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ORIGINAL PAPER

Preliminary studies evaluating the condition of *Quercus robur* acorns infected with *Ciboria batschiana* through electronic nose measurements

Piotr Borowik[(1)], Tomasz Oszako[(2)]✉, Tadeusz Malewski[(3)], Miłosz Tkaczyk[(2)], Sławomir Ślusarski[(2)], Rafał Tarakowski[(1)]

[(1)] Faculty of Physics, Warsaw University of Technology, Koszykowa 75, 00-662, Warszawa, Poland

[(2)] Forest Protection Department, Forest Research Institute, Braci Leśnej 3, 05-090, Sękocin Stary, Poland

[(3)] Museum and Institute of Zoology, Polish Academy of Science, Wilcza 64, 00-679, Warszawa, Poland

ABSTRACT

The possibility of using gas sensors in the portable electronic nose to detect the proportion of acorns colonized by *Ciboria batschiana* was examined. The present research has demonstrated that for such a task a portable electronic nose can be used as fungus-infected acorn tissue emits different volatile organic compounds from healthy tissue. However, the gas sensors used in commercially available the PEN3 electronic nose are not selective, therefore the measurements did not provide accurate information about the chemical composition of the odor. It was found that the electronic nose sensors responded to the presence of the fungus to different degrees. There was a difference in the response of the sensors to the presence of different compositions of the measured volatile compounds at different percentages for acorns colonized by *C. batschiana*. The correlation coefficients between acorn infection level and the response of the sensors were found to be statistically significant. The R^2 coefficient of the linear regression model reached a value of 0.19 in the cases where the slope coefficients of three predictors were statistically significant.

KEY WORDS

English oak, fungi, fungus molecular identification, seed pathogen, volatile organic compounds

Introduction

The concept of the electronic nose (Persaud and Dodd, 1982; Gardner and Bartlett, 1994; Nagle *et al*., 1998) involves the application of a set of nonspecific gas sensors, usually with an overlapping range of gas detection, with machine learning pattern recognition algorithms. Several applications for such a non-invasive and rapid diagnostic tool have been proposed. Multiple research papers address applications of electronic noses, focusing on forestry and agriculture (Wilson, 2013; Cellini *et al*., 2017; Ray *et al*., 2017; Cui *et al*., 2018; Wilson, 2018; Cheng *et al*., 2021). The identification of fungal species by electronic noses was reported by Mota *et al*. (2021). The odors of various fungus-infected seeds were investigated using electronic noses, *e.g*., in cereal grain samples

✉e-mail: T.Oszako@ibles.waw.pl

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(Paolesse *et al*., 2006; Presicce *et al*., 2006), rice (Gu *et al*., 2019; Men *et al*., 2022), rapeseed (Gancarz *et al*., 2017a, b) and wheat (Lin *et al*., 2022). Recently, the detection of fungal infection of silver fir seeds was investigated using the PEN3 electronic nose (Borowik *et al*., 2022a).

However, the electronic nose (E-nose) cannot be the only tool for the early detection of all plant diseases, even with its high level of efficacy, as other highly reliable molecular biology methods should always be used in tandem. Rather, the E-nose should allow for field pre-screening and if it indicates the presence of pathogens then it should be further confirmed by DNA analyses (PCR, qPCR or NGS) for example.

In recent years, many technological advances have been made in agriculture, including E-noses, for which new applications have been found. Fruits and vegetables are increasingly sold, sorted and labeled, making it easier for customers to identify the quality of the product. Therefore, a need arose to develop sorting with easy-to-use and cost-effective tools. According to the latest state of the art food technology, E-noses can be used in food quality control systems, for example, for *Capsicum annuum* L. peppers (Rasekh *et al*., 2022). Using this method, it is possible to reliably separate sweet and hot peppers based on olfactory parameters and to develop sorting machines based on olfactory characteristics (Rasekh *et al*., 2022). An E-nose applying nine MOS sensors was also used to test different potato varieties for which the carbohydrate content, sugar content and hardness of the potatoes were measured (Khorramifar *et al*., 2022). Overall, the E-nose can be used as a rapid and non-destructive method for detecting different potato varieties. Researchers in the food industry find this method extremely useful for selecting the desired product and samples (Khorramifar *et al*., 2022). Volatile compounds in coffee green beans varieties could also be tested with E-noses. Chlorogenic acids, trigonelline, caffeine, total lipids, total protein and color parameters were measured in *Coffea robusta* L. Linden in China. Seventy-nine volatile compounds were confirmed by Headspace Solid-Phase Microextraction coupled to Gas Chromatography-Mass Spectrometry (HS-SPME/GC-MS) and showed significant differences among all tested coffee varieties (Dong *et al*., 2015).

Agriculture and forestry is an important source of economic development for many communities. However, diseases affecting crops, including forest plantations, significantly reduce primary production. It is becoming increasingly difficult for plant pathologists to detect disease symptoms that are masked by pesticide use (Chang *et al*., 2014). Infected asymptomatic plants carry a variety of plant pathogens such as bacteria, fungi and viruses from nursery to crop, country to country or continent to continent in international trade. Therefore, a variety of new approaches should be used to solve this problem in order to, for example, detect infected seeds or seedlings more quickly.

Polish nurseries produce about 800 million seedlings annually, most of which are pine seedlings (58%) while birch and oak represent 10% and 7%, respectively. Due to the irregular fruiting periods of forest trees, continuous long-term storage of propagation material in nurseries is needed. This is very important, particularly for oaks, which produce seeds every four to eight years (Schermer *et al*., 2019). In most cases, acorns are collected in barrels in the years of harvesting, where they are stored for the following years. Unfortunately, infected acorns could be a source of infection for neighboring ones. Detection of acorn pathogens can be done through visual inspection which can be automated using computer visual methods (Przybyło and Jabłoński, 2019). However, such an approach requires inspection of each acorn and would be time intensive. A more promising method could be to monitor the health status of stored acorns by examining the odor emissions. The idea being that pathogenic fungi as well as healthy and infected plant tissues should have different odors. Undoubtedly, the human nose is not sufficient to detect decomposition odors in the early stages, therefore, much more sensitive sensors are needed. Consequently, artificial

E-noses need to be built and appropriate applications, such as machine learning in the context of artificial intelligence, need to be used to analyze the results. Ideally, it would be possible to verify not only whether infection of acorns has occurred but also to what extent. Such information is critical for forest nurseries when considering a particular seed lot for spring sowing in nurseries. If the sowing fails, unnecessary costs are incurred, moreover, fungal diseases are introduced into the soil causing further losses even if new, healthy seeds are sown. To date, there are no other methods for evaluating acorns stored in barrels, except through visual inspection. Therefore, testing the odor of spoiled seeds is a novel innovation. In the tests conducted for this research we used combinations with different amounts of rotten seeds.

Acorns are most frequently and severely damaged by the fungus *Ciboria batschiana* (Zopf) N.F. Buchw. with thermotherapy most commonly applied to treat the infection (Knudsen *et al.*, 2004). A 2.5-hour bath in water at 41°C was developed to rid the surface of acorns of pathogens, particularly of the fungus *C. batschiana*, which mummifies these seeds, *i.e.*, turns them into black sunken shells (Knudsen *et al.*, 2004). After the seeds are bathed and dried (but not 'desiccated', the humidity should not fall below 40%) another treatment follows called fungicide dressing. Acorns are surrounded by a mixture of fungicides and thus protected from fungal diseases (Knudsen *et al.*, 2004). After these treatments, the acorns are placed in containers – 110 l barrels with drainage tubes and again placed in cold storage (–3°C). However, the barrels may contain secondary infections caused, for example, by molds or oomycetes (Knudsen *et al.*, 2004). The risk of infection increases with the length of storage time as the seed is weakened by a sharp decline in moisture content. Pesticide use can also affect seed viability. Conversely, spores or spore-forming organs of pathogens may survive protective treatment or enter stored seeds at a later stage of storage.

The main objective of the research conducted was to test a new application of an E-nose for forest seeds especially during storage. In our studies we focused on distinguishing between healthy and damaged oak seed lots based on odor differences. We made a series of measurements with the E-nose to determine the correlation between the infection status of the samples and the sensory response patterns recorded. To the best of our knowledge, this was the first attempt to assess the infection status of acorns in seed lots using an E-nose. In these preliminary studies we also used molecular methods to identify the fungus *C. batschiana* on the collected acorns that resulted in cotyledon rot and embryo damage which was an additional objective. To achieve this goal, DNA analysis was performed using specific primers and a qPCR technique to confirm the causal agent of the disease symptoms.

Materials and methods

Samples of acorns. Acorns were collected in May 2022 in Chojnów Forest District (52°05'54.6" N, 20°52'10.7" E). We collected about 2 kg of acorns that had fallen spontaneously to the ground from English oak *Quercus robur* L. aged 75 years. The acorns were transported to the laboratory at the Forest Research Institute where they were carefully washed off soil and organic residues. Then the washed samples were sorted into two categories: (i) healthy acorns (without visible symptoms of fungal infection) and (ii) acorns with visible symptoms of infection by *C. batschiana*. There were a total of 42 healthy ungerminated acorns and 30 acorns infected with *C. batschiana* (Fig. 1). The number of acorns used for measurements was much smaller than the total number of collected acorns as we decided to exclude acorns which developed infestation symptoms different than those of *C. batschiana* from the analysis as well as those that had already germinated or were desiccated.

Fig. 1.
Samples of acorns. Healthy acorns on the left. Colonized by *C. batschiana* on the right

Ciboria batschiana infection

Visual identification by symptoms. All collected acorns were carefully sorted for the occurrence of symptoms typical of *C. batschiana* infection. *C. batschiana* is the main cause of black rot of *Q. robur* acorns and the cause of serious economic losses during storage (Kowalski, 1999). Infection is thought to occur on fruits after falling to the ground but before harvesting through ascospores issued from apothecia formed during the winter on infected fruits (Delatour and Morelet, 1979). The first symptom of the disease is the appearance of yellow-orange spots with a brown border which merge over time on the surface of the cotyledons of infected acorns. The cotyledons then darken, shrink and wrinkle eventually becoming completely black and dry (Kowalski, 1999). Based on the symptoms described above, a group of *C. batschiana*-infected acorns was selected and then several seeds were genetically analyzed to confirm the presence of the fungus in the infected tissues.

Molecular identification of pathogens. To confirm the presence of *C. batschiana* infection on acorns, ten healthy and ten infected acorns were randomly selected for qPCR analysis. Genomic DNA was extracted from acorns using a NucleoSpin Plant II kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions.

For each sample, DNA was eluted in 50 μL of H_2O and the extracted DNA was stored at –20°C.

Primers F218 5'-TTGTAGAACTCCTAGTCGTA-3' and R347 5'-ACCGAGATTCTC-GAATTTGTCTTTA-3' and probe T2696 FAM-ATCTCTAATTGTTGTCGAACAGATGGT--HBQ1 against the *Hsp60* gene were used for identification of *C. batschiana* (Lamarche *et al.*, 2015).

PCR primers were synthesized by Sigma-Aldrich (Milwaukee, WI, USA). Real-time PCR was performed in a total volume of 20 μl and consisted of 10 μl LuminoCt qPCR Ready Mix (Sigma-Aldrich, St. Louis, MO, USA), 2 μl forward and 0.5 μM reverse primers, 1 μl, 0.2 μM probe, 2 μL DNA and 7 μl water. PCR amplification was performed using the 7500 Real-Time PCR system (ThermoFisher Scientific, Waltham, MA, USA). Thermocycling conditions consisted of initial denaturation at 95°C for three minutes followed by 40 cycles at 95°C for 30 seconds and 60°C for 90 seconds. Fluorescence was measured at the end of the extension step for each cycle. Ct values below 35 were considered a positive detection reaction. Negative controls (no DNA) did not produce amplification products (Lamarche *et al.*, 2015) for 40 cycles. The possible presence of inhibitors in the analyzed samples was verified by amplification of ITS in *Q. robur* using the forward primer 5'-ACCTGCACAGCGGAACG-3' and the reverse primer 5'-ATTCATTAGA-CGCCGACCG-3' as described in a previous paper (Oszako *et al.*, 2021). All reactions were repeated twice.

Ten healthy acorns and ten acorns with visible symptoms of *C. batschiana* infection were used for qPCR analysis. The qPCR analysis performed with the *Hsp60* primers specific for *C. batschiana*

yielded positive results (Ct 28.17-32.54) for all analyzed DNA samples isolated from acorns with visible symptoms of infection. None of the DNA samples isolated from healthy acorns yielded a positive result for *C. batschiana* (Ct>40). To rule out the possibility that DNA extracted from acorns contained inhibitors, qPCR analysis was performed using primers specific for *Q. robur*. The qPCR yielded positive results for all samples analyzed (Ct 16.71-20.06). This indicates that the extracted DNA was free of inhibitors and a positive signal indicated infection of acorns with *C. batschiana* (Table 1). In this way, the genetic analyses proved that the visual selection of acorns was performed correctly, *i.e.*, they were divided accurately into healthy and diseased (infected with *C. batschiana*) acorns.

Measurements by the electronic nose

PEN3 electronic nose device. The PEN3 E-nose used in our experiment has been successfully used in several research projects in forestry and agriculture on fungal infection. Zhou and Wang (2011) used the E-nose to identify rice infection by *Nilaparvata lugens* (Stĺl). Baietto *et al.* (2013, 2015) detected root rot in shade tree species. Biondi *et al.* (2014) reported the detection of potato brown rot and ring rot. Liu *et al.* (2018) reported studies on the fungal contamination in peaches. Guo *et al.* (2020) reported classification for *Penicillium expansum* Link spoilage in apples. Our team reported results of pathogen detection on ash saplings *Hymenoscyphus fraxineus* (Borowik *et al.*, 2021d) and fir seeds (Borowik *et al.*, 2022a).

The E-nose system PEN3 used in our work was commercially available and widely used in the laboratories (Airsense Analytics GmbH, Schwerin, Germany) with Airsense WinMuster 1.6.2 software. It uses ten high-temperature metal oxide sensors as detection units. All sensors

Table 1.
qPCR analysis of acorn samples

	Ct value			
	C. batschiana		*Q. robur*	
Sample	repeat 1	repeat 2	repeat 1	repeat 2
Negative control	>40	>40	>40	>40
Healthy 1	>40	>40	18.35	17.81
Healthy 2	>40	>40	19.38	44732
Healthy 3	>40	>40	17.44	16.89
Healthy 4	>40	>40	18.39	19.45
Healthy 5	>40	>40	16.71	17.32
Healthy 6	>40	>40	17.02	17.88
Healthy 7	>40	>40	18.87	17.41
Healthy 8	>40	>40	16.89	17.62
Healthy 9	>40	>40	19.91	18.52
Healthy 10	>40	>40	18.12	19.04
Infected 1	29.43	29.28	17.82	16.92
Infected 2	30.18	29.87	20.01	19.57
Infected 3	28.69	29.41	18.76	19.41
Infected 4	31.52	30.96	19.16	18.33
Infected 5	32.08	31.62	16.98	17.72
Infected 6	31.72	32.54	19.36	18.63
Infected 7	28.17	28.98	17.75	17.01
Infected 8	30.32	29.48	18.33	19.37
Infected 9	31.48	31.44	18.69	17.81
Infected 10	32.31	31.72	17.11	18.56

have a very wide range of detectable gasses with the list of PEN3 E-nose sensors and their target gasses presented in Table 2.

The instrument also has efficient air pumps that transport ambient air and odor samples to the sensors. The ambient air is purified by an activated charcoal filter. The signal recorded by the sensors is the conductance of the sensors in the ambient air divided by the values in the reference gas (clean air).

Measurement procedure. The measurement procedure was carried out according to the work described in our previous publications (Borowik *et al.*, 2021c). The experiment was performed using the PEN3 E-nose which was turned on at least one hour before the start of measurement taking to warm up the sensors properly. To ensure that no particles from prior measurements remained in the sensor chamber, the PEN3 automatically purged the sensors with filtered air for 180 seconds before each new measurement. The instrument then measured the level of the sensors' reference point in the presence of clean air (G_0) and after which it recorded the sensors' response to the constant gas flow in the headspace of the measured sample during the next 120 seconds. The signals from the sensors recorded by the software were G/G_0. The conductivity of the sensors (G) during the measurement were divided by the conductivity of the ambient air G_0. In addition, all measurements were performed in a laminar flow booth (Telstar, Bio II Advance) at 21°C with the air supply turned on. This made it possible to maintain controlled temperature and humidity conditions throughout the experiment.

Acorn samples were stored in 200 mL jars at room temperature (Fig. 2). Four experimental variants, as listed in Table 3, and three replicates for each variant were chosen for the study.

Table 2.

Sensor array details in PEN3 electronic nose device as reported in the menu of options of the electronic nose software

Sensor	Main Gas Targets
W1C	Aromatic organic compounds
W5S	Very sensitive, broad range sensitivity, reacts to nitrogen oxides, very sensitive to negative signals
W3C	Ammonia, also used as sensor for aromatic compounds
W6S	Detects mainly hydrogen gas
W5C	Alkanes, aromatic compounds and nonpolar organic compounds
W1S	Sensitive to methane. A broad range of organic compounds detected
W1W	Detects inorganic sulfur compounds, *e.g.*, H_2S. Also sensitive to many terpenes and sulfur-containing organic compounds
W2S	Detects alcohol, partially sensitive to aromatic compounds, broad range
W2W	Aromatic compounds, inorganic sulfur and organic compounds
W3S	Reacts to high concentrations of methane (very selective) and aliphatic organic compounds

Fig. 2.

Prepared acorn samples during PEN3 electronic nose measurement

On each day of the experiment, after the measurements were completed, all acorns were removed from the jars, carefully wiped so that no fragments of infected tissue remained on the surface of the healthy seeds and then randomly placed back into the jars, maintaining the procedure described above. Samples prepared in this manner were left overnight to collect volatile odorants components (VOCs). The air above the seeds was collected by inserting a PEN3 E-nose tube into prepared holes in the lid of the jar. Between measurement collection times, the jars were closed and the holes were sealed with parafilm. Three jars of samples of each variant (as listed in Table 3) were prepared for measurements.

One to three series of measurements were performed daily for three weeks. In each series, 12 samples were measured (4 variants with 3 independent samples of each). Before the start of each series, a random number generator was used to determine the order of the measurements. When more than one series of measurements were made per day, the holes in the jar lids were taped with parafilm immediately after the measurement to allow the accumulation of VOCs from the seeds. A total of 20 measurement series were performed throughout the experiment.

Data analysis. In analyzing the data, we addressed the problem of evaluating the status of pathogenic infection in stored *Q. robur* acorns. We used a fixed number of acorns (*Ntotal*) with different proportions of acorns with visible infection symptoms (*Ninfected*) as measurement samples using the proportion *Ninfected*/*Ntotal* as an indicator of sample status.

To explain the analysis of the data collected by the PEN3 E-nose, we show in Figure 3 a diagram with an example of the sensors responses during measurement collection procedures. The data was collected over 120 seconds starting from the time the sensors were exposed to the measured odor. The response level of each sensor at the end of the observation period was extracted and used as a feature for further statistical data analysis. Averaging over the last 10 seconds was performed to reduce noise.

The collected data was analyzed and visualized using statistical methods. The Pearson correlation coefficient between each sensor response at the end of the observation period and the

Table 3.
Variants of numbers of infected and healthy acorns used in measured samples

Variant	Variant description
0/6	6 healthy acorns
1/6	1 infected and 5 healthy acorns (6 in total)
3/6	3 infected and 3 healthy acorns (6 in total)
6/6	6 infected acorns

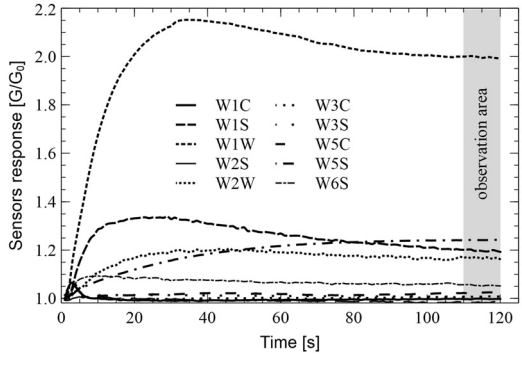

Fig. 3.
An example of the PEN3 electronic nose sensor response captured during one series of sample measurements. The colored region in the last phase of the observation time indicates the region from which modeling features are extracted as the average value of the sensor response. The sensor's symbols are listed in the figure legend. Description of the sensor's target gases are available in Table 2

percentage of infected acorns in the sample were calculated. It must be admitted here that the distribution of the collected data did not pass the statistical test for normal distribution. One of the reasons was that we could collect only a limited sample of data with discrete proportions of healthy and infected acorns in a sample along with the proportion being confined to the [0,1] range. We, however, decided to follow these preliminary analyses despite not fulfilling these conditions.

Linear regression models were calculated with the proportion of infected acorns in the sample as the dependent variable and sensor responses as independent variables (predictors). Different numbers of predictors used in the models were tested, and for a selected number, the model with the maximum R^2 value was chosen. The p-value of each regression coefficient was calculated to estimate whether the predictors were non-zero at $p<0.05$.

The Mallows statistic C_p (Mallows, 1973; Gilmour, 1996) was also calculated to address the problem of model selection and to assess the fit of the estimated regression models. The statistic is defined as $C_p=SSE_p/s^2-N+2(p+1)$, where N is the total number of available predictors, p is the number of predictors in the candidate model, SSE is the sum-of-squares error for a model with p parameters and s^2 is the mean squares error for the full model. This statistical analysis was proposed by Mallow as a criterion for selecting among many alternative subset regressions and is often used as a stopping rule of stepwise regression when the predictors are added to a series of models and their performance is compared. The model with the lowest C_p value, approximately equal to $p+1$, is considered the most 'adequate' model.

Data processing and statistical analysis of the data was performed with SAS 9.4 software (SAS Institute, Cary, NC, USA) using the SAS Enterprise Guide user interface and SAS/Stat procedures (Cody, 2011). PROC CORR was used to calculate Pearson correlation coefficients with corresponding p-values. PROC REG was used to estimate linear regression coefficients with the corresponding p-values and R^2 values, model selection and C_p statistics. In evaluating the analysis, a p-value below 0.05 was considered an indicator of a statistically significant result.

Results

Correlation analysis of data collected by electronic nose measurements. The VOCs in a sample containing healthy and infected acorns consist of a mixture of chemical compounds. E-nose sensors respond nonlinearly to the presence of gases as the sensors used are not selective therefore the measurement does not provide accurate information about the chemical composition of the odor. Typically, all sensors respond to the presence of a sample, however, to varying degrees.

Figure 4 shows the Pearson correlation coefficient between the sample status variable and each sensor response. The absolute value of the correlation coefficient is plotted whether it is a positive or negative value. This is due to the fact that we are concerned with the difference in sensor response to the presence of different compositions of measured VOCs between different statuses of pathogen-infested acorns in our analysis.

As can be seen in Figure 4, the correlation coefficients between acorn infection status and sensor response are in the range of 0.2 to 0.3 for sensors W1S, W1W and W2W. The correlation coefficients calculated for these sensors have an associated p-value below 0.02 indicating that these correlations, although rather weak, are nevertheless statistically significant. For other sensors, the correlation coefficient is smaller with $p>0.05$. However, such a result does not exclude a correlation between the infection status and the response of the sensor if this correlation is not linear.

TRENDS IN ELECTRONIC NOSE SENSOR'S RESPONSE. The main features of the response distribution of the W1S sensor are presented in Figure 5 grouped according to the different proportion levels of infected and healthy acorns in the samples. We chose this sensor because, as we showed in Figure 4, the response of this sensor had the strongest correlation with the infection status of the acorns.

In Figure 5 it can be seen that the variability of the data for each category of infection status show quite a wide range which is understandable given the natural variability of both healthy and infected biological samples. Nevertheless, a trend in sensor response can be detected with

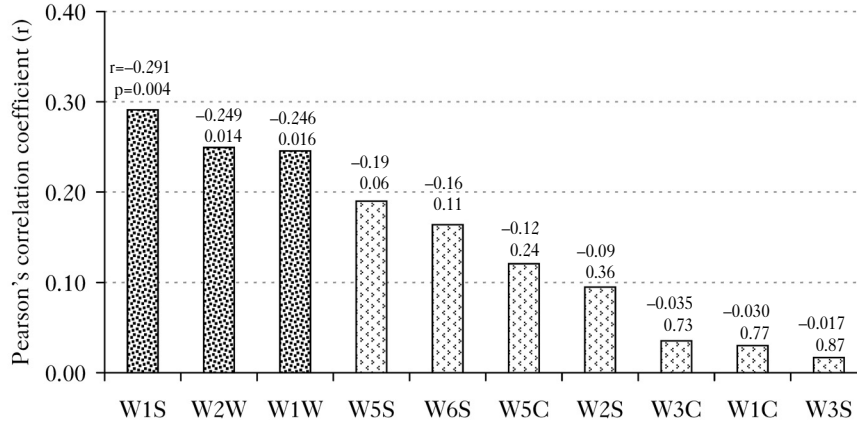

Fig. 4.

Pearson correlation coefficient between the number of infected acorns in a measured sample and the sensor response magnitude at the end of the observation time. The absolute value of the correlation coefficient is plotted. The magnitude of the correlation coefficient and corresponding p-value is indicated above the bars. The blurred bars indicate that the correlation coefficient is not statistically significant at the level $p<0.05$

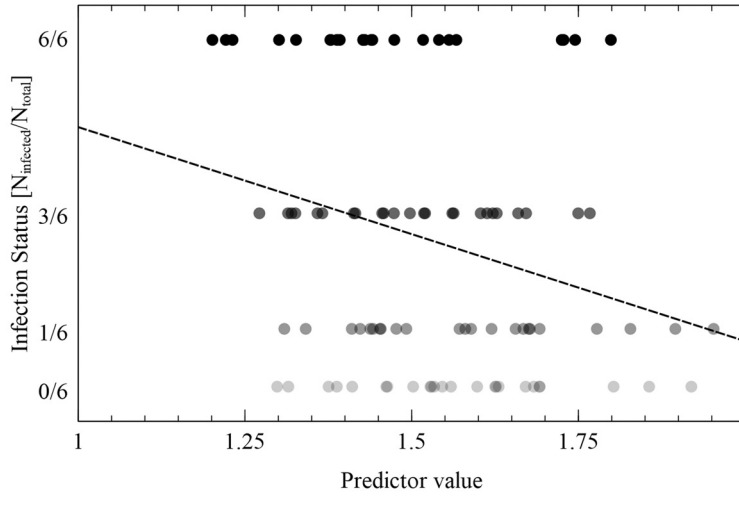

Fig. 5.

The W1S sensor's response at the end of the observation time (predictor value) versus the number of infected acorns in the measured sample. The proportion of acorns with symptoms of pathogenic infection to the total number of acorns in a sample is indicated in the labels. The dashed line represents a linear regression fit. The p-values of the regression coefficients are below 0.004 and the model's R^2 of 0.085

the observed change in the ratio of infected to healthy acorns. This trend exhibits statistical significance of the slope parameter in the linear model with a p-value of 0.004. Analysis of the coefficient of determination (R^2) in the model shows that the measurements of the single-variable model using the W1S sensor data explains about 9% of the data variability.

Linear regression analysis. Figure 6 compares the R^2 of a series of linear regression models using responses collected from multiple PEN3 sensors as predictors. The fusion of data from different sensors was tested and the model that maximized R^2 was selected for a given set of predictors.

In this analysis, we applied two approaches to determine the best model in terms of the number of predictors used. Firstly, Mallow's C_p statistics are calculated and the model with a minimum in this parameter could be selected. This gives the model using as predictors data collected by six PEN3 sensors with a corresponding R^2 of 0.26. Another approach is to examine the slope coefficients for the predictors and choose the models where the coefficients are statistically significant for all factors used. This approach suggests that we should choose a model with data collected from the three PEN3 sensors, W1S, W2S and W3C. This model reached the R^2 value of 0.19.

In Figure 7 we present a scatter-plot chart comparing predictions of a linear regression model versus the actual value of sample infection status (dependent variable). The model using predictor responses of six sensors is chosen for which the Mallow's C_p coefficient reaches a minimal value as presented in Table 4. As can be observed, the trend of data points is rather weak and there is a high level of deviation in data points from the diagonal. This result with low statistical significance reflects the presented above R^2 value which reaches the presented model magnitude of 0.24.

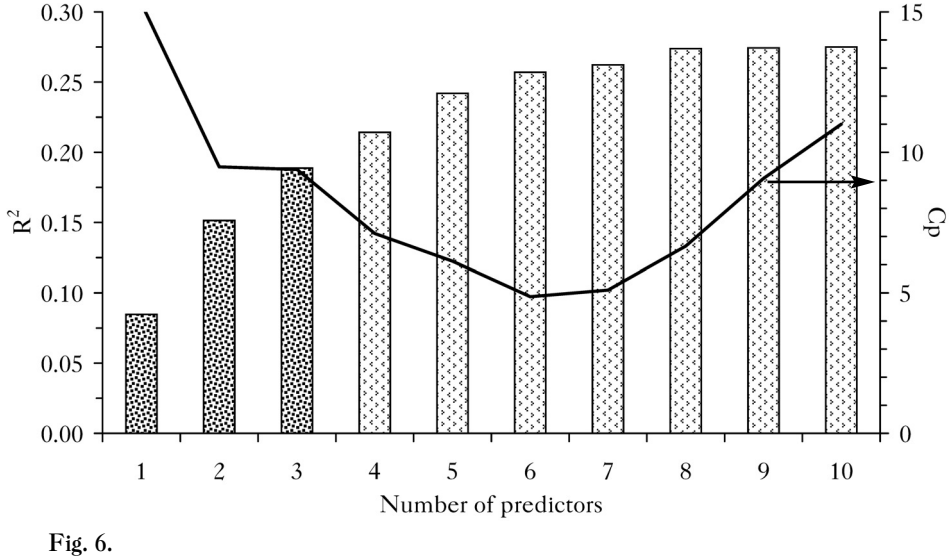

Fig. 6.

The coefficient of determination (R^2) of the linear regression models built with various numbers of predictors. The model with a maximum magnitude of the R^2 is selected for a given number of features. The list of sensors from which data is used is indicated. The models, for which the regression coefficients for all factors are statistically significant at the level of $p<0.05$, are indicated as full-color bars. Line represents a trend of Mallow's C_p statistics. The list of sensors from which predictors values were extracted and magnitudes of presented coefficients are presented in Table 4

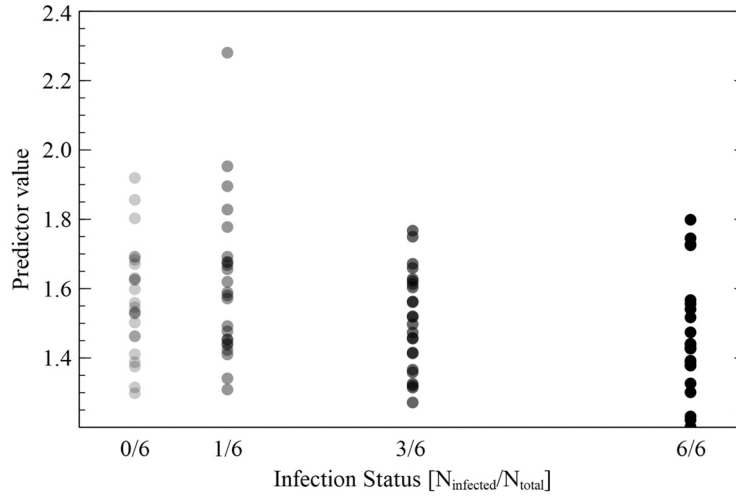

Fig. 6.

Results of linear regression prediction vs actual value of dependent variable, for model applying as predictors responses from seven electronic nose sensors as listed in Table 4

Table 4.

Sensors, from which data is selected by linear regression modeling, for various number of predictors, with corresponding magnitudes of coefficient of determination R^2 and Mallow's C_p statistics. The horizontal line separates the models for which all slope coefficients are statistically significant at p-value level of 0.05, to more complex models for which coefficient's p is at least $p>0.05$

#	Sensors	R^2	C_p
1	W1S	0.085	15.3
2	W1S, W3S	0.15	9.5
3	W1S, W2S, W3C	0.19	9.4
4	W1S, W2S, W3C, W5C	0.21	7.1
5	W1S, W2S, W3C, W5C, W1C	0.24	6.1
6	W1S, W2S, W3C, W5C, W1C, W6S	0.26	4.9
7	W1S, W2S, W3C, W5C, W1C, W2W, W1W	0.26	5.0
8	W1S, W2S, W3C, W5C, W1C, W2W, W1W, W6S	0.27	6.7
9	W1S, W2S, W3C, W5C, W1C, W2W, W1W, W6S, W3S	0.27	9.1
10	W1S, W2S, W3C, W5C, W1C, W2W, W1W, W6S, W3S, W5S	0.28	11.0

Discussion

Detection of acorn and oak pathogens by Volatile Organic Compounds. Multiple reported VOCs have been found emitted from leaves, bark and acorns of *Quercus* species (Burlacu *et al.*, 2020). However, most of this research focuses on the analysis of healthy organism samples driven by application in various industries.

Studies using an E-nose had been conducted prior but these usually involved detecting the odor of pure *in vitro* cultured individual pathogenic fungi of the genera: *Fusarium*, *Rhizoctonia*, *Armillaria*, and *Hymenoscyphus* or the oomycetes *Phytophthora* or *Pythium* (Loulier *et al.*, 2020; Borowik *et al.*, 2021d). They secreted specific VOCs representing their secondary metabolites which were identified using gas chromatography techniques and a GC-MS mass spectrophotometer. The related oomycetes *Phytophthora plurivora* Jung & Burgess and *Pythium intermedium*

de Bary were successfully distinguished (Borowik *et al*., 2021a) but differed in their degree of pathogenicity. For tree nursery workers the availability of information related to plant health problems and early warnings allow them to prepare in advance and take protective measures. Due to the necessity of implementing an early warning system for pests and pathogens, it would be quite useful (perhaps in subsequent studies) to examine infected acorns by an E-nose to detect the early stage of infection or the smallest proportion of infected acorns. Similar results can be obtained by identifying the species *Fusarium oxysporum* Schltdl and *Rhizoctonia solani* J.G. Kühn which have different hosts and are characterized by different levels of harmfulness in forestry practices (Borowik *et al*., 2021b). Previous experiments with infections in germinating acorns confirmed the possibility of distinguishing between the individual pathogens (Borowik *et al*., 2021c) but did not help to develop specific sensors for the individual VOCs. The production of these types of sensors would have been too expensive, thus a change in strategy became necessary. At present, we are not interested in identifying specific compounds that are excreted, but rather the effect they have, as we have shown in the study of the root fungus *Armillaria gallica* Marxm. & Romagn. which is a so-called 'oak-weakening pathogen' (Borowik *et al*., 2021d). The odors are transformed into images that, when arranged separately, confirm that we are able to distinguish between species without knowing the specific VOCs behind them. In seed storage this is particularly important because different fungi at different stages of decomposition can be responsible for destroying seeds as has been observed in the case of fir seeds (Borowik *et al*., 2022a). To our knowledge, this is the first attempt to detect diseased acorns in seed lots collected and stored in barrels using an E-nose.

Data collected in the current experiment. The current experiment showed an interesting trend. The more diseased acorns were present, the greater the possibility of discerning their health status by analysis of odors. Some discrepancies in the control were identified that resulted in not being able to correctly select completely healthy acorns. Only after being cut open did some of them (19%, *i.e*., 8 out of 42) show damage to the cotyledons which had not been visible prior as the damage was hidden under the seed coat (Fig. 1). However, after completion of the daily measurements, all healthy acorns were randomly selected for their respective variant with the aforementioned 8 'diseased' acorns assigned to a different variant each day. However, this may have interfered with the experiment by affecting the sensitivity of the E-nose sensors.

In any case, the experiments proved to be instructive and worthwhile due to a lack of this type of non-invasive device use for forest conservation that facilitates the detection of fungus-infected seeds. This provides new decision-making tools such as whether a seed lot is suitable for sowing or whether it should first be selected or sorted out altogether to avoid introducing pathogens into the nursery soil (substrate).

Selection of the electronic nose gas sensors. In Table 2 can be found with the description of the target gases of the PEN3 sensors provided by the manufacturer. As can be seen, all sensors selected as best predictors by the regression model are broadly sensitive to organic or aromatic compounds. This is consistent with the general idea of designing E-noses using nonspecific sensors with overlapping sensing regions and using machine learning models to analyze patterns in the data. In our experiment, the W1S sensor proved to be the one whose response correlated most strongly with the proportion of infected acorns in the measured samples. Other sensors that also showed a correlation with this target variable were W1W and W2W. We can conclude that in the study by Labanska *et al*. (2022), in which the same type of E-nose was used, W1S and W1W sensors were also found to be useful in assessing onion spoilage status. In a study by Jia *et al*. (2019), PEN3 was used to detect moldy apples and it was found that W1W, W2W and W5S

provided the best discriminating signals. However, the biological samples used in our experiment and the aforementioned reference examples (Jia *et al.*, 2019; Labanska *et al.*, 2022) are quite different, therefore, it is not surprising that the lists of significant sensors are different.

Applications of electronic nose. In the research presented in this paper we applied a commercial PEN3 E-nose device to detect infection of acorns by *C. batschiana* pathogenic fungi. These were preliminary studies to verify the possibility of continuous monitoring of the health status of stored acorns. An E-nose detection system can also be used for the early detection of invasive species by plant protection services. Specifically, it could be used in tree nurseries or at borders to monitor the presence of invasive organisms as the first line of defense against the introduction of an unwanted fungus, oomycete or insect in quarantine. Such unapproved plant material could be subject to further testing, such as genetic testing, to confirm or identify the invasive species. Such a tool would be beneficial to quarantine services and may slow the spread of harmful agrophages in the European Union.

Challenges in the use of E-noses. It should be noted that when the samples under investigation are moist (Slimani *et al.*, 2020), they become a challenge for artificial odor. The NeOse Pro odor analyzer used in this experiment, based on surface plasmon resonance imaging and biological sensors, provided multidimensional data and improved statistical discrimination of odor patterns (Slimani *et al.*, 2020). It was found that the presence of a high background signal such as water vapor from aqueous samples degraded discrimination ability. For this reason, preconcentrators (filled with hydrophobic adsorbent) were used in the gas analysis method to improve the detectability and increase the selectivity by reducing the background signal of water (Slimani *et al.*, 2020). For example, the results of the cited studies showed that coupling a silicon PC unit with NeOse Pro resulted in an improvement in the detection limit of n-nonane by at least a factor of 125. In seed storage, especially in that of acorns, moisture is of great importance because it ensures the viability of stored seeds. At the same time it allows pathogenic fungi to thrive which is why our E-nose is equipped with moisture and temperature sensors. In our case, all measurements were performed in a chamber with controlled temperature and humidity. Likewise, the acorns were stored during the measurements.

In forestry, especially as related to seed storage, our research is groundbreaking. However, it has already found wider application in agriculture. A current challenge for many countries is the widespread use of chemical nitrogen fertilizers, raising concerns about the dangerous accumulation of nitrogen compounds in food and agricultural soils as a result of excessive nitrogen fertilization. Consumption of food from crops with high nitrate content may pose a risk to human health. Therefore, Tatli *et al.* (2021) investigated the effect of different doses of urea fertilizer on VOC emissions from cucumber crops using the experimental device MOS E-nose. Urea fertilizer was applied at rates of 0, 100, 200, 300 and 400 kg/ha. Cucumbers were harvested 4 to 5 months after planting and differences in odor signatures were evaluated. This new monitoring tool could be useful in adjusting future urea fertilizer rates to avoid over-fertilization with nitrogen (Tatli *et al.*, 2021).

Nitrogen fertilizers are commonly applied to increase yields of *Ocimum basilicum* L. (sweet basil), a member of the Lamiaceae family (Khodamoradi *et al.*, 2021). Nitrogen fertilizer increases the accumulation of nitrate in plant tissues which is dangerous for human health. Therefore, the classification of plants based on different amounts of consumed nitrogen fertilizer was carried out using a machine odor system or E-nose. For this purpose, four different amounts of urea fertilizer (0, 50, 100, and 150 kg/ha) were successfully tested with high accuracy (between 96.7% and 97.8%).

In this case, the moisture content of the samples was similar. The collected data were analyzed using linear discriminant analysis and quadratic discriminant analysis, among others, as is the case in our research (Khodamoradi *et al.*, 2021).

Recently, developments have been observed in the field of statistical software that allows food scientists to perform a variety of mathematical-statistical analyses. Consequently, not only have advanced analytical methods increased significantly but also the use of multivariate statistical methods. The use of principal component analysis (PCA) and hierarchical cluster analysis (HCA) in food chemistry research have increased because the results are easy to interpret and discuss. However, their uncritical use to evaluate the relationship between bioactive compounds and functional properties *in vitro* have been criticized as providing a qualitative view of the data. If justified, it should be noted that the correlation between compound content and bioactivity can be adequately discussed using correlation coefficients (Granato *et al.*, 2018). Due to our awareness of this, we will focus on machine learning methods instead of R^2 from classical linear models in future studies and evaluate the sensitivity and specificity for the constructed algorithm. Although the correlation found was weak, perhaps later researchers knowing our results, will modify their experiments to obtain more significantesults. Now we would like to share our experience with the scientific community, not to repeat our way but instead to look for an improved path, as we believe this is the purpose of scientific progress.

Conclusions

The development of new technologies in agriculture (which in the EU includes forestry) also concerns the increasing use of E-noses. The first attempts to use them in the storage of forest seeds, acorns in our case, are encouraging.

The PEN3 electronic portable nose which uses sensors to detect VOCs is able to assess, to some extent, the health status of stored acorns. However, further field trials are needed to refine both the sampling method for acorn preparation and the measurements themselves (duration) to make the most of the device's capabilities (sensitivity and selectivity).

We are well on our way to developing an E-nose (Borowik *et al.*, 2021a, b, 2022b, 2023) that can distinguish between healthy seeds (which are suitable for sowing) and diseased seeds (which should be rejected). We hope that it will also be able to detect the presence of dangerous acorn disease pathogens such as *C. batschiana*.

Furthermore, the presented study genetically confirms the causal agent of acorn mummification, the fungus *C. batschiana*, which is the main causal agent of acorn damage during storage including within the forest and after seeding in the forest nursery.

Authors' contributions

P.B. – research concept, data analysis, manuscript preparation and corrections; T.O. – research concept, manuscript preparation; T.M. – research concept, fieldwork, data analysis, manuscript preparation; M.T. – fieldwork, manuscript preparation; S.Ś. – fieldwork; R.T. – fieldwork.

Competing interests

The authors have no competing interests to declare that are relevant to the content of this article.

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STRESZCZENIE

Wstępne badania oceny stanu żołędzi *Quercus robur* porażonych przez *Ciboria batschiana* za pomocą pomiarów nosem elektronicznym

Drzewa leśne owocują nieregularnie, dlatego ich nasiona muszą być przechowywane, aby zapewnić ciągłą produkcję materiału rozmnożeniowego w szkółkach. Dotyczy to zwłaszcza dębów, owocujących co 4 do 8 lat, których żołędzie atakowane są przez patogeny, a w szczególności przez grzyb *Ciboria batschiana* (Zopf) Buchw, powodujący ich zamieranie i mumifikację (ryc. 1). Żołędzie zgromadzone w latach urodzaju przechowywane są w beczkach. Monitorowanie zdrowotności żołędzi na podstawie wydzielanego zapachu ma charakter nowatorski. Należy zweryfikować nie tylko, czy doszło do zakażenia żołędzi, ale także w jakim stopniu. W niniejszych testach prowadzono próby z różną ilością zepsutych nasion (tab. 1). Zastosowany system e-nosa PEN3 wykorzystuje jako jednostki detekcyjne 10 wysokotemperaturowych czujników z tlenków metali (ryc. 2). Koncepcja użytego e-nosa polegała na zastosowaniu zestawu niespecyficznych czujników gazu, o pokrywającym się zakresie ich detekcji (ryc. 2). Wybrane czujniki są wrażliwe na związki organiczne lub aromatyczne (ryc. 3). Opis gazów wykrywanych przez czujniki podano w tabeli 2.

Celem niniejszych badań było sprawdzenie nowego zastosowania e-nosa do rozróżniania zdrowych i uszkodzonych partii nasion na podstawie wytwarzanych lotnych metabolitów wtórnych.

Wykonano serię pomiarów, aby określić korelację pomiędzy stanem zakażenia próbek a zarejestrowanymi wzorcami reakcji sensorycznych (ryc. 4). Z dostępnej literatury wynika, że jest to pierwsza próba oceny stopnia infekcji żołędzi w ten sposób.

Czujniki e-nosa w różnym stopniu reagowały na obecność metabolitów wtórnych grzybów sygnalizujących zepsucie się żołędzi (ryc. 5). Między innymi stwierdzono różnicę w reakcji na obecność lotnych związków przy różnym procencie żołędzi zasiedlonych przez patogen (tab. 3). Określono, że współczynniki korelacji pomiędzy odsetkiem porażonych żołędzi a reakcją czujników są statystycznie istotne. Współczynnik R^2 modelu regresji liniowej osiągnął wartość 0,19 dla przypadku, gdy współczynniki nachylenia 3 predyktorów były statystycznie istotne (tab. 4). Okazało się, że przenośny e-nos PEN3 był w stanie ocenić zdrowotność przechowywanych żołędzi. Powietrze nad nasionami zbierano, wkładając rurkę PEN3 do przygotowanych otworów w pokrywce słoika (ryc. 2). Między pomiarami słoiki były zamknięte, a otwory uszczelnione parafilmem. Do pomiarów przygotowano po 3 słoiki z próbkami każdego wariantu (tab. 1). Na ryc. 4 przedstawiono współczynnik korelacji Pearsona pomiędzy zmienną stanu próbki a reakcją czujnika. Analizowano różnice w reakcji czujników na obecność kompozycji mierzonych lotnych substancji a liczbą zaatakowanych żołędzi. Współczynnik korelacji pomiędzy stanem porażenia żołędzi a reakcją czujnika mieścił się w zakresie (0,2, 0,3) dla czujników W1S, W1W i W2W (ryc. 4). Dla współczynników korelacji obliczonych dla tych czujników związane z nimi wartości *p*-value mieściły się poniżej 0,02, co wskazuje, że korelacje te, choć raczej słabe, były jednak istotne statystycznie. Dla pozostałych czujników współczynnik korelacji był słabszy (również $p>0,05$). Taki wynik nie wyklucza korelacji między zakażeniem a odpowiedzią czujnika, jeśli korelacja ta nie jest liniowa. Główne cechy rozkładu odpowiedzi sensora W1S przedstawiono w postaci wykresu pogrupowanego według różnych poziomów udziału zainfekowanych i zdrowych żołędzi w próbach (ryc. 5). Wybrano ten czujnik, ponieważ wykazano, że jego odpowiedź wykazywała najsilniejszą korelację ze statusem infekcji żołędzi (ryc. 4).

Indywidualna zmienność danych w każdej badanej kategorii statusu infekcji wykazała dość szeroki zakres, co jest zrozumiałe ze względu na naturalną zmienność próbek biologicznych, zarówno zdrowych, jak i zainfekowanych (ryc. 5). Rycina 6 porównuje współczynnik determinacji (R^2) serii modeli regresji liniowej wykorzystujących odpowiedzi zebrane z wielu czujników PEN3 jako predyktory. Na ryc. 7 przedstawiono wykres typu scatter-plot, porównujący przewidywania modelu regresji liniowej z rzeczywistą wartością stanu zakażenia próbki (zmienna zależna). Wybrano model wykorzystujący jako predyktory odpowiedzi 6 czujników, dla których współczynnik C_p Mallowa osiąga minimalną wartość (tab. 4).

Badania potwierdziły, że grzyb *C. batschiana* był głównym patogenem badanych żołędzi, przy czym im więcej było chorych żołędzi, tym większa była możliwość określenia ich stanu zdrowotnego za pomocą analizy zapachów. Szkółkarze potrzebują skutecznych i szybkich narzędzi do monitorowania przechowywanych nasion za pomocą nieinwazyjnego urządzenia ułatwiającego wykrywanie nasion porażonych. Konieczne są dalsze próby terenowe w celu dopracowania zarówno metody pobierania próbek żołędzi, jak i samych pomiarów (czas trwania), aby w pełni wykorzystać możliwości urządzenia (czułość, selektywność).