International Journal of Innovative Technology and Exploring Engineering (IJITEE)
ISSN: 2278-3075 (Online), Volume-9 Issue-4, February 2020

Pathogen Detection in Khasi Mandarin Orange using Serological and Electronic Nose Diagnostic Technique

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Abstract*: *The inherent ability of most living organisms to perceive their immediate environment based on sensory responses has immensely contributed to their survival in the harshest of conditions. Animals rely on their olfactory sense to assess the quality of food before intake. This paper addresses a technique of using the electronic nose for distinguishing Khasi Mandarin orange plants infected by a virus called Citrus Tristeza Virus (CTV) in terms of their degree of infection. Leaves from 16 plants were collected and, tested for CTV infection using the standard serological test, Enzyme-linked Immunosorbent Assay (ELISA), prior to electronic nose (e-nose) analysis. Essential oil was extracted from the leaves using hydro distillation and the extracted oils were analyzed with commercial e-nose system Alpha MOSFOX 3000 system. Bootstrapped ensemble of support vector classifier was used for classifying the samples. The classifier model was optimized with the best parameters and a kernel specific performance evaluation was done for finding out the best model for classification. Among the linear, radial basis function and polynomial kernels, the linear kernel of the classifier performed the best among all the kernels with an accuracy of 97.67% and a Cohen's Kappa score of 95.25%. Dimensionality reduction techniques like principle component analysis and linear discriminant analysis were also used for graphical visualization of the classification boundaries. The dimensionally reduced dataset was also fitted to the optimized bootstrap ensemble support vector classifier and the performance of the classifier was analyzed. The performance scores of the classifier models reveal the possibility of using e-nose technique in detecting CTV infected plants.

Keywords: Volatile Organic Compounds (VOC), Support vector classifier (SVC), Citrus Tristeza Virus (CTV).

I. INTRODUCTION

Citrus species having its probable origination in the warm southern slopes of the Himalayas in the north-eastern region of India slowly spread to China and eventually found its way to other parts of the world [1].

Revised Manuscript Received on February 28, 2020.
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The Orange industry, employing a large section of farmers, is in peril at its native land, cursed by diseases and inefficient use of technology for better management of plants in both pre and post-harvest stages. One such disease, largely impacting citrus production is caused by Citrus Tristeza Virus (CTV). The disease caused by the virus affects both the quality and quantity of citrus yield. An epidemic caused by the virus in the 1920s, is still considered to be one of the most economically destructive diseases in citrus [2]. Tristeza literally means sadness in Spanish and Portuguese, and is nicknamed as the quick decline in the United States. Infected bud wood grafting and movement of virus affected plant materials by aphids helps in transmission of the diseases to long distances. Citrus brown aphids, (Toxoptera Citricida) are considered to be the most effective vectors, capable of transmitting CTV for up to 24 hours after acquisition. However, it is highly improbable for its transmission through seeds , contaminated tools and equipment [3]. Development of a rapid disease and stress diagnostic systems for plants and proper quality inspection systems to provide better sorting and grading of citrus yield, post-harvest has been attracting the interest of the scientific community in recent years.

In response to a pathogen attack, plants defend themselves by inducing resistance mechanisms, where the plant sets into motion a signal transduction pathway activating resistive responses like programmed cell death and defense gene expression[4]. Plants release some complex organic compounds of carbon and hydrogen, volatile in normal temperature and pressure with vapor pressure ranging from 0.13kPa to 101.3 kPa at 293 K [5], called Volatile Organic Compounds (VOC). Most VOCs have enough vapor pressure to get released into the atmosphere in significant amounts from various tissues both above and below the ground. The role of their emission is however not much understood, but still can be linked to many functions like protection of the photosynthetic system as an antioxidant, to get rid of excess carbon, or as a defense compound against pathogens and herbivores. VOCs range from a host of compounds like terpenes, fatty acid derivatives, benzenoids, phenylpropanoids, and amino acid-derived metabolites. These are emitted as a result of the complex interplay of different metabolic, biochemical pathways and alterations of hundreds of gene expressions, thus containing vital information about the metabolic status of plants. Plants on being stressed, respond by altering their metabolism, which in turn leads to modifications in their VOC emission pattern. These VOCs also serve as communication signals for preparing themselves as well as plants in the vicinity of possible attacks.

Several compounds like green leaf volatiles (GLV), terpenes, methanol and the phytohormones (ethylene, methyl jasmonate (MeJA), methyl salicylate (MeSA)), have been reported to be functioning as communication signals.

For instance, GLVs are emitted upon mechanical damage of plants and herbivore feeding, in not only the wounded leaves but also systemically in the distant leaves. Terpenoids, accounting for more than half of the total emission of VOCs, provides herbivore related damage signals. MeJa has been reported as an integral component of the defense response of a plant to insect feeding and mechanical damage. MeSA was reported for pathogen-induced defense response and aphid feeding damage. Ethylene helps in resisting necrotrophic pathogens and submergence and drought tolerance. It is reported to be actively involved in regulating growth, reproduction, and senescence of most parts of the plant, especially leaves, flowers, and fruits, thereby controlling the plant's affinity to pollinators[6]. The rise in Methanol emission in response to mechanical damage have extensively been reported by Dorokhov et.all [7]. Mechanical injuries or herbivory are responded defensively by the emission of VOCs which act as an herbivore repulsion agent and also instigates defensive responses in neighboring plants[8]. These chemical clues could be investigated to device a rapid diagnostic test for stress attacks on plants.

Apart from traditional molecular methods for disease detection like ELISA (Enzyme-linked immune-sorbent assay), RT-PCR (Real-time polymerase chain reaction), which require ample time, elaborate laboratory and equally experienced operators, machine sensing combined with machine learning techniques are being widely researched for their ability to automatically, rapidly, frequently and non-invasively monitor the status of plants [9]–[11]. Machine olfaction, though less ventured comparatively, is emerging as a potential technique for application in the field as well as at storage for plant science. Two common methods practiced for accessing volatile metabolite profiles emitted by plants are analytical instruments like GC-MS (Gas Chromatography-Mass Spectrometry) and electronic nose (e-nose) system based techniques. Healthy and Huanglongbing (HLB) infected trees were distinguished using Capillary electrophoresis. Infected plants were reported to have higher concentrations of compounds like naringenin, quercetin ,and hesperidin [12]. Another study reported identifying the major peel oil odorants responsible for the VOC profile of an HLB tolerant mandarin hybrid using GC-MS/O (Gas Chromatography-Mass Spectrometry/Olfactometry) associated with the Aroma Extraction Dilution analysis technique [13]. Application of e-nose in this field has been a new domain, though many works have been attempted, a few works have been reported related to its application in the citrus industry. Akakabe et al. used GC-MS (gas chromatography-mass spectrometry) to evaluate and identify VOCs and e-nose to discriminate the odor of essential oil extracted from different species of Japanese sour oranges [14]. The maturity of mandarin oranges during different harvest periods was evaluated using e- nose technique and pattern recognition technique [15]. Application of e-nose in storage was reported by Di Natale et. al where aroma transformation of oranges in storage over a period of one month was measured in addition to detection of post-harvest ripening defects and skin damage in apples [16]. The effectiveness of e- nose system to detect quality and genuineness of bergamot essential oil, while using GC-MS for chemical composition analysis was reported in another study [17]. In another study, plants suffering from water stress were effectively diagnosed by e-nose technique[18]. Classification of citrus juice using e-nose was also recently reported[19].

In this paper, we explore the effectiveness of the e-nose technique in diagnosing CTV in Khasi Mandarin orange plants from the essential oils extracted from their leaves. The results of the traditional serological diagnosis test were used for training the classifier models and these models were used for testing the performance of the e-nose technique on unseen samples.

II. MATERIALS AND METHODS

Orange leaves were collected from 16 trees from Citrus Research Station, Tinsukia, Assam (India). The leaves were packed in pre-labeled moisture barrier packets to be transported in an ice chest. For maintaining homogeneity in the samples all leaves collected were properly sunlight exposed, fully expanded and roughly belonged to the same age group. All the samples were tested for CTV infection using the standard serological ELISA test. 250g of these leaves were hydro distilled using Clevenger's apparatus yielding 3ml of essential oil. The oil was collected and fed to the commercial e-nose system ALPHA MOS FOX 3000 for VOC analysis.

A. Double-Antibody Sandwich Enzyme-linked immune-sorbent assay (DAS-ELISA):

The midrib tissue samples of the raw leaf samples were tested using DAS ELISA as described by Clark and Adams[20]. The midrib tissues were chosen as they contain the maximum concentration of CTV as reported in [21].The antibodies for DAS-ELISA were obtained from Bioreba AG, CH4153, Reinach BL1, and Switzerland. The change in colors in the wells of the plate was read by the ELISA plate reader Bio Rad using 450 nm wavelength[22]. The ELISA tarter values (ETV) obtained from the test formed the basis of classifying the samples as mildly and moderately infected samples as shown in Table I.

Table I: Sample specifications for e-nose analysis

Infection	No of sample plants	ETV
Mild Infection	9	<0.12
Moderate infection	7	>0.12

B. E- nose analysis

The Alpha MOS FOX 3000 e- nose system consists of two sensor chambers, each containing 6 MOX sensors (LY2/LG, PA/2, T30/1, T70/2, P40/1, P10/2, P10/1, LY2/G, LY2/gcT, LY2/gcTL, LY2/GH, LY2/AA). Initially, the acquisition parameters like the acquisition time, chamber temperature, headspace generation time, flow rate, etc.., are set and the system waits until these parameters are reached. Contamination free dry air is used as a carrier gas for feeding the odor from the sampling chamber to the sensor chamber.

The essential oil samples were fed to the sample holder of the Alfa-MOS FOX 3000 e-nose system, where they were agitated for 5 minutes at a temperature of 35°C for headspace generation.

The headspace is then fed to the sensor chamber at a flow rate of 150ml/min. The data acquisition time for each sample was fixed to be 2 minutes, with data being recorded each second to be stored in a computing system for further pattern analysis. Between each acquisition, the sensor chambers were purged with clean dry air and the time between acquisitions was 20 minutes to ensure sensor recovery.

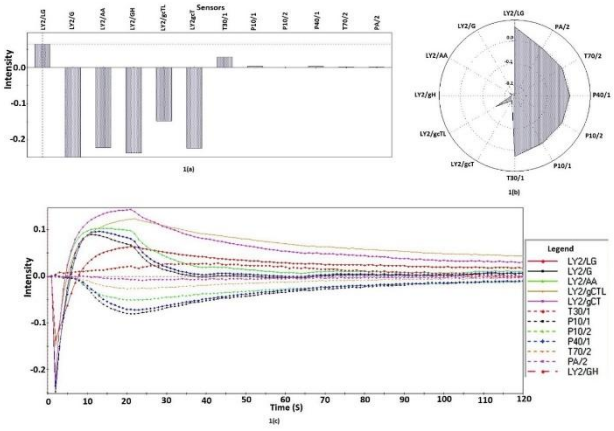

Fig.1. Sensor responses. 1 (a), (b): Peak response of each sensor, 1(c) Response of the sensors over the sampling time of 120s.

The intensity of the sensor responses as shown in Fig. 1 is also referred to as the sensitivity of the sensor and is given by the relative resistance change defined by, sensitivity = (Rt-R0)/Rt, where, R0 is the resistance across the sensor at time zero seconds, and Rt is the resistance at t sec. From Fig. 1(c), it is observed that most of the sensor responses seem to vary within 0 to 40 seconds, after which the responses start decaying. Hence, only these data points are considered for pattern analysis.

C. Data analysis:

The data stored in the computing system was used for pattern recognition analysis by using classifier algorithms. The data was first split into 80 % training and 20% testing set. 10 fold cross-validation was used for hyper tuning and training the models. The trained models were tested with the untouched 20% test set and the performance scores revealed the diagnostic capability of e-nose technique. Bootstrap aggregation ensemble of the Support vector classifier was used as the classifier model. For visualization of the classification, the multidimensional dataset was dimensionally reduced into two dimensions and the reduced dimensions were plotted in 2D scatter plots.

III. RESULTS AND DISCUSSION

The data set generated from the e-nose system was analyzed using the bootstrap ensemble (bagging) of support vector classifier (bagSVC). It is a supervised classifier and is trained based on the ELISA test results, which labeled samples as mildly infected or moderately infected samples. Table II shows the performance scores of the bag SVC for different kernel functions of the SVC. The linear kernel performed the best among the three kernels with the highest accuracy and Cohen's Kappa score greater than 90% reveal perfect agreement between the predicted values and the true values(as observed from the ELISA test). The models were hyper tuned to find the best parameters, for each kernel. For graphical visualization of the data, and the classification, the multidimensional data was dimensionally reduced into two dimensions using kernel Principal Component Analysis (kPCA) and linear discriminant analysis (LDA). The reduced dimensions were then fitted to the classifier models analyzing the performance of these models on the reduced dataset, as shown in Table III. The classifier decision boundary is shown in Fig.2.The performance of the classifier models on the reduced data set was significantly lower than that of the original dataset. This is due to the fact that there is a loss of information due to dimensionality reduction.

Table II: Performance metrics of the SVC kernels

SVC Kernel	Accuracy	F1-score	Cohen's Kappa score	Confusion Matrix	
RBF	90.69	90	80.58	44	12
				0	73
Linear	97.67	98	95.25	54	2
				1	72
Polynomial	91.47	91	82.23	45	11
				0	73

Table III: Performance matrix of the SVC kernels on dimensionally reduced features kPCA features

SVC Kernel	Accuracy	F1-score	Cohen's Kappa score	Confusion Matrix	
RBF	79.06	79	58.43	52	8
				19	50
Linear	77.51	55.62	78	49	7
				21	51
Polynomial	77.5	55.25	78	50	10
				19	50

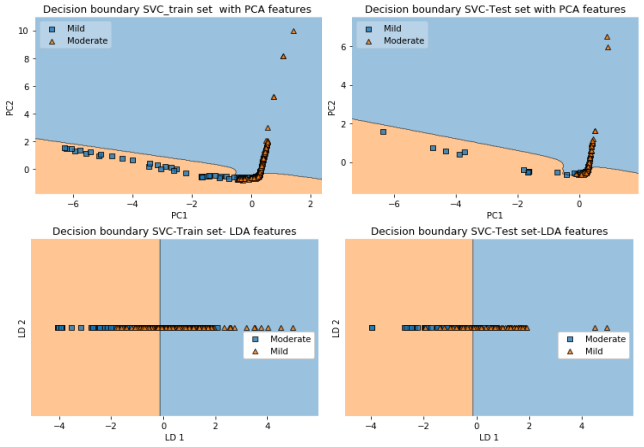

Fig.2 Decision boundaries of the RBF kernel bagSVC for kPCA reduced features and LDA reduced features.

The ROC curve for the linear kernel of the bagged SVC classifier is shown in Fig. 3, where the sensitivity (true positive rate) and the specificity (False positive rate) of the classifier are plotted. The Area under the curve scores as depicted in the figure shows the performance of the classifier in separating the two classes of samples.

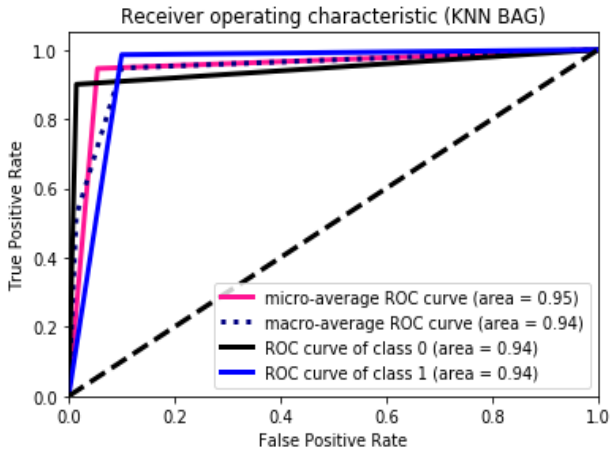

Fig.3 ROC curve of the linear kernel of bag SVC

IV. CONCLUSION

The results from the above study reveal the successful classification of the mildly infected Khasi Mandarin orange plants from moderately infected plants. Both the techniques employed for CTV detection viz. serological test (ELISA) and the e-nose sensing technique exhibited conformity. The study considers the ELISA test results to be the standard disease detection technique and uses the result to train classifier models, which can diagnose unseen data. The bag SVC was optimized with the best parameters during the 10-fold cross validation. Among the kernel functions such as RBF, Polynomial and linear kernels, the linear kernel performed the best. Dimensionality reduction techniques were used for visualizing the data in 2D space and the reduced data set was also analyzed with the bag SVC model. The performance of the classifier in this case decreased with respect to the original data set as there was loss of information during the dimensionality reduction. The e-nose technique could discriminate between mildly and moderately infected plants. It has to be noted that, although serological or molecular diagnostic techniques are accurate and reliable, they requires ample time and resources for diagnosis. E-nose technique on the other hand, can provide a firsthand alternative diagnosis for monitoring large scale orchards.

ACKNOWLEDGMENT

The author acknowledges the Department of Science & Technology, Govt. Of India, for providing the INSPIRE fellowship which provided the financial support for carrying out the work. Special thanks to Dr Palash Debnath, Professor, Assam Agriculture University for the most required assistance in the field. CSIR-CEERI (Central Electronics Engineering Research Institute, Pilani, Rajasthan) needs to be mentioned with lots of gratitude for providing all the necessary infrastructure for conducting the experiments in the institute. The Citrus Research Station Tinsukia, Assam has also rendered their vast knowledge and offered their technical guidance for carrying out the work.

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