoes in ships.

Fig. 4(a) reports the PCA score plot of data collected after sensorial analysis of potato samples sealed in 1 L jars. The first principal component explained ca. 91% total variance of the data. Healthy samples were clustered on the left in the score plot whereas diseased samples were localized in two different score plot regions: 5 infected samples fell in the healthy cluster, 13 were highly dispersed on the right region of the score plot. As already observed using active sampling for the discrimination between healthy and infected potatoes, the disease severity was the parameter which influenced the data distribution: the points outside the cluster of healthy samples contained at least one tuber with a disease severity ≥ 3. Accordingly to what has been already reported in Blasioli et al. [19], tubers with medium or high disease symptom level were able to produce volatile compounds which could be used by e-nose to recognize infected potatoes. Looking at the loading plot, it was evident that only sensors MOS2 and MOS8 contributed to the discrimination between healthy and diseased samples. MOS2 and MOS8 sensors, as reported in Section 2 , respond to a broad range of aliphatic and alcohols substances: among volatiles and markers identified by GC–MS [18,19], alcohols and aliphatic compounds represented the most relevant detected molecules. A new processing of data—after exclusion of all sensor responses except for those from MOS2 and MOS8—did not modify the distribution of points in the score plot (data not shown), but a slight improvement was obtained by applying the LDA classification model (Table 2). Using LDA, in fact, samples were correctly assigned (with "leave-one-out" cross-validation) to "healthy" and "diseased" groups with a total recognition percentage of 81.3% after exclusion of negligible sensor response values: the total recognition percentage calculated taking in account all sensor responses was 78.1%. These findings are in agreement with those which have been obtained by Stinson et al. [18], indeed, applying neural networks, they discriminated Rs diseased samples and their negative controls with 100% of accuracy and with 70% for Cms ones. PCA and LDA results (Fig. 4 and Table 2) highlighted the passive sampling was the more effective sampling method for the discrimination between healthy and diseased tubers, compared with active sampling (Fig. 3 and Table 1): passive sampling was therefore used for the experiments at intermediate and real scale.

3.2.3. *Passive sampling of volatile compounds at intermediate scale*

The study at intermediate scale was carried out on Cms infected tubers only, due to the lack of a sufficient number of Rs infected tubers with a disease severity higher than 3, according to the phytopathometric scale (Table S1).

Firstly, the effect of storage temperature and confinement of volatile compounds emitted from healthy tubers was assayed. In Fig. 5(a), score plot of data collected by sensorial analysis of volatile compounds emitted from healthy potatoes placed in net and PE bags both at 4 °C and room temperature are reported. The 98.8% total variance was explained by the first two principal components, 94.1% of which was by the first principal component. Nevertheless, the PCA plot allowed to visually discriminate

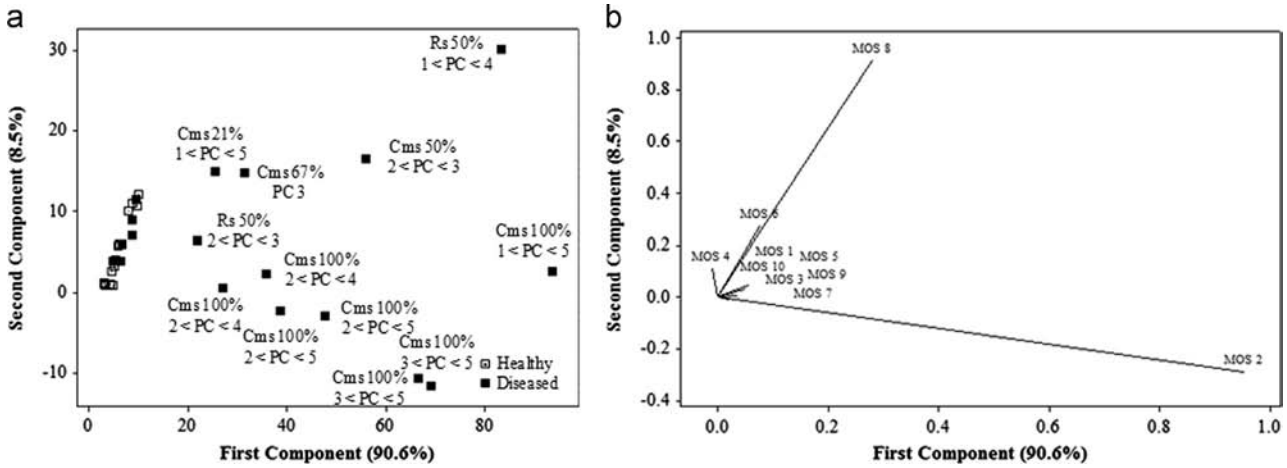

Fig. 4. (a) Score plot in the plane defined by the first two principal components of volatile compounds from potato samples analyzed by e-nose using a Radiello™ cartridge as a volatile compound trap (passive sampling at laboratory scale) placed inside the jars. For diseased samples discriminated from healthy ones, percentage of disease incidence and the higher phytopathometric class (PC), which the tubers composing the sample belongs to, are reported; and (b) loading plot of 10 MOS variables in the plane defined by the first two principal components.

Table 2
Classification of potato tuber samples placed in jars at room temperature, in net bags at 4 °C, and in 1.25 t bags at 4 °C by LDA with application of "leave-one-out" cross-validation method. Volatile compounds were collected by passive sampling (Radiello™ cartridge was used as an adsorbent material). Total recognition percentages before sensor selection are reported between brackets.

Put into group	True group					
	Jar		Net bag		1.25 t bag[a]	
	Healthy	*Diseased*	*Healthy*	*Diseased*	*Healthy*	*Diseased*
Diseased	0	12	3	8	2	8
Healthy	14	6	4	0	10	0
Total *N*	14	18	7	8	12	8
N correct	14	12	4	8	10	8
Recognition percentage (%)	100	66.7	57.0	100	83.3	100
Total number of samples	32		15		20	
Total recognition with cross-validation (%)	81.3 (78.1)		80.0 (n.a.)		90.0 (80.0)	

n. a.: not applicable.

[a] Data from potato samples collected in 1.25 t bags stored at 4 °C are compared to those from samples kept in net bags at 4 °C.

samples in net and PE bags located in different region of the score plot (left and right area, respectively). On the contrary, the storage temperature had no significant effect on discrimination between open and sealed tuber samples. These findings allowed continuing investigation under intermediate conditions consisting in potato samples into net bags (Fig. 2(d)).

Volatile compounds from experimentally Cms infected potato samples were collected at 4 °C to validate the e-nose method in terms of: (i) evaluation of e-nose efficacy to discriminate between healthy and diseased tubers placed in net bags at the temperature simulating refrigerated storage conditions and (ii) determination of e-nose sensitivity threshold using diluted infected samples (net bags containing 50%, 75% and 100% infected samples; on the average 24%, 34% and 47% of which, respectively, presented the symptoms of ring rot). Score plot of related data is reported in Fig. 5(b), where the first principal component explained 92.7% total variance indicating that the sensors were highly correlated among them. Nevertheless, healthy and diseased samples were localized in two different regions of the score plot. The data distribution of Cms diseased tubers agreed with the disease incidence value: the sample containing the highest percentage of symptomatic tubers was placed on the borderline of diseased cluster. On the contrary, and differently from what it was observed in the laboratory scale experiments (Figs. 3(a) and 4(a)), no effect of disease severity in score plot representative of intermediate conditions was observed as reported in Fig. 5(b). The discrepancy could be explained by lower temperature (4 °C) and volatile compounds unconfinement (net bag) conditions adopted in the intermediate scale experiments when compared to the laboratory scale experiment conducted at room temperature in sealed jars.

The loading plot (in Supporting information, Fig. S2) of MOSn variables highlighted that only MOS2, MOS6, and MOS8 contributed to discriminate healthy from diseased samples. As reported in the technical data of e-nose, MOS2, MOS6, and MOS8 are generic sensors, sensible to alcohols and aliphatic substances. The higher contribution of these sensors to the discrimination process was in agreement with what was already found in the study of Blasioli et al. [19], where the increase of concentration of a mixture of volatile aliphatic compounds and alcohols confirmed the presence of Rs and Cms pathogens in potato tubers.

After exclusion of sensor responses that were negligible from the data matrix, the new data processing by PCA did not decrease the value of first principal component but significantly improved the data classification. Indeed, due to the high correlation among sensors, the LDA was not applicable before sensor exclusion; on the contrary, after the data matrix simplification, the LDA correctly assigned all diseased samples (0% error rate) to the corresponding group whereas about 70% of samples were correctly assigned to healthy group. The total recognition percentage with cross validation was 80.0% (Table 2).

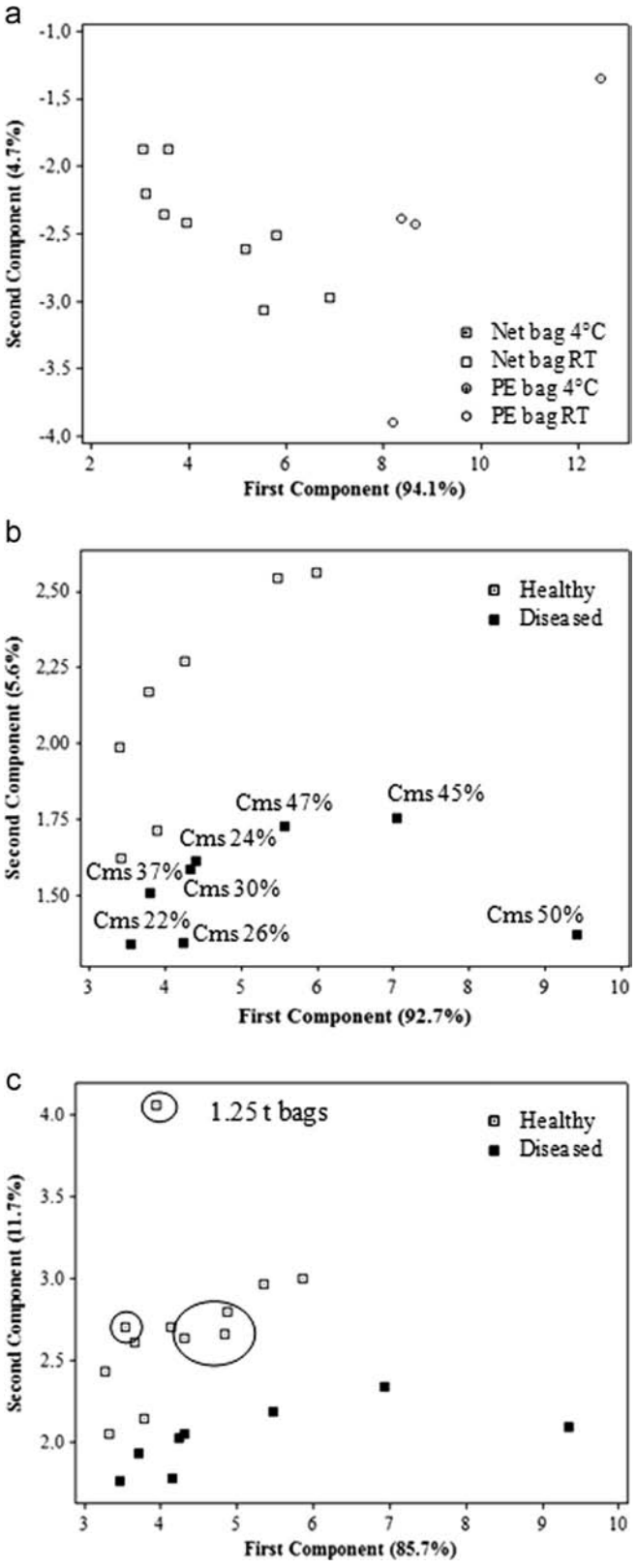

Fig. 5. Score plots in the plane defined by the first two principal components of volatile compounds, analyzed by e-nose using a Radiello™ cartridge, emitted from (a) healthy potato samples placed in net bags and net bags sealed in PE bags stored both at 4 °C and room temperature (passive sampling at intermediate scale), (b) healthy and diseased potatoes (percentage of sample disease incidence is reported) placed in net bags at 4 °C (passive sampling at intermediate scale) and (c) healthy potato samples placed in 1.25 t PP bags stored at 4 °C (data were compared to those from samples in net bags at 4 °C) (passive sampling at real scale).

3.2.4. Passive sampling of volatile compounds at real scale

Usually, imported tubers are stored within the ship hold in units of 25 kg sacks, 1.25 t PP bags or 20 t containers. When, ships from outside of Europe (e.g. Israel and Egypt) arrive in Europe, potatoes are automatically inspected by Phytosanitary Services for quarantine pathogens at the point of entry. Potatoes used for this study, contained in 1.25 t PP bags, had been unloaded from a ship, and stored in a refrigerated room. Before e-nose analysis, potatoes have been analyzed by the local Phytosanitary Service and claimed healthy.

In Fig. 5(c), the score plot of data collected by sensorial analysis of potatoes stored in 1.25 t bags at 4 °C is reported. These data were compared with those from potatoes stored in net bags at 4 °C. Healthy and diseased samples were well discriminated in the plane defined by the two first principal components which explained 97.4% total variance. As expected, the data related to potato samples collected in 1.25 t bags were included in "healthy" cluster confirming the reliability of method in real scale conditions. Loading plot of MOSn variables (in Supporting information, Fig. S3) highlighted that MOS2, MOS4, MOS6, and MOS8 contributed to the discrimination among the samples more than the other sensors. As already observed at intermediate scale, the sensors, responding to volatiles emitted from potatoes, were MOS2, MOS6 and MOS8 (selective for aliphatic compounds and alcohols) and in addition, in real scale conditions, MOS4 specific for the hydrogen. The new data processing, excluding negligible sensors, did not modify the PCA score plot, but positively affected the LDA classification: healthy samples were correctly assigned to their group for 83.3% whereas the diseased ones for 100% (Table 2). The total recognition percentage was raised to 90.0% instead of 80% before sensor exclusion, confirming the goodness of method also under real scale conditions.

In conclusion, the suggested method based on the use of e-nose to detect potato brown and ring rot, showed good potentials for practical use: e-nose was able to recognize healthy potato samples from diseased ones under real scale conditions (potatoes stored in 1.25 t bags in refrigerated chamber) with a high prediction capacity.

However, even if the method seems promising, it still needs to be improved (i.e., the number of real samples analyzed is not enough to validate the method) for being ready to be used by phytosanitary inspectors. Indeed, it is not able to detect latent tuber infections as well as very low severity disease. In addition, the high complexity of volatile compound profiles from both healthy and diseased potato samples [16,18,19,29], and the small relative abundance of the most specific markers [19] in infected tubers with medium severity (class 3), contributed to the variability of sample analysis.

At the moment, since the analysis is non-destructive and can be repeated several times without supplementary costs, the method, if improved, might be considered as a complementary tool to speed up the subsequent classical diagnostic analyses suggested in the EU directives.

Acknowledgments

We gratefully thank U. Mazzucchi and C. E. Gessa for their precious work and suggestions which helped us in setting up the experiments. We also thank A. Nastri and S. Vecchi for the excellent management of the fields used for the tuber production, and S. Brigati and P. Bertolini for the use of the refrigerated cells. We are grateful to the Phytosanitary Service of Emilia Romagna Region that provided special permissions to export infected tubers to project partners: in particular we thank G. Tallevi, who allowed

us to collect volatile compounds from PP bags stored in the port of Ravenna. Finally, we acknowledge A. Bertaccini for her constructive comments related to the manuscript.

This work was financed as Q-Detect project which was a part of the EU's 7th Framework Programme (FP7-KBBE-2009-3).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.04.057.

References

[1] K. Persaud, G. Dodd, Nature 299 (1982) 352–355.
[2] M. Peris, L. Escuder-Gilabert, Anal. Chim. Acta. 638 (2009) 1–15.
[3] A. Branca, P. Simonian, M. Ferrante, E. Novas, R.M. Negric, Sens. Actuators B 92 (2003) 222–227.
[4] L. Zhu, R.A. Seburg, E. Tsai, S. Puech, J.C. Mifsud, J. Pharm. Biomed. Anal. 34 (2004) 453–461.
[5] J. Yinon, Anal. Chem. 75 (5) (2003) 98 A–105 A.
[6] D.A. Wilson, Proced. Technol. 1 (2012) 453–463.
[7] J.W. Gardner, M. Craven, C. Dow, E.L. Hines, Meas. Sci. Technol. 9 (1998) 120–127.
[8] A.K. Pavlou, N. Magan, D. Sharp, J. Brown, H. Barr, A.P.F. Turner, Biosens. Bioelectron. 15 (2000) 333–342.
[9] S. Blasioli, E. Biondi, I. Braschi, U. Mazzucchi, C. Bazzi, C.E. Gessa, Anal. Chim. Acta 672 (2010) 20–24.
[10] F. Spinelli, M. Noferini, J.L. Vanneste, G. Costa, Bull. OEPP/EPPO Bull. 40 (2010) 59–67.
[11] M.T. Momol, M.O. Balaban, F. Korel, A. Odabasi, E.A. Momol, G. Folkes, J.B. Jones, Plant Health Prog. (2004), http://dx.doi.org/10.1094/PHP-2004-0405-01-HN.
[12] Anonymous, Bull. OEPP/EPPO Bull. 34 (2004) 173–178.
[13] Anonymous, Bull. OEPP/EPPO Bull. 36 (2006) 99–109.
[14] J.L. Varns, M.T. Glynn, Am. Potato J. 56 (1979) 185–197.
[15] D.R. Waterer, M.K. Pritchard, Can. J. Plant Pathol. 7 (1985) 47–51.
[16] L. Lui, A. Vikram, H. Hamzehzarghani, A.C. Kushalappa, Potato Res. 48 (2005) 85–96.
[17] J. Laothawornkitkul, R.M.C. Jansen, H.M. Smid, H.J. Bouwmeester, J. Muller, A.H. vanBruggen, Crop Prot. 29 (2010) 872–878.
[18] J.A. Stinson, K.C. Persaud, G. Bryning, Sens. Actuators B 116 (2006) 100–106.
[19] S. Blasioli, E. Biondi, D. Samudrala, F. Spinelli, A. Cellini, A. Bertaccini, S.M. Cristescu, I. Braschi, J. Agric. Food Chem. 62 (2014) 337–347.
[20] B.P.J. de Lacy Costello, R.J. Ewen, H.E. Gunson, N.M. Ratcliffe, P.T.N. Spencer-Phillips, Meas. Sci. Technol. 11 (2000) 1685–1691.
[21] A. Kelman, Phytopathology 44 (1954) 693–695.
[22] E.E. Wilson, F.M. Zeitoun, D.L. Fredrickson, Phytopathology 57 (1967) 618–621.
[23] K.R. Beebe, R.J. Pell, M.B. Seasholtz, Chemometrics, A Practical Guide, John Wiley and Sons, New York, 1998.
[24] M. Meloun, J. Militky, M. Forina, Chemometrics for Analytical Chemistry, Ellis Horwood, New York, 1992.
[25] S.J. Dixon, R.G. Brereton, Chemometr. Intell. Lab. Syst. 95 (2009) 1–17.
[26] F. Bianchi, M. Careri, A. Mangia, M. Mattarozzi, M. Musci, I. Concina, M. Falasconi, E. Gobbi, M. Pardo, G. Sberveglieri, Talanta 77 (2009) 962–970.
[27] S.E. Seal, L.A. Jackson, J.P.W. Young, M.J. Daniels, J. Gen. Microbiol. 139 (1993) 1587–1594.
[28] K.-H. Pastrik, Eur. J. Plant Pathol. 106 (2000) 155–165.
[29] B.P.J. de Lacy Costello, P. Evans, R.J. Ewen, H.E. Gunson, N.M. Ratcliffe, P.T.N. Spencer-Phillips, Plant Pathol. 48 (1999) 345–351.