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Preface

We would like to present, with great pleasure, the inaugural volume-3, Issue-8, August 2017, of a scholarly journal, *International Journal of Environmental & Agriculture Research*. This journal is part of the AD Publications series *in the field of Environmental & Agriculture Research Development*, and is devoted to the gamut of Environmental & Agriculture issues, from theoretical aspects to application-dependent studies and the validation of emerging technologies.

This journal was envisioned and founded to represent the growing needs of Environmental & Agriculture as an emerging and increasingly vital field, now widely recognized as an integral part of scientific and technical investigations. Its mission is to become a voice of the Environmental & Agriculture community, addressing researchers and practitioners in below areas

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Environmental science and regulation, Ecotoxicology, Environmental health issues, Atmosphere and climate, Terrestric ecosystems, Aquatic ecosystems, Energy and environment, Marine research, Biodiversity, Pharmaceuticals in the environment, Genetically modified organisms, Biotechnology, Risk assessment, Environment society, Agricultural engineering, Animal science, Agronomy, including plant science, theoretical production ecology, horticulture, plant, breeding, plant fertilization, soil science and all field related to Environmental Research.

Agriculture Research:

Agriculture, Biological engineering, including genetic engineering, microbiology, Environmental impacts of agriculture, forestry, Food science, Husbandry, Irrigation and water management, Land use, Waste management and all fields related to Agriculture.

Each article in this issue provides an example of a concrete industrial application or a case study of the presented methodology to amplify the impact of the contribution. We are very thankful to everybody within that community who supported the idea of creating a new Research with *IJOEAR*. We are certain that this issue will be followed by many others, reporting new developments in the Environment and Agriculture Research Science field. This issue would not have been possible without the great support of the Reviewer, Editorial Board members and also with our Advisory Board Members, and we would like to express our sincere thanks to all of them. We would also like to express our gratitude to the editorial staff of AD Publications, who supported us at every stage of the project. It is our hope that this fine collection of articles will be a valuable resource for *IJOEAR* readers and will stimulate further research into the vibrant area of Environmental & Agriculture Research.

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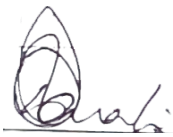

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Table of Contents

Trypanosoma cruzi discrete typing units in patients of Chagas disease and *Triatoma infestans* after insecticide spraying in Chile

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Abstract— *In this study, we evaluate mixed discrete typing units (DTUs) of Trypanosoma cruzi present in 69 patients of Chagas disease and 92 Triatoma infestans collected under the entomological surveillance program after more than 20 years of intervention with insecticide spraying in Chile. Our aim is to identify T. cruzi DTUs still circulating in T. infestans and chronic patients to evaluate their impact on the environmental health in endemic areas of the country. Blood DNA or triatomine DNA was used as DNA template for PCR assays. For genotyping, different T. cruzi stocks were used to generate the DNA probes to determine four parasite DTUs or mixtures infecting each patient or vector by means of hybridization assays. We found different frequencies of T. cruzi DTUs in patients and in T. infestans. Tc I was the most frequent found in T. infestans, but was less frequent in humans. In contrast, in humans Tc V was most frequent but was less frequent in T. infestans. In conclusion, there were significant differences between the T. cruzi DTUs circulating in patients and vectors. We discussed these results in the context of what has being reported in Chile before the vector control, in neighboring countries, and the selection pressures existing for T. cruzi populations within the invertebrate and vertebrate hosts.*

Keywords— Chagas disease in Chile, Trypanosoma cruzi DTUs, Triatoma infestans, kinetoplast minicircles.

I. INTRODUCTION

Chagas disease is widespread in Chile, distributed in rural and peri-urban areas in the seven endemic regions of the country. Human Chagas disease presents two distinct phases: the acute phase, which appears just after infection, and the chronic phase, which may last several years. After a long asymptomatic phase, around 30% of infected individuals develop chronic disease with severe damage to the heart and digestive system [Arribada et al., 1986]. Microscopic examinations of fresh or stained blood smears, xenodiagnosis and hemoculture are methods used for detected *T. cruzi* during the acute phase. In contrast, the detection is by circulating antibodies during the chronic phase. *Triatoma infestans* (Hemiptera, Reduviidae), a strictly hematophagous and almost exclusively domestic species, is the main vector of *Trypanosoma cruzi*, the causative agent of Chagas disease, in the southern cone of South America [Lent and Wygodzinsky 1979, Zeledón 1983]. Domestic animals are excellent hosts for these insects. The peri-domestic area around human houses in rural villages is very important, because it usually includes heavily infested goat corrals, chicken coops, rabbit cages and storerooms, which in many cases are just a few meters from the house [Gürtler 1993, 1997, Cecere et al., 1997, López et al., 1999, OMS 2002, Catalá et al., 2004]. In Chile, it attempted disruption of the domestic cycle of transmission of *T. cruzi* by means such as health education, improvement of housing and vector elimination by applying insecticides to human dwellings. Along northern areas where the domiciliary vectors were present systematic insecticide spraying with the support of health authorities it was applied during the last 20 years. Goat corrals are the main refuge for the peri-domestic populations of *T. infestans* and one of the ecotopes in which pyrethroid insecticides show low efficiency against these insects [Cecere et al., 1997, Gürtler et al., 2004]. Chicken coops are also very frequent and maintain abundant vector populations. Infested peri-domestic places could act as sources of house re-infestation after insecticide application [Gürtler et al., 2004], because of the movement of insects between habitats [Schofield 1985]. Although *T. cruzi* infection in peri-domestic *T. infestans* is not as frequent as in intra-domestic insects, the peri-domestic structures may be important sources of vector specimens. Chagas disease represents a major public health

problem in America, with an estimated 16-18 million people infected by *Trypanosoma cruzi* [Moncayo, 2003]. In the most endemic areas of this disease of Chile, the Norte Grande and Norte Chico, there are an estimated 150,000 infected people, even though the only domiciliary vector *Triatoma infestans* has been controlled since 2000 [Lorca et al., 2001]. However, there are still geographic areas with infection rates of 0.55% and very low *T. infestans* infestation rates [Jercic et al., 2011]. The clinical symptoms of chagasic patients are cardiological or/and digestive dysfunction in about 1/3 of the chronic infected subjects. In Chile most cases are reported as diseases in other organs (50.6%), followed by cardiological dysfunction (44.6%) and digestive dysfunction (4.7%) [Moncayo, 2003]. The etiologic agent *T. cruzi* is composed of six DTUs (TcI-TcVI). Efforts to find the association between the infective *T. cruzi* DTUs with clinical manifestations have been made all over Latin America [Zingales et al., 2012]. In Chile there are reports indicating that TcI, TcII, TcV and TcVI, studied with different molecular markers, are prevalent (including mixed infection) [Sanchez et al., 1993; Rozas et al., 2005; Coronado et al., 2006; Arenas et al., 2012]. *T. cruzi* belongs to the order Kinetoplastida, a group of parasitic organisms with an organelle called a kinetoplast, which contains DNA in concatenated minicircles and maxicircles. The minicircles are very abundant (10,000-20,000 copies/cell), and therefore represent a perfect DNA target for diagnosis by means of PCR-DNA amplification. These amplicons are also useful for genotyping the *T. cruzi* DTUs, since minicircles are composed of different classes which may be used to characterize *T. cruzi* lineages by DNA-DNA hybridization methods [Veas et al., 1991; Brenière et al., 1998; Torres et al., 2004]. Each minicircle (1400bp) contains four constant regions intercalated with four hypervariable regions, which vary among the different minicircle classes present in each *T. cruzi* DTU [Arenas et al., 2012]. The medical entomology laboratory of the Institute of Public Health, analyzed samples of *T. infestans*. The Regional Ministerial Secretariats (SEREMIS) of Atacama, Valparaíso and Metropolitana provided triatomines captured in the period 2005-2010 of which 28.5% were infected. Parallel to the studies with the vector, there were a series of studies of serological screenings in children under 5 years old and their families between the regions of Arica and Parinacota and O'Higgins. Five thousand one hundred eleven screenings were preformed, with 28 positive cases (0.55%), a value close to the 0.7% prevalence obtained by the National Health Survey (NHS) 2009-2010 (Jercic et al., 2011). Our study, framed by the recommendations of the Panamerican Health Organization Initiative of Countries of the Southern Cone, sought to evaluate the effectiveness of the programs of vector elimination carried out in Chile [OPS, 2002]. The Chilean Health Ministry and the Public Health Institute (PHI) confirmed each chagasic patient by means of serological methods, as well as the infected or non-infected status of the triatomines, by means of PCR diagnosis. This epidemiological information is relevant to direct the necessary efforts to spray human dwellings with insecticides and try to eradicate *T. infestans.* In this study, we evaluate the *T. cruzi* DTUs present in random representative samples of these patients and *T. infestans* collected in all endemic areas of the country under the entomological surveillance program of the Ministry of Health. Our aim is to identify *T. cruzi* DTUs currently circulating in *T. infestans* and non-treated chronic adult patients to analyze the *T. cruzi* DTUs circulating in the survivors in both hosts in an extensive area of Chile. We pretend to analyze the interactions between *T. cruzi* parasites and its hosts in order to evaluate their impact on the environmental health. To accomplish this goal we used a direct method to genotype *T. cruzi*, to avoid selection of *T. cruzi* clones from mixed infections during the parasite isolation and amplification processes.

II. MATERIALS AND METHODS

2.1 Patients

Blood samples of 69 chagasic patients were obtained from regions XV (Arica-Parinacota), I (Tarapacá), II (Antofagasta), III (Atacama), IV (Coquimbo), V (Valparaíso), RM (Región Metropolitana) and VI (O'Higgins). Fig.1 shows a map of Chile describing the geographic regions. The whole area remained untreated with insecticides, except in the cases of re-infestation, during the 20 years before the present study. To ascertain chagasic patients we applied two of the three serological methods (ELISA, indirect inmunofluorescence and/or Western blot) at PHI. An infected case gives positive result with two methods.

FIGURE 1. MAP OF CHILE (GRAY AREAS) INDICATING THE REGIONS FROM WHICH THE SAMPLES CAME.

2.2 Triatomines

Intra-domiciliary Triatomines (n=92) were from regions III (Atacama), IV (Coquimbo), V (Valparaíso) and RM (Región Metropolitana). Triatomines from the Norte Grande and the VI region were not available due to absence of notifications from local health authorities. Local personnel of the surveillance program of the Ministry of Health transported the dead insects to the PHI for species identification and determination of infection status with *T. cruzi* by PCR assays as described [Jercic et al., 2011]. Hindgut of each triatomine was used to DNA extraction according to the manufacturer´s instructions (EZNA Blood DNA Mini Kit OMEGA biotek, Nercross, GA) and used as DNA template for PCR.

2.3 Blood samples

The samples of peripheral blood of the patients were preserved in Guanidine-EDTA as described [Wincker et al., 1994] and boiled for 15 minutes at 98 ºC before extraction and purification of DNA using the Favorgen kit according to the manufacturer´s instructions, and maintained at -20 ºC until use (Biotech, Corp., Selangor, Malaysia).

2.4 Minicircle PCR assay

A blood DNA or triatomine DNA sample (5 µL) was used as DNA template for PCR. The reactions were performed in triplicate with oligonucleotides 121 and 122, which anneal to the four conserved regions present in minicircles of *T. cruzi* [Wincker et al., 1994], including a positive and negative control in each test. The 330-base pair PCR product was separated by electrophoresis in a 2% agarose gel and visualized by staining with ethidium bromide.

2.5 DNA blot assay

T. cruzi DTU genotyping in the patients and vectors was performed by DNA blot of minicircle amplicons, as described previously [Veas et al., 1991]. Briefly, 10 µl of each PCR product was subjected to electrophoresis, transferred onto a Hybond N+ nylon membrane (Amersham, Little Chalfont, United Kingdom) and cross-linked with ultraviolet light to fix the DNA. The membranes were pre-hybridized for at least 2 hours at 55 °C and hybridized with different probes of *T. cruzi* minicircle P^{32}-labeled DNA (1 x 10^6 cpm/membrane). Nylon membranes were then submitted to successive washing at different conditions of stringency [10]. For genotyping, different *T. cruzi* stocks were used to generate the DNA probes to determine the parasite DTUs or mixture infecting each patient or triatomine. Construction of specific probes sp104 c11 TcI, CBB c13 TcII, NR c13 TcV, and V195 cl1 TcVI was performed by amplification of the variable region of *T. cruzi* minicircles; primers for probe generation were CV1 (5'GATTGGGGTTGGAGTACTAT-3') and CV2 (5'-TTGAACGGCCCTCCGAAAAC-3'), which produced a 270-bp fragment [Veas et al., 1991].The specificity of the probes was previously set in DNA blot experiments with *T. cruzi* clones genotyped by other methods [Arenas et al. 2012]. The DNA probes were labelled using the random primer method with [α^{32}P] dATP and the hybridization profiles were analyzed. Finally, the membranes were scanned in a P-Imager (BioRad, USA) for 4-12 hrs. We used the chi-square test for comparing the *T. cruzi* DTUs circulating in both hosts and for comparing *T. cruzi* DTUs detected and no detected in both hosts.

III. RESULTS

We obtained different *T. cruzi* DTUs frequencies in patients and in *T. infestans* (Table 1). Tc I was the most frequent DTU in *T. infestans* but less frequent in humans. In contrast, Tc V was the most frequent DTU found in humans and less frequent in *T. infestans* ($\varkappa^2$= 63.75; d.f. = 1; p=0.05). These two DTUs were the most prevalent; however, we also found two other *T. cruzi* DTUs (Tc II and occasionally Tc VI) which are circulating in *T. infestans* and humans in all the endemic areas of Chile, with no evident geographical distribution (Table I). We found *T. cruzi* DTUs alone or combined with other different DTUs, representing mixed infections. Fig.2 shows representative results of single and mixed infection of patients and Fig. 3 the same in *T. infestans.* We detected more mixed infections in humans than in *T.infestans.* As shown in the rate mixture of lineages/total studied, and the relative percentage of each. The average frequency of TcV detected in humans was very similar in the different geographic areas studied. Even though the space of this longitudinal study covered about 2000 kms and included the Atacama Desert, which splits the country into two endemic areas, the Norte Grande and the Norte Chico. The principal endemic area is the semiarid Norte Chico, in the III, IV, and V political regions. Other *T. cruzi* DTUs different to Tc I, Tc II, TcV and Tc VI are circulating in both kinds of hosts in all endemic areas studied. We found these unknown *T. cruzi* DTUs more frequently in humans than in *T. infestans* (43.4% vs. 11.9%) ($\varkappa^2$= 11.48; d.f. = 1; p=0.05). Frequency (%) of mixed infections with more than one *T. cruzi* DTUs, including those not determined, in patients exceeds that found in *T. infestans* (156.2 versus 120.5 respectively).

TABLA 1
***TRYPANOSOMA CRUZI* DTUS (TcI, TcII, TcV, TcVI) IN CHILEAN PATIENTS AND IN *TRIATOMA INFESTANS* (T.I.)**

Geographic region N>S	Patiens TcI	TcII	TcV	TcVI	ND	M/TE	*T. i.* TcI	TcII	TcV	TcVI	ND	M/TE
XV Arica	4	3	2	0	6	3/10	*	*	*	*		
I Tarapacá	3	5	6	0	1	3/10	*	*	*	*		
II Antofagasta	1	1	5	1	4	2/8	*	*	*	*		
III Atacama	0	2	2	1	5	1/8	21	2	5	0	7	4/24
IV Coquimbo	0	1	4	2	5	2/7	12	4	0	0	1	0/17
V Valparaíso	1	2	7	4	2	4/9	24	9	0	0	3	4/28
R. Metropolitana	3	3	5	1	4	3/9	23	0	0	0	0	0/23
VI O'Higgins	2	3	4	0	3	2/8	*	*	*	*		
Total	14	20	35	9	30	20/69	80	15	5	0	11	8/92
Frequency(%)	20.2	28.9	50.7	13.0	43.4	28.9	86.9	16.3	5.4	0	11.9	8.6

N>S: north to south ND: non determined * without samples M/TE: mixture of lineages/ total studied

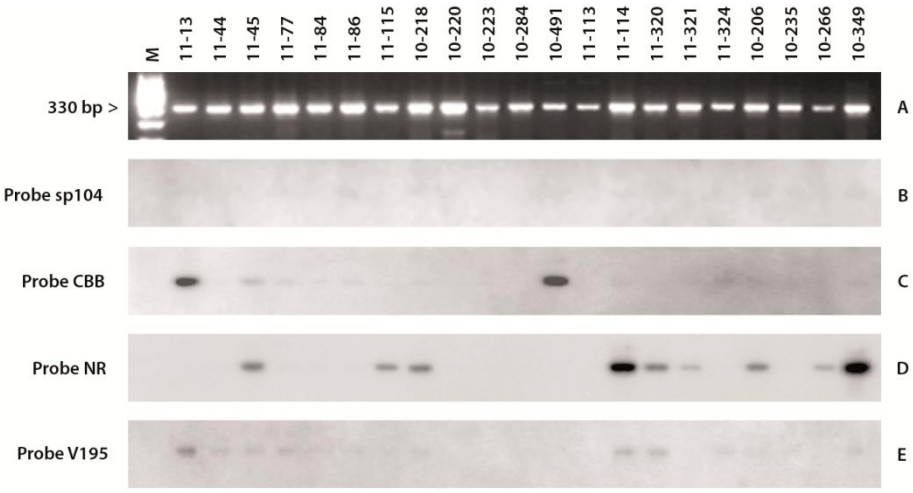

FIGURE 2. RESULTS WITH PATIENTS. TRYPANOSOMA CRUZI AMPLICONS STAINED WITH ETHIDIUM BROMIDE (A). HYBRIDIZATION PROFILES OBTAINED WITH GENOTYPE SPECIFIC PROBES CORRESPONDING TO TcI (B), TcII (C), TcV (D), and TcVI (E). THE 330 BASE PAIR (bp) PRODUCT REPRESENTS A POSITIVE ASSAY

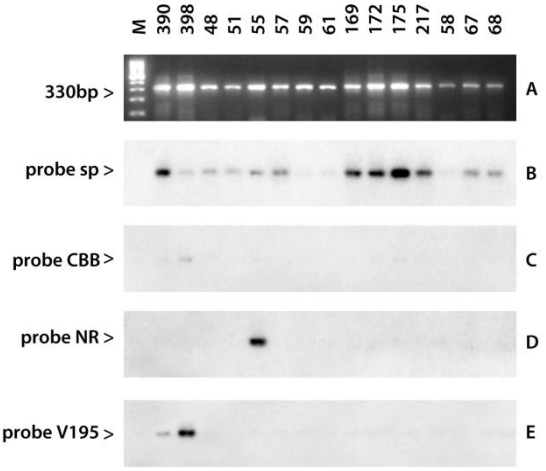

FIGURE 3. RESULTS WITH TRIATOMA INFESTANS. TRYPANOSOMA CRUZI AMPLICONS STAINED WITH ETHIDIUM BROMIDE (A). HYBRIDIZATION PROFILES OBTAINED WITH GENOTYPE SPECIFIC PROBES CORRESPONDING TO TcI (B), TcII (C), TcV (D), and TcVI (E). THE 330 BASE PAIR (bp) PRODUCT REPRESENTS A POSITIVE ASSAY

IV. DISCUSSION

Previous epidemiological surveys of *T. cruzi* lineages isolated from patients and triatomines of the Norte Chico have been performed [Miles et al., 1984]. However, the studies in the Norte Grande involved small sample sizes [Arribada et al., 1986; González et al., 1995]. In the present study a large number of mixed infections in humans (TcV plus another DTU) were found in the less endemic arid areas of Norte Grande (XV, I and II regions), with levels of mixed infections similar to the most endemic ones (III, IV, and V regions). The *T. cruzi* DTUs composition in *T. infestans* (mainly TcI) also presented a very homogeneous distribution all over the studied area, as previously described [Bacigalupo et al., 2012]. Interestingly, unknown genotypes were not as frequent as in human patients. Probably these unknown genotypes are not well adapted to this invertebrate host or are eliminated in *T.infestans* under starvation, since the insects studied were dead. These unknown genotypes probably are variants of TcI and TcII for which there is great heterogeneity as determined by cytochrome b gene sequencing [Arenas et al. 2012]. There studies performed with *T. cruzi* stocks isolated in patients of Chile [Miles et al., 1984; Apt et al., 1987; González et al., 1995]; which select specific *T. cruzi* DTUs from a natural mixture as demonstrated [Deane et al., 1984; Miles and Cibulskis, 1986]. The frequency order of *T. cruzi* DTUs isolated from humans in the IV region was TcV, TcII, TcI [Miles et al., 1984; Apt et al., 1987]. However, a high prevalence of Tc I *T. cruzi* stocks was only detected in *T. infestans* of Tarapacá (I region) [Allen, 1984]. Other studies reported the presence of Tc I and only very few Tc V in *T. infestans* of southern Peru [Allen, 1984]. However, the most complete studies are from Bolivia, with surveys of *T. cruzi* stocks from *T. infestans* in diverse geographic areas close to the Chile border. Tibayrenc et al. 1986 reported TcI and only very few Tc V in the north (La Paz), TcI, TcV, TcVI and TcII in the south (Tupiza and Tarija), and TcI and TcV in eastern Bolivia (Santa Cruz). The *T. cruzi* DTUs composition in the west side of Argentina close to the Chilean border is also available. In the north area near the Andes the *T. cruzi* DTUs found in humans are TcV, but other DTUs such as TcI, TcII and TcVI also have been described [de Luca D'Oro et al., 1993; Macina et al., 1987; Diosque et al., 2003]. However, other surveys in northwest Argentina including domestic animals and *T.infestans* recorded mainly TcVI [Cardinal et al., 2008]. Results obtained with the same methods used here of *T. cruzi* DTUs in Argentinean patients confirmed the presence of TcV and TcVI alone or mixed [Diez et al., 2010]. In conclusion, the analysis of *T. cruzi* DTUs composition between patients and *T. infestans* in Chile found significant differences, even though the route of transmission to humans is through *T. infestans* than by vertical transmission or blood transfusion. TcV was most often found in patients infected several years ago, while the most often found currently in *T. infestans* is TcI A similar conclusion was reached in an area of Cochabamba, Bolivia. With the same direct genotyping method with no or little triatomine intervention; Tc I and TcV are prevalent in *T. infestans* and Tc V in humans, respectively [Brenière et al., 1998]. The results in Chile may account for the effectiveness of the method of flow interruption of *T. cruzi* DTUs between the vector and the human host, but this conclusion should be considerer with caution. The same differences were obtained in Bolivia without or very few insecticide applications. This difference suggests differential adaptation of the *T. cruzi* DTUs to different hosts. In Chile and from some areas of Bolivia the major DTUs Tc I and TcV alone or combined were prevalent; the former appears to be better adapted to the triatomine and the latter to the human host and distributed over a wide geographic area. These results also suggest that both *T. cruzi* DTUs are successfully circulating in nature, one more prevalent in the vertebrate and the other in the invertebrate host. Thus, both *T. cruzi* DTUs coexist due the alternation of parasites in the two kinds of hosts. This observation has pivotal importance, since a digenetic parasite such as *T. cruzi* will have more opportunities to amplify than in a monogenic one. Intrestingly Tc V seems to be more pathogenic compared with Tc I evaluated in a murine experimental model [Wallace et al., 2001].

V. ACKNOWLEDGEMENTS

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Occupational Safety and Health Practices In Agricultural and Livestock Research Organisations, Western Kenya Region

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***Abstract*—** *The Kenya agricultural and livestock research organisation (KALRO) western region is divided into KALRO-Kakamega (non-ruminant) and KALRO-Kitale (food crops) mandated to innovate, improve technological activities that touch on the environment and the livelihood of people. Such practices should comply with occupational safety and health Act (OSHA, 2007) standards. The aim of the study was to assess safety awareness at KALRO-Western Kenya region during the months of April to June 2016. Structured questionnaires, checklist, photographs and observation tools were used for data collection and analyzed using statistical package (SPSS). Study shows that75% of the respondents in KALRO-Kakamega and 79% of the respondents in KALRO-Kitale (χ^2=.187, df=1, p=.665) agreed that both institutes had safety and health policy. Respondents in KALRO-Kakamega (55%) and respondents in KALRO-Kitale (63%) (χ^2=.813, df=1, p=.367) had access to such policies necessitating requirement for sensitization to access policy document. Respondents in KALRO-Kakamega(55%) and respondents in KALRO-Kitale (52%) (χ^2=9.482, df=4, p=.050) indicated that only qualified service engineers maintained machines and equipment. The respondents in both KARLO institutions read labels before using the chemicals KARLO Kakamega 88.7% and KARLO-Kitale 84.2% (χ^2=.511, df=1, p=.475).Compliance to Safe work procedure as per institution (KALRO-Kakamega 70%, KALRO-Kitale 63%) (χ^2=.570, df=1, p=.450). From the research findings, there were no significant differences in predictor factors for safety awareness at both KALRO-Kakamega and KALRO-Kitale. Training of workers to identify, classify and quantify hazards should be enhanced at the two institutes in order to raise their safety awareness levels as per (OSHA, 2007) standards.*

***Keywords*— food crops research, health, KALRO, Non-ruminant research, safety.**

I. INTRODUCTION

The KALRO Western Kenya Research Institutes were created under the Kenya Agricultural and Livestock Research Act of 2013.The Institutes' main focus is to develop improved technologies that support the upgrading and commercialization of both the non-ruminant livestock and food crops value chains. . KALRO-Western research Institutes conducts focused research on non-ruminants (pigs, poultry, and rabbits) and food crops (cereals, grain legumes, and root and tuber crops) with a potential for commercial farming (KALRO, 2017).

The agricultural production carried out in KALRO-Western Kenya involves crops and live stocks activities. The interaction relationship between people, machines, work environment activities bring about occupational safety and health issues (Kohn, P.J. :Friends, A. M. :Winterberger, 1996). There is no known study carried out to establish the extent of implementation of OSHA standards at the two research institutions since incorporation of KALRO- Mandate – Nationally.

An occupational safety and health plan, anticipates and prevents health problems or hazards that are caused by the work which people do or innovate (Rukunga, 2001). Promotion of safety and health to workers is part of the battle against major scourges of poverty, ignorance and diseases that is still handicap to many developing countries such as Kenya (Glanville, Schilling, & Wood, 1979). The owners or occupiers of Agricultural Production institute in Kenya are required by the Occupational Safety and Health Act (OSHA, 2007) to carry out initial risk assessments in order to manage their risks at the source. Systematic application of management policies, procedures and practices to tasks help identifying, analyzing, evaluating, treating and monitoring Risk (Hughes & Ferrett, 2008). Process whereby decisions are made to accept known or assessed risk and/or the implementation of actions to reduce the consequences of probability of occurrence is referred as risk management (Jeremy, 2006). Risk management covers a wide range of hazards and these can be conveniently categorized under the general headings of environment, technical/economic and social/people hazards. Thus risk management is a system of managing risks at work (GOK, 2004). KALRO-Western, like any other production system in Kenya, is expected to create a safe work environment and ensure workers are not affected by workplace hazards in their operations, for efficient and effective unit achievement of their mandate. The purpose of this study was to determine safety awareness among workers and managers in KALRO - Western Kenya region.

II. MATERIAL AND METHOD

2.1 Study area

The study was carried out at KALRO - Western Kenya which comprises of Non-ruminant research Institute (Kakamega) and Food crop research Institute (Kitale) during the months of April to June of 2016.

Purposive sampling technique was used for selection of the two KALRO institutions. Both research institutes areas cover approximately 100ha of land for research, pastures and commercial farming. The target populations were managers, supervisors and workers at all levels of production. The organisations had 500 workers hired on casual and contract basis during planting and harvesting activities. The technical team of 142 officers (scientists, laboratory technicians, research officers, and research assistants) as indicated in records of each institutional administration.

2.2 Study design

Descriptive cross sectional design was applied in the study which involved structured questionnaires, checklist, photographs and observation tools to collect the required data. The questionnaires were delivered to the respondents. Checklists and observation tool were used to assess the location, process, environment, structure and related factors (hazards) at the workplace.

2.3 Analysis of data

Data were analysed using Statistical Package for Social Scientists (SPSS)-version 21.0. The program was used to analyze questionnaires from respondents, edited to completeness, relevance and accuracy. The data was then coded to enable the responses to be grouped into categories for both closed and open ended questions. The statistical data analysis was done using descriptive statistics and displayed using bar charts, tables, frequencies. Inferential analysis conducted for goodness of fit and contingency analysis. Goodness of fit was used for testing what is recommended, expected and what was observed.

III. RESULTS

3.1 Safety and health policy

Majority of the Workers were aware that safety and health policy was present at their institutions as illustrated in figure 1 (Kakamega 75%, Kitale 79%,(χ^2=.187, df=1, p=.665)

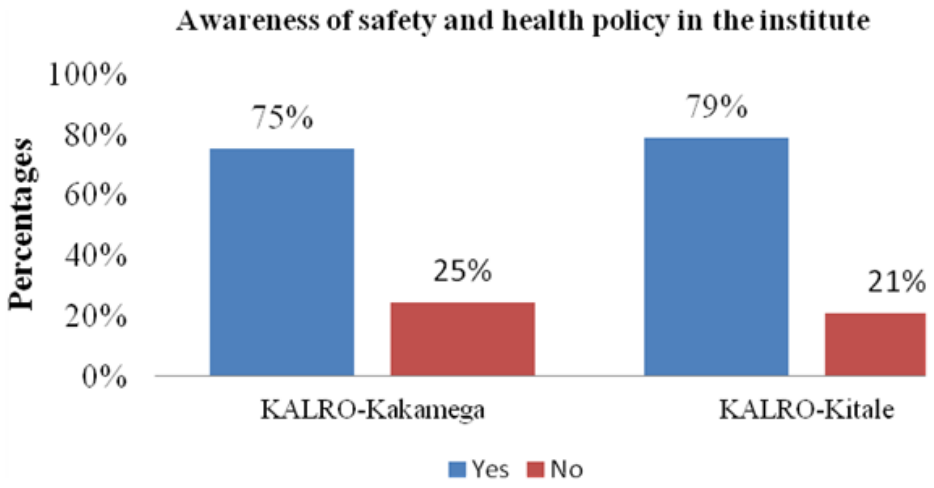

FIGURE 1: EMPLOYEE AWARENESS OF SAFETY AND HEALTH POLICY

Awareness of safety and health policy will enhance the performance of an organization through personal development of workforce in avoiding accidents to reduce Insurance financial premium losses (Hughes & Ferrett, 2008).

3.2 Workers access to safety and health policy

Majority of workers in KALRO-Western Kenya; Kakamega 55%, Kitale 63%,have access to a copy of the safety and health policy, while 45% of workers in Kakamega research institute and 37% in Kitale research institute have no access (χ^2=.813, df=1, p=.367) as illustrated in Figure 2.

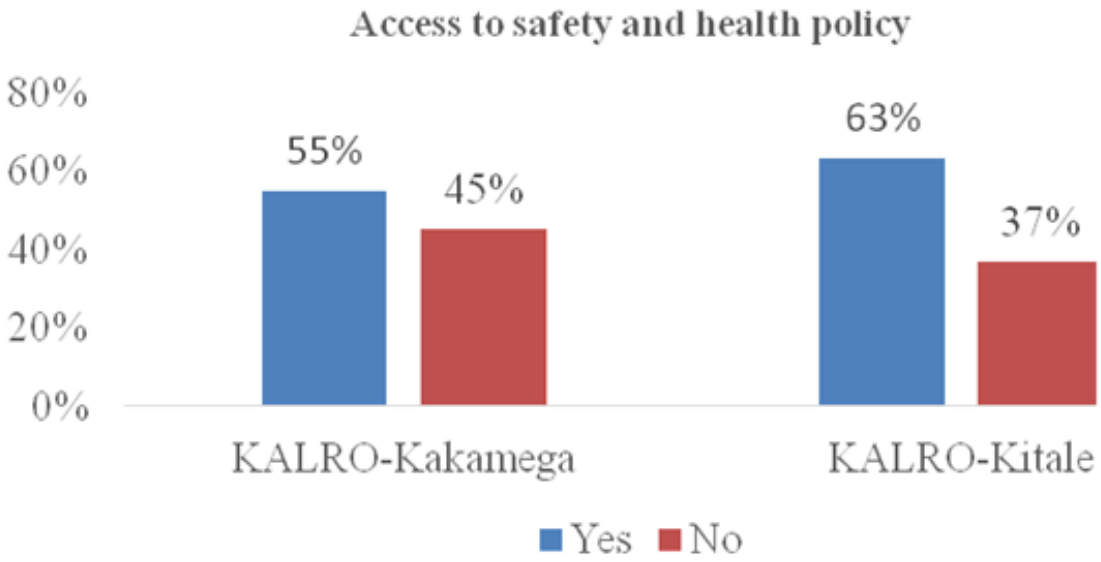

FIGURE 2: ACCESSIBILITY TO SAFETY AND HEALTH POLICY

3.3 Awareness of policy provisions on health and safety

The employee awareness on safety and health policy provided per age are presented in the table 1.

TABLE 1
EMPLOYEE AWARENESS ON SAFETY AND HEALTH POLICY PROVISIONS AS PER AGE

		Respondents awareness of safety and health policy in the institute		Total	Chi-Square Tests
		Yes	No		value 13.674
Age of respondents	18-28 Years	38%	62%	100%	df=3 Asymp. Sig. (2-sided) .003
	29-39 Years	83%	17%	100%	
	40-49 Years	94%	6%	100%	
	Above 50 years	81%	19%	100%	
Total		76%	24%	100%	

Although there is a large number of workers saying they have access to a safety and health policy, the low significance (χ^2=13.674, df=3, p=.003) which is less than .05 suggest that there is a big difference between those who access and those who do not access as per age. The chi square value asymptotic significance indicates the awareness of safety and health policy is significant in raising safety awareness.

3.4 Pesticides procurement, storage and disposal

KALRO-Western-Kenya procures their Chemicals (pesticide) through the procurement department as requested by the user sections. Research findings revealed that pesticide storage in farm store faces a myriad of challenges including excessive purchase of chemicals/pesticides and storage of excessive unused chemicals/ pesticides which is not a good practice as per occupational safety and health regulation. Chemicals/Pesticides are supposed to be stored in a well ventilated and proper system of lighting for easy retrieval of the same to minimize aerosol exposures to them which is a good practice as per occupational safety and health regulation Act (2007). From the interviews, excess chemicals/pesticides not used is often sprayed on barren land and containers perforated before disposal. Safe disposal of pesticides should involve decontamination of unutilized toxicants, proper disposal methods and Label reading before using chemical to be sought.

3.5 Awareness of safe work procedures

Among the total number of employees in KALRO-Western Kenya, Kakamega 70%, Kitale 63% (χ^2=.570, df=1, p=.450) were aware of safe work procedure in their respective research institute for routine operations in the workplace activities.

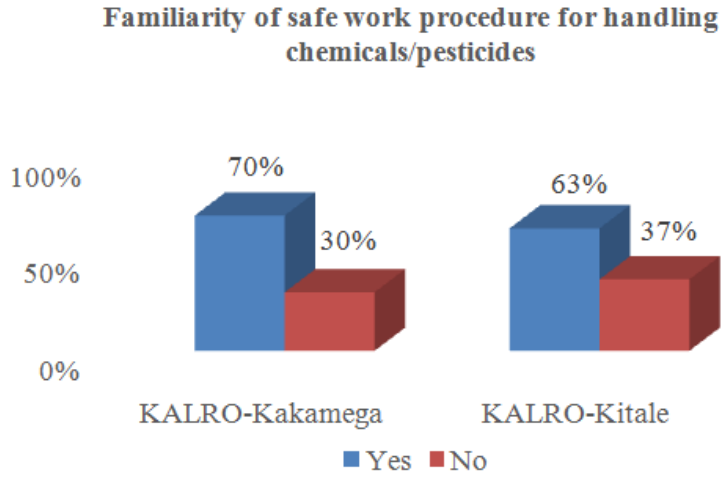

FIGURE 3: EMPLOYEES AWARENESS OF SAFE WORK PROCEDURES

Workers who do not follow safe work procedure observed in the population did not go through either secondary, college or university education were prone to accidents; Kakamega 30% , Kitale 37% . During safe work procedures development, hazards that are likely to cause injuries are identified, assessed, evaluated and controlled, having positive impact towards raising level of safety awareness at workplace. The high percentage of safety and health awareness in research institutions is due to high pronouncement of safe work procedures in their daily activities.

TABLE 2
AGE OF RESPONDENTS AND COMPLYING TO WRITTEN WORK PROCEDURES

		Availability of Written work procedures to be followed while working		Total
		Yes	No	
Age of respondents	18-28 Years	46%	54%	100%
	29-39 Years	83%	17%	100%
	40-49 Years	88%	12%	100%
	Above 50 years	61%	39%	100%
Total		68%	32%	100%

KALRO-Western Kenya employees (18-28 years) 54% do not follow written work procedure at their workplace hence are required to be sensitized to follow written work procedures in order to avoid accidents at the workplace.

3.6 Label reading before using pesticides/Chemicals

The respondents in both KARLO institutions read lebels before using the chemicals (KARLO-Kakamega 88.7% and KARLO-Kitale 84.2%, (χ^2=.511, df=1, p=.475, table 3).

TABLE 3
EMPLOYEES READING LABELS ON CHEMICALS /PESTICIDES BEFORE USE

	Reading labels on chemicals/pesticides before use by respondents		Total	chi square tests
	Yes	No		p value = .511
KALRO-Kakamega	88.7%	11.3%	100.0%	df = 1
KALRO-Kitale	84.2%	15.8%	100.0%	assymp sig (2 sided) = .475
Total	87.5%	12.5%	100.0%	

3.7 Population that is aware of procedures for mixing categories of Pesticides

From the results table 4, 51% in KARLO-Kakamega and 42% in KARLO-Kitale (χ^2=.875, df=1, p=.350) indicated that they mix various categories of pesticides before using them. The statistics tests indicate that there is no significant difference between the two centres when it comes to knowledge of mixing pesticides to achieve sound results. Mixing and loading remain a potential source of pesticide exposures (Kariathi *et al*., 2016). This is supported by Cornell (1992) study that pesticides must only be issued to staff who has appropriate training. The trained operators will be aware of the pesticides being used, able to carry out spraying safely and take precaution in case of a spill.

TABLE 4
EMPLOYEES DIRECTLY EXPOSED TO CHEMICALS THROUGH MIXING THEM

	Mixing of various categories of pesticides by respondents		Total	Chi square tests
	Yes	No		value = .875
KALRO-Kakamega	51%	49%	100%	df=1
KALRO-Kitale	42%	58%	100%	Assymp sig (2 sided) = .350
Total	49%	51%	100%	

3.8 Knowledge of handling plant and animal waste

The level of agreement that animal and plant waste are highly handled in a hygienic manner was high in KALRO Kakamega with a percentage of 62% followed with KALRO Kitale 42% (χ^2=5.986, df=4, p=.200).

TABLE 5
EMPLOYEES KNOWLEDGE OF HANDLING PLANT AND ANIMAL WASTE

		Animal and plant wastes handled in a highly hygienic manner in the institute					Total	Chi square tests
		Strongly disagree	Disagree	Neutral	Agree	Strongly agree		value 5.986
Respondents institute of work	KALRO-Kakamega	6%	19%	11%	53%	9%	100%	df=4
	KALRO-Kitale	16%	21%	21%	37%	5%	100%	Assymp sig (2 sided) = .200
Total		10%	19%	16%	47%	8%	100%	

The overall agreement is at 55% in KALRO western Kenya in handling wastes and protection of workers from biological agents while working. The protection may be rated as moderate/fair in KALRO western Kenya. There was no significance difference between the two centres when it comes to knowledge in handling plant and animal wastes.

3.9 Chemicals/Pesticide poisoning and emergency action plan awareness

Workers are not aware of emergency action plan in case of chemicals/ pesticide poisoning and any other form of emergency Kakamega 51%, Kitale 63%. (χ^2=1.681, df=1, p=.195).

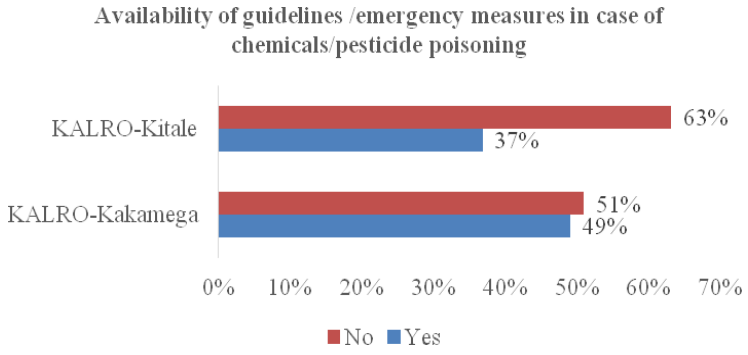

FIGURE 4: AVAILABILITY OF EMERGENCY MEASURE AND GUIDELINES

There are only Kakamega 49%, Kitale 37% workers that are aware of emergency measures in case of emergency or chemical/pesticide poisoning due to their specialized task undertaking at their workplace. There was no significant difference between the two centres when it comes to chemical emergency action plan. In the event of chemical/pesticide poisoning, the first aid procedure arrangement for calling ambulance and rescue team is not well known to the staff in KALRO-Western Kenya. There is a need to put in place a more detailed emergency plan which may take account of findings of initial risk assessment for incidents/accidents at work place. Emergency procedure is about control procedure and equipment to limit the damage to people and property caused by an incident/accident, for instance pesticides should always be applied in the direction of the flow of wind. Application of pesticides should be started near downward edge of the field and should proceed upward with the back to the wind so that the operator is always in an untreated area. If there is substantial drift/windy period, pesticides should not be applied. Mathew G. A. (1985) noted that if pesticides are accidently swallowed, medical advice should be sought without delay.

3.10 Machinery and equipment safety

TABLE 6
KNOWLEDGE ON MAINTENANCE OF EQUIPMENT AND TOOLS

	Equipments provided are well maintained by qualified service engineers					Total	Chi square tests
	Strongly disagree	Disagree	Neutral	Agree	Strongly agree		value = 9.482
KALRO-Kakamega	9%	17%	19%	42%	13%	100%	df = 4 Assymp sig (2-sided) = .050
KALRO-Kitale	26%	11%	11%	47%	5%	100%	
Total	14%	15%	17%	43%	11%	100%	

Workers agree that equipment and tools used on the farm are well maintained 54%. The level of agreement was high in KALRO-Kakamega 55%, KALRO-Kitale was 52% (χ^2=9.482, df=4, p=.050).There is significant difference in terms of maintenance of equipment and tools in the two centres due to administrative priority put to safety of machines equipment at two centres. This was due to the nature of specialized tasks at workplace. Work equipment needs to be properly maintained so that it continues to operate safely and in the way it was designed to perform. The amount of maintenance will be stipulated in manufacturers' instructions and will depend on the amount of use, the working environment and type of equipment. High speed, high-hazard machines, which are heavily used in adverse environment, may require very frequent maintenance, whereas simple hand tools may require very little maintenance.

KALRO-Western Kenya workers and managers are aware as to why equipment maintenance is important in order to control machinery risks and poor ergonomic issues at KALRO-Western which should be enhanced further.

IV. CONCLUSION

From the research findings, the management and workers in KALRO-Western Kenya region access the safety and health policy which is evidence based for decision making in creating safety awareness. There were no significant differences in predictor factors for safety awareness at both KALRO-Kakamega and KALRO-Kitale. Positive predictors of safety awareness in the two research organizations included compliance to safe work procedures, reading pesticides labels before handling or mixing, good machinery maintenance practices, knowledge of handling plant and animal waste, in line with(OSHA, 2007) standards.

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Meteorological Conditions: Influence on Yield, Sanitary Status and Grape Composition

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Abstract— *The current study aimed to establish which meteorological conditions have the strongest impact on grapevine yield, sanitary status and berry composition, as well as checking their relative importance in relation to management practices and grapevine variety. Weather data was correlated to yield, sanitary status and grape composition of three varieties (Cabernet-Sauvignon, Merlot and Tannat) under two trellis systems (lyre and vertical shoot positioning), with or without yield control (pruning type and cluster thinning) over four seasons throughout the south of Uruguay. Principal component analysis showed that weather variables explained, respectively, 57.3%, 64.3% and 57.8% of the variance in yield, sanitary status and grape composition within the studied dataset. Hierarchical Clustering grouped years, confirming that the relevance of weather interannual variability was greater than that of genetics and management practices. Yield depended on bunch number, which was determined by rainfall and temperature. Water statuses during the first stage of the growing cycle are determinant for bunch rot infection, as well as thermal and hydric conditions that prevail during maturation. Grape compounds were positively correlated to thermal sum at the beginning of the growing cycle and negatively with high temperatures and water availability in maturation. Our results suggest that the favourable intervals of atmospheric conditions for yield and bunch rot are different from those for berry quality.*

Keywords— Berry quality, climate, genotype, viticultural practice, yield components.

I. INTRODUCTION

The main climatic elements that explain variations in grapevine performance and oenological quality are sunlight, temperature and precipitation. Among these, temperature and precipitation have the most marked effect on yield components and berry composition, which are sensitive to their magnitude, variations and distribution over the crop cycle [1,2]. Bunch number per plant explains about 60-70 % in the interannual variation in grapevine yield [3]. Initiation – induction of inflorescences and floral differentiation take place in the period of budbreak - fruit set, in two consecutive seasons, hence temperature and water availability during this phase are determinant factors for quality and yield in two harvests [4-6]. Water deficits in the season previous to harvest produce yield declines by reduction in number of bunches per plant. For the harvest year, water deficit influences the differentiation of flowers, fruit set or abortion of flowers, fruit, and berry size, leading to variations in yield [4, 7]. Vine water requirements depend on phenological stage, being flowering - veraison (48.2 %) the most demanding period over a total of 750 mm required during the growing season [8]. *Botrytis cinerea* is a serious threat to grapevine and has a negative impact on grape and wine quality. Weather conditions during pre-harvest (frequent precipitations, high relative humidity, mild temperatures and low wind intensity) are key elements for the development of this disease [9,10]. Meteorological conditions accounted for 88 % of the total variability on grape composition, a higher percentage than that explained by variety or soil [11]. Wine grape quality between years depends on temperature variability that determines whether grape ripening would be completed, due to its impact on sugar content, acidity degradation and berry anthocyanins balance [12]. The optimum diurnal temperature ranges from 25°C to 30°C. Values over 37°C inhibit sugar accumulation and induce a negative balance in anthocyanins; while the respiration of malic acid accelerates starting from 35°C [12, 13]. During maturation, optimal levels of acidity and a positive balance in anthocyanins require a temperature range night / day of 16 / 25°C; lower temperatures promote high levels of malic acid [13]. Meanwhile, thermal sum in that period, expressed as degree-day base 10ºC, is strongly associated with anthocyanin content [1]. In general, it is recognized that a progressive and moderate water stress from flowering to fruit set favors the accumulation of sugars and anthocyanins, and decreases the acidity associated with the reduction of vegetative growth in the fruit ripening stage [2,14]. Post veraison water stress is responsible for the largest increase in polyphenolic content.

In this context, the current study aimed to establish which meteorological conditions have the strongest impact on grapevine yield, sanitary status and berry composition. An additional objective was to assess when and how weather interannual variability affects grapevine yield and berry quality, as well as checking its relative importance in relation to management practices and variety.

II. MATERIAL AND METHOD

2.1 Description of the study sites

Ten plots distributed throughout the south of Uruguay (34°35'12.43" S; 56°15'2.26'' W), which comprises 76.4% of vineyard surface in the country (INAVI, 2013), were established in commercial vineyards. The climate of this area is Temperate warm, with Temperate nights and Moderately dry according to the Multicriteria Climate Classification [MCC, 15,16]. To cover the full range of situations representative of the conditions present in the region, during four years (2001-2004), three different *Vitis vinifera* L. varieties were studied: Tannat, Cabernet Sauvignon and Merlot (accounting for 47.2% of the surface of red varieties). They were either trellised to lyre or Vertical Shoot Positioning (VSP), representing 98% of vineyard trellis systems in the area, and subjected to yield control: with or without cluster thinning at veraison or type of pruning (spur or cane pruned). Row orientation was north to south and the rootstock was SO4 in all plots For data collection, three rows with ten vines each were randomly selected within the whole vineyard, for each situation. Each individual vine was considered as an experimental unit (30 repetitions). In those plots were cluster thinning was undertaken, the number of clusters left on the vines was 50% of those left in the unthinned vines. Cluster thinning was always performed when grapes attained 5% veraison [Eichhorn-Lorenz Stages, 35 E-L]. In order to make all the plots comparable, the number of buds left at winter pruning was the same (Table 1).

TABLE 1
PLOTS USED IN THE CURRENT STUDY AND ABBREVIATIONS USED FOR REFERRING TO EACH ONE OF THEM

Variety	Management practices	Year of plantation	Plant density (vines/ha)	Abbreviation (*)
Merlot	Lyre spur pruned and no cluster thinning	1994	3300	ML year
Merlot	Lyre spur pruned and cluster thinning	1994	3300	MLT year
Merlot	VSP spur pruned and no cluster thinning	1996	3200	MV year
Merlot	VSP spur pruned and cluster thinning	1996	3200	MVT year
Cabernet-Sauvignon	Lyre spur pruned and no cluster thinning	1994	3320	CSL year
Cabernet-Sauvignon	VSP spur pruned and no cluster thinning	1996	4000	CSV year
Cabernet-Sauvignon	VSP spur pruned and cluster thinning	1996	4000	CSVT year
Tannat	Lyre spur pruned	1992	3472	TLS year
Tannat	Lyre cane pruned	1992	3472	TLC year
Tannat	VSP spur pruned	1996	3478	TVS year

(*) Year refers to the studied season: 2001, 2002, 2003 or 2004.

2.2 Climate conditions

Weather data for the years 2001 to 2004 were collected at a meteorological station (34°40'S - 56°20'W; 32 m above sea level, Davis Instruments, Hayward, CA, USA) located at about 5 km from the studied plots. Historic meteorological data (years 1972-2002) were used for determining the climate class, according to MCC, adapted by Ferrer [16] using specific conditions for Uruguay (growing cycle from September to February and available soil water of the studied plots). Hence, three synthetic and complementary climatic indices were computed: Heliothermal index (HI), Dryness index (DI) and Cool Night index (CI) [15]. Pre-dawn leaf water potential (ΨPD) was determined with a pressure chamber (Soil moisture equipment, Santa Barbara, CA, USA). These measurements were made before dawn at fruitset, veraison and harvest, in 20 adult, healthy leaves per plot. Threshold ΨPD values to evaluate the level of water stress (WS) experienced by vines were: -0.2 MPa no WS; -0.2 MPa > ΨPD ≥ 0.4 Mpa mild to moderate WS; -0.4 > ΨPD ≥ - 0.6 MPa moderate WS; ΨPD < -0.6 MPa severe to high WS.

2.3 Yield components and bunch rot incidence determinations

At harvest, the yield of the 30 plants per plot was individually weighed, the number of clusters was counted and the average weight per bunch was calculated by dividing yield per vine to the number of clusters. Rot incidence was estimated by weighing separately bunches with at least 5% of berries affected and expressed as percentage from the total yield per vine.

2.4 Grape samples and analysis

The harvest was done at "technological maturity" for each treatment, considering pH values, the relation between sugar content and titratable acidity of grapes, and berry weight. These parameters were analyzed periodically according to OIV [17] methods. For this purpose, replicated 250-berry samples from all vines in each plot were collected weekly from veraison to harvest. Berry composition was determined after manually destemming the berries and obtaining the juice by crushing the pulp with an electric blender (HR2290, Phillips, The Netherlands). Sugar contents were measured using a refractometer (Atago N1, Atago, Tokyo, Japan); pH was determined with a pH meter (HI8521, Hanna instruments, Villafranca Padovana, Italy) and acidity, expressed as g sulfuric acid/L juice, was measured by titration. The potential in total (ApH1) and extractable anthocyanins (ApH 3.2) was measured according to the spectrophotometric method (Shimadzu UV-1240 Mini Shimadzu, Japan) proposed by Ribéreau-Gayon and Stonestreet, [18]. The phenolic richness of the grapes (A280) were determined by measuring the absorbance at 280 nm of the pH 3.2 extract according to Glories and Augustin [19]. The indexes were calculated considering the respective dilution of the grape extracts, according to González-Neves et al. [20].

2.5 Statistical analyses

Data were analyzed using multivariate techniques, such as Principal Component Analysis (PCA) and Hierarchical Clustering (HC), to determine significant correlations between meteorological conditions and yield, berry composition and sanitary status. Moreover, a correlation analysis was performed using Pearson's correlation coefficient. Analysis of variance was performed on the surveyed composition variables, followed by the Tukey test for mean separation. All the statistical analyses were carried out using the InfoStat software.

2.6 Variables used in the study

For every variable, the corresponding abbreviation is listed in Table 2.

TABLE 2
ABBREVIATIONS OF THE VARIABLES USED IN THE CURRENT STUDY

Variable	Abbreviation
Rainfall during growing season (mm , 1 september -28 february)	RG
Rainfall from 1 september to harvest (mm)	Rsh
Rainfall from budbreak to fruitset (mm)	Rbf
Rainfall from 1 september to flowering (mm)	Rsfl
Rainfall at flowering stage (mm)	Rfl
Rainfall from budbreak to veraison (mm)	Rbv
Rainfall from budbreak to veraison of previous year (mm)	Rbv-1
Rainfall from veraison to harvest (mm)	Rvh
Rainfall during january (mm)	RJ
Rainfall during february (mm)	RF
Rainfall during ripening period (mm)	Rm
Rainfall 10 days before harvest (mm)	Rh-10
Rainfall 20 days before harvest (mm)	Rh-20
Rainfall 30 days before harvest (mm)	Rh-30
Dryness index during growing season (mm)	DIG
Water deficit of growing season (mm, september at february)	WD
Water deficit during ripening period (mm, january and february)	WDm
Pre-dawn leaf water potential at fruitset (bars)	Ψf
Pre-dawn leaf water potential at veraison (bars)	Ψv
Pre-dawn leaf water potential at harvest (bars)	Ψh
Heliothermal Index (°C, september at february)	HIG
Heliothermal Index from budbreak to veraison (°C)	HIbv
Heliothermal Index from veraison to harvest (°C)	HIvh
Minimum temperature from veraison to harvest (°C)	Tmvh
Cool night Index (°C, Minimum temperature 15 february –15 march)	CI
Maximum temperature at bloom stage (°C)	TMbl
Minimum temperature at bloom stage (°C)	Tmbl
Maximum absolute temperature in January (°C)	TMJ

Maximum average temperature in January (°C)	TMxJ
Minimum temperature in January (°C)	TmJ
Maximum temperature in February (°C)	TMF
Minimum temperature in February (°C)	TmF
Maximum temperature 10 days before harvest (°C)	TMh-10
Maximum temperature 20 days before harvest (°C)	TMh-20
Maximum temperature 30 days before harvest (°C)	TMh-30
Minimum temperature 10 days before harvest (°C)	Tmh-10
Minimum temperature 20 days before harvest (°C)	Tmh-20
Minimum temperature 30 days before harvest (°C)	Tmh-30
Relative Humidity at bloom stage (%)	RHb
Relative Humidity during ripening period (%)	RHr
Wind from veraison to harvest (m/s)	W
Yield/ha (kg)	Y
Clusters number/vine	CN
Cluster weight (g)	Cw
Berry weight at veraison (g)	Bwv
Berry weight at harvest (g)	Bwh
Bunch rot (% yield)	By
Sugar content (g/L)	S
Total acidity (g/L H2SO4)	TA
Total anthocyanins (ApH1)	ApH1
Extractable anthocyanins (ApH3.2)	ApH 3.2
Phenolic richness (ua 280)	A280

III. RESULTS

3.1 Climatic conditions

The classification of each one of the years studied, according to MCC, showed that two years (2001 and 2003) were different from the rest.

2001: Temperate warm, Warm nights, Humid.

2002: Temperate warm, Temperate nights, Moderately dry.

2003: Temperate warm, Temperate nights, Sub-humid.

2004: Temperate warm, Temperate nights, Moderately dry.

Over the 2001 and 2003 growing seasons, HIG values were on the threshold between the Temperate-Warm and the Warm classes (>2400 ºC). The cool night index was lower than the normal value (1972-2002) in three out of the four years studied; however, in 2001, it was higher, corresponding to the class of Warm nights. In 2001 and 2003, the dryness index was higher than the historical average for the site, corresponding to Humid and Sub-humid classes, respectively. When calculated between veraison and harvest, HIvh varied amongst years and in respect to the historical average. In 2002, this index was 17.7% lower than the historical average, whereas in 2004 this reduction was 2.6%. In contrast, the historical value was surpassed by 24.9% in 2001 and no deviations were observed in 2003. Veraison to harvest represented 48.7% of the heat accumulated over the whole growing season (HIvh/HIG) in 2001 (higher than the historical average that is 41.1%), whereas it represented 32.6%, 38.4% and 38.9% in 2002, 2003 and 2004, respectively.

The average maximum temperature in January and the absolute maximum temperature in 2002 and 2004 were below historical values, while they were higher in 2001 and 2003, respectively.

Historical records of rainfall over the growing season (RG) and during fruit ripening were 586.6 mm and 205.5 mm, respectively. In the years of study, RG exceeded the historical average in 2001 by 41.4%, in 2002 by 4.0%, in 2003 12.0% and 24.0% in 2004. In flowering-veraison, rainfall accounted for 26.8% of the cycle in 2001 and between 46.4% and 48.1% in the other 3 years studied. Rainfall during fruit ripening exceeded the historical record in 2001 by 125.0%, while the other three years were below this record. Water deficit over the growing season was higher than the historical record for 2004 and lower for the rest of the studied years. During grape ripening in 2001 there was no WDm deficit, whereas the rest of the years

exceeded the historical deficit for this period; in more than 65 mm in 2002 and 2004 Vine water status reflects the evolution and accumulation of soil water content. At bloom - fruitset, Ψ_{PD} showed values of no WS, a situation that continued in 2001 during the whole growing season. Vine water status at veraison and harvest (Ψv_{PD}, Ψ_{HPD}) varied depending on the year. In 2003, the ranks of restriction registered were of no WS at veraison and mild to moderate WS at harvest. At veraison, in 2002, values of moderate to severe WS were recorded, evolving to no WS at harvest. In 2004 at veraison, mild to moderate WS was recorded, evolving to severe and high WS at harvest (Table 3).

TABLE 3
CLIMATE CONDITIONS OF THE STUDIED YEARS AND NORMAL VALUES FOR THE 1972-2002 PERIOD

Year	HIG (°C)	HIvh (°C)	CI (°C)	DIG (mm)	TMxJ (°C)	TMJ (°C)	RG (mm)	Rm (mm)	WD (sep at feb) (mm)	WDm (mm)
2001	2390	1136.3	18.8	157.1	29.2	35.6	829.4	462.3	-117.5	90.2
2002	2306	751.4	16.0	35.3	27.9	34.1	610.0	111.5	-309.3	-240.5
2003	2391	918.1	15.3	120.3	29.5	38.8	657.0	169.4	-282.0	-198.6
2004	2285	889.3	14.2	47.4	28.6	34.0	715.8	152.0	-336.9	-242.9
1972-2002	2220	913.0	16.75	47.5	28.9	34.8	586.6	205.5	-313.4	-172.5

3.2 Yield components

The first two principal components (PC) explained 57.3% of the total variance in the dataset; PC1 and PC2 accounted for 37.6% and 19.7%, respectively. Load vectors of yield and its components contributed to PC2. Load vectors of climatic elements (CE) contributed to the highest values of PC1. The plots of 2001 are separated in PC1 (CE) and those of 2003 in PC2 (lower CN), while those from 2002 and 2004 are relatively close (Fig. 1)

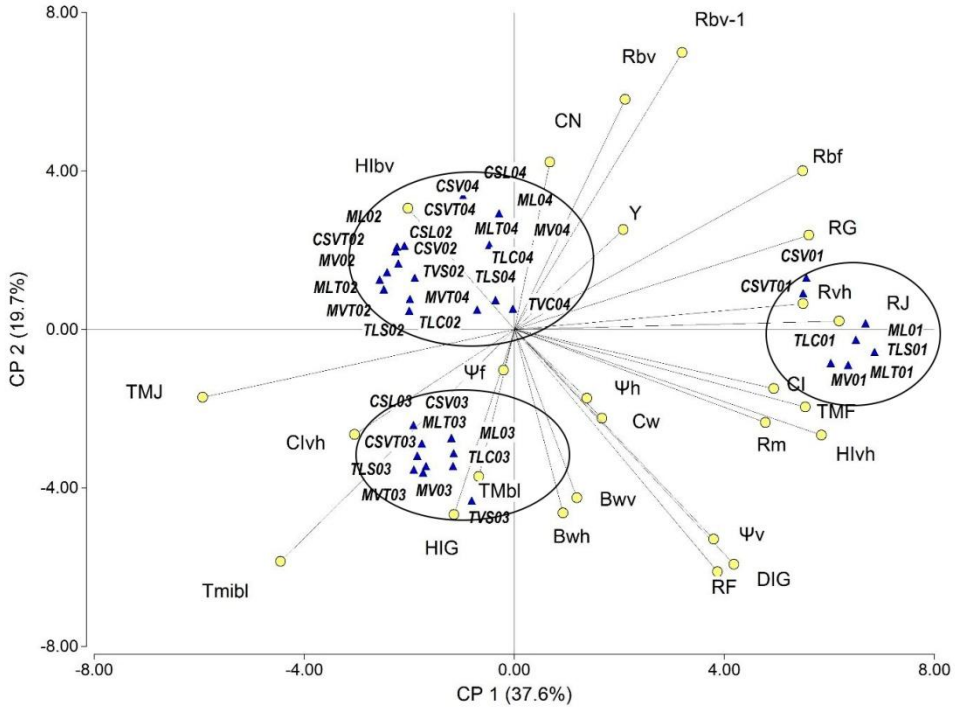

FIGURE 1: PRINCIPAL COMPONENT ANALYSIS OF THE STUDIED PLOTS ACCORDING TO YIELD COMPONENTS AND CLIMATE ELEMENTS. ABBREVIATIONS OF THE VARIABLES ARE LISTED IN TABLES 1 AND 2

The yield and its components were positively correlated with the hydric conditions of the current cycle and of the previous cycle and negatively with the thermal conditions.

Cluster number ($r = 0.56$, $p < 0.001$) and bunch weight ($r = 0.49$, $p < 0.001$) were the overriding factors contributing to yield variation. Nevertheless, berry weight at veraison or harvest did not show correlation with yield. The years 2003 and 2004 had a significantly different number of clusters per plant (16.50 vs 25.45, respectively) and the hydric and thermal conditions were also different in those years; 2004 recorded higher volumes of rainfall compared to 2003. In contrast, 2004 thermal conditions were cooler compared to 2003.

It was noticed that 2002 and 2003 were the most similar years, while 2001 was the most different amongst the four (Fig. 2), confirming the PCA results. In addition, within each year, the effect of variety on yield was greater than that of crop management.

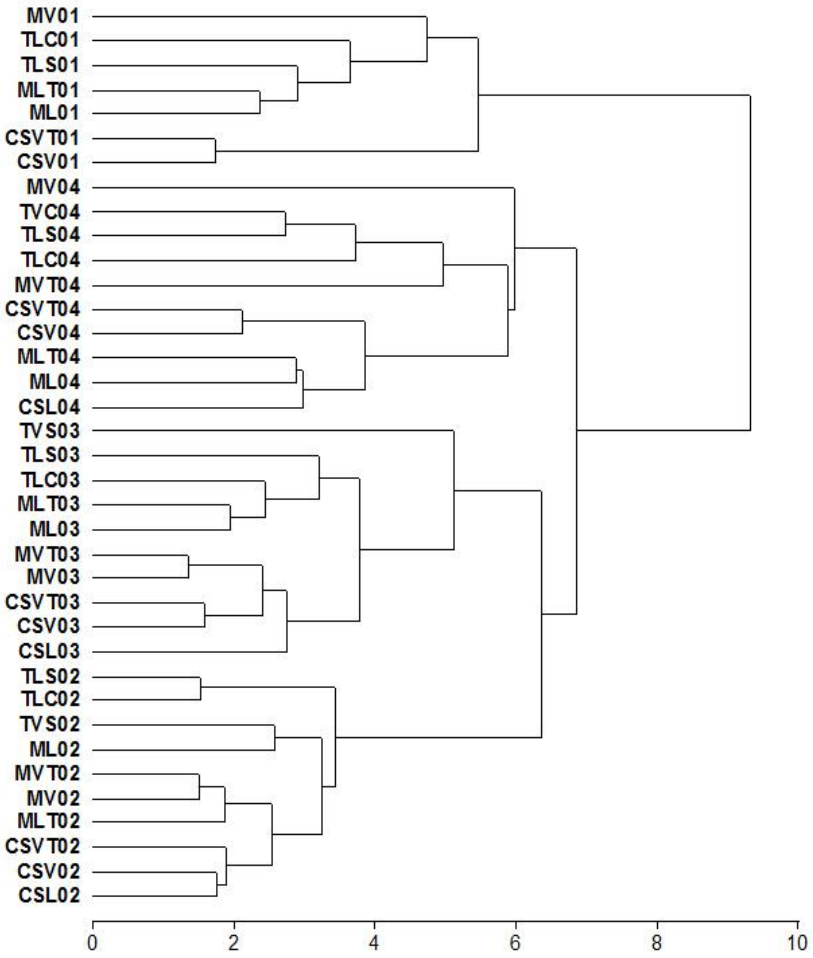

FIGURE 2 ASCENDING HIERARCHICAL CLASSIFICATION (EUCLIDIAN MEAN) WHICH REGROUPS THE YEARS - VARIETY-CULTIVATION TECHNIQUES FOR SIMILARITY OF YIELD AND ITS COMPONENTS. ABBREVIATIONS ARE LISTED IN TABLE 1

3.3 Berry sanitary state: rot incidence

The clusters affected by rot contributed significantly in reducing grape yield ($r = -0.79$, $p < 0.001$). The first two PC included 64.3% of the total variance in the dataset. Respectively, PC1 and PC2 explained 46.6% and 17.7% of the variance in the data set. The vector load bunch rot incidence (By) contributed to PC2. The plots of 2001 are clearly separated from the rest in PC1. PC2 separated plots of 2004 from the rest, while the plots of 2002 and 2003 were relatively close and had the lowest incidence of bunch rot (Fig. 3). Load vectors of thermal and hydric conditions of the month prior to harvest and the period from September to flowering contributed positively to PC1 with the highest values. September rainfall at harvest, the minimum temperatures in January and relative humidity during ripening contributed positively to PC2.

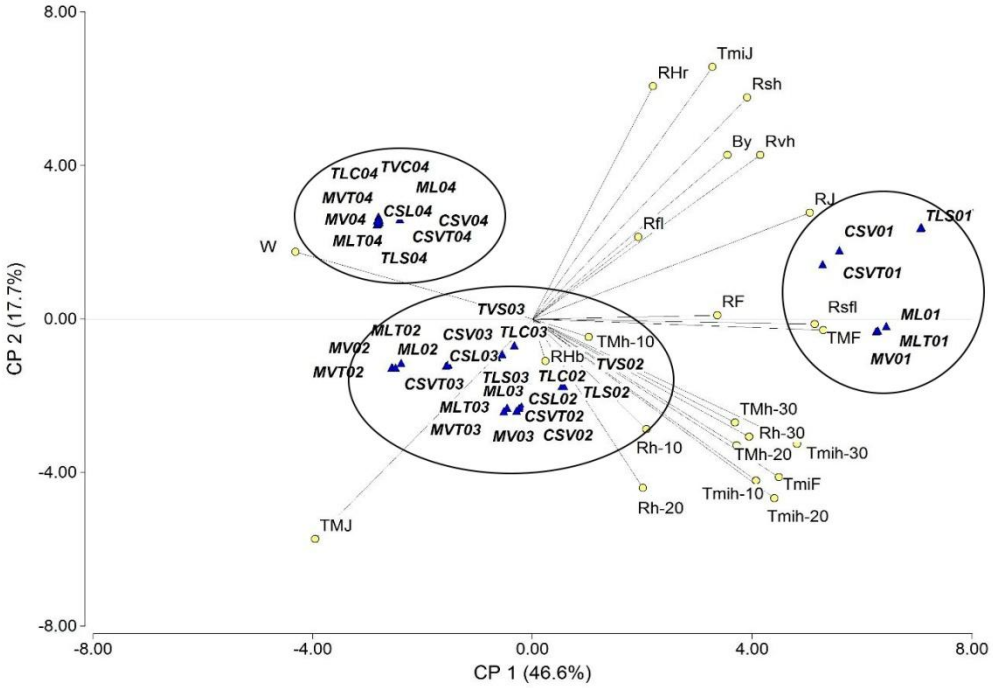

FIGURE 3: PRINCIPAL COMPONENT ANALYSIS .DISTRIBUTION OF PLOTS DEPENDING ON WEATHER AND BUNCH ROT INCIDENCE. ABBREVIATIONS OF THE VARIABLES ARE LISTED IN TABLES 1 AND 2

In 2001 there was a significantly higher incidence of bunch rot (53.4%) compared to 2003 (6.0%). In 2001, weather conditions related to rainfall exceeded to their corresponding values in 2003. In contrast, thermal conditions were higher in 2001 when compared with 2003. However, wind intensity during the ripening period of 2001 was lower than in 2003.

The four years studied were clearly differentiated in the cluster analysis (Fig. 4). In addition, within each year, the plots of a given variety are perfectly grouped, differing from other varieties. The plots of 2001 are those separated by a greater distance from the rest.

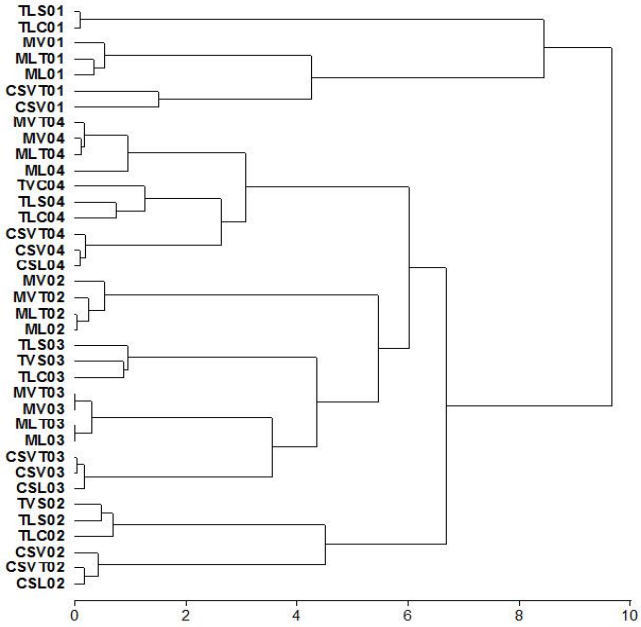

FIGURE 4 ASCENDING HIERARCHICAL CLASSIFICATION (EUCLIDIAN DISTANCE) WHICH REGROUPS THE YEARS - PLOTS BY SIMILARITY OF BUNCH ROT INCIDENCE. ABBREVIATIONS OF THE VARIABLES ARE LISTED IN TABLE 1

3.4 Berry composition

Load vectors of the chemical attributes of grape composition contributed to PC2 (Fig. 5).

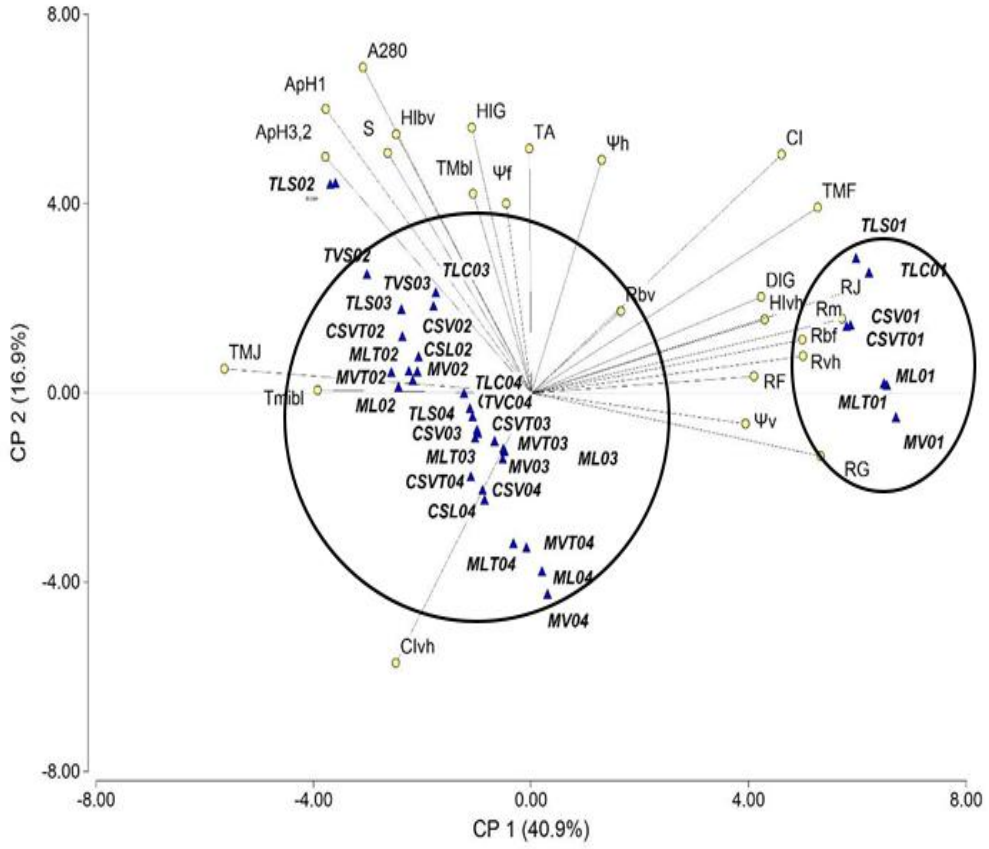

FIGURE 5: PRINCIPAL COMPONENT ANALYSIS. DISTRIBUTION OF PLOTS ACCORDING TO BERRY COMPOSITION AND CLIMATE ELEMENTS. ABBREVIATIONS OF THE VARIABLES ARE LISTED IN TABLES 1 AND 2.

The first two main components include 57.8% of the overall variance of the dataset. PC1 explains 40.9% and PC2 16.9% of the variance. As in previous cases, the plots of 2001 are clearly separated from the rest through PC1. In contrast, the plots of 2002, 2003 and 2004 are grouped. Load vectors of hydric and the TMF contributed negatively to PC1 with the highest values, whereas TMJ did it positively. Vector load of thermal sums and vine water status at harvest contributed positively with the highest values to the PC2, whereas night temperatures contributed negatively. All of the considered grape composition attributes were significantly higher in 2002 than in 2001, except for titratable acidity (Table 4).

TABLE 4
GRAPE COMPOSITION ATTRIBUTES AT HARVEST AS A FUNCTION OF YEAR.

Year	S (g/L)	TA (g/L)	ApH1 (mg/L)	ApH3.2 (mg/L)	A280
2001	202.6 a	4.6 ns	984.9 a	587.0 a	42.7 a
2002	220.8 b	4.50 ns	2270 d	1200 c	70.1 b
2003	211.3 b	4.25 ns	1853.5 c	977.2 b	61.0 b
2004	217.2 b	4.53 ns	1584.3 b	994.0 b	48.1 a

Abbreviations of the variables are listed in Tables 2

In 2002 the maturation occurred in cooler and drier conditions than in 2001, as evidenced by the values of the thermal and hydric conditions. The HC according to grape composition (Fig. 6), as in the previous cases, separated the four years, being 2001 the one with a greater distance from the rest.

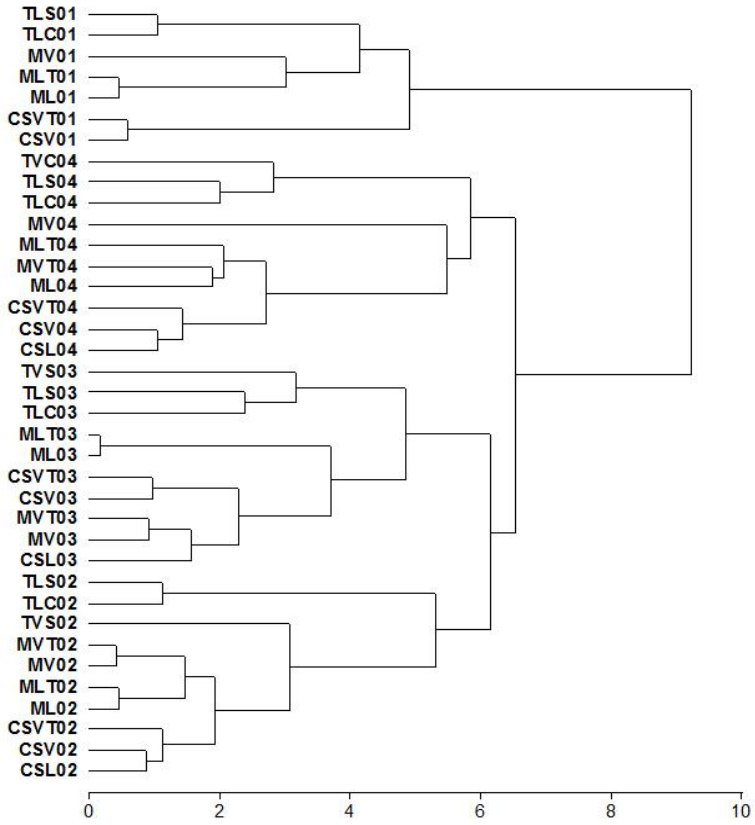

FIGURE 6 ASCENDING HIERARCHICAL CLASSIFICATION (EUCLIDEAN DISTANCE) WHICH REGROUPS THE YEARS - PLOT ACCORDING TO SIMILARITY OF THE GRAPE COMPOSITION. ABBREVIATIONS OF THE VARIABLES ARE LISTED IN TABLE 1

IV. DISCUSSION

Weather interannual variability was evidenced by different thermal and hydric conditions amongst years and with respect to historical values. This proved that MCC is a good method to characterize the climate of a given region, as suggested by Santos et al. [5].

4.1 Yield components

The two variables with the greatest influence on yield were cluster number and weight, in accordance with Dry et al.[2] Clingeleffer [3], and Jones et al. [21]. A positive correlation was detected between cluster number and rainfall over the growing season, rainfall between budbreak and veraison occurred on the previous cycle and the same period of the current season; whereas this relationship was negative for the thermal conditions of the current season. Warm temperatures, high solar irradiation and adequate water availability are needed for induction, formation and development of inflorescences, as well as for fruit set [4-6,36]. Appropriate water availability favors root activity, which has an essential role in the aforementioned stages [22]. Jones et al. [21] reported that temperatures higher than 15ºC favor cell division in the inflorescences. In the current study, the low yield observed in 2003 was a consequence of a low number of clusters; caused by water deficits during the two aforementioned phenological stages (Rbv -1 and Rbv), as well as minimum temperatures (11.1°C) under the appropriate values for the correct development of the inflorescences. According to Cuevas et al.[8], vine water requirements during flowering - veraison in 2003 were not satisfied (48.2% of the required over total growth).

Cluster weight, which also explained part of the variation in yield, was positively correlated with berry weight. The link between cap fall and germination of the pollen provides a possible explanation for the fact that weather conditions at flowering influence fruit set since they determine its percentage and also the number of seeds, two traits directly related to berry weight [4,7]. As shown in the present study, weather conditions were appropriate for fruit set, since maximum temperatures (between 20-25ºC) and rainfall (greater than 25.9 mm) values were in accordance with those reported by Heazlewood et al. [23]. These conditions also occurred during the year with the lowest yields, although it showed the greatest

berry and cluster weight when compared with the rest of the years, this was not enough for compensating the low yield caused by a low number of clusters.

Berry weight at harvest was significantly correlated with that at veraison, in accordance with Ferrer [16] and Ollat et al.[24]. In this study, berry weight was positively related to water availability before veraison, as reported by Ferrer et al. [25] and Niculcea et al.[26]. In the years when berry weight was high (2001 and 2003), no water restrictions were detected at veraison, and the dryness index was higher than the historical average. During this stage of maturation, the negative effect of maximum temperature of January on berry weight can be explained by berry dehydration, in accordance with Rogiers et al. [27]. In our study, this effect was likely attenuated because the highest temperatures occurred in years with high water availability (2001 and 2003).

It was expected that HIG would be positively correlated with yield and its components, due to its positive influence on the induction process and on the number of clusters, as reported by Jones et al. [21]. However, we found a negative effect of the thermal sum in the first stage of the season (HIbv), accompanying the negative effect of high temperatures reported by Santos et al.[5]. From veraison onwards, this index (HIvh) was positively correlated with yield and cluster and berry weights.

The effect of year prevailed over those of the variety and the management practices, as shown by HC; however, within the same year, the effect of the variety on yield and its components was greater than that of the management practices. This can be explained by the differences in components such as cluster number and weight. In Australia, Dry et al. [2] reported that Merlot and Cabernet Sauvignon present a high bunch number and a low bunch weight; whereas Ferrer et al. [28] found that, for Uruguayan conditions, Tannat showed a high bunch number and weight, in accordance with the findings of the current study.

4.2 Berry sanitary state: rot incidence

Bunch rot reduces grape yield and quality affecting vineyard economic revenues [10]. In the current study, in years of low yields (2002 and 2003) the incidence of this disease was lower compared with that observed in years of high yields (2001 and 2004), because, presumably, high yields facilitate the spread of bunch rot or water condensation within bunches, thus creating a favorable microclimate for fungal colonization [9].

Diseased berries at harvest may be the result of latent infections that occur during bloom and early stages of berry growth or direct infections during ripening. In both periods, rainfall and relative humidity were strongly correlated with bunch rot incidence, in accordance with Fermaud et al. [9] and González-Domínguez et al. [29].

Wind intensity was negatively correlated with bunch rot incidence because as wind speed increases, relative humidity is reduced. Wind speed at maturation in the year of greatest bunch rot incidence was, on average, below that of the rest of the years (0.85 m/s vs. 1.53 m/s).

Maximum temperature of January was negatively related with bunch rot incidence since it surpassed the threshold for being favorable to fungus development [9,29]. However, the minimum temperatures of the same month and those of the maturation period were positively correlated with bunch rot incidence and they were between the intervals reported as favorable by the same authors (Table 3).

Within the same year, the tested varieties showed different behavior in relation to bunch rot infection. On average for the four years, 26.2%, 5.6% and 7.4% of clusters were infected for Tannat, Merlot and Cabernet Sauvignon, respectively. These results are in agreement with the susceptibility ranking to bunch rot reported by Ferrer et al. [28]. This different sensitivity associated to variety can be explained by combined effects, such as cluster compactness [30] and resveratrol content [31]. Tannat clusters are more compact than those of Merlot and Cabernet Sauvignon [28]. In addition, Merlot grapes presented higher resveratrol content than Cabernet Sauvignon and the lowest content was observed for Tannat.

4.3 Berry Composition

The compounds that responded more markedly to the atmospheric conditions of the year were total poliphenols, total and extractable anthocyanins, as well as sugars and titratable acidity, in accordance with van Leeuwen et al. [11]. Meteorological conditions during ripening determine berry composition at maturity. Rainfalls after veraison and during maturity were negatively correlated with total and extractable anthocyanins, total polyphenols and sugars. The dilution effect explains the reduction in compounds linked to berry enological quality [2,14], whereas water restriction showed a positive correlation because of its related impact on phenolic biosynthesis. This response is related to the competition for photo-assimilates

between vegetative and berry growth [33]. Appropriate water availability during maturation maintains vegetative growth and, consequently, this competes with the accumulation of sugars and other chemical compounds in the berries [2,14]. A positive correlation between grape composition and spring temperatures, as well as with the sum of degree days between flowering and veraison was established in accordance with Nicholas et al.[34]. In the current study, total soluble solids were strongly correlated ($p < 0.001$) with those traits accounting for quality (ApH1 $r = 0.79$; ApH3.2 $r = 0.79$; A280 $r = 0.77$), in accordance with Keller [4]. Temperature during the last stage of maturation was negatively correlated with grape composition, as previously reported [1,12]. However, this relationship was positive with temperature of January. Therefore, it can be suggested that flowering thermal conditions, which are strongly correlated with maturation temperatures, would exert a great influence on berry composition. In a previous research, Gonzalez-Neves et al. [35], indicated that Tannat grapes had the highest sugar contents and titratable acidity during all years. Merlot and Cabernet Sauvignon grapes did not differ between them in the majority of the years. On the other hand, Tannat grapes are characterized for the highest values of phenolic richness and total anthocyanin potential. Moreover, anthocyanin potentials were significantly higher in Cabernet Sauvignon than Merlot grapes in all the years studied. These might be the reasons why the effect of the variety prevailed over that of the management practices within the same year.

V. CONCLUSIONS

Annual variability on grapevine performance, sanitary state and berry composition was greater than that produced by variety and management practices. The number of clusters was the main component of the annual variation in yield; this number was defined by thermal and hydric conditions occurred in the first stage of the previous growing cycle and those of the current year.

Hydric conditions during the first stage of the growing cycle were determinant for bunch rot infection, as well as thermal conditions and water availability during maturation.

Compounds related to berry quality were positively influenced by thermal sum during the first stage of the growing cycle and negatively affected by high temperature and water availability during maturation.

Within each year, the effect of the variety was more relevant for yield, sanitary status and berry composition than that of the management practices considered in this study.

Our results suggest that the intervals of atmospheric conditions that favor yield and bunch rot are different from those that favor berry quality

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Effect of Copper Foliar Spray upon the Contents of Other Elements in Apple Leaves

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***Abstract*—** *Apple leaves of cultivars Topaz and Golden delicious, organically grown upon spindly shaped apple trees and grafted at 5 dwarfing rootstocks, were analyzed for 42 main and trace elements. Spraying a Cu-oxychloride particle suspension plus an adherent as a fungicide, induced some leaf concentration changes with respect to untreated control groups, though inputs of other elements from spraying were negligible. Cu- treatment tended to increase concentrations of Fe, Si and J, and to decrease Zn, Co and Cd in the leaves, because these effects appeared for both cultivars at all rootstocks. Other changes might be rather due to fertilization regime and climate.*

***Keywords*— apple leaves, Cu-spraying, trace elements, year-to-year variation.**

I. INTRODUCTION

In order to cope with fungal diseases during organic viticulture and fruit farming, use of foliar copper spraying up to 6 kg/ha.a has been generally permitted within the EU [1]. Fixation of copper-hydroxide or copper-oxychloride particles at leaf surfaces can be achieved by emulsions together with adherents like ethoxylated rapeseed oil or soy oil. Simulation of elution by rain drops showed that adherents decreased Cu-losses from leaves within the first 30 mm of rain got decreased from 75-90% down to 10-25%, and increased the covered surface area per particle. Washout to the soil left the particles rather unchanged [2,3]. Within the soil, the Cu-containing particles get irreversibly adsorbed at humics and pedogenic oxides within a few hours, leaving exchangeable fractions within a few percent of total [4].

Because Cu is much more toxic towards fungi than towards bacteria, Cu inputs decreased fungal biomass, whereas bacterial phospholipids and xylanase were not affected, but effects upon the mycorrhiza remained unclear. Thus, effects upon bacterially driven N-, P- and C- cycles seem less pronounced [5].

Though numerous studies about Cu-speciaton and mobilities in soil, as well as toxicity symptoms in green plants are available, effects upon the metabolism of other elements after Cu-spraying are largely unknown.

In green plants, about 98% of total Cu is bound to various organic molecules. Cu shows high affinities to thiol groups of peptides, and thus to proteins rich in cystein. But it may also form stable chelates with carboxylic and hydroxylic groups, often assisted by groups containing basic nitrogen. Cu-containing enzymes catalyse electron transfer reactions, like photosynthesis, respiration, perception of ethylene, metabolism of reactive oxygen, and remodeling of cell walls. Many Cu-proteins have a functional counterpart that uses Fe [6,7].

Whereas divalent Cu shows high affinity to histidine, monovalent Cu favors cystein or methionine. In case, metals are transported by a common transport protein, Cu can displace other essential metals in metallo-proteins because of its high stability of its thiol complexes. According to the Irving-Williams-series, the stability of bondings between metallo-proteins and metals increases within $Ca^{2+} < Mg^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+}$-$Cu^{2+}$-$Cu^{+}$ [7,8].

Small organic molecules like mugeinic acid or nicotianamine (N-N-(3-amino-3-carboxypropyl)-3-amino-3-carboxypropyl) azetidine-2-carboxylic acid) are utilized to transport essential metals like Cu, Fe, Mn, Ni or Zn within the green plant. In vitro, the complexation capability of nicotianamine increases within the sequence Mn-Fe-Co-Zn-Ni-Cu, and peaks at pH 6,5. In case of Cu, Fe or Zn deficiencies, nicotianamide get increasingly formed, but this necessitates, however, high supply of nitrogen [6]. At Cu-deficiency, Cu-treatment induces the formation of metallothioneins, which assist the reconstruction of plasma membranes and act as anti-oxidants [9]. Within the roots, both Cu and Fe get reduced by root cell ferric reductase. When given in excess, plants have reduced Fe uptake, and vice versa [6]. Excess Cu concentrations tend to decrease root growth because of preferential Cu- accumulation in that organ.

In case Cu is taken from the soil, the most frequent symptom of Cu- intoxications is chlorosis as well as reduced uptake of Fe. In addition to chlorosis, excess Cu causes symptoms like necrosis, and reduced growth. Inside the cells, excess Cu can disrupt protein structures, reduce enzyme activities, interfere in the biosynthesis of photosynthetic pigments and membranes, cause deficiencies of other essential elements, and induce oxidative effects [8]. Cu-induced Fe deficiency, replacement of Mg by Cu, or destruction of the oxygen transporting polypeptide decreases chlorophyll content.

Therefore, the Cu- tissue levels are regulated within a narrow physiological range by homeostasis. Frequently, green plants have specific metal sensors, which start a cascade of signals to induce corresponding reactions. The green plant can protect itself against excess Cu by stimulation of excretion, increase of chelators, and separation into a vacuole [8].

Immissions of toxic amounts of Cu can be caused by industrial and residential wastes, pig manure and poultry dung, and also Cu foliar sprays. Most papers about metal tolerance deal with elevated soil or hydroponic Cu levels. Among green plants, tolerance versus excess Cu is highly variable. Cu tolerant plants are mainly excluders, reacting by reduced secretion of root exsudates, and immobilisation inside the root. Excess soil Cu gets at first enriched within the roots, lowers root growth, promotes root damage and lowers transport processes inside the plant. Transport of excess Cu from roots to shoots gets prevented by adherence to cell walls, reduced flux across plasma membranes, increased outflow from the cytoplasma as well as intracellular chelation by organic acids, special phytochelatins, and metallo-thioneins [7].

The reverse transport of Cu from the leaves back to stalks and roots takes place at leaf aging. Increased N-supply delays aging and affects availability and mobility inside the plant by binding more Cu to amino acids and proteins. Cu gets hardly redistributed from old leaves to younger ones [6,7].

In case of Cu-deficiency, because of limited mobility from soil, foliar Cu-spray acts much faster and more effectively than additions to the soil. Cu levels applied as fungicides, however, are 10-100 times higher than usually needed for fertilization in case of deficiencies [7]. A hydrophobic cuticula protects leaves from external damage. Because the cuticula of young leaves is not so strong, effects of foliar spraying are higher in this case. The adherence of sprayed solutions depends on genotypical differences of leaf surface properties, like hairs and smoothness of surface.

A known interlement effect facilitates the decision to spray each element separately, or to use a mixture and thus safe work. The uptake of foliar-sprayed Cu, Mn, and B into apple leaves was higher in May than in June and September, and elevated levels of Cu lasted longer than of Mn and B. In combination with Mn, the leaves adsorbed less Cu than without [10]. Cu addition to sandy soil also increased the uptake of Ba, Ca, Sr and Fe into the leaves of MacIntosh apple seedlings, but decreased Mn and Mo. Addition of peat to this sandy soil increased soil adsorption, but also decreased Cu uptake into the seedlings [11].

In order to document differences in root trace element uptake and foliar spray, young nursery-grown apple trees were grown in pot experiment on quartz sand. Spraying increased the Cu content of the components that were directly exposed, but hardly increased the Cu content of other tree components, like roots. Differences in plant growth were marginal. The levels of N, P, K, Ca, Mg and Na in the leaves were about the same after sufficient spraying or soil additions of Cu, Mn, Zn and B [12].

Within a field trial at the experimental orchard Jedlersdorf (Vienna/Austria), run by the University of Natural Resources Vienna, apples of „Topaz“ cultivar have been grafted upon different rootstocks, beneath a lot of other fruit items. Rootstocks M9 with and without „Rubinola“ as interstem, M26, M7 grafted at 25 cm and at 55 cm, MM111 and Bittenfelder seedlings, were used, trained as spindles. Growth, yields and mean fruit weights have been reported elsewhere [13]. This enabled us to investigate the effect of rootstocks and the year of growth upon the composition of fruits and leaves at the same site and fertilization regime [14]. In 2012, young leaves were sampled in early June, which had got no Cu-treatment, whereas in 2015 and 2016, young leaves from the same trees were sampled after Cu treatment. At the same site, apple leaves of Golden delicious variety were available with and without Cu treatment, grown in 2016.

For Cu treatment, Cuprofor liquid, containing copper oxichloride, was used, diluted at 0,03% solution (%v/v).

II. MATERIAL AND METHODS

At the experimental orchard Jedlersdorf (Vienna/Austria) in autumn 2008, 5 rows of spindly shaped apple trees were grafted by variety Topaz at dwarfing rootstocks M9, M26, M7 and M111, as well as on seedling (Bitterfelder). At present, Topaz is the most utilized apple cultivar grown by organic farming in Austria. It has favorable storage properties, high vitamin C

contents and a balanced acid-sugar proportion, early flowering stage and late harvest [15]. The trees have been organically grown within 5 rows, each containing 4 trees of each kind randomly distributed.

The soil is of calcaric chernozem type, pH7.6 -7.8, containing about 7% of total Ca, and low mobile K (in 0,16M acetic acid 1 to 20: 137 mg/kg in the upper 25 cm, and 42 mg/kg below). Fertilization was done by addition of the organic fertilizer "Biofert" (Austria) to supply N at 30 kg/ha.a .

At the day of sampling, leaves of each kind were taken separately within each row, wearing gloves, preferably the third leaf off the sprout tops. All 4 trees within one row were sampled all around, to average light and shadow sites. This enabled to compare the uncertainties between data obtained from different rows and rootstocks, respectively. In the evening after sampling, the leaves were rinsed with de-ionized water upon nylon gauze, put into new poly-ethylene bags, submitted to freeze drying, and finely crashed inside the bags in order to avoid further contacts.

All samples were submitted to two different digestion procedures, at least in duplicate. About 0,25g sample was digested with 3,8 ml suprapure HNO_3 plus 0,1 ml HF p.a. in closed vessels by microwave heating, and finally made up to 25 ml. In addition, about 1 g sample was digested with 8 ml of a nitric acid – potassium chlorate solution (20g $KClO_3$ p.a. + 200 ml H_2O + 80 ml HNO_3 suprapure), and finally made up to 25 ml also [16]. Ultrapure water and polythene volumetric flasks were used throughout.

The resultant digestion solutions were submitted to multi-element analysis by ICP-OES (Perkin-Elmer Optima 3000XL) operating with a horizontally mounted torch, as well as ICP-MS (Perkin Elmer Sciex ELAN DRC II) for selected low level trace elements. $KClO_3$ digestion solutions were analyzed at the ICP-OES versus matrix matched calibrants, others with calibrants containing K, Ca and P within expectable ranges. For ICP-MS measurements, samples were diluted 1+9, indium added as internal standard, and read for the elements Bi, Cd, Co, Mo, Ni, Pb, Tl, Y and Rare Earth elements). Total iodine was determined in special runs at higher plasma power than the default, after dilution with 1/80 diluted digestion reagent solution, and standard addition of iodate calibrants.

The $KClO_3$ digest is especially useful for the determination of non-metals B, Si, S and I, because they get partially volatilized from conc. HNO_3 [16]. Unexpected purity of the $KClO_3$ permitted the determination of all main and trace elements, except K, Rb and Cs.

The fungizide Cuprofor was refluxed with HNO_3 or HCl, made up to 100 ml, and measured at the ICP after various dilutions and for Cu by flame-AAS. An oily precipitate was filtered off. HNO_3 and HCl yielded the same results.

Total nitrogen contents of the leaves were obtained by combustion utilizing a LECO FP-528 Nitrogen-Analyzer.

III. RESULTS AND DISCUSSION

The Cu-containing fungicide was quite pure with respect to other inorganics, and thus contributed negligible loads of other elements to the leaves. It contained less than 5% of Mg, Ca, Sr and Mn concentrations encountered in dry leaves, as well as Li, Al, Fe, Pb and Ni in slight excess (Table 1). This could not increase leaf concentrations substantially.

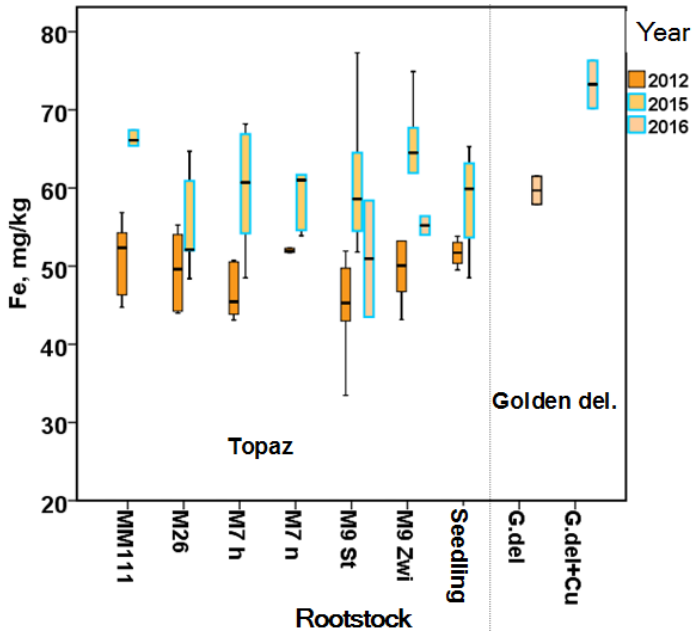
TABLE 1
CONCENTRATIONS FOUND IN CUPROFOR PER WEIGHT

Cu 28.9 ± 1.7 %			
	mg/kg		**mg/kg**
Na	1535 ± 187	**Cr**	8.5 ± 1.1
Ca	613 ± 43	**Cd**	2.0 ± 0.5
Fe	287 ± 35	**Pb**	1.7 ± 0.5
Zn	198 ± 20	**Co**	1.7 ± 0.5
K	158 ± 103	**Mn**	1.1 ± 0.4
Al	154 ± 54	**Ni**	1.1 ± 0.4
Mg	47 ± 16	**Sr**	0.85 ± 0.15
		Li	0.32 ± 0.10

Whereas in leaves taken in 2012, Cu levels were ambient, Cu in leaves taken 2015 ranged about 100-150 mg/kg, which would be highly toxic, if the same load had come via the soil, because of irreversible root damage. It exceeds the legal limit of 90 mg/kg, given in the Austrian standard ÖNORM S 2203, entitled "Requirements for manufactured soils from compost" [17]. Leaves sampled in 2016 contained about 50-70 mg/kg Cu.

Trends of element concentrations induced by Cu-spraying seem to be real, if the same effects occur upon all rootstocks, as well as within several growing seasons and cultivars.

When concentration ranges met in apple leaves and grown upon various rootstocks, are depicted as boxplots, possible differences get easily visualized, but contrary to Table 2, medians and the within 50%-ranges are shown. Figs. 1-7 show some boxplots assorted to rootstocks, year and cultivars, for those elements, which yielded equal patterns in subsequent years. These patterns were shifted in parallel with respect to 2012 data to higher values in 2015 and 2016 in case of Fe, Si and I, and to lower values in case of Cd, Co, Zn, and possibly Mo and Ti (table 2; figs 1-7). The Rare Earth elements are generally too low to detect such effects. Adverse trends in the two cultivars appeared for N, P as well as for Na, Sr, Ni, Pb, and V, if data from leaves grown upon rootstocks M9 are compared (Table 2); they are surely no effect of Cu-spray but of the fertilization regime. Other elements did not show uniform trends and thus do not seem to be affected by Cu spraying. Also, element proportions like Ca/Mg, Ca/Mn, and B/Mo did not show common trends.



M7 h: M7 grafted at 55cm height

M7 n: M7 grafted at 25 cm height

M9 n: M9 normal

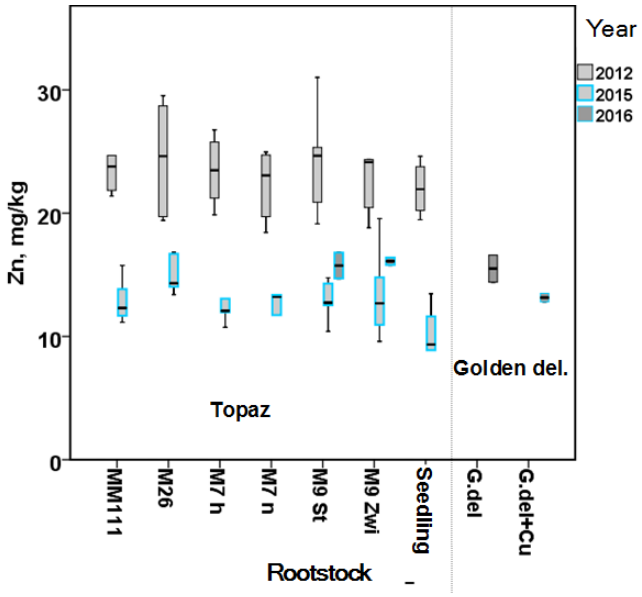
M9 Zwi: M9 grafted with Rubinola as interstem

Seedling: Bittenfelder seedling

Golden delicious grafted at M9

FIG 1. Fe CONTENTS IN APPLE LEAVES!

blue framed boxes: with Cu; black framed boxes: without Cu



M7 h: M7 grafted at 55cm height

M7 n: M7 grafted at 25 cm height

M9 n: M9 normal

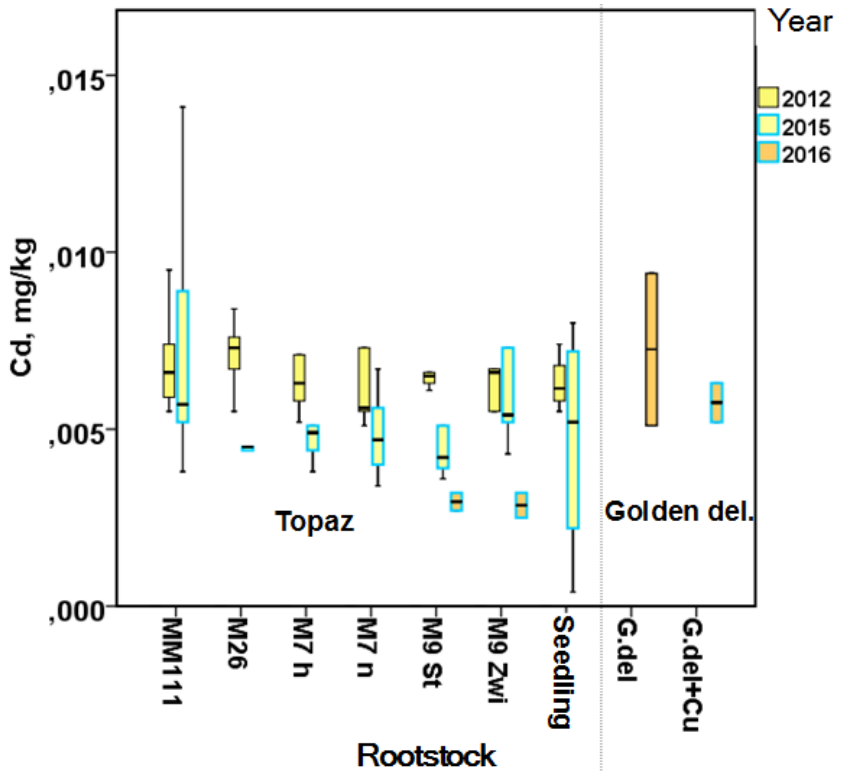
M9 Zwi: M9 grafted with Rubinola as interstem

Seedling: Bittenfelder seedling

Golden delicious grafted at M9

FIG. 2 Zn CONTENTS IN APPLE LEAVES!

blue framed boxes: with Cu; black framed boxes: without Cu



M7 h: M7 grafted at 55cm height

M7 n: M7 grafted at 25 cm height

M9 n: M9 normal

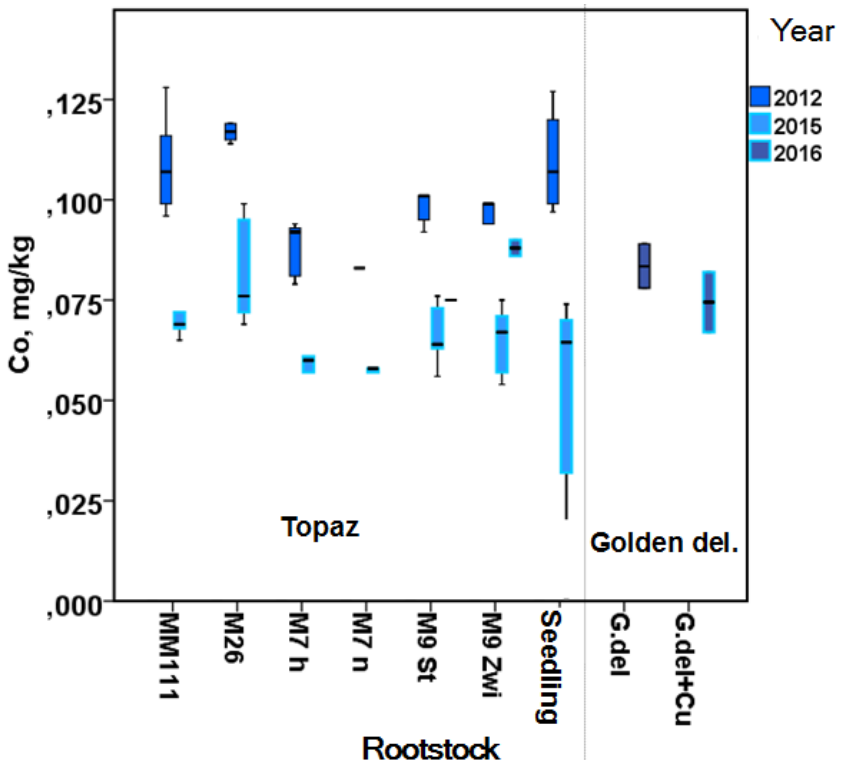
M9 Zwi: M9 grafted with Rubinola as interstem

Seedling: Bittenfelder seedling

Golden delicious grafted at M9

FIG 3. Cd CONTENTS IN APPLE LEAVES!

blue framed boxes: with Cu; black framed boxes: without Cu



M7 h: M7 grafted at 55cm height

M7 n: M7 grafted at 25 cm height

M9 n: M9 normal

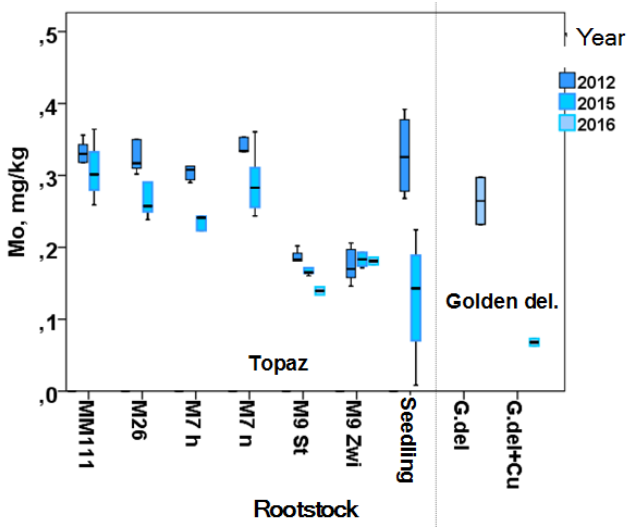
M9 Zwi: M9 grafted with Rubinola as interstem

Seedling: Bittenfelder seedling

Golden delicious grafted at M9

FIG 4. Co CONTENTS IN APPLE LEAVES

blue framed boxes: with Cu; black framed boxes: without Cu



M7 h: M7 grafted at 55cm height

M7 n: M7 grafted at 25 cm height

M9 n: M9 normal

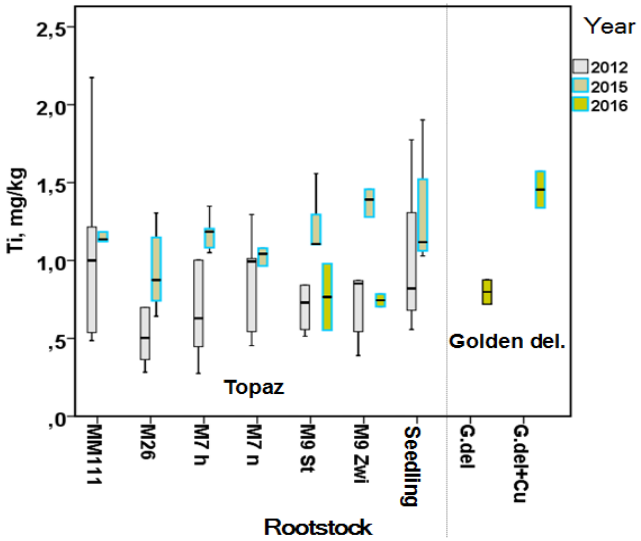
M9 Zwi: M9 grafted with Rubinola as interstem

Seedling: Bittenfelder seedling

Golden delicious grafted at M9

FIG 5. Mo CONTENTS IN APPLE LEAVES

blue framed boxes: with Cu; black framed boxes: without Cu



M7 h: M7 grafted at 55cm height

M7 n: M7 grafted at 25 cm height

M9 n: M9 normal

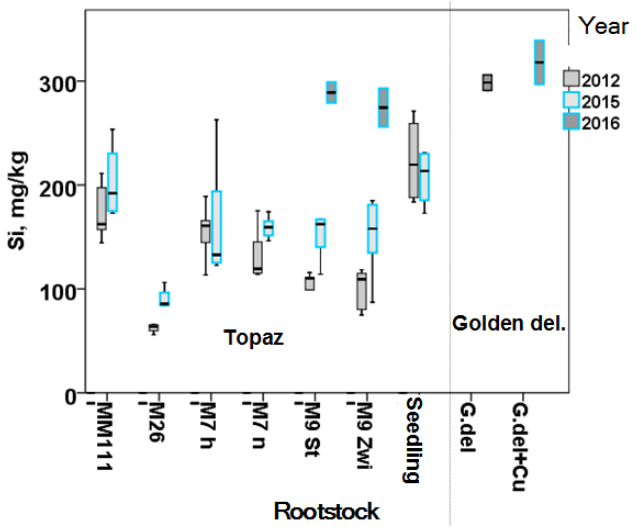
M9 Zwi: M9 grafted with Rubinola as interstem

Seedling: Bittenfelder seedling

Golden delicious grafted at M9

FIG 6. Ti CONTENTS IN APPLE LEAVES

blue framed boxes: with Cu; black framed boxes: without Cu



M7 h: M7 grafted at 55cm height

M7 n: M7 grafted at 25 cm height

M9 n: M9 normal

M9 Zwi: M9 grafted with Rubinola as interstem

Seedling: Bittenfelder seedling

Golden delicious grafted at M9

FIG 7. Si CONTENTS IN APPLE LEAVES

blue framed boxes: with Cu; black framed boxes: without Cu

TABLE 2
CONCENTRATIONS IN APPLE LEAVES, mg/kg DRY MASS, GRAFTED AT ROOTSTOCK M9 ASSORTED FOR MAIN ELEMENTS, Cu, ESSENTIALS, AND NON-ESSENTIALS

	Topaz 2012	Topaz 2015	Topaz 2016	Golden del. 2016	Golden del. 2016
% N	2.82 ±0.15	2.65 ±0.22	2.19 ±0.04	2.37 ±0.04	2.50 ±0.04
% Ca	1.313 ±0.135	1.331 ±0.227	1.974 ±0.184	1.673 ±0.155	1.529 ±0.035
% K	0.707 ±0.082	0.684 ±0.140	1.122 ±0.127	1.909 ±0.542	1.925 ±0.075
% Mg	0.388 ±0.035	0.358 ±0.043	0.391 ±0.038	0.266 ±0.016	0.299 ±0.004
% S	0.230 ±0.037	0.193 ±0.008	0.139 ±0.012	0.148 ±0.003	0.149 ±0.010
% P	0.200 ±0.015	0.192 ±0.013	0.162 ±0.024	0.139 ±0.004	0.148 ±0.001
mg/kg					
Cu	10.42 ± 1.16	131.0 ±39.2	50.6 ±4.5	8.83 ±0.35	71.0 ±1.1
Fe	48.3 ±8.5	62.5 ±9.3	53.1 ±6.6	59.7 ±2.5	73.3 ±4.3
Mn	37.8 ±4.1	40.1 ±6.7	27.5 ±2.0	42.9 ±0.3	35.7 ±0.4
Zn	24.1 ±4.6	13.2 ±2.9	15.9 ±0.9	15.5 ±1.6	13.2 ±0.5
B	27.9 ±2.0	23.4 ±3.5	26.7 ±0.8	30.8 ±0.1	30.2 ±0.9
Mo	0.180 ±0.020	0.189 ±0.039	0.160 ±0.025	0.265 ±0.046	0.068 ±0.007
Co	0.099 ±0.009	0.066 ±0.008	0.082 ±0.008	0.084 ±0.008	0.075 ±0.011
Li	0.326 ±0.064	0.308 ±0.049	0.641 ±0.029	1.573 ±0.170	0.665 ±0.025
Na	35.1 ±13.4	22.8 ±11.8	9.8 ±0.7	11.8 ±4.2	15.4 ±0.3
Rb	3.48 ±0.45	3.95 ±2.46	3.80 ±1.06	0.88 ±0.29	4.00 ±0.49
Cs	0.018 ±0.003	0.023 ±0.007	0.020 ±0.001	0.005 ±0.001	0.013 ±0.003
Be	< 0.003	< 0.003	< 0.003	< 0.003	0.004 ±0.001
Sr	26.0 ±4.7	28.0 ±5.4	32.3 ±2.8	42.6 ±13.8	24.6 ±0.1
Ba	44.5 ±7.9	37.9 ±7.0	52.8 ±2.5	25.7 ±1.6	28.6 ±0.7
Al	32.8 ±4.6	32.7 ±8.3	68.9 ±5.5	61.3 ±6.7	84.1 ±5.6
Sc	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Y	0.014 ±0.002	0.017 ±0.004	0.015 ±0.001	0.015 ±0.004	0.029 ±0.014
La	0.027 ±0.011	0.029 ±0.007	0.023 ±0.003	0.031 ±0.003	0.058 ±0.015
Ce	0.051 ±0.008	0.057 ±0.016	0.044 ±0.005	0.061 ±0.000	0.121 ±0.023
Pr	0.006 ±0.002	0.007 ±0.002	0.005 ±0.001	0.006 ±0.001	0.013 ±0.003
Nd	0.021 ±0.009	0.026 ±0.007	0.019 ±0.002	0.025 ±0.002	0.050 ±0.014
Sm	0.004 ±0.002	0.005 ±0.001	0.004 ±0.001	0.004 ±0.001	0.009 ±0.003
Eu	0.010 ±0.001	0.007 ±0.002	0.011 ±0.001	0.007 ±0.003	0.007 ±0.001
Gd	0.004 ±0.001	0.004 ±0.001	0.004 ±0.001	0.004 ±0.0001	0.008 ±0.003
Tb	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Ho	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Er	0.0015±0.0003	0.0018±0.0004	0.0013±0.0005	0.0010 ±0.0000	0.0070 ±0.0071
Lu	< 0.0005	< 0.0005	< 0.0005	< 0.0005	< 0.0005
Ti	0.89 ±0.54	1.27 ±0.48	0.76 ±0.18	0.80 ±0.11	1.46 ±0.16
V	0.052 ±0.014	0.031 ±0.028	0.031 ±0.008	0.037 ±0.006	0.078 ±0.025
Cr	0.065 ±0.041	0.174 ±0.082	0.056 ±0.014	0.058 ±0.022	0.099 ±0.003
Ni	0.29 ±0.07	1.63 ±0.27	0.69 ±0.09	2.12 ±0.18	0.92 ±0.01
Cd	0.0064±0.0014	0.052 ±0.0016	0.0029±0.0004	0.0073 ±0.0030	0.0058 ±0.0008
Si	101 ±18	156 ±40	282 ±19	299 ±11	318 ±30
Pb	0.18 ±0.02	0.24 ±0.03	0.26 ±0.02	0.24 ±0.05	0.22 ±0.06
J	0.263 ±0.087	0.487 ±0.094	0.421 ±0.057	0.317 ±0.001	0.400 ±0.022

IV. CONCLUSION

Cu- spraying as a fungicide increased Cu contents in apple leaves to levels, which would be toxic, if this load would have passed through the roots. Though the input of other elements to the leaves was negligible, Cu-spraying might influence the contents of other trace elements in the leaves, like Fe, Si, I, Cd, Co and Zn. This pilot study has to be confirmed by sampling of treated and untreated leaves for the same cultivars at the same sites within the same years. Future results would be interesting for other fruits and vine as well.

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Ultrastructural Analysis of Pseudorabies Virus Infection in IB-RS-2 Cell Line and with Treatment by *Persea americana* Extract

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***Abstract*—** *In this study, we focused on the cycle of replication of the Pseudorabies virus (PrV) in the swine IB-RS-2 cell line in absence or in presence of an infusion from Persea americana leaves. The ultrastructure of the Nova Prata strain virus presents the typical characteristics of Varicelovirus as well as lytic replication with total cell destruction between 18 and 24 hours post-infection (pi). Adsorption is immediate followed by fusion penetration of the virus membranes with those of the host. The capsid once inside the cell migrates to the nucleus where it disappears. Precursor viral particles appear from 4 hours and will form the nuclear crystalloids. The capsids with the viral DNA incorporated migrate to the nuclear membranes where they receive viral envelope after de-envelopment and re-envelopment constituted by cytoplasmic membranes. Then the virions appear grouped in vesicles that merge with the plasma membrane and finally are released out of the cell and become associated with it. Infected cells in the presence of an infusion of Persea americana leaves show few viral particles and many cells without signs of infection. In the initial stages of replication, they are shown pleomorphic with a different morphology when compared with the control. At 7 h post-infection double core with a single envelope are found both between the nuclear membranes and in the cytoplasm of the cell. These findings indicate that the extract may be interfering with virus replication as well as useful in detecting possible targets of inhibition.*

***Keywords*— PrV; Aujeszky's disease virus; IB-RS-2 swine cell line; viral ultrastructure; antiviral; Persea americana .**

I. INTRODUCTION

The Aujeszky's disease virus (ADV) or pseudorabies virus (PrV), also called swine herpesvirus (SuHV-1) and belonging to the family Alfaherpesviridae, occurs in several species of mammals and attacks mainly the central nervous system. It is considered a disease primarily of swine; its control is difficult and causes many economic losses in the stock. The pigs are one of the largest reservoirs of the virus; however, in several mammalian species, such as in cattle, sheep, goats, dogs and cats the disease can also occur (Pomeranz et al., 2005; Nauwynck et al., 2007).

In this study, the strain Nova Prata isolated in Brazil in 1956 from cattle infected with PrV was investigated (Fonseca et al., 2010).

Studies in the literature describe the morphology of the virus mainly in infected cells (Granzow et al., 1997; Johnson and Baines, 2011; Goldsmith et al., 2013). The herpesviruses consist of four distinct structural components: the viral core composed of a double-stranded DNA genome and enclosed by icosahedral capsid to form the nucleocapsid, and the tegument that is a protein matrix surrounded by the viral membrane or viral envelope (Boldogköi and Nógrádi, 2003; Pomeranz et al., 2005; De Clerq and Li, 2016). The replication cycle of the virus, have been well studied in cultured cell systems and can serve as a template for didactic and illustrative purposes, as well as for the study of targets for blockade using both synthetic and natural antiviral drugs (Goldsmith et al., 2013; Kalu et al., 2014; Paulini, 2015).

Among the natural products with antiviral properties, aqueous leaf extract (infusion) from *Persea americana* showed strong action on PrV with effective inhibition when the extract was added simultaneously to the virus inoculation in IB-RS2 cell line (Koseki et al., 1990; Almeida et al., 1998). In addition, Simoni et al. (1996) showed a reduction of viral infectivity of 1.5 log after 30 minutes of direct incubation with the virus.

The present study aimed to illustrate by electronic microscopy the steps of the PrV replication in IB-RS-2 infected cell line and to compare it with the replication of the same treated with *Persea americana* extract.

II. MATERIAL AND METHOD

The Pseudorabies virus (PrV) strain Nova Prata (Fonseca et al., 2010) was provided by the Desidério Finamor Veterinary Research Institute (IPVDF-RS, Brazil) and had 20 passages in the RK-13 rabbit kidney line. Then, it was propagated in the IB-RS-2 clone 13 porcine and the titer obtained was 10^8 $TCDI_{50}$/ml.

The plant extract used in this study is an infusion of *Persea americana* leaves was prepared as described by Simoni et al. (1996). Dried and powdered leaves were extracted by infusing with boiling distilled water at 10% (w/v) and were used at the 1.25% non-cytotoxic concentration (MNTC). This MNTC determined by viewing under the Olympus inverted optical microscope the appearance of treated cells in comparison with that observed for control cells without extract.

IB-RS-2 C-13 porcine kidney cells maintained in minimal Eagle medium (MEM) plus 10% fetal bovine serum (SFB) were seeded for 24 h with 1.2 x 10^5 cells per flask of 25 cm^2 capacity. The confluent cell monolayers were then washed with Hanks physiological solution and subjected to the following treatments:

1) Cells infected with 200 µL of viral suspension;
2) Cultures controls where 200 µL of MEM were added;
3) Control cultures where 200 µL of plant extract were added
4) Cells infected with 100 µL of viral suspension and added with 100 µL of plant extract
5) Cells infected with 200 µL of a mixture composed by 100 µL of viral suspension incubated at 37 °C with 100 µL of plant extract for 1 h.

At 0 min, 1, 2, 4, 6, 7, 8, 18 and 24 hours post-infection (pi) intervals at 37 ^oC, the cells were washed twice with physiological solution, carefully collected from the surface of the flasks by scraping and prepared to be examined under the transmission electron microscope. The flasks of 0 min pi were collected shortly after inoculation and rinsing with physiological solution and the remaining flasks were washed with Hanks's solution. After 1 h of incubation, MEM without SFB (treatments 1, 2 and 5) was added to cell monolayers or plant extract (treatments 3 and 4).

Prior to microscopy processing all cell cultures were observed under the inverted optical microscope to check for possible cellular changes as well as the cytopathic effect (CPE) caused by the virus on the infected cells and also to compare with the infected cells in the presence of extract.

The cells harvested for ME were centrifuged at low speed and each procedure was washed with physiological solution. A solution containing 3% glutaraldehyde in 0,1M phosphate buffer, pH 7.2, was added to the pellet over night at 4 ^oC. The cells were then washed and fixed with 1% aqueous osmium tetroxide for 2 h at 4 °C, dehydrated and soaked with Spurr's resin at 65 ^oC for 72 h. The materials were sectioned with anultratome LKB III and contrasted with 2% uranyl acetate for 20 min. and Reynold's lead citrate for 5 min. prior to observation under the Philips EM 300 electron microscope.

III. RESULTS AND DISCUSSION

The steps of the PrV replication in IB-RS-2 infected cells observed by means of Transmission Electronic Microscopy (TEM), in the presence or not of a *Persea americana* leaf infusion (plant extract), are shown in Figures 1-5 and commented below.

Figure 1 shows the IB-RS-2 lineage observed under the light microscope where uninfected cells (treatment 2) form a monolayer (Fig. 1a). Uninfected cells and cells in the presence of plant extract (treatment 3) did not exhibit a cytotoxic effect. They presented a similar appearance to that observed for control cells without plant extract with the preserved organelles

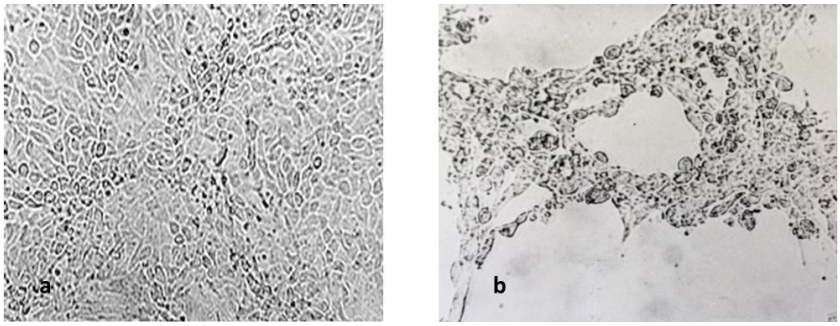

FIGURE 1: MONOLAYER OF IB-RS-2 CELL LINE A) NON INFECTED CELL LINE. B) INFECTED CELLS WITH PSEDORABIES VIRUS (PRV). MAGNIFICATION ABOUT 100X

The infected cells without plant extract, when observed under the light microscope (treatment 1), did not show changes in their morphology before 6 h pi, while the infected cells in the presence of plant extract (treatments 4 and 5) did not show changes in their morphology after 7 h pi.

The monolayers with no plant extract (treatment 1) started to show morphological changes after 6 h pi, initially with the formation of some groups presenting a bulky aspect, contrasting with the rest of the monolayer cells. Mello & Koseki, 1998 also observed discernible changes at 6 h after infection studying GBK cells infected with the same strain Nova Prata seen in nuclear phenotypes using Feulgen-stained preparations.

Foci of cytopathic effect were observedat light microscope with 8 h pi showing many rounded swollen cells and some empty spaces caused by cellular retraction. The evident degeneration process occurred at 18 h pi where a large number of cells (75% of the monolayer) was already detached from the surface of the flask (Fig. 1b).

Uninfected cells (treatment 2) when observed at TEM showed the ultrastructures are well preserved with evident and numerous mitochondria, in addition to several cellular polysomes, indicating intense protein synthesis (Fig. 2a).

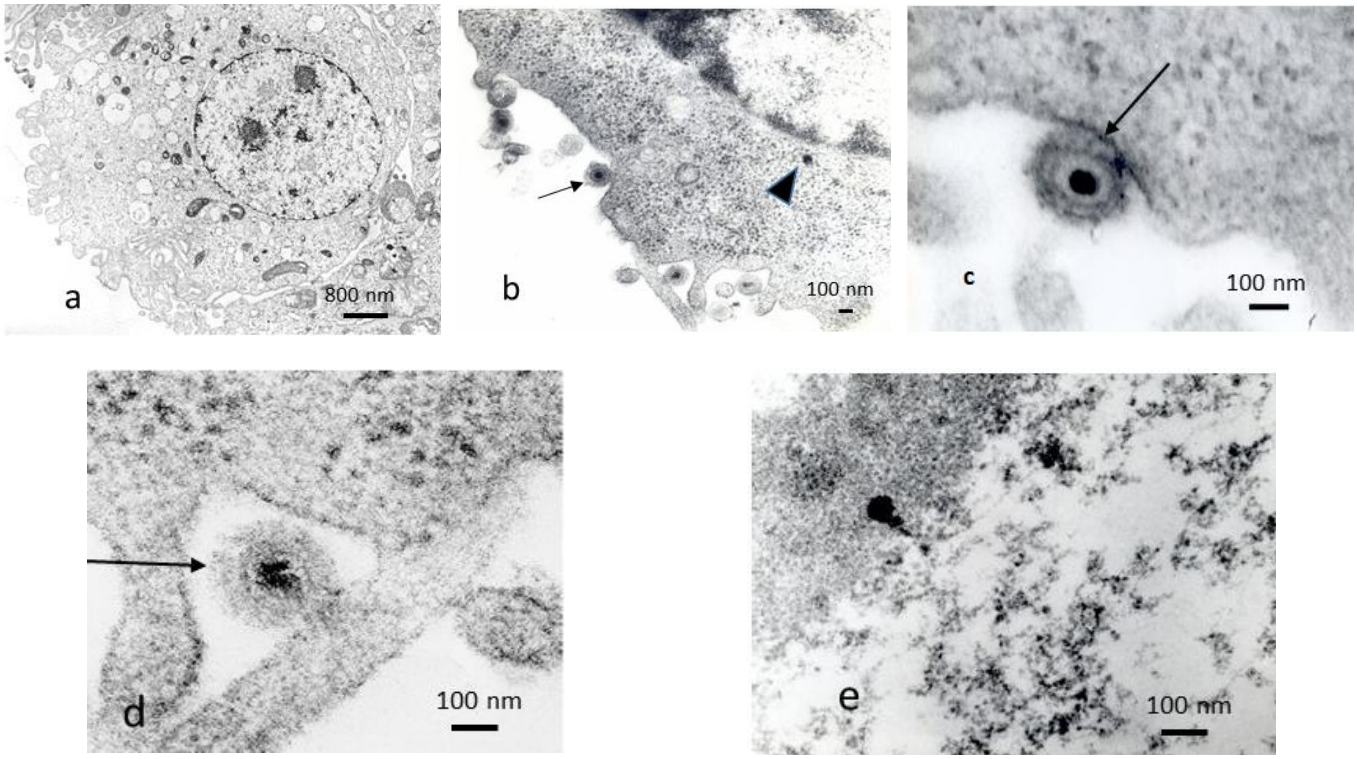

FIGURE 2: THIN SECTION ELECTRON MICROGRAPHS OF UNINFECTED IB-RS-2 CELL LINE. (a) PRV IMMEDIATELY AFTER THE VIRUS INOCULATION. (b) SHOWING VIRUS PARTICLES ATTACHED TO PLASMA MEMBRANE OF INFECTED CELLS (ARROW) AND INTRACYTOPLASMIC NUCLEOCAPSID (ARROWHEAD). HIGHER MAGNIFICATION OF A CELL IN (b) SHOWING ELECTRON-DENSITY OF PLASMA MEMBRANE (ARROW) (c). HIGHER MAGNIFICATION OF A CELL IN (b) SHOWING MEMBANE FUSION (ARROW) (d) NUCLEOLUS OF INFECTED IB-RS-2 CELL WITH PRV AT 4 H POST-INFECTION (e).

Infected cell cultures exhibited extracellular mature virions (Fig. 3g) measuring around 200 nm. This size corresponds to those described in other studies (Pomeranz et al., 2005; Nauwynck et al., 2007; Cardone et al., 2012).

Ultrastructural examination of infected cells at the very beginning of the infection in the first 10 min reveals the immediate adsorption of virions that appear to be bound to the plasma membrane (Fig. 2b), or even electron-dense virus particles inside the cells without the viral envelope (Fig. 2b). Holmes & Watson (1963) also observed in the early stages of herpes virus infection in BHK-21 cells viral particles inside and outside the cell. Granzow et al. (1997) found that the PrV entry into the cell is very fast and occurs in the first minutes. Frampton et al. (2010) report that after the fusion of the equine herpesvirus and its penetration into the cell, the viral particle in the cytoplasm migrates to the nucleus by using the microtubules such as dinein for its transport and efficient arrival to the nucleus. In addition, EHV-1 induces acetylation of this tubulin within the first 15 min of infection.

A virion can be seen in contact with the cell membrane with a higher electron-density region indicating a change caused by the association of the viral particle with the host cell (Fig. 2c). A virioncan be seen in an extension of a cell in which the fusion of a virion membrane with the cellular membrane is occurring (Fig. 2d). Morgan et al., 1968 also described the first stage of HSV-1 entry in Hela cell line characterizedby disintegration of the viral envelope adjacentto the cell wall.

Karasneh& Shukla (2011) reported that herpesviruses enter into the cell in various ways and these steps comprise the binding of the viral particle to the host cell surface, interaction with the specific entry receptor, particle internalization, and membrane fusion.

A detail of a cell after 4 h pi with no typical viral structures, but showing electron-dense structures close to the nucleolus in the nucleus of the infected cell can be observed in (Figure 2e). Holmes & Watson (1963) also reported the disappearance of the virus at penetration soon after entry into the nucleus, in addition to the marked increase of particles after 4 h of infection.

Wild et al. (2002) described intranuclear nucleocapsids in bovine herpesvirus infected MDBK cells with 4 h of incubation. The authors also reported that all phases of morphogenesis could be found at any time in the infected cell.

In this study, from 4 h pi the electronic micrographs also show precursor particles of viruses in the nucleus, which are getting more numerous in the course of the infection. Initially, they appear to be associated with electron-dense intranuclear bodies that resemble nucleoli or are still scattered in the nuclear matrix. These nucleolar structures are due to chromatin marginalization (Fig. 3a, b, c).

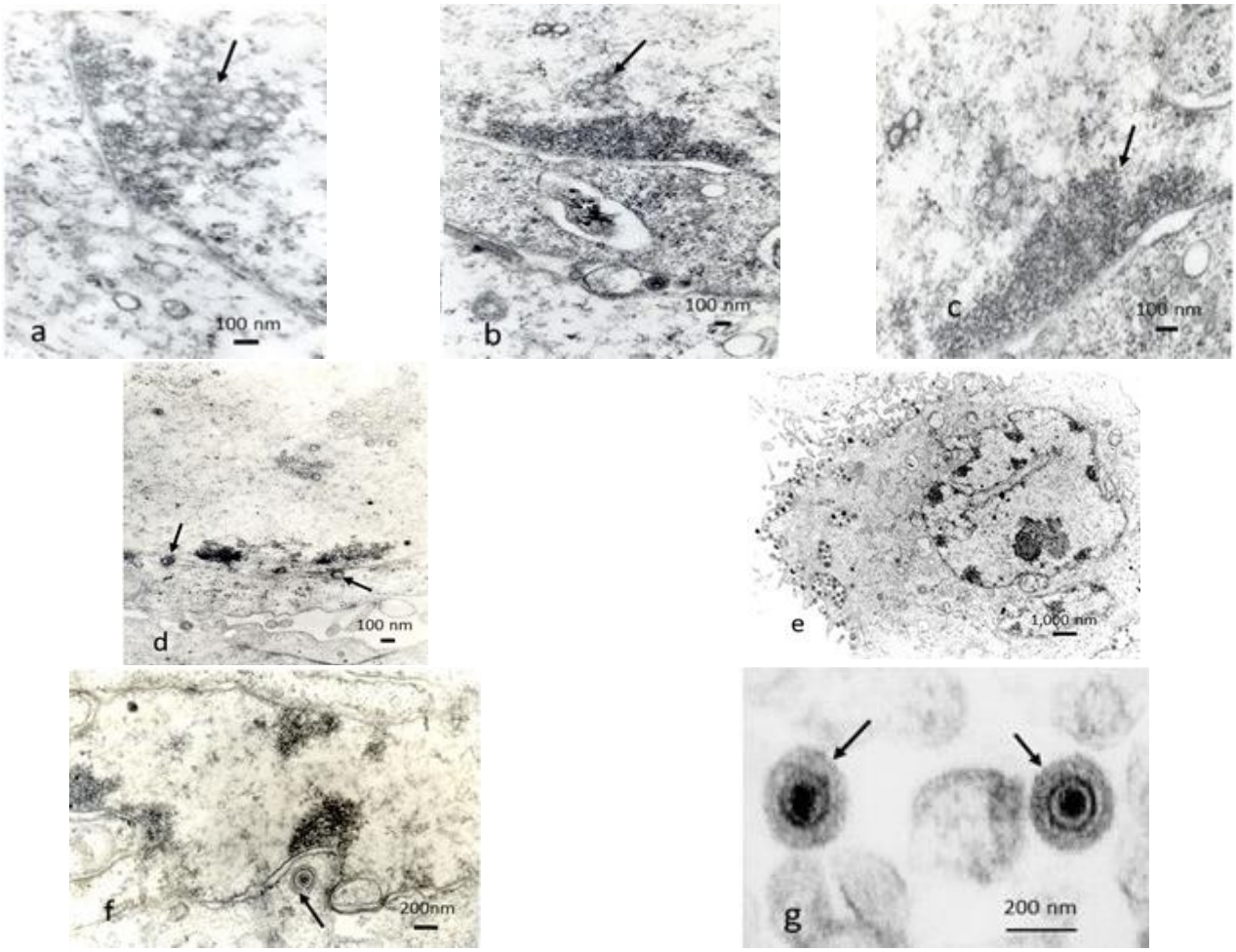

FIGURE 3: ELECTRON MICROSCOPY OF IB-RS-2 INFECTED WITH PrV AT 4-8 H pi, ARROWS INDICATED MORPHOGENESIS OF INTRANUCLEAR CAPSIDS (a) AND (b). HIGHER MAGNIFICATION OF A CELL IN (b) SHOWING MARGINALIZED CHROMATIN (c). INFECTED CELL SHOWING DISTENDED GAP OF THE NUCLEAR MEMBRANE AND NUCLEOCAPSID IN THE PERINUCLEAR SPACE INDICATED BY THE ARROW (d). INFECTED CELL WITH PrV AT 8 h pi (e) HIGHER MAGNIFICATION OF A CELL IN (e) SHOWING PARTIALLY ENVELOPED NUCLEOCAPSID UNDERGOING RE-ENVELOPMENT INTO THE CYTOPLASM (f) ULTRASTRUCTURE OF MATURE VIRAL PARTICLE (ARROW) (g).

Marginalization of chromatin is also observed in the replication of other herpesviruses such as HSV-1. Myllys et al. (2016) related an increase in the nuclear volume and relocation of the host chromatin into the nuclear periphery and in this place, viral nucleocapsid assembly occurs. They also described that there is the formation of channels across the chromatin layer and in this gaps frequently contained viral nucleocapsids allowing for the passage of progeny viruses to the nuclear envelope (Myllys et al., 2016).

Between 4 and 8 h pi, cells exhibit capsids and pleomorphic cores mostly with little electron density (Fig. 3d). These capsids increase in number and size by forming the crystalloids in the nucleus (Fig. 4a).

In the same infected cell, a series of events occurring at the same time can be observed such as a viral particle that appears in an invagination of the nuclear envelope and seems to be engulfed by membranes in the cytoplasm (Fig. 3e, f).

Capsids with dense cores can be seen between the membranes of the nucleus of the infected cell, suggesting that they may be acquiring envelope from nuclear membranes (Fig. 3 d).

We can also observe the presence of many complete virions in vesicles or at the periphery of cells as well as extracellular viruses adhered to the plasma membrane (Fig. 3 e,f,g).

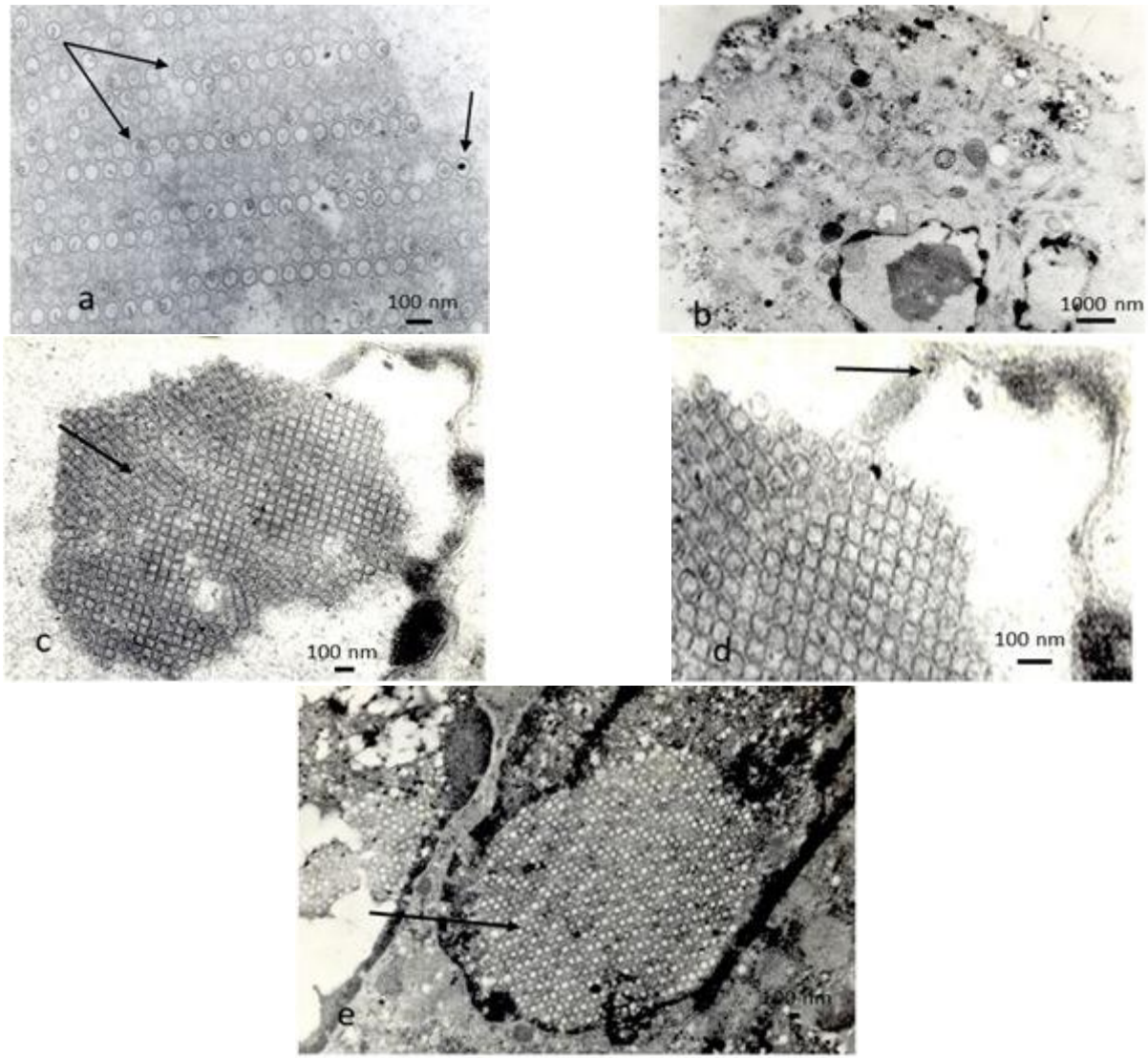

FIGURE 4: ELECTRON MICROGRAPH OF IB-RS-2 INFECTED WITH PrV AT 18-24 h pi, ARROWS INDICATED LARGE NUMBER OF CAPSIDS AGGREGATED AND ARRAYED AS CRYSTALOIDS (a). INTERNAL FEATURES OF INFECTED CELL AT 18 h pi (b). HIGHER MAGNIFICATION OF A CELL IN (b) SHOWING A PSEUDOCRYSTAL (ARROW) (c) HIGHER MAGNIFICATION OF A CELL IN (c) SHOWING A NUCLEOCAPSID IN A CONNECTION EXTENSION WITH THE NUCLEAR MEMBRANE INDICATED BY A ARROW (d). INFECTED CELL AT 24 pi SHOWING ABSENCE OF ORGANIZED STRUCTURES WITH CELL DESTRUCTION AND A LARGE CRYSTALLOID (ARROW) (e).

The nuclei of the cells exhibit irregular condensation and marginalization of the chromatin, which become more intense in the later stages of infection. In these nuclei chromatin spraying (Fig. 4b), nucleolus disappearance and invaginations of the nuclear envelope also occur. Such alterations compromise and decharacterize the structures that constitute the nucleus (Fig. 4b, c, d, e).

In the late stages of infection (18 - 24 h pi), we can observe a great destruction of the typical cellular structures and the presence of aggregates of capsids, the crystalloids, which almost occupy all the space that was previously the nucleus and even the cell.

Most of the micrographs of IB-RS-2 cells infected in the presence of *Persea americana* extract (treatments 4 and 5) exhibit similar morphology to that observed in uninfected control cells even 7 h pi (Fig. 5a). In few cells, we verified the presence of infection, but the structural aspect was different from that observed in the infected cells without plant extract. Figure 5c shows a cell after 1 h of virion infection with an increased core and amorphous capsid exhibiting structure different from that observed in virus control (Fig. 2c; Fig. 3g). Some infected cells present virions and cellular changes resulting from the presence of viral replication, but with different aspects from those observed in infected control cells. Many viral particles exhibit non-electron-density bundles and adhered to the plasma membrane of the cell (Fig. 5b); some 7 h pi cells show viral particles in vesicles in the nucleus or cytoplasm of the infected cell in pairs or not (Fig. 5d, e).

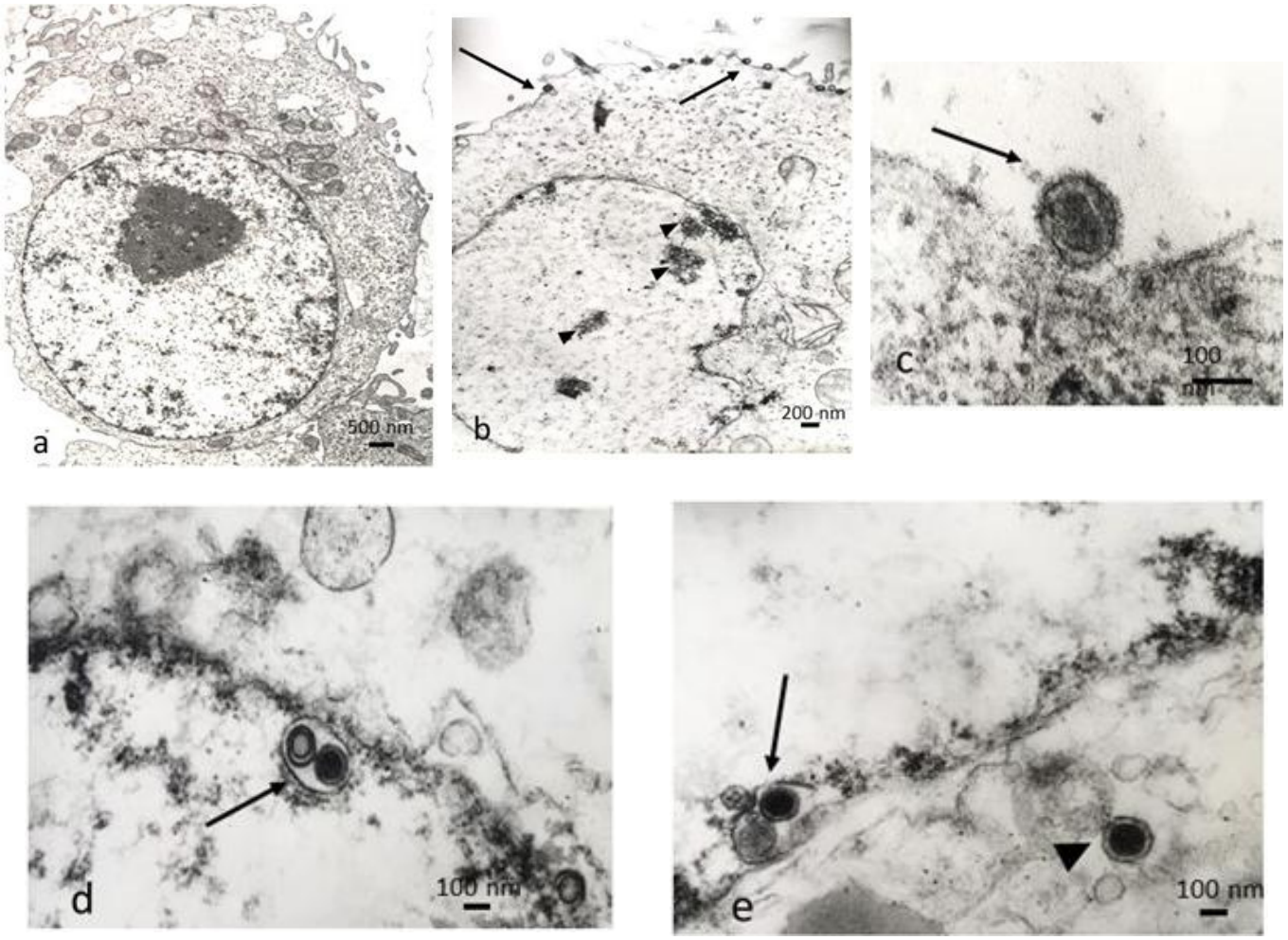

FIGURE 5: ELECTRON MICROGRAPH OF PrV INFECTED CELL AT 7 h pi AND TREATED WITH CRUDE EXTRACT OF PERSEA AMERICANA LEAVES. INFECTED CELLS TREATED WITH EXTRACT SHOWING MORPHOLOGICAL APPEARANCE SIMILAR TO UNINFECTED CELLS (a). INFECTED CELLS TREATED WITH EXTRACT DISPLAYING VIRAL CAPSIDS IN THE NUCLEUS (ARROWHEADS) AND PARTICLES ADHERED TO THE PLASMA MEMBRANE (ARROWS) (b). INFECTED CELLS TREATED WITH EXTRACT SHOWING DEFECTIVE VIRAL PARTICLE (ARROW) (c). INFECTED CELLS TREATED WITH EXTRACT SHOWING DOUBLE CORES IN A SINGLE WRAP IN THE CORE (ARROW) (d). CELL EXHIBITING DOUBLE CORES IN A SINGLE ENVELOPE IN THE SPACE PERINUCLEAR (ARROW) AND A VIRAL PARTICLE INTO THE CYTOPLASM OF THE INFECTED CELL (ARROWHEAD) (e).

The replication cycle of PrV in IB-RS-2 lineage cells shows the same replication strategies observed in the genus Varicelovirus with a lytic replication (Johnson and Baines, 2011). The IB-RS-2 strain Nova Prata was able to destroy in a few hours the cell monolayer. In the first few minutes of infection, virions are already found inside the cells. Morgan and Howe (1968) have also observed adsorption and penetration of the Sendai virus into the cells at 5 min of inoculation. Soon after the adsorption, the virions underwent various types of changes such as the fusion of the viral envelope in the cellular membrane, dissolution of the capsid and the consequent release of the viral core in the cytoplasm. The viral core in the cytoplasm is not detected because it is difficult to distinguish it from other granules present in both the cytoplasm and the nucleus.

Precursor viral particles appear starting at 4 hours and will form the nuclear crystalloids.The capsids with the viral DNA incorporated migrate to the nuclear membranes where they receive viral envelope after de-envelopment and re-envelopment constituted by cytoplasmic membranes. Then the virions appear grouped in vesicles that merge with the plasma membrane and finally are released out of the cell and become associated with it.

In this study, we confirmed that *Persea americana* extract inhibited the PrV infections, as described in our previous reports (Koseki et al., 1990; Simoni et al., 1996; Almeida et al., 1998). We report here that *Persea americana* can affect the virus particle in the adsorption stage, and probably at late stages of virus maturation related with capsid assembly, envelopment and release of infectious virus from the cell.

Antiviral drugs can act at various stages of viral replication and many of them have been described as being against herpesviruses, for instance, the drug Nelfinavir inhibits the maturation and export of HSV-1 (Kalu et al., 2014). However, there are few articles describing the action of plant extracts against herpesvirus on the replication steps studied by TEM (Kerr and Pennington, 1984).There are few reports on the effects of plant extracts on virus infected cells from the point of view of electron microscopy. Studies are needed to better elucidate the mechanisms of action of complex mixtures such as plant extracts and to identify the chemical components responsible for these effects.

IV. CONCLUSION

Persea americana leaf extract inhibited the replication of PrV, a virus of great importance in animal health. Electron microscopy of wild-type PrV infection generated results that corroborated the results found by other authors for both HSV-1 and those observed in the replication of PrV.

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Mycobacterium gordonae infection in freshwater fish from lakes and ponds in a park at São Paulo city, Brazil

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***Abstract*—** *In recent year's fish farming has greatly increased in Brazil, favoring the development of diseases such as mycobacteriosis. This is a chronic progressive disease that affects temperate and tropical fish, both freshwater and marine. Mycobacteriosis can occur in several species of fish and amphibian. In addition, some species of Mycobacterium spp. can be transmitted to humans by occupational or recreational source. A total of 54 fishes from lakes from São Paulo city, were collected and examined for mycobacteriosis. Granulomas were visualized in 5 fishes via histopathology (H&E), and acid-alcohol resistant bacilli were visualized in 8 animals by electron microscopy and 8 were positives using the Fite -Faraco technique. In this study, we isolated acid-fast bacillus from one fish which were identified as M. gordonae by molecular methods: PCR and sequencing.*

***Keywords*— *mycobacteriosis, pathology, aquaculture, sanity, disease*.**

I. INTRODUCTION

In recent years fish farming has greatly increased in Brazil, favoring the development of diseases such as mycobacteriosis (ISHIKAWA et al., 2001; ROMANO et al., 2012). This is a chronic progressive disease that affects temperate and tropical fish, both freshwater and marine (JACOBS et al., 2009).

Mycobacteria spp. can cause serious and costly diseases in different vertebrates and invertebrates, such as humans (tuberculosis, leprosy, Buruli ulcer), livestock (bovine tuberculosis) and ectotherms (reptiles, amphibians and fish) (BIET et al., 2005; GRANGE & YATES, 1986; JACOBS et al., 2009; REAVILL & SCHMIDT, 2012; SHINNICK & GOOD, 1994; TORTOLI, 2003; TURENNE et al., 2007).

The first *Mycobacterium spp.* was identified in carp in 1897 (BATAILLON, DUBARD, TERRE, 1897). This was named as *Mycobacterium piscium* and was shown to be highly pathogenic to frogs and some endothermic animals. The main species identified in captive and wild fishes *are M. marinum, M. fortuitum and M. chelonae* (mainly in marine fish). New species have also been proposed, including M. salmoniphilum. Other organisms related to *M. ulcerans* and the *M. tuberculosis* complex have also been recently implicated (GAUTHIER & RHODES, 2009; JACOBS et al., 2009).

Transmission typically occurs by ingestion of contaminated food and water, but transovarian transmission can also occur in viviparous species (ASTROFSKY et al., 2000; GAUTHIER & RHODES, 2009; JACOBS et al., 2009).

Granulomas are mainly located in the spleen, liver and kidney during the initial stages of the disease, but can spread to any other organs, leading to terminal illness. At the beginning of the infection, macrophages are invaded and become epithelioid cells. Giant cells may or not be present (GAUTHIER & RHODES, 2009; JACOBS et al., 2009).

In fish, the severity of mycobacteriosis ranges from chronic infection, without major changes in tissues and few losses, to severe and acute infection, with high mortality, depending on the mycobacteria and fish species involved. Clinical signs include weight loss, apathy and lethargy, decreased fertility spine defects, exophthalmia, abnormal behavioral, changes in skin color, and ulcerative lesions in the skin, gills, fins and musculature. There may be enlargement of liver, spleen, kidney and nodular lesions in internal organs (ASTROFSKY et al., 2000; GAUTHIER & RHODES, 2009; JACOBS et al., 2009; ROMANO et al., 2012).

There are few reports of mycobacteriosis in fish and amphibian species in Brazil. Studies are needed to understand the occurrence and consequences of the disease in animals maintained in captivity in lakes, ponds and parks (FERREIRA et al., 2006; ISHIKAWA et al., 2001; LEITE et al., 1998; ROMANO et al., 2012).

The aim of this study was to study this disease of fishes in the lakes and ponds park in São Paulo city and from decorative lake, unfit for consumption.

II. MATERIAL AND METHOD

2.1 Experimental Design

A total of 54 fishes (41 carps, 9 tilapias, 2 curimbatas (Prochilodus lineatu)s, 2 pirapitingas (Piaractus brachypomus), both male and female, were randomly collected from lakes and pounds in Jardim da Luz, located in downtown area at São Paulo City, Brazil. Samples of spleen, hepatopancreas, kidney and gills were fixed in 10% neutral buffered formalin or frozen. The sampled fishes varied in length from 10 cm to 52cm. Macroscopically, 2 carps showed lesions suggestive of granulomas.

2.2 H.E. Technique

Serial sections were prepared from the fixed material: fragments embedded paraffin. 5µm sections were cut using a microtome and adhered to the glass slides and stained by hematoxylin-eosin.

2.3 Fite- Faraco Ziehl-Neelsen technique (Z-N) (we used Fite-Faraco staining protocol, since the classic staining protocol of Ziehl Neelsen may result in false negatives).

Serial sections were prepared from the fixed material: fragments embedded paraffin. 5µm sections were cut using a microtome and adhered to the glass slides. The sections will be de-paraffinize in a solution composed of two parts of xylol and one part of peanut oil (or almond oil) for 15 minutes. The sections are then washed in tap water to remove the remaining xylene / oil mixture. Filter on carbol fuchsin solution, DO NOT HEAT, for 20 mins. Wash in running tap water. Differentiation will be done by means of 10% sulfuric acid for 2 minutes. Wash well in running tap water, rinse distilled water. Counterstain in 0.25% methylene blue for 20 seconds. Wash and blot dry. DO NOT DEHYDRATE IN ALCOHOL. Clear in xylene. Repeat the blotting-xylene treatment until section is clear. Mount in a DPX type mountan (FITE ET AL., 1947).

2.4 Negative Contrasting

The samples were suspended in 0.1M phosphate buffer pH 7.0 and placed in contact with metal grids previously coated with collodion and carbon film drained with filter paper. They were negatively contrasted with ammonium molybdate to 2% and pH 5.0 and observed using a Philips EM 208 (BRENNER & HORNE, 1959; HAYAT & MILLER, 1990) TEM.

2.5 PCR

For mycobacterial isolation, approximately one gram of each clinical specimen was ground with sterile sand, decontaminated by the classical Petroff method and seeded in four tubes containing medium of Stonebrink and four tubes with Petragnani medium. Two tubes of each seeded medium were incubated at 37°C and the remaining tubes at room temperature. All tubes were observed weekly for checking the growth of the colonies (KANTOR, 1988). To PCR, the isolated colonies were resuspended in 1.5mL sterile ultrapure water. DNA extraction was performed by inactivation of the samples by boiling at 100°C for 5 minutes, after which they were subjected to freezing at -20°C for at least one hour (BEMER-MELCHIOR & DRUGEON, 1999).

Thawed samples were PCR amplified using TB11-TB12 primers, designed for identification of the *Mycobacterium genus* (TELENTI et al., 1993). These generated a final product of 439 bases pairs. DNA amplifications were held in thermal cycler, submitting samples to the initial treatment of 95°C for 5 minutes, followed by 45 cycles of three temperatures: denaturation at 94°C for 1 minute, annealing at 65°C for 1 minute and extension at 72°C for 1 minute. After the last cycle, was held a final extension at 72°C for 7 minutes after which the product remained at 4°C until its analysis by electrophoresis in horizontal vat. PCR products were observed in 1.5% agarose gel containing 0.01% ethidium bromide, viewed under UV light and photographed with the aid of molecular gel doc system. To sequencing, the almost sequences of the 16S rDNA gene were obtained as described by CAMPOS et al. (2012).

III. RESULTS AND DISCUSSION

Of the 54 fishes examined, 8 were positives when stained using the Fite -Faraco Z-N technique (Fig 1). In the H&E staining, 5 animals presented numerous granulomas (Fig 2 a and b) of numerous sizes, with caseous necrosis in the center, eosinophilic cells and surrounded by inflammatory cells and fibroblasts (1 animal in the spleen, 3 kidneys and pancreas, 1 in hepatopancreas).

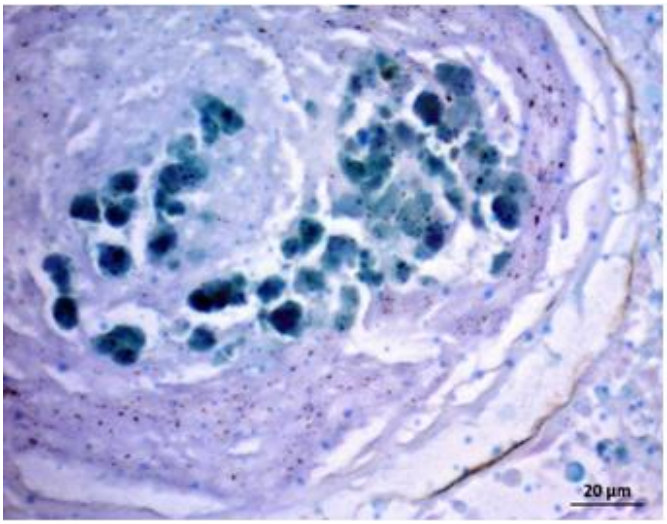

FIG 1 - PHOTOMICROGRAPH OF HEPATOPANCREAS SHOWING NUMEROUS RED MYCOBACTERIA (SMALL POINTS) INTO A GRANULOMA USING STAINED BY FITE- FARACO ZIEHL NIELSEN TECHNIQUE. (Bar: 20µm).

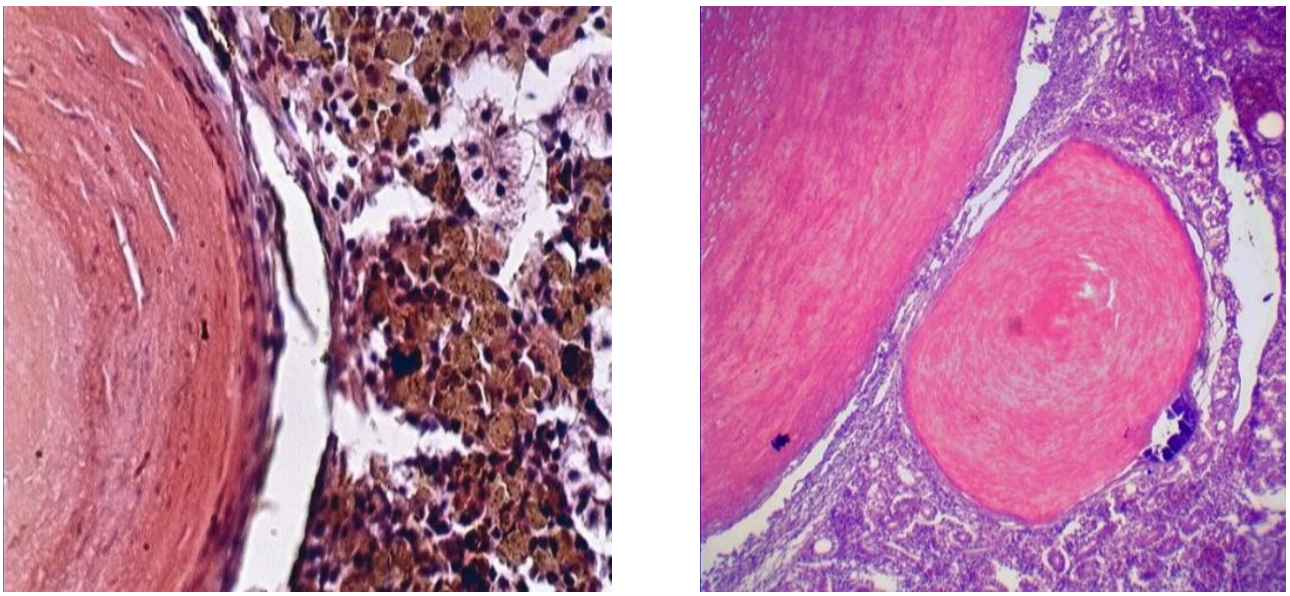

FIG 2 a and b - PHOTOMICROGRAPH SHOWING AT RIGHT 2 GRANULOMAS WITH CASEOUS NECROSIS IN THE CENTER, SURROUNDED BY EOSINOPHILIC AND FIBROBLASTS AND INFLAMMATORY CELLS (LYMPHOCYTES, NEUTROPHILS, HETEROPHILES AND MELANOMACROPHAGES) AND AN AREA OF CALCIFICATION (ARROW). HE. BAR = 200µm. ON THE LEFT, AT HIGHER MAGNIFICATIONS, A GRANULOMA AND NUMEROUS MELANOMACROPHAGES (BROWN). HE. (Bar: 50 µm).

It was observed, thus, lymphocytes, neutrophils and heterophiles. Some macrophages alone or in groups, were filled with golden-yellow substance (melanomacrophage) next to the granulomatous or degenerative lesions. The most severe changes were observed in the kidney that showed convoluted tubules in vacuolar degeneration or necrosis. Glomeruli were also visualized in degenerating, necrotic or deformed, hypo- or hyperplastic and presenting increased Bowman's space. Nephrocalcinosis was observed in 2 cases.

With the transmission electron microscope, *Mycobacterium* spp was also observed (Fig 3 a and b) in 8 fishes, these same animals that were positive for the Z-N technique.

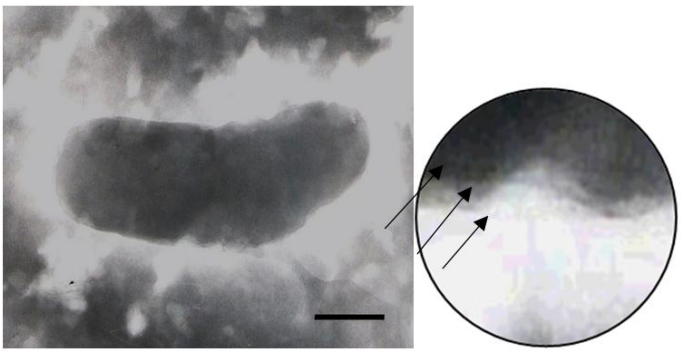

FIG 3 a and b - ELECTRON MICROGRAPH OF MYCOBACTERIUM SPP IN FISH LIVER AND VISUALIZED BY NEGATIVE STAINING TECHNIQUE. AT THE ARROWS, THE TRIPLE LAYER ENVELOPE. (Bar: 140 nm)

Slow-growing scotochromogenic colonies were obtained from one fish (Fig 4). These colonies were catalase positive and did not reduce nitrate. The nearly entire 16S rDNA gene (1456pb) sequence obtained showed 99.65% identity with *M. gordonae* type strain (ATCC 14470). This was deposited in GenBank (accession number JN899581). We also sequenced the 16S rDNA gene of the type strain of *M. gordonae* ATCC 14470, (accession number JN899579) for comparative identification purposes, as in the sequence GenBank (X52923) had 8 unidentified (N) bases.

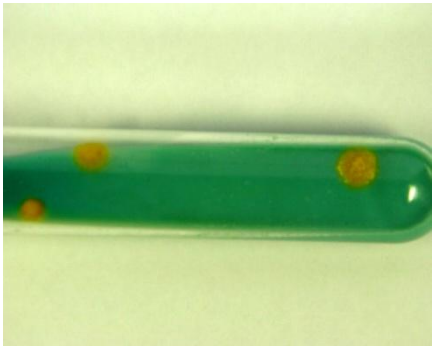

FIG 4 - COLONIES OF MYCOBACTERIUM GORDONAE ISOLATED FROM FISH IN PETRAGNANI MEDIUM

IV. DISCUSSION AND CONCLUSION

Brazil has enormous potential for animal and fish farming production, given its vast land, water sources and favorable weather conditions. In Brazil, fish are typically raised in lakes for consumption, although in this study they were not intended for consumption.

High concentrations of fish can favor the onset epizootic disease outbreaks caused by *Mycobacteria* spp, although in natural environmental conditions spontaneous disease outbreaks can also occur (GAUTHIER & RHODES, 2009; HECKERT et al., 2001; RAMSAY et al., 2009).

Mycobacteriosis can occur in several species of fish. In addition, some species of Mycobacterium spp. can be transmitted to humans by occupational or recreational source (BHATTY et al., 2000; GAUTHIER & RHODES, 2009; JACOBS et al., 2009; REAVILL & SCHMIDT, 2012).

In Brazil, there are a few studies on mycobacteriosis in ectotherms. MOK and CARVALHO in 1984, described the presence of *M. chelonei* in *Bufus marinus* and *B. granulosus,* and although mycobacteriosis outbreaks in frog farms have been reported by FERREIRA et al., 2006. In fish, was notification by ISHIKAWA et al., 2001 and ROMANO et al., 2012.

Histopathological examinations are important for early diagnosis of mycobacterial infection in fish. Granulomas are suggestive of mycobacteriosis but are not pathognomonic of the disease; acid–fast bacilli must be visualized in the lesions. A positive culture will provide a definitive diagnosis, but it is not very easy to isolate mycobacterias at 37° C, the optimum temperature for human pathogens. Fishes isolate are well-characterized by molecular methods.

In this study, we isolated acid-fast bacillus from one fish which were identified as *M. gordonae* by molecular methods. It is recommended that further, more in-depth, studies are undertaken to gain a better insight of the impact of this disease in cultured and wild fish species in Brazil.

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Evaluation of knowledge, attitude and practices in hand hygiene of students in biological sciences from Felix Houphouët-Boigny University of Cocody (Abidjan-Côte d'Ivoire)

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Abstract— *The purpose of this paper is to identify risk factors, analyze and compare their effects on student hygiene behavior at Félix Houphouët-Boigny University. It is a descriptive and analytical cross-sectional study, initially covering 333 students in the second and third year of Training and Research Unit of Biosciences from University Félix Houphouët-Boigny enrolled during the university year in 2015-2016. A total of 318 out of 333 students Interviewed or 95.49% attending the toilets. The majority of the students questioned, 219 (65.76%), had poor hand hygiene practices. There is a non-significant difference between hand hygiene practice and gender (p = 0.16). A staff of 325 students questioned out of 333 or 97.59% denounced a lack of toilets. 95.49% of them are aware of the handwashing procedure. The toilets are also used by girls and boys. We note an insignificant difference between toilet use and sex (p = 0.76).*

The correct practice of handwashing is not practiced by the students of Training and Research Unit of Biosciences; this was confirmed by direct observation. In the area of toilet surveys, lacks of hygiene equipment and inadequate toilets have been reported.

Understanding the challenges of hand hygiene practice in academic may help in the development of hand hygiene promotion strategies for the prevention of infections, especially those that are handled. The promotion of hand hygiene should start with health education.

Keywords— Hand, Hygiene, university, Abidjan, Côte d'Ivoire.

I. INTRODUCTION

Poor hygiene is a major concern in many low-income countries, especially those in sub-Saharan Africa (IFRCS, 2007; CSRS, 2013). This situation leads to the proliferation and spread of several germs responsible for pathologies (Racurt, C. *et al.*, 2006; Adoubryn *et al.*, 2005; Rasso *et al*., 2005; Mouchet *et al.*, 1987; SOLIDARITE International, 2017). The hand represents the tool most often used by humanbeing and serves him to interact with his environment (Santé Canada, 2010). This environment is colonized by a varied flora. In contact and colonized by these germs, the hand constitutes a potential vector of these. Hence, hand hygiene is an essential element for the public health mission to reduce the transmission and the consequences of pathologies, especially man-made diseases. Optimal hand hygiene behavior will be considered the cornerstone of prevention (Red Cross Côte d'Ivoire, 2014; SOLIDARITE International, 2017; WHO, 2017a). Infectious diseases of parasitic, bacterial or viral origin are often the cause of high absenteeism among students. This often leads to a drop in their academic performance (Eau-vive, 2010; Diallo, 2015). Many of these infectious diseases are handled and their eradication requires hygienic behaviors, including handwashing (Delphine, 2008; SFHH, 2009). Investment in the water and sanitation sector produces considerable economic benefits. It is estimated that a dollar invested in these services would be a profit of $ 4.3 (WHO, 2017a). Ebola haemorrhagic fever has plagued many countries neighboring Côte d'Ivoire in 2014

(WHO, 2013). The man becomes contaminated when he comes into contact with the excretions of a man, an animal (bat, chimpanzee ...) sick or healthy carrier. As far as contamination between man is concerned, it is a horizontal or human-to-human transmission. In this type of transmission, the disease spreads rapidly in high density populations. The promiscuity of the student population at the Félix Houphouët-Boigny University is worrying. In addition, it was found that the illustration poster of the handwashing procedure does not exist in our toilets. The aim of this study is to evaluate the hygiene practices of students studying animals (domestic and wild) in order to assist in the promotion of hand hygiene.

II. MATERIAL AND METHODS

2.1 Material

Three categories of materials were used in our study. These include the survey equipment, the toilet survey sheet and the hand-held observation record of hand hygiene practice.

2.1.1 Survey equipment

It is a questionnaire which covers the main themes concerning general hygiene: Knowledge of hand hygiene, knowledge of handwashing procedures and self-assessment of hygienic behavior. The questionnaire, to be easily accepted by the students surveyed, was reduced to the basic hygiene notions for everyday practice. It was subjected to the critical reading of students who were not part of the survey and was validated by a statistician of ENSEA (National School of Statistics and Applied Economics).

It consists of five parts:

The first part concerns the student himself: his name, level of study, nationality, age, sex, ethnicity, religion, and place of residence. The second part concerns the knowledge of hygiene at university. There are six questions in this section. The student is asked if he attends university toilets, whether there are mixed toilets, whether the toilets are cleaned regularly, what equipment and materials are available for hand washing: present a washbasin in the consultation room, type of soap used, use of hydro-alcoholic gel, wiping equipment, trash. The third part contains 21 questions on the knowledge of procedures. The expected answers are yes or no. They include hand washing, use of hand sanitizer, sneezing in public places and the general presence of bacteria. The fourth part deals with the self-assessment of student's hygienic behavior and the importance they attach to different hand hygiene behaviors.

Finally the fifth and last part includes seventeen questions on the cleaning of computer systems.

2.1.2 Prospecting equipment

To find out about the toilets at Training and Research Unit of Biosciences, we used a digital camera to take pictures, a muffler to prevent bad odors and a pair of disposable gloves for safety measures.

2.1.3 Equipment for direct observation

Direct observation of the practice of hand hygiene was carried out by means of a card containing the identification of the subject, observation time (Morning, Afternoon and evening), hand washing behavior (None Washing, rinsing hands and washing hands with soap) and hand washing time.

2.2 Methods

This study was carried out over the period from May 2016 to April 2017. It is not intended to judge students but to try to know their habits in order to make them aware of this topical issue and to inform them about the latest recommendations. The first part of the work was the development of a questionnaire, a toilet survey and a good hand hygiene record.

2.2.1 Population and study site

The study was carried out at Félix Houphouët-Boigny University in Cocody-Abidjan (Côte d'Ivoire) (Figure 1). Demography is growing. It is estimated at more than 60,000 students (UFHB, 2016). The study population for the survey is made up of

students in the second and third year of Bachelor of Animal Biology for the academic year of 2015-2016 of Training and Research Unit of Biosciences.

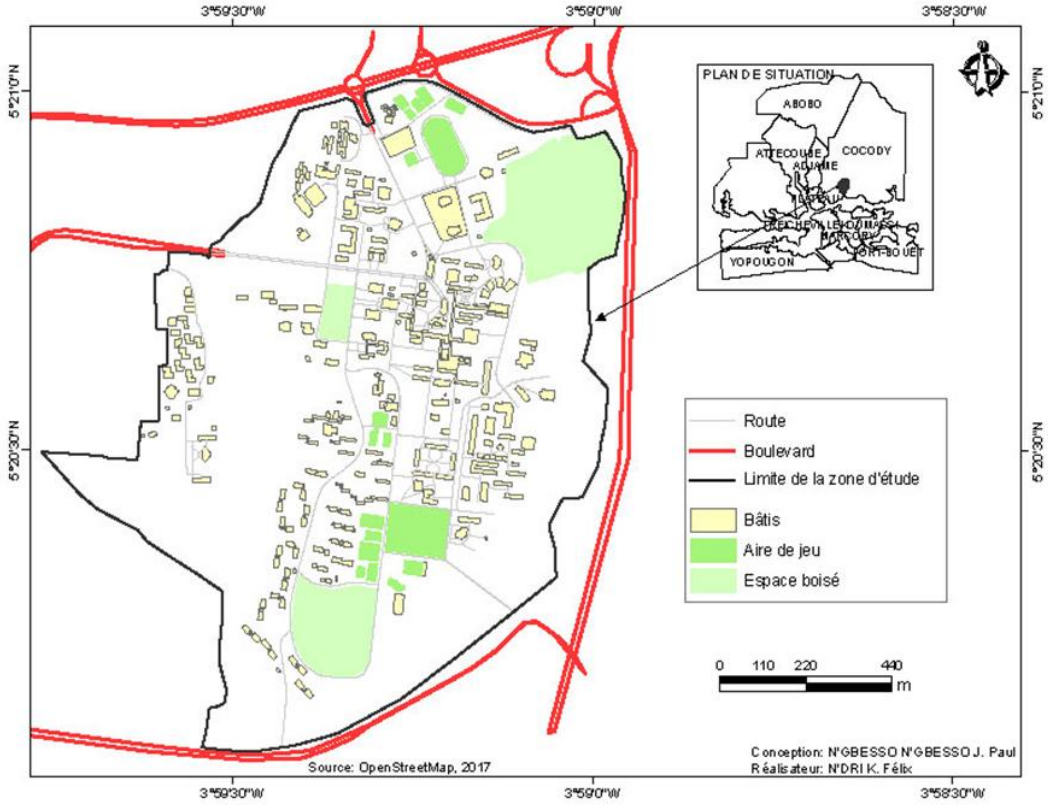

FIGURE 1. MAP OF STUDY SITE

These are students specially for study animals. These students carry out by their specific practice gestures "at risk" from the point of view of transmission of germs on a daily basis. The observation to the good practice of the hygiene of the hands to concern the students of Training and Research Unit of Biosciences attending the toilets.

2.2.2 Questionnaire survey

A sample of all students in the second and third year of Training and Research Unit of Biosciences was interviewed as part of the survey. To do this, we listed the selected students. Then, based on their workforce, individual questionnaire forms were printed. After a presentation of the subject of the survey, a copy of the questionnaire was given to each student to fill it "anonymously", sometimes asking for help in understanding certain items. Participants are those who have given their verbal and written agreements (Figure 2). Then, the list and the number of corresponding cards were given to the delegate. The latter was responsible for handing over a questionnaire sheet to each volunteer student interested in the survey. The cards were received by the delegate after 48 hours, after a second clearance.

2.2.3 Prospecting toilets

The toilets were surveyed by direct observation in order to count the number of functional toilets, determine the available equipment and sanitation conditions. The surveys were carried out over a dozen months, from May 2016 to April 2017. They consisted of going around the toilets points for the students of the UFR Biosciences. At each point, the number of functional and non-functional toilets was counted. Then the existing hygienic material was identified.

2.2.4 Direct observation of hand hygiene practice

The observation was made discreetly by concealing the observations of hands washing behaviors. All observations were recorded using a standard coding form. The coding form consisted of the subject's ID, date, observation time, and hand washing behaviors. Washing behaviors were recorded in three categories: no washing (leaving the toilet without washing or rinsing hands), attempting to wash hands (wet hands without using soap) and washing hands with soap. Handwashing time

was discreetly based on how many seconds the subjects' hands were placed under running water during washing, foaming and rinsing. Observation time was collected and the nominal time categories were formed at the end of the analysis.

2.2.5 Data analysis

The data were checked, coded and entered in Excel and analyzed using Software R version 3, 3, 3**.** For each respondent, the percentage of correct answers on a given theme allowed to classify it in the scoring grid developed from 0 to 100. A score of positive responses less than or equal to 49, between 50 and 100 is considered respectively as Insufficient and acceptable. This score at the thematic level is reflected on each chapter according to the same principle.

For direct observation to good hand hygiene practice, the data were compiled and analyzed using Chi-square analysis. More specifically, Chi-square analysis was used to identify statistically significant differences in the demographic variables of the subjects, environmental variables in toilets and hand washing behaviors.

Data from the toilet survey to a good observation of hand hygiene practice and from the survey were captured in the Excel software. Then, the percentage of students who participated in the survey and the percentage of the affirmative responses of the survey questions were calculated. The rates obtained were used to assess the participation of students in the survey and to assess their hygienic practices.

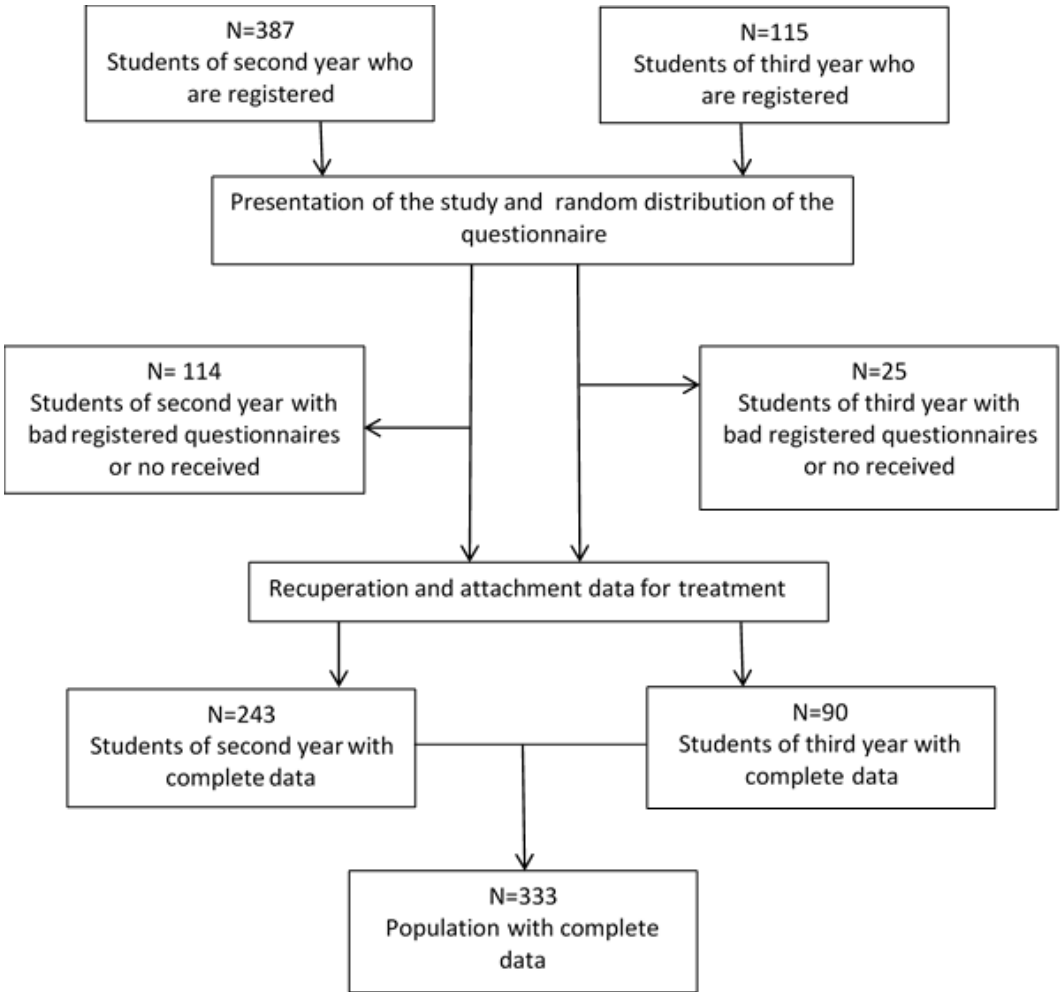

FIGURE 2. FLOW CHART OF QUESTIONNEMENT'S METHODOLOGY

III. RESULTS

3.1 Survey results

3.1.1 Sociodemographic characteristics of the population studied

Three hundred and thirty-three (333) students were studied, including 243 (72.97%) male and 90 (29.02%) females [95% CI 0.62 - 0.70]. The average age is 24 ± 6 years. The study participation rate was 66.33% (Table 1).

3.1.2 Knowledge and Habit of Hand Hygiene of Students Questioned

A total of 318 out of 333 students surveyed or 95.49% attended the toilets. The majority of students surveyed, 219 (65.76%), had poor hand hygiene practices, compared to 114 (34.23%) who had an acceptable hygiene practice (Figure 3). There is a non-significant difference between hand hygiene practice and gender (p = 0.16).

TABLE 1
DISTRIBUTION OF PARTICIPANTS BY CHARACTERISTICS SOCIO-DEMOGRAPHIC

Variables		Frequencies	Percentage %	95 % CI
Gender	Male	243	72. 97	[0.62 – 0.70]
	Female	90	27.02	[0.22 - 0.32]
Total		333		
Nationality	Ivoirian	326	97.89	[0.95 - 0.99]
	Burkinabe	3	0.9	[0.001 - 0.026]
	Béninoise	3	0.9	[0.001 - 0.026]
	Malienne	1	0.3	[7.602656e-05 -0.016]
Total		333		
Level of study	Second year	241	72.37	[0.67 - 0.77]
	Third year	92	27.62	[0.22 - 0.32]
Total		333		
Ethnicity	Baoulé	81	24.32	[0.19 - 0.29]
	Sénoufo	40	12.01	[0.08 - 0.15]
	Agni	31	9.30	[0.06 - 0.12]
	Malinké	20	6	[0.03 - 0.09]
	Attié	15	4.5	[0.02 - 0.07]
	Other	146	43.84	[0.38 - 0.49]
Total		333		
Religion	Christian	235	70.57	[0.65 - 0.75]
	Moslem	71	21.32	[0.17 - 0.26]
	Animist	1	0.3	[7.602656e-05 -0.016]
	None	26	7.80	[0.05 - 0.11]
Total		333		

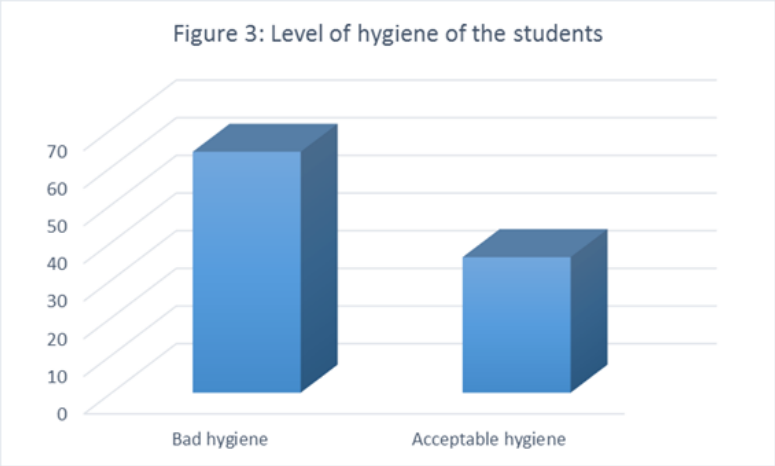

FIGURE 3: FREQUENCY DISTRIBUTION DIAGRAM ACCORDING TO SCORES IN HYGIENIC PRACTICE FOR STUDENTS OF TRAINING AND RESEARCH UNIT OF BIOSCIENCES

TABLE 2
DISTRIBUTION OF FREQUENCY ACCORDING TO SCORES IN KNOWLEDGE OF STUDENTS OF TRAINING AND RESEARCH UNIT OF BIOSCIENCES

Thematic	Score (%)		
	70 - 100	50 - 69	≤ 49
Hands washing	95 (28.52%)	207 (62.16%)	31 (9.30%)
Using hands desinfectant	129 (38.73%)	133 (39.93%)	71 (21.32%)
Sneezing in public site	254 (76.27%)	54 (16%)	25 (7.50%)
Presence of germs pathogenic	317 (95.19%)	14 (4.20%)	2 (0.6%)

At the level of the various themes, hand washing, the use of hand sanitizer, sneezing in public places and the presence of pathogenic germs, we note average knowledge in general of students (Table 2).

3.2 Results of the prospecting of the different toilets of the students

At the level of toilets surveys, a lack of hygiene equipment (PH, soap or disinfectant liquid ...) and inadequate toilets have been reported. The toilets are the main sites for hand washing of students.

At the circumference of the Training and Research Unit of Biosciences, we counted nine (9) toilets area.

Of the 9 student washrooms area, five (5) or 55.55% were accessible at the start of the survey to stabilize at four (4) toilets area by the end of the survey (44.44%). We note that there were 16 functional toilets on a total of 26 toilets or 61.53%, for all students of Training and Research Unit of Biosciences. In addition, there is no hygienic material (Figure 4)

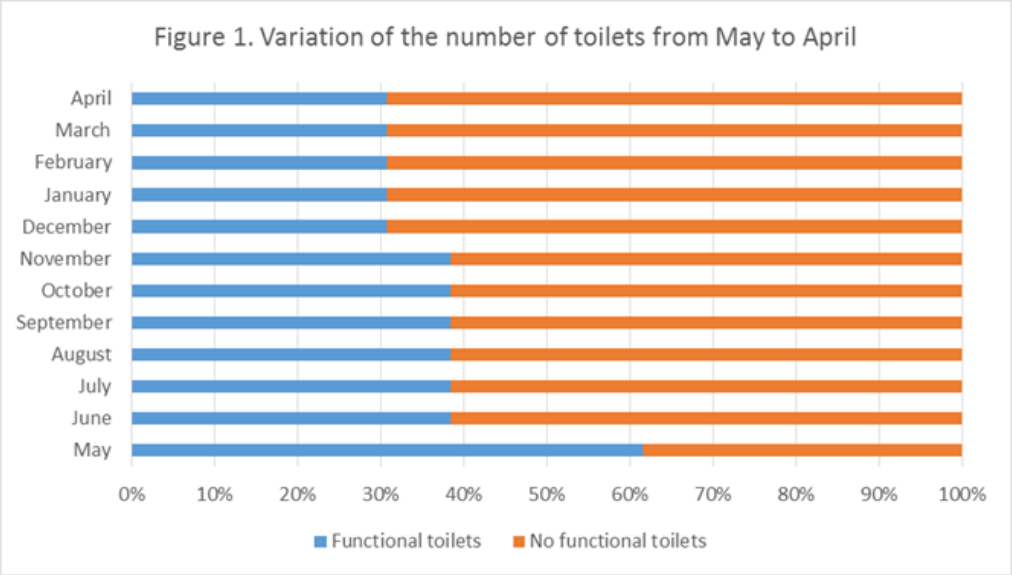

FIGURE 4: VARIATION OF FUNCTIONAL AND NON-FUNCTIONAL TOILETS BY MONTH

3.3 Direct observation of hand washing at toilet points

Direct observation on the ground revealed that men were more likely to use toilets than women, with a proportion of 92.86% compared to 7.14% (Table 3).

Chi-square analysis revealed statistically significant differences in handwashing behavior between length of hands washing timeand sex, and hand washing behavior and sex (Table 3). For example, about 96.10% of students do not wash their hands, compared to 3.9% of students. Male subjects frequent toilets at any time more than female subjects. The gender difference was confirmed with women using soap and engaging in proper handwashing behavior significantly

Conventional handwashing with soap and water is not practiced by any student and the time of passage of the under-running water during the washing does not exceed 15 seconds.

TABLE 3
CHI-SQUARE TEST: COMPARISON OF HAND WASH BEHAVIOR ACCORDING TO SEX (N = 6 16)

Variable	Sex		X^2	*p*
	Male 92.86% (n=572)	Female 7.14% (n=44)		
Observation time	%	%	1.328	p = 0.5146
Morning	93.18	6.82		
After-noon	90.48	9.52		
Evening	95.24	4.76		
Washing behavior			35.066	p <0.05
No washing	96.10	3.90		
Rinsing	80.47	19.53		
Washing hands with soap	0	0		
Length of hands washing time			52.569	p <0.05
0 seconds	96.10	3.90		
1 – 8 second(s)	83.05	16.95		
9 – 14 seconds	50	50		
15 seconds or longer	0	0		

IV. DISCUSSION

This study revealed that there are only four (4) toilets areas accessible to students out of the nine (9) that exist in the academic space of Training and Research Unit of Biosciences. At these four points, only sixteen (16) toilets are functional. When reporting the number of registered students (1498 individuals) out of sixteen (16) available toilets, we note a toilet for eighty-four (94) individuals.

This increases the number of people defecating in the open air. This has been demonstrated by WHO as this practice evolves as the number of people concerned increases (WHO, 2017b). It is then necessary to rehabilitate the points of the flawed toilets in order to put a larger number of toilets at the disposal of the students and to carry out a health education for a change of behavior.

In this momentum, we will be able to meet the objective of sustainable development, which stipulates that by 2030, ensure equitable access to sanitation and hygiene services for all and with a focus on the needs of women, girls and vulnerable people (UNICEF, 2017).

The exponential decrease of the toilets undoubtedly demonizes a very strong attendance of the university toilets. Heavy attendance may make these toilets at risk of infection. It should be noted that all toilets are mixed. This demonstrates the importance of interventions in the water, sanitation and hygiene (WASH) sector that can help prevent a wide range of diseases (diarrhea…) (WHO, 2017b). According to the WASH (Water Sanitation and Hygiene) charter, which stipulates that a toilet for every twenty (20) male and one toilet for fifteen (15) female individuals is required (Red Cross Côte d'Ivoire, 2014). In this context, it would be possible to ensure hygiene and sanitation favorable to all students.

Moreover, the toilets are only regularly washed with soap and rarely with bleach. This may be to avoid waiting for some students to defecate and urinate in the open air.

In general, the toilets were clean during our surveys despite their high attendance. It may be because they were cleaned 3 times a day that they looked clean. But the fact that they are often washed with soap and rarely with bleach demonstrates that they are not completely disinfected. Because soap alone is not enough to kill the majority of germs. Thus, relieving oneself in toilets could be a risk (WHO, 2017c). There is a greater risk of leaving the toilet because there is no toilet paper or soap to wash the hands after relieving so the students do not wash their hands with water and soap. We believe that even if 93.96% of the students know that washing their hands with water alone is useless, they can not do otherwise because the accessories (toilet paper, soaps, disinfectants ...) do not exist toilet. As 79.33% of respondents know that disinfectants are effective in cleansing hands may be that they use it.

These results demonstrate that the risk of infection in the heads of faucets, handles of toilet doors is not negligible in the study area because water alone is used to wash hands after the need. Thus, preventive measures such as hand washing with

soap and hand sanitizing recommended by WHO (WHO, 2010) are not practiced by students at Training and Research Unit of Biosciences.

Hand washing is the most effective way to reduce the spread of infectious diseases according to CDC (CDC, 2012; Mead *et al*., 1999). Our study provided detailed information on the duration and in which environments different groups engaged in various handwashing behaviors. Our study recognizes the importance of environmental factors that promote proper hand washing behaviors. To our knowledge, our study was one of the first studies to focus on hand washing behaviors and wash time while incorporating environmental factors and observation time

The study revealed that none was washing hands with soap and water. This is an important discovery because a high percentage of people do not wash properly and signs that include messages highlighting proper hand washing or reminders to use soap can increase compliance. It seems that this type of explicit recall may be particularly useful in men's toilets (Larson *et al*., 1997; Larson, 1991).

The study of the effect of time of day on the behavior of hand washing showed that hand washing generally decreased during the evening. The most important results of our research concern the distinction between hand washing behaviors and hand length were washed. Specifically, no individual in the sample approached the recommended hand washing time.

V. CONCLUSION

We note that the students interviewed know the hygienic practices of handwashing. But they can not apply it entirely because they can only wash their hands with water alone because hand soaps and disinfectants do not exist in the toilet. Thus, preventive measures are not practiced by students at Training and Research Unit of Biosciences.

Understanding the challenges of hand hygiene practice in academia may help in the development of hand hygiene promotion strategies for the prevention of infections, especially those that are handled.

LIMITS OF THE STUDY

It should be noted that the observations took place only in an academic environment in a Training and Research Unit (Department of Chemistry Animal Biology). It is therefore necessary to take care to generalize the results throughout the university. It should be recognized, however, that even an apparent discreet observation may influence hand washing behaviors because the simple presence of others in a toilet may lead to increased compliance (Bittner *et al*., 2002; Drankiewicz, 2003; Edwards *et al*., 2002; Nalbone, 2005). It would be good to include in the future studies the drying act because studies have shown that transfer of microorganisms is more likely to occur from moist than dry skin (Mackintosh, 1984; Merry *et al*., 2001; Patrick, 1997).

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Physicochemical Properties and Selected Heavy Metals in Tin-Mine Spoil Soils around Jos Plateau Nigeria

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Abstract— *The physicochemical properties and heavy metals in both vegetated and non-vegetated spoil soils in Jos Plateau were determined. Three dug soil profiles and thirty three composite soil samples were collected with auger at depths of 0 – 15cm, 15 – 30cm and 30 – 50cm and analysed using standard methods. The results showed mean value ranges of 4.58 – 5.28 pH, 0.09 – 1.46 Organic matter, 42 84% Sand, 6 – 46% clay, 5 -29% silt, 0.022 - 0.110% total nitrogen, 14.80 – 41.44ppm available phosphorus, 3.13 – 5.00meq/100g exchangeable calcium, 1.45 – 2.35meq/100g exchangeable magnesium, 0.04 – 0.51meq/100g exchangeable potassium, 0.15 – 0.20meq/100g exchangeable sodium, 8.16 – 12.28meq/100g CEC126.80 – 778.33ppm Fe, 0.16 – 0.44ppm Al and 5.50 – 23.50ppm Mn. The soils were found to be predominantly Sandy Clay Loam, acidic and deficient in nutrients, Nitrogen, Phosphorus and Exchangeable Bases. The concentrations of most parameters were higher in the cultivated spoil soils than in the uncultivated spoil soils. Land amendment materials such as organic wastes and town refuse ash should be applied to the soils in the area.*

Keywords— Tin mine, Spoil soils, Vegetated, Non-Vegetated, Jos Plateau, Nutrients, Cultivated.

I. INTRODUCTION

Nigeria's tin mining industry is situated on the Jos Plateau more than 1300m above sea level.

Mining activities have been identified as a major cause of land degradation the world over (World Bank, 1995). The physical and chemical characteristics of mine soils affect reclamation efforts. These characteristics vary depending on the quality of the original soil, the amount and size of pulverized bedrock included in the spoil and the method of placement (Reuter, 2001). Generally, however, the properties of mine soils make them a poor medium for plant growth (Sengupta, 1993). This is because removal and crushing of bedrock exposes geologic material that is not stable at earth surface conditions and the alteration of this material impacts the chemistry of the environment (Reuter, 2001).

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About 320km^2 land area of the Jos Plateau has been degraded as a result of open cast tin mining since 1904. Only about 30km^2 has been reclaimed thus leaving nearly 90% of the affected area derelict to some degree and only little reclamation has taken place since 1982 (Alexander, 1992).

The reclamation strategy adopted by the MLRU was a straightforward strategy involving the leveling of the spoil mounds and the infilling of the flooded excavations with the intention of returning the land to immediate agricultural production (Wimbush, 1963). Reclamation was rendered more difficult because at the beginning of mining, no attempt was made to adopt the normal strip-mine policy of removing and storing separately top soil, sub-soil and overburden. Consequently, the spoil mounds comprise a complex mixture of these various strata, which produces extremely acid and nutrient deficient soil parent material (Alexander, 1992).

Open cast tin mining in the area, among other factors, has led to shortage of arable lands. Subsequently, encroachment into marginal lands and the use of unproductive soils for farming is commonplace. Alexander (1989) reported that population pressure and land shortage reduced the period of fallow or even caused complete abandonment.

This study will examine soil constraints to natural vegetation growth on the tin mine spoils of the Rayfield area and Barkin Ladi LGA, both within the Jos Plateau. The soil categories include: unreclaimed / non-vegetated spoils, unreclaimed /

vegetated spoils, reclaimed / vegetated spoils, cultivated spoils and about seven decades undisturbed soils. This study is aimed to assess the physicochemical properties of non-vegetated tin mine spoil soils on Jos Plateau.

II. MATERIALS AND METHODS

2.1 Study Area

Jos Plateau lies about 10^o North of the equator in the 'middle belt' of Nigeria and is a highland area of approximately 8,600km^2 occupying the northern part of Plateau State (Alexander and Kidd, 2000). It is bounded by latitudes 10^o 11'N and 8^o 55'N and longitudes 8^o 21' E and 90^o 30' E with an average elevation of about 1,250m above mean sea level (Ajaegbu, 1992) (Fig. 1a).

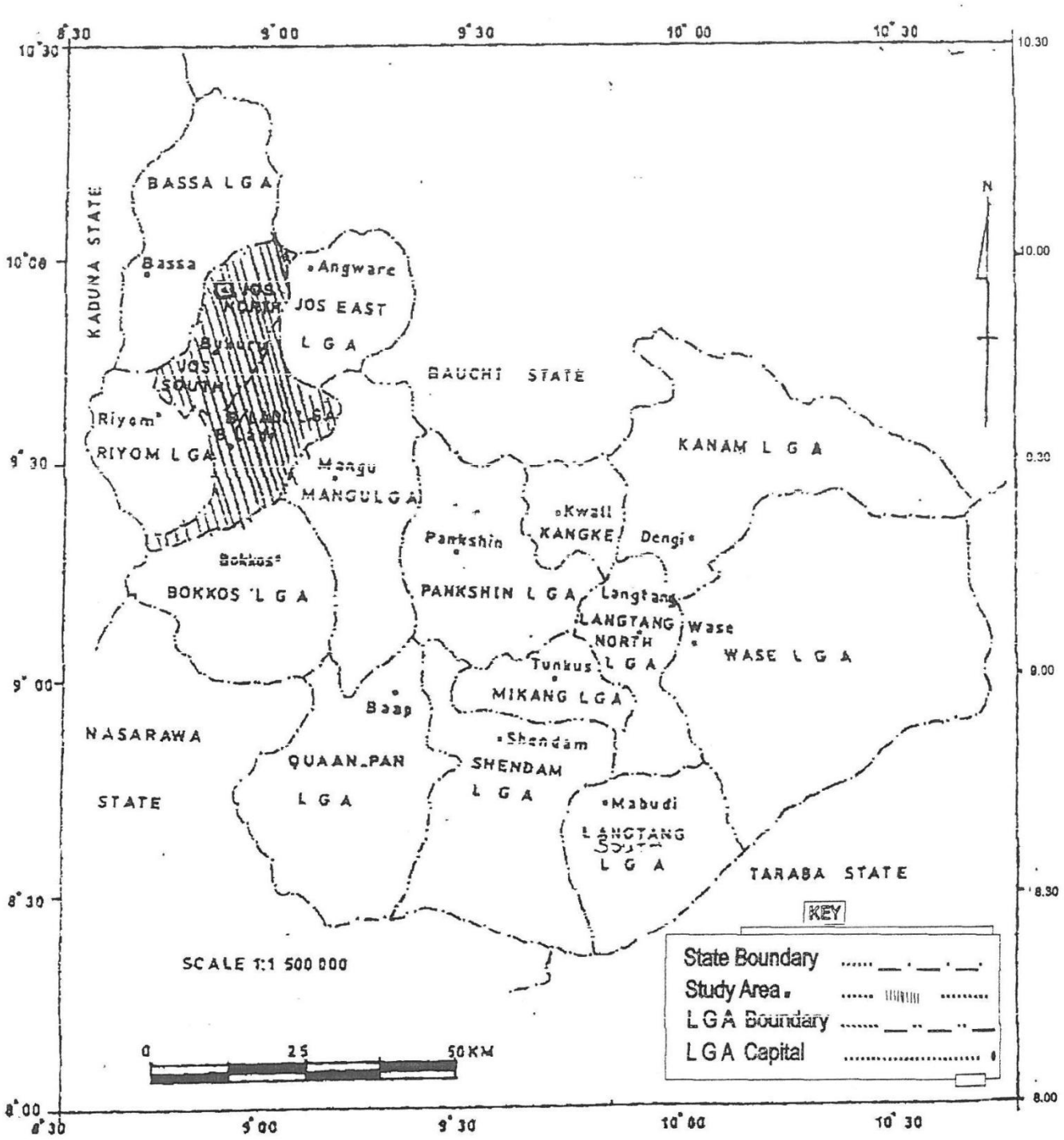

FIGURE 1A: MAP OF PLATEAU STATE SHOWING THE STUDY AREA

The Jos Plateau is located within the Northern Nigerian Cystalline Complex (Macleod et al, 1971). In relation to the rest of the Northern Nigerian Crystalline Complex, it is its elevation above sea level (1,250m) that makes it stand out (Ajaegbu, 1992). The area has a mean monthly temperature range of 20-24^{o}C (table 3.1) and annual total rainfall of 1400mm which falls primarily over a period from April to October (Alexander and Kidd, 2000).

Three distinct soil associations have been identified on the Jos Plateau by Alexander (1986). These are; soils associated with rock outcrops or lateritic pans, soils on newer basalts and soils on granite.

The Jos Plateau falls within the Northern Guinea Savannah zone which is open woodland with tall trees but the native vegetation has been considerably altered by human activities. Even where the natural vegetation is not disturbed by agriculture or mining, soil erosion is a hazard (Alexander, 1986).

2.2 Description of Sampling Locations

Soil samples were collected from:

- **Category 1:-** Unreclaimed / vegetated spoils: vegetated mine dumps
- **Category 2:-** Unreclaimed / Non-vegetated spoils: mine dumps completely devoid of vegetation
- **Category 3:-** Reclaimed / vegetated spoil soils: mine dumps that were leveled and planted with Eucalyptus trees during reclamation. Eucalyptus is exotic species from Australia.
- **Category 4:-** Cultivated spoil soils: mine spoil soils under cultivation
- **Category 5:-** Undisturbed soils: unmined and uncultivated soils with grasses and few trees

Forty five soil samples were collected from dug profile pits by auger borings. For each auger boring, sample collection was at three pre-determined depths (0-15cm, 15-30cm and 30-50cm). For each of these depths, samples were collected from four spots to obtain composite samples. After collection, the soil samples were stored in well labeled polythene bags before being sent to the laboratory for further preparation and analysis. Figure 1 shows map of the study area showing sample collection spots.

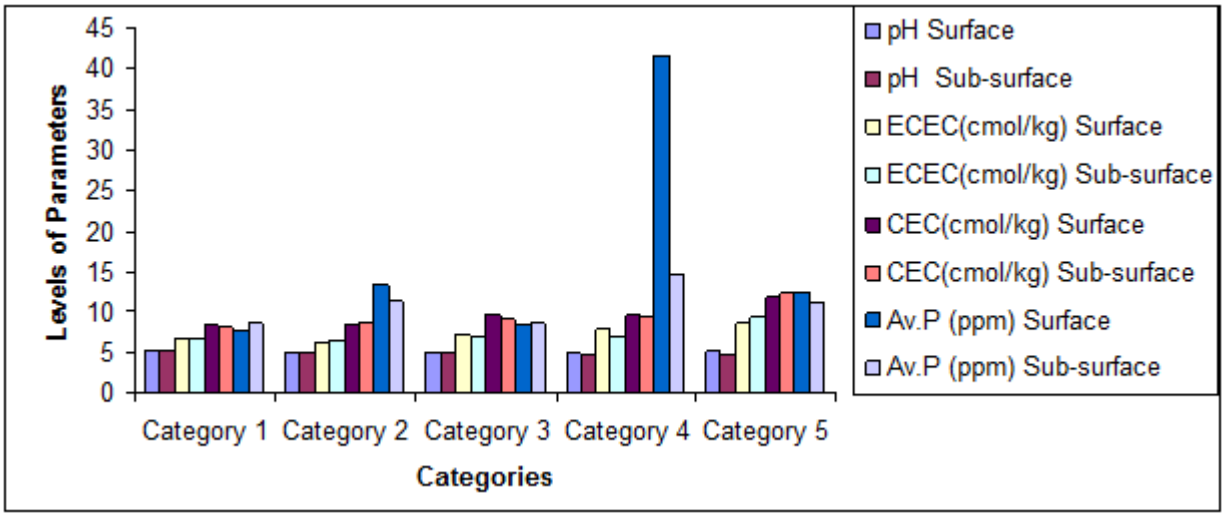

FIGURE 1: VARIATIONS IN LEVELS OF PHYSICOCHEMICAL PARAMETERS IN SOILS AT THE STUDY AREA

2.3 Analytical Methods

The soil samples were air-dried for five days and screened through a 2mm sieve. Particle size was determined by the hydrometer method (Day, 1965), modified by Gee and Bauder (1986). Soil reaction was determined in 1:2.5 soil/water ratio by the use of glass electrode pH meter. Organic matter was determined following the Walkey and Black (1934) method. Total Nitrogen was determined following the wet oxidation procedure modified by Bremner (1965). Available phosphorus was extracted using dilute HCl/NH_4F (Bray-1) described by Bray and Kurtz (1945). The extraction of Ca, Mg and K was made using in ammonium acetate at pH 7.0 (USDA, 1972). Ca and Mg were measured by using an atomic absorption spectrophotometer (AAS) while K and Na were measured with the aid of flame photometer. Effective Cation Exchange Capacity was determined by summation method following the extraction of exchangeable acidity in IN KCl. Pyrophosphate

extractable Fe, Al and Mn were determined by atomic absorption spectrophotometry (AAS). Base saturation percentage (BSP) was calculated using the formula

$$BSP = \frac{(Ca^{++}+Mg^{++}+K^{+}+Na^{+})}{\text{Cation Exchange Capacity}} \times 100 \quad (1)$$

III. RESULTS AND DISCUSSION

3.1 Physicochemical Properties of the Tin Mine Spoil

The results of the physico-chemical parameters measured from the tin mine spoil soils are presented in Table 1 and Figs 1 - 3.

TABLE 1
MEAN LEVELS OF PHYSICO-CHEMICAL PROPERTIES MEASURED IN THE STUDY AREA

		Mean Levels				
Parametres	**Sampled**	**Category 1**	**Category 2**	**Category 3**	**Category 4**	**Category 5**
pH	Surface	5.08	4.95	4.82	4.99	5.28
	Sub-surface	5.06	4.93	4.82	4.79	4.58
OM(%)	Surface	0.09	0.36	0.32	1.41	0.28
	Sub-surface	0.23	0.51	0.38	0.56	1.46
CLAY(%)	Surface	28.75	22.00	18.00	19.00	12.00
	Sub-surface	28.50	24.71	23.50	21.13	24.75
SAND(%)	Surface	59.25	63.00	65.00	66.00	64.00
	Sub-surface	59.00	61.71	60.50	66.00	56.50
SILT(%)	Surface	12.00	15.00	17.00	15.00	24.00
	Sub-surface	12.50	13.57	16.00	13.00	18.75
TOTAL N (%)	Surface	0.022	0.041	0.044	0.076	0.105
	Sub-surface	0.027	0.040	0.035	0.059	0.110
AV.P (PPM)	Surface	7.57	13.37	8.26	41.44	12.20
	Sub-surface	8.65	11.30	8.65	14.80	11.02
Exch.Ca (cmol/kg)	Surface	3.20	3.13	3.30	4.13	5.00
	Sub-surface	3.33	3.40	3.15	3.40	3.75
Exch.Mg (cmol/kg)	Surface	1.70	1.50	1.45	1.97	2.35
	Sub-surface	1.58	1.79	1.53	1.79	2.23
Exch.K (cmol/kg)	Surface	0.07	0.11	0.07	0.51	0.34
	Sub-surface	0.09	0.04	0.05	0.10	0.20
Exch.Na(cm	Surface	0.15	0.17	0.20	0.16	0.16
ol/kg)	Sub-surface	0.15	0.17	0.19	0.18	0.17
Exch.Al+H (cmol/kg)	Surface	1.40	1.27	2.20	2.07	0.80
	Sub-surface	1.50	0.94	2.05	1.43	2.35
ECEC (cmol/kg)	Surface	6.52	6.18	7.22	7.83	8.64
	Sub-surface	6.64	6.49	6.97	6.89	9.45
CEC (cmol/kg)	Surface	8.38	8.30	9.65	9.47	11.85
	Sub-surface	8.16	8.71	9.10	9.39	12.28
BSP	Surface	61.29	58.79	51.86	73.18	65.70
	Sub-surface	59.21	61.92	53.69	58.06	52.10
Fe (ppm)	Surface	137.50	281.70	256.25	778.33	342.50
	Sub-surface	201.69	126.80	162.50	629.06	705.00
Al (ppm)	Surface	0.20	0.43	0.29	0.44	0.37
	Sub-surface	0.24	0.21	0.16	0.36	0.42
Mn (ppm)	Surface	6.76	9.98	11.26	23.50	7.43
	Sub-surface	5.50	13.08	7.49	10.34	9.58

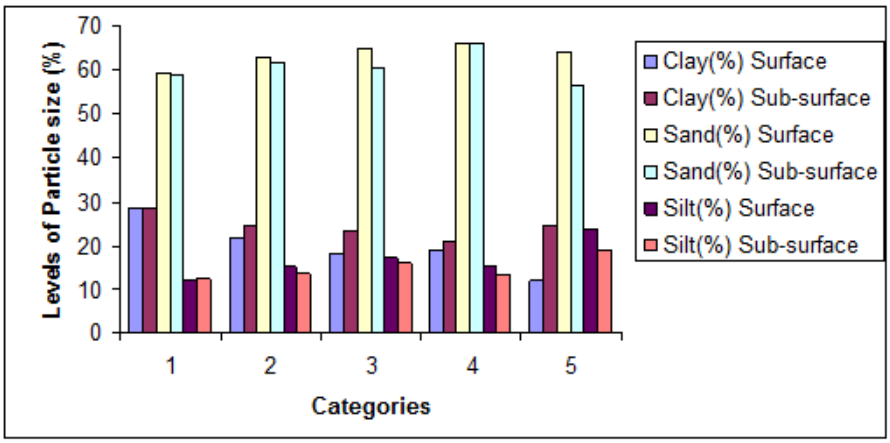

FIGURE 2: VARIATIONS IN LEVELS OF SOILS PARTICLE SIZE AT THE STUDY AREA

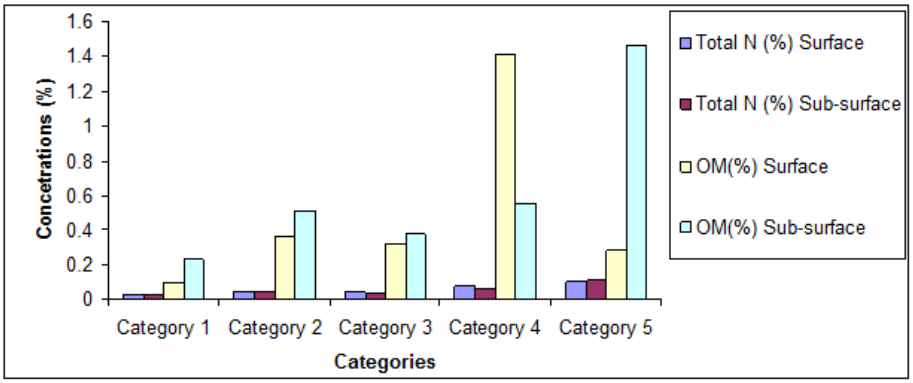

FIGURE 3: VARIATIONS IN CONCENTRATIONS OF CHEMICAL PARAMETERS IN SOILS AT THE STUDY AREA

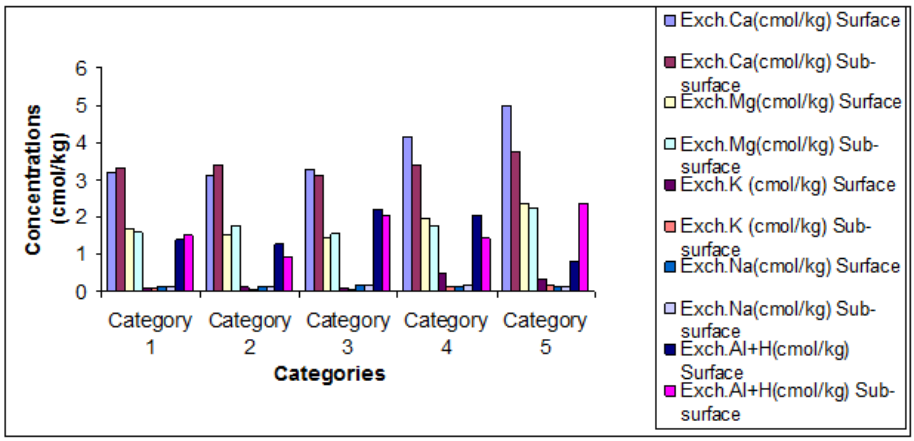

FIGURE 4: VARIATIONS IN CONCENTRATIONS OF EXCHANGE CATIONS IN SOILS AT THE STUDY AREA

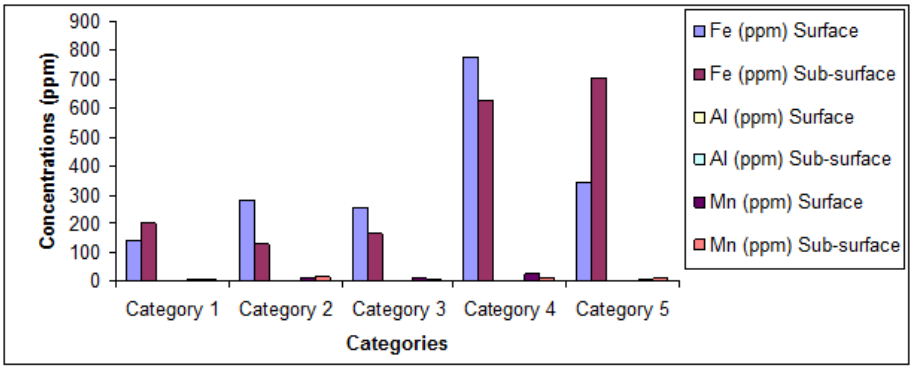

FIGURE 4: VARIATIONS IN CONCENTRATIONS OF HEAVY METALS IN SOILS AT THE STUDY AREA

3.2 Soil pH

Table 1 shows that irrespective of soil category or depth, all the samples analyzed were acidic. Mean pH values range from 4.58 – 5.28. The soils were strongly acidic to very strongly acidic in reaction based on pH ratings by Truog (1948) and Brady and Weil (1994). The acidic nature of the granitic parent rock that underlies the Jos Plateau tin mining area (Olowolafe, 2002) as well as the abundance of laterites in the area may be responsible for the acidity of the soils. Low pH implies increased solubility and availability of toxic metals (Fe, Al, Mn) and this could lead to reduced vegetation growth, reduced population of N-fixing bacteria and allows for leaching of essential nutrients.

Remediation processes that can be employed towards proper management of the acidity of the soils of the study area include the use of organic amendment (plant and animal remains) and liming – which eliminates Al and H ions thereby raising soil pH and could also supply Ca and Mg if dolomite [Ca, Mg $(CO_3)_2$] is used (Hue and Ikawa, 2006).Acid-tolerant crops can thrive well in the area. While pineapples thrive within a pH range of 4.7-5.7, Irish potatoes require pH of 4.8 to 5.2 to thrive well (Hue and Ikawa, 2006).

3.3 Organic Matter

Organic carbon levels below 2% (organic matter equivalent of 3.44%) are taken to be very low for tropical soils (Metson, 1961; Landon, 1991). Based on this rating, all the soil samples analyzed are low in organic matter. This is characteristic of mine soils (Haigh, 1999; Reuter, 2001) and in the case of the study could be the result of the non-adoption of the normal strip-mine policy of storing separately the different strata of the excavated spoil materials. This may have resulted to the acidic and nutrient – deficient materials at the deeper horizons of the soils being brought to the surface. The high temperature and high rainfall of the area which leads to rapid organic matter decomposition and disappearance as well as poor soil management practices (over cultivation, over-grazing, bush-burning etc) are other factors which may be responsible for the low level of organic matter in the area. Mele and Carter (1993) observed that precipitation and temperature affect the formation and distribution of organic carbon.

Fig. 3 shows that except for the cultivated soils, the sub-soils of all soil categories contain more organic matter than the surface soil. Cultivation and fertilization could be responsible for more organic matter for the surface horizons of cultivated soils while erosion and leaching keep surface organic matter levels low for other sites.

According to stocking (1997), when soil organic carbon declines, plant nutrients such as nitrogen and phosphorus are mostly at risk. Adoption of better soil management practices and intensifying the use of organic manures (plant remains, animal droppings and town refuse ash) will go a long way towards bettering the organic matter status of the area thereby making more nutrients available for plant uptake.

3.4 Particle Size Distribution

The textural classes of the samples are predominantly sandy clay loam and sandy loam. This implies that the soils are mainly loamy textured and is consistent with findings by Olowolafe (2002) and Anaryu (2005). The soils contain more of sand (a range of 42-84%) and then clay (6-46%) with silt as the least (5-29%). Also, most of the samples have less than 30% clay content. This reflects the coarse texture and the slow rate of weathering of some mineral contents of granite (Olowolafe, 2002) e.g. quartz and muscovite (Ilaco, 1985). This coarse-textured nature implies that the soils cannot hold much water or nutrients. Water infiltration and water-holding capacity are expected to be poor as a result with surface capping being experienced mainly on the non-vegetated mine dumps. Revegetation and organic amendments will improve the textures of these soils and improve water holding capacity and nutrient availability.

3.5 Total Nitrogen

Landon (1991) rated soils with total Nitrogen levels from 0.1 – 0.2% as low and <0.1% as very low for tropical soils. The mean values of total Nitrogen for the different soil categories as presented on table 4.1 show that while the total nitrogen values (surface and sub-surface) for the undisturbed soil fell within the 'low' range going by the rating by Landon (1991), the values for all other categories fell within the 'very low' range. In other words, the soils of the area are low to very low in their total nitrogen content. Daniels and Zipper (1988) reported that Nitrogen was initially the plant-growth limiting factor on young mine soils in the Appalachian region, but through time, phosphorus became more limiting. Torbert *et al* (1988) and Reuter (2001) reported that Nitrogen is usually deficient in mine soils and this limits vegetation establishment and sustained productivity.

The total amount of Nitrogen required to sustain plant growth over time must come from initial fertilization and subsequent symbiotic microbial N-fixation by legumes (Torbert *et al*, 1988) Organic amendments should be used as a source of mineralizable material to enhance nitrogen levels and extend Nitrogen availability through cycling (Reuter, 2001) since Nitrogen is primarily combined in organic matter in soils (Torbert *et al*, 1988).

3.6 Available Phosphorus

According to Ilaco (1985) and Landon (1991), available phosphorus value below 15ppm is regarded as low for tropical soils. The results of this study show that the surface soils of the cultivated spoils had available phosphorus values that exceeded the critical limit of 15ppm with the value for the sub-surface soils within the limit. Soils of the other four categories were deficient in available phosphorus. Cultivation and fertilizer application may be responsible for the trend in the cultivated areas.

The tendency of mine soils to fix phosphorus increases over time and because organic bound phosphorus is to subject to P-fixation, it is critical to establish and build an organic – P reservoir in the soil to supply long term plant needs through P-mineralization (Torbert *et al*, 1988). Low phosphorus levels may hinder nitrogen accumulation since symbiotic N-fixing bacteria have a high phosphorus demand. Therefore, mine soil nitrogen and phosphorus must be managed together, and not as independent factors (Torbert *et al*, 1988).

3.7 Exchangeable Bases

Ilaco (1985) and London (1991) rated soils with <2.0meq/100g as very low in calcium, 2-5meq/100g as low and 5-10 meq/100g as medium. The levels of exchangeable calcium show that the soils of the study area are very low to medium in exchangeable calcium.

The soils were found to range from low to medium in exchangeable magnesium based on the ratings by Ilaco (1985) and Landon (1991). According to these authors, exchangeable magnesium values from 1.5 – 3.0 meq/100g is medium, 0.5-1.5meq/100g low and <0.5 meq/100g very low.

According to Ilaco (1985), levels of exchangeable potassium between 0.1 – 0.3 meq/100g soil are regarded as low for tropical and sub-tropical soils and below 0.1 meq/100g soil very low. Based on this rating, the soils analyzed in this study ranged between low to moderate in their exchangeable potassium content with most of the samples falling within the 'low' and 'very low' ranges. According to Jones and Wild (1975), amounts of exchangeable potassium in the tropical savannah is low. The surface horizons of the cultivated spoils have the highest mean exchangeable potassium value . This could be attributed to cultivation and enrichment of the soils with fertilizers.

Also, Ilaco (1985) and Landon (1991) rated exchangeable sodium levels below 0.1 meq/100g as very low and 0.1 – 0.3 meq/100g of soil as low. Based on this, all the soils studied are low in exchangeable sodium. Landon (1991) observed that although sodium could serve as a substitute for potassium, it is not an essential plant nutrient. For this reason, he added that its absence or presence in only small quantities is not usually detrimental to plant nutrition. He observed that when sodium is present in soil in significant quantities, particularly in proportion to other cations present, it can have an adverse effect, not only on many crops, but also on physical conditions of the soil.

The generally low content of exchangeable bases in the spoil soils of the study area could be attributed to the parent material that underlies the area (biotite-granites), the predominance of the low activity clay mineral (kaolinite) in the area, the lateritic nature of the area, the non-adoption of the normal strip-mine policy/the method of placement of excavated materials as well as leaching of essential plant nutrients. Increasing the pH and organic matter levels will make exchangeable bases more available in the soils.

3.8 Cation Exchange Capacity

London (1991) rated soils with CEC value of 5-15 meq/100g as low. Based on this rating, the soils analyzed in this study are low in CEC. The low level of organic matter obtained from this study and the dominance of the low activity clay mineral kaolinite in the study area may have contributed to the low CEC and ECEC values of the soils. The Base saturation percentage values obtained in this study generally ranged from 33.16% - 90.12%. With a general improvement of pH and organic matter levels, more basic cations can be made available to satisfy the CEC of the soils as against acidic cations.

3.9 Fe, Al and Mn

Low pH (below 5.5) makes metals such as Zn, Cu, Mn, Fe and Al more soluble for plant uptake (Torbert *et al*, 1988; Donahua *et al*, 1990; Reuter, 2001). With the low pH values obtained in this study, the Fe, Al, and Mn values obtained show that the acidic condition of the soils increased the solubility and availability of these metals. This situation encourages leaching of nutrient elements and is best tackled by pH amelioration.

IV. CONCLUSION

Whether vegetated or non-vegetated, the tin mine spoil soils of the Jos Plateau are acidic and nutrient-deficient. Although the cultivated spoils generally have higher values for most of the soil parameters, most of these values were below permissible limits.

The findings from this study have shown that cultivation is one way through which the "waste-lands" of the Jos Plateau tin mine fields can be better utilized. The soils need application of land amendment materials such as organic wastes (from plants and animals) and town refuse ash as the soil physico-chemical properties show that all the soils of the area are acidic and deficient in Organic Matter, Total Nitrogen, Available Phosphorus and Exchangeable Bases.

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Productivity and Quality of Hybrid Canola Oil and Seeding Time

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***Abstract*—** *Biofuels are the main alternative for changing the world's energy matrix, which is now centralized in fossil fuels. The characterization of alternative sources of biomass, mainly regionally, shapes database for decision making.For this purpose, a factorial experiment was carried out with three canola cultivars (Hyola 43, 61 and 571), seeded in four times (April 4th, April 16th, May 2nd and May 14th).As biomass characterization, grain yield, oil content and yield, specific mass, oxidative stability, acidity and lipid profile were determined. There was significant difference for the hybrids in the variables oil content, induction time and in the stearic, linoleic and linolenic contents. The highlight was the hybrid Hyola 43.There was significant difference for the periods in the grain yield, oil content, oil yield, and induction time and in the palmitic, stearic, oleic and linoleic contents. Considering values of dependent, quantitative and qualitative variables, the best seeding season of canola would be between the second fortnight of April and the first week of May.*

***Keywords*— Biodiesel, vegetable oils, fatty acid.**

I. INTRODUCTION

Directly or indirectly biomass is the basis for alternative energies. The biomass is responsible for 14% of world energy used [1].

For the production of biodiesel, biomass (raw material) is the most important and costly part of the process. In Germany, the main producer and consumer of biodiesel in the world, and in the European Union, canola accounts for 80% and 60%, respectively, of the cultivation of oilseeds for this purpose[2].The planted area with canola in Brazil in 2015 was 53,610 ha, an expansion of 8% in relation to the previous harvest [3].

Oil crops for the production of biofuels must offer good oil yield and physicochemical characteristics consistent with use and current standards [2]. The determination of the fatty acid profile is one of the main characterizations of an oilseed.The main fatty acid found in canola oil is the oleic acid, also known as Omega-9[4].

An issue in biofuels, oxidative stability is closely related to the structure of fatty acid chains, being more susceptible those with a higher proportion of unsaturated lipids[5].Canola oil has a high percentage of unsaturated fats, close to 93% [6].

The natural decomposition of triglycerides can be accelerated by light and heating, and rancidity is almost always followed by the formation of free fatty acids. High levels of acidity reflect in negative effects on oils, which may make them inappropriate for food or even for fuel purposes.

The mentioned characteristics may vary according to genetics and the environment agricultural management and environmental, such as water availability and frost, are control variables in the qualitative and quantitative yield of canola[7, 8].

II. MATERIAL AND METHOD

The field experiment was conducted in the municipality of Tibagi, state of Paraná, with the approximately coordinates 24º 16' 29" South and 50º 05' 42" West, and average elevation of 952 m. The experiment consisted of two factors, seeding times (four) and canola (*Brassica napus*) hybrids (three). The experiment was conducted in randomized blocks. The seeding times were: April 4th, April 16th, May 2nd and May 14th.The used hybrids were Hyola 43, Hyola 61 and Hyola 571.

At the seeding time, we used 18 seeds per meter and spacing between rows of 0.45 m. There was applied, in the furrow, 100 kg $ha^{-1}$of fertilizer (NPK formula 13-33-00). On the 45th day after seeding, there were applied, on total area,100 kg $ha^{-1}$of urea and100 kg ha^{-1}ofKCl. During the crop cycle no fungicides, insecticides or herbicides were applied.

For the monitoring of the climatic conditions during the crop development, information on minimum, maximum, daily mean and cumulative rainfall index were used. They were recorded in a meteorological station of IAPAR - Instituto Agronômico do Paraná, located in the TelêmacoBorba city (approximate coordinates: latitude 24° 20' S - longitude 50° 37' W).

For oil and biodiesel characterization, 150 g of seeds were ground in a Willey knife mill, with 20 mesh sieve.For lipids extraction, a Soxhlet apparatus was used, using n-hexane as solvent, for 6 hours.

The obtained oil was submitted to an aqueous degumming process, aiming to remove phosphatides, by adding 5% of water mass in relation to the total lipid mass. The mixture was stirred for 30 minutes, at 65 °C, in a refrigerated ultracentrifuge, Hitachi Himac, model CR21GII.The mixture was subjected to 5000 rpm at a constant temperature of 4 °C for 15 minutes.

After degumming the lipid specific mass was determined, at 20 °C, using the Anton Paar digital densimeter, model DMA 4500 M.

The oxidative stability was determined in an accelerated oxidation test, under a temperature of 110 °C and 10 L h^{-1} airflow, with RancimatMetrohm apparatus, model 893.The acid index was determined according to [9].

The biodiesel production process was carried out by mixing 0.0675 g of sodium methoxide dissolved in 5 ml of methanol and added in 10 ml of oil. The reaction mixture was kept under constant stirring and heating at 55 °C for one hour. After resting for 30 minutes the glycerol was removed, were performed two steps of aqueous washing and drying with anhydrous calcium chloride and washing with organic solvent (petroleum ether)[10].

The biodiesel was evaluated by Nuclear Magnetic Resonance Spectroscopy^{1}H – RMN, with deuterated chloroform as solvent, with a BRUKER ASCEND spectrometer, of 400 MHz.

Chromatographic analyzes were performed on a Perkin-Elmer Gaseous Chromatograph, Clarus 580, with FID detector, capillary column type Elite-Wax, 60 m long, 0.25 mm in diameter and with a stationary phase of 0.5 mm thickness. Heating ramp with initial temperature of 190 ° C, heating rate of 10 ° C min^{-1} to 250 °C was used. An injection volume of 1.0 mL of sample in hexane and internal standard methyl nonadecanoate was used.

All dependent variables were submitted to the F test for analysis of variances and, when pertinent, Tukey's regression analysis and mean test were performed. The Sisvar software was used for the analysis, version 5.3[11].

III. RESULTS AND DISCUSSION

The temperatures observed during the experiment conduction are shown in Fig. 1. In the initial stage, from emergence to flowering, temperatures of 13 to 22 °C are recommended [6]. At this stage, for the first and second seeding season, temperatures were higher, which may have led to lower productivity.

According to the stadium basal temperatures are different, for example, there were determined for canola - 0,8 ° C for vegetative stage stadium and 10,0 ° C for flowering [8]. Therefore all seeding times were harmed. Low temperatures, with frost occurrence, at the start of flowering can reduce up to 50% of flowering, whereas frostings at the end of flowering can reduce from 80 to 100% of flowering [7]. The only negative temperatures cataloged were recorded on July 25th, the only day

where the mean temperature was also below 5 °C (Fig. 1). This may have adversely affected the first and second seeding seasons

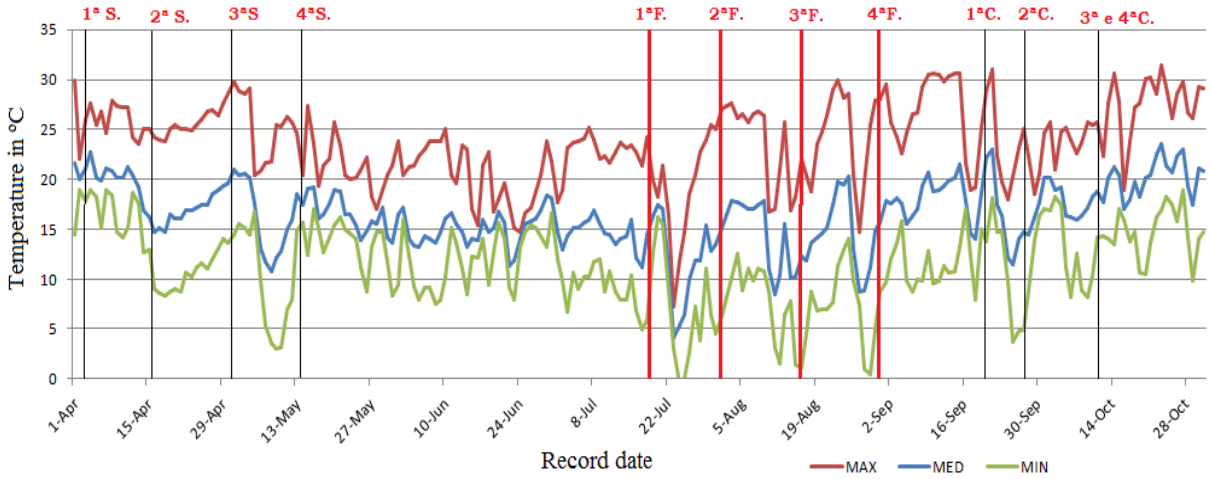


FIGURE 1- Minimum, maximum and mean temperatures from April to October 2013, meteorological station of the InstitutoAgronômico do Paraná, in TelemacoBorba, latitude: 24 ° 20'S - longitude: 50 ° 37' W - altitude 768m.

Note: "S"- seeding time; "F"- approximate date of end of flowering; "C" –crop date

When the temperature of the air rises above 27 °C, thermal stress occurs in the crop, reducing or even inhibiting the canola processes of growth and development, mainly in the final period of flowering and initial filling of the grains [12]. This is what happened to the fourth seeding season. Water stress at the end of flowering and beginning of grain filling has negative effects on the concentration of oil in the grain [13].As the beginning of flowering and the duration of this period may vary a few days, it was considered that there was no water stress (Fig. 2).

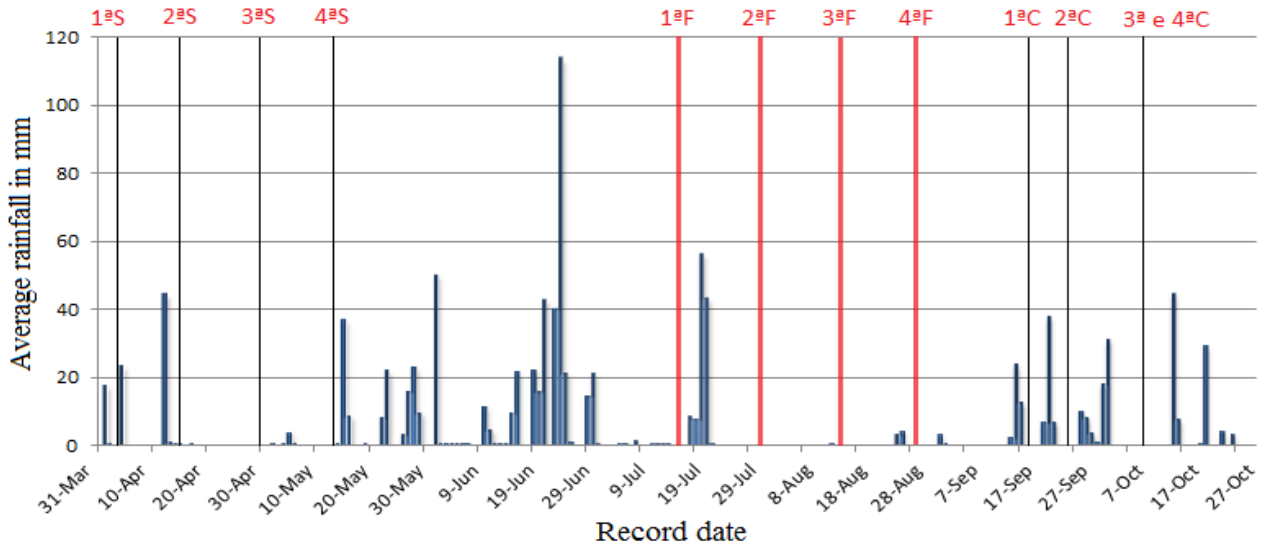


FIGURE 2- Daily precipitation from the months of April to October 2013, collected from themeteorological station of the InstitutoAgronômico do Paraná, in TelemacoBorba, latitude: 24 ° 20'S - longitude: 50 ° 37' W - altitude 768m.

Note: "S"- seeding time; "F"- approximate date of end of flowering; "C" –crop date

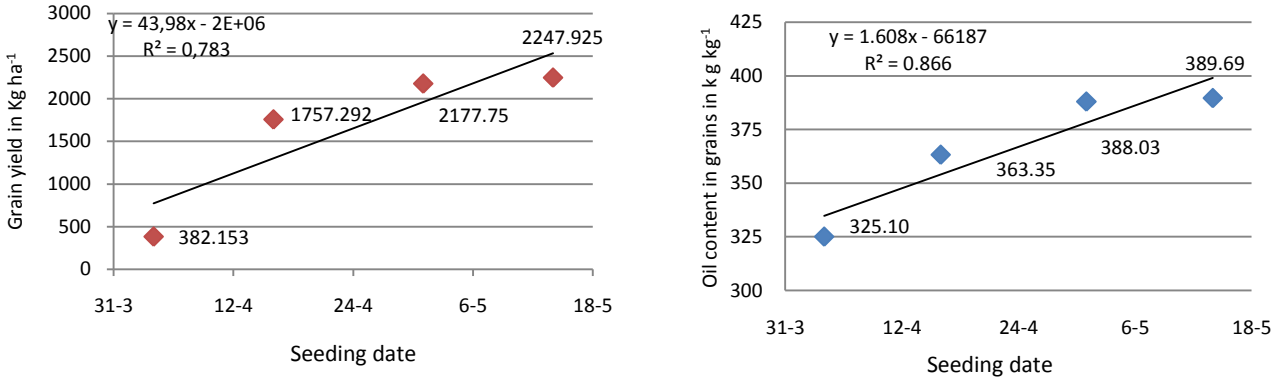
The productive potential of an agricultural crop is defined through genotype-environment interaction.According to analysis of variance, the grain yield variable did not present a significant difference for the hybrid factor, but there was a significant difference ($p<0.01$) for the seeding time factor, and no significant difference was observed for the interaction of the factors and for the blocks (Table 1).

TABLE 1
Grain Yield, Oil Content, Oil Yield, Oil Specific Mass, Induction Time (Oxidative Stability) and Acidity of the Oil and Test of Means, When Pertinetes, of the Three Canola Hybrids

Hybrid	Productivity kg ha^{-1}	Oil content* g kg^{-1}	Oil yield kg ha^{-1}	Specific mass kg m^{-3}	Induction time* h	Acidity mg KOH g^{-1}
Hyola 43	1,556.90	382.7 a	641.8	901.0	9.95b	1.70
Hyola 61	1,703.17	350.4b	616.9	898.0	8.66 a	1.72
Hyola 571	1,663.76	370.6 ab	716.2	899.0	9.50b	1.61

means followed by equal letters in the columns do not differ from each other to 0.05 of probability by the Tukey test.

Linear regression analysis showed a positive relationship between time and yield (Fig. 3a). The canola seeded on April 4th reached the lowest productivity, with an mean of 382kg ha^{-1}. The effect of the high temperatures recorded for this month and the occurrence of frost at the end of flowering/beginning of filling caused a negative impact (Fig. 1).



(a) (b)

FIGURE 3 – Values found and adjusted regression for grain yield (a) and oil content (b) for Hyola hybrids 43, 61 and 571, according to seeding dates. Tibagi, PR.

The mean yield of canola grains in the State of Paraná in the 2011 harvest was 1,368 kg ha^{-1}, while the 2012 harvest was 813 kg ha^{-1}, due to strong frost. The harvest of 2013 had an average of 1,436 kg ha^{-1}[14].

In contrast, in experiments performed in Aw climate, with mild temperatures, with nine canola varieties, using flowering irrigation, yields ranged from 1,494 kg ha^{-1} to 2,268 kg ha^{-1}[15]. In this case, the authors related terminal flower abortion and lower filling of the siliquas with elevation of temperature.

The variable oil content showed a significant difference for the hybrid factors and time ($p<0.01$), showing no difference for the interaction of the factors. The mean lipid value expected in Brazil is 380 g kg^{-1}[6]. In Cfa climate, varying sowing dates, [16] obtained, for two hybrids, values between 346 and 399 g kg^{-1}.This variation, according to the authors, was correlated with water stress.

The mean for the Hyola 43 hybrid was statistically equal to the Hyola 571 hybrid and higher than the Hyola 61 hybrid.The Hyola 571 hybrid was statistically equal to the Hyola 61 hybrid (Table 1).In this case, there was a positive linear relationship between grain oil content and seeding time (Fig. 3b).The mean temperature increase in the filling stage of the grains can provide deformities and decrease in oil content [17].As was the case with seeding seasons 1 and 2.

The canola seeded in the first season may have its oil content reduced due to the low temperatures, below 5 °C, observed in the second half of July, which also presented negative temperatures (Fig. 1). At that time, the crop was at the end of the flowering phase and beginning of the grains filling, critical phases to the crop [13].

According to analysis of variance, the variable oil yield per area did not present a significant difference for the hybrid factor (Table 1), but there was a significant difference($p<0.01$) for the seedingtime factor (Fig. 4a), with no difference for the interaction between the factors. There was a positive linear correlation between the oil yield per area and the seeding times. These values are consequences of the productivities and oil content present already presented and discussed.

The variance analysis showed a significant difference for the induction time (oxidative stability) for the factors hybrid (Table 1) and time ($p<0.01$) (Fig. 4b). There was no significant difference for the factors interaction.The hybrids Hyola 43 and

Hyola 571 were statistically equal and superior to the hybrid Hyola 61. The values can be considered coherent compared to [18] that obtained a mean of 7.2 hours. There was a tendency to increase the induction time with seeding times. Oxidative stability was affected by climatic conditions, especially in the case of the first seeding season.

The specific mass did not present significant difference for the hybrid factors and times, with mean value of 899.3 kg m^{-3} (Table 1).According to [19] the value of the specific mass of canola lipids is 878 kg m^{-3} (25 ºC). For different temperatures, [20] found a variation of 908 kg m^{-3} (10 ºC) to 921 kg m^{-3} (30 ºC).

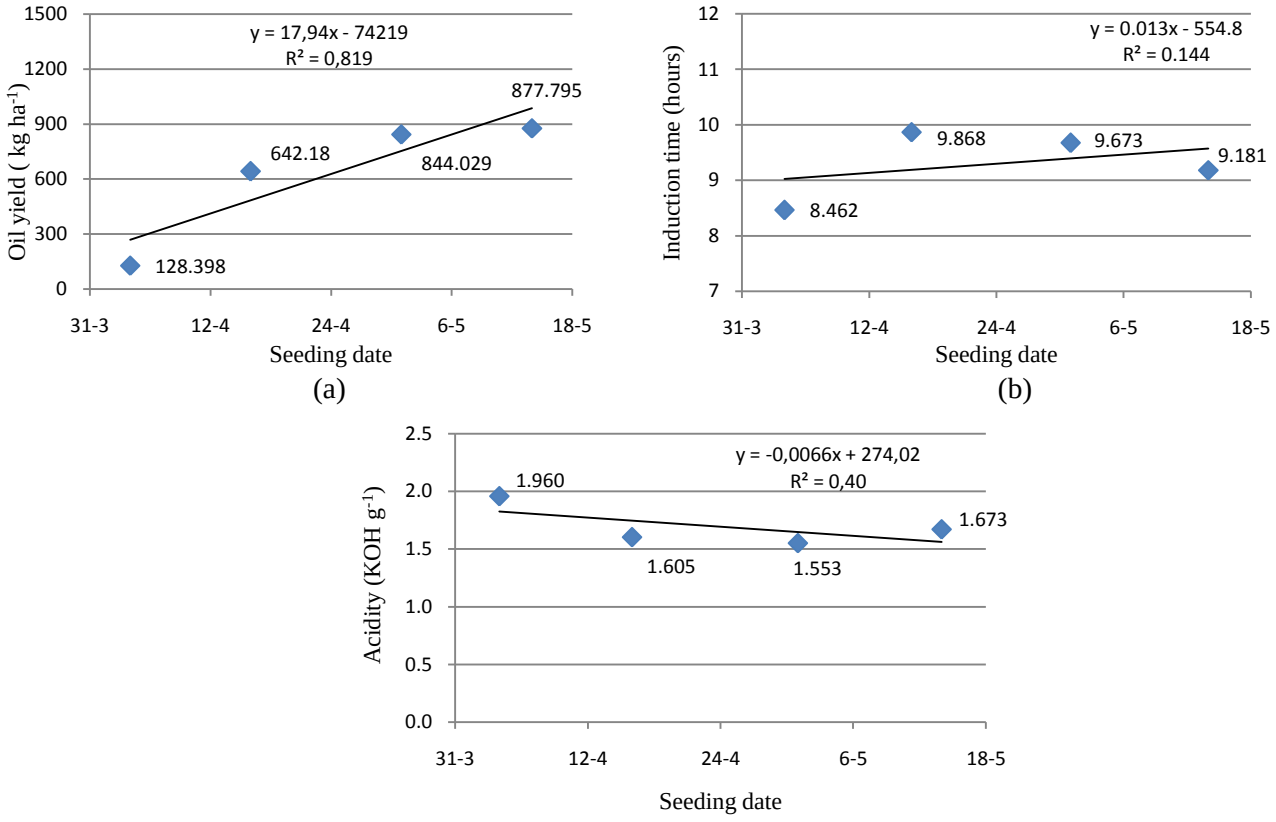


FIGURE 4 -Values and adjusted regression for oil yield (a), induction time (b) and acidity (c) for Hyola hybrids 43, 61 and 571, according to sowing dates. Tibagi, PR.

The variable acidity did not present significant differences for the hybrid factor (Table 1), while there were significant differences for the time factor ($p<0.05$), and no significant difference was observed for the interaction (Fig. 4c).The values found may be high. Evaluating crude and degummed canola oil, [21] obtained values of 1.08 mg KOH $g^{-1}$and 0.53 mg KOH g^{-1}, respectively.

The fatty acids determined were palmitic, stearic, oleic, linoleic and linolenic. The palmitic acid variable (C 16:0) did not present a significant difference for the hybrid factor (Table 2), whereas there was a significant difference for the time factor ($p<0.01$).

TABLE 2
Relative Mass of Palmitic, Stearic, Oleic, Linoleic and Linolenic Fatty Acids and Test of Mean (Tukey), When Applicable, of Three Hybrids of Canola. Tibagi, PR.

Hybrid	Palmitic (C 16:0) g kg^{-1}	Stearic (C 18:0)* g kg^{-1}	Oleic (C 18:1) g kg^{-1}	Linoleic (C 18:2)* g kg^{-1}	Linolenic (C 18:3)* g kg^{-1}
Hyola 43	46.7	28.7 a	644.8	195.4 a	77.3a
Hyola 61	46.2	25.5 b	652.1	172.4 b	92.8b
Hyola 571	43.5	28.3 a	654.2	176.1 b	86.3ab

means followed by equal letters in the columns do not differ from each other to 0.05 of probability by the Tukey test.

Regarding the temporal evaluation, there was a tendency to decrease the palmitic content (C16:0) with advance in the seeding dates (Fig. 5a).Evaluating two varieties of canola, and two varieties of rapeseed, [22] found values of 80.8 g $kg^{-1}$and 59.0 g kg^{-1}in canola and 47.8 g kg^{-1} e 39.8 g kg^{-1}in rapeseed. [23]found the presence of 39.0 g kg^{-1}palmitic acid in canola oil.

The stearic acid variable (C18:0) presented a significant difference for the hybrid factors and times ($p<0.01$), and no significant difference was observed for the interaction.The highest concentrations of stearic acid were found in the Hyola 43 and 571 hybrids, 28.71 and 28.29 g kg^{-1}, respectively. The hybrid Hyola 61 obtained the lowest concentration; 25.5 g kg^{-1} (Table 2).Fig. 5b shows the concentrations of stearic acid according to sowing time.

Comparing stearic acid in canola and rapeseed, [22] obtained 16.9 and 16.5 g kg-1 for canola, and 20.2 and 25.0g kg^{-1} for rapeseed. [23] Found 16.0 g kg^{-1} canola.

The variable oleic acid (C18:1) showed a significant difference for the time factor ($p<0.01$), whereas no significant differences were observed for the hybrid factor, and no significant difference was observed for the interaction.The mean concentration of this lipid found in the hybrids was 650.4 g kg^{-1} (Table 2).

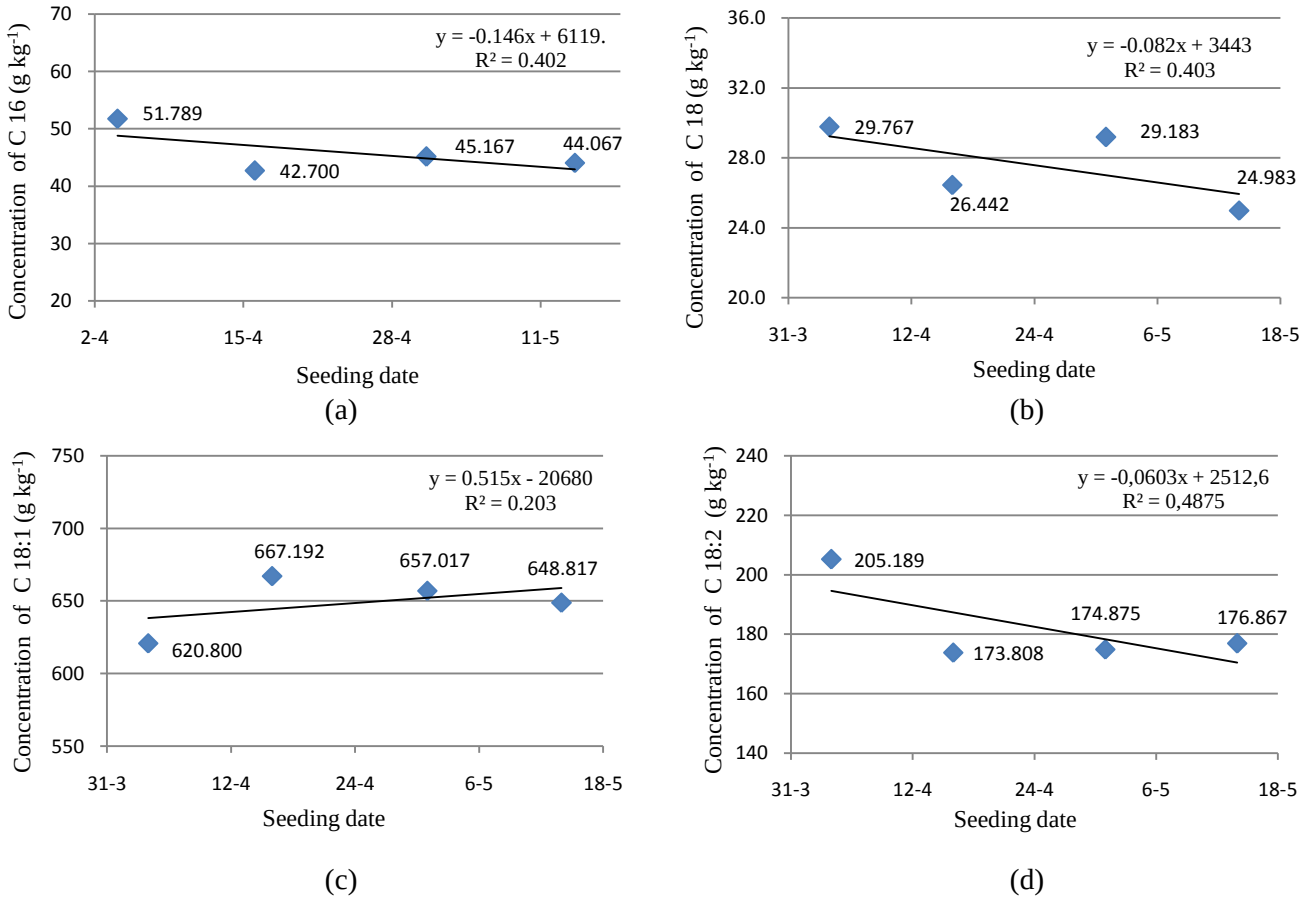


FIGURE 5 -Values and adjusted regression for C16:0 (a), stearic (C18:0) (b), oleic (18:1) (c) and linoleic acid (18:2), 61 and 571, according to sowing dates. Tibagi, PR

Although the equations represented (Fig. 5) were significant, the mathematical adjustments presented a low R2 value, so the seeding time can influence in approximately 20 g $kg^{-1}$for oleic (18:1) and 30 g kg^{-1} linoleic (18: 2).

The concentration of oleic tended to increase as the seeding dates progressed (Fig. 5c).Studying canola and rapeseed, [22] observed oleic acid concentrations of 570.9 g kg^{-1} and 599.2 g kg^{-1} for canola and 604.3 g kg^{-1} and 612.4 g kg^{-1} for rapeseed. [23] Studying commercially available oils (Illinois, USA), found for canola 600 g kg^{-1}. [24] evaluated twenty edible oils observed the presence of 620 g kg^{-1} oleic in canola.

Oleic acid, also known as omega-9, has only one unsaturation, being one of the healthiest sources of dietary fat [25]. The oleic acid values found in this experiment are higher than most cited in the correlative literature.

According to analysis of variance, the variable linoleic acid (C 18:2) presented significant differences for the hybrid and period factors, whereas no significant difference was observed for the interaction. The hybrid Hyola 43 had the highest concentration of this fatty acid, mean of 195.4 g kg^{-1}. The concentrations in Hyola 61 and 571 hybrids were 172.4 and 176.0 g kg^{-1}, respectively (Table 2). Linoleic acid also known as omega-6 plays a key role in maintaining human health.

Qualifying canola and rapeseed varieties, [22] found 231.2 and 230.7 g kg^{-1} of linoleic acid to canola and 232.7 and 236.3g kg^{-1} to rapeseed, and [23] found 220 g kg^{-1} of linoleic acid in canola. The linoleic acid values found in this experiment are lower than those reported.

The linolenic acid variable (C 18:3) presented a significant difference for the hybrid factor, and no significant differences were observed for the time factor and for interaction.

For the linolenic acid, the hybrid Hyola 43 presented the lowest concentration of this variable, reaching the percentage of 77.3 g kg^{-1}, while Hyola 61 showed the highest percentage of this fatty acid, obtaining 92.7 g kg^{-1}. Hyola 571 had a mean of 86.3 g kg^{-1} and was statistically significant to Hyola 43 and 61 (Table 2).

Linolenic acid or omega-3 has three unsaturations in its molecule and this lipid is also part of the group of essential fatty acids in the human diet because it is not synthesized by the human body.

Searching for various oils, [23] verified the presence of 101.0g kg^{-1} of linolenic acid in canola oil.[22]obtained values of 100.2 g kg^{-1} and 94.6 g kg^{-1} of linolenic acid in two varieties of canola, and 95.0% and 116.5% in two varieties of rapeseed. The values of linolenic acid found in this experiment are lower than those mentioned.

According to the time evolution, there was a tendency to exchange values between oleic (one saturation) that increased and linoleic (two saturations) that decreased, expected physiological process. This process is interesting for the use of the oil as fuel and would harm the oil if the final destination was the human food. Considering the results of dependent, quantitative and qualitative variables, the best sowing time of canola would be between the second fortnight of April and the first week of May.

IV. CONCLUSION

There was a significant difference for the hybrids in the variables oil content, induction time and in the stearic, linoleic and linolenic contents. Highlighting Hyola 43.

There was a significant difference for the periods in the grain yield, oil content, oil yield, and induction time and in the palmitic, stearic, oleic and linoleic contents. The most interesting seeding season would be between April 16th and May 2nd.

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Regional Disparity Level at West Papua Province

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Abstract— *A region possesses different potentials, conditions, and characteristics. Those aspects generate disparities between regions. Several factors that causing regional disparities related to physical and economic variables. Based on Gross Domestic Regional Product (PDRB) of West Papua Province 2012-2016, there were several sectors that significantly raised, i.e. mining and excavation, processing industry, and construction. Sorong City and Manokwari District had a domination role to the surrounding areas, and it affected into the imbalance growth speed of each areas, which in turn, it triggered the regional disparities. Manokwari is a capital of West Papua Province and a central of governmental activities, so the district got a fairly complete facilities, such as health, education, transportation, etc. The same condition was also applied to Sorong City. Therefore, the research objectives was to identify the regional disparity level reviewed from the population,, facilities and infrastructures, and regional economic based on Gini Index and Williamson Index. The results showed that the regional disparities on the scale of a province were medium level. On District scale, the results showed Pegunungan Arfak District was included in high level of disparity. Whereas the low level of disparity was concluded on Fakfak District, Kaimana District, Teluk Wondama District, Teluk Bintuni District, Manokwari Selatan District, Sorong District, and Raja Ampat District.*

Keywords— West Papua Province, disparity level, regional.

I. INTRODUCTION

West Papua Province is consisting of twelve districts. Based on Gross Domestic Regional Product (PDRB) of West Papua Province 2012-2016, there were several sectors that significantly developed, i.e. mining and excavation, processing industry, and construction. Those sectors were closely related to the effort of West Papua Province to develop the infrastructure on land, air, and sea at the last five years. The increased accessibility of areas has the role to stimulate the development level of an area. Level of social services could be reviewed from education sector; the distributions of colleges were concentrated at Sorong City, Manokwari District, Fakfak District, and Teluk Bintuni District. The comprehensiveness of educational facilities was linier with the development of human resources. Facilities and infrastructures aspects that made Sorong City and Manokwari District had a domination role to the surrounding areas, and it affected into the imbalance growth speed of each areas, which in turn, it triggered the regional disparities. The research objectives was to identify the regional disparity level reviewed from the population,, facilities and infrastructures, and regional economic based on Gini Index and Williamson Index.

II. METHODS

2.1 Research Variables

The research variables were formulated from theoretical review related to area's characteristic and disparities, also by reviewing previous research with similar themes.

2.2 Research Methods

Evaluative analysis method was used to discover the disparity level of a region in West Papua. The analysis was done using Gini index and Williamson index techniques.

2.2.1 Gini Index

Gini Index is a brief index about the inequality of income distribution's level in a country. It can be obtained by counting the area of a region or diagonal line (perfect equality) with Lorenz curve compared to the half area of where the Lorenz curve is located. [7]

TABLE 1
RESEARCH VARIABLES

References	Variables	Data types
There are factors that could cause regional disparities, which are related to physical and social economic variables. [1]	• physical • social • economy	• physical • social • economy
The development level of a region is essentially a function from natural environment, population, economic and social activities, that in turn will affect the development level of the region.[2]	• the development level of the region	• population • economy • social
The components of region's development consist of [3]: • The amount of economic and social facilities: educational facility, health facility, worship facility, and economical facility. • Population: The amount of the population and the density of the population. • Region's Accessibility: The width of the region, the distance to the district and the length of the road.	• Social Facility • Population • Accessibility	Social Facility • Educational facility • Health Facility • Worship facility Population • The amount of the population • The density of the population. Accessibility • The width of the region. • The distance to the capital. • The length of the road.
The public facility and infrastructure is a facility that is needed by many people and the provision is done simultaneously. The level of fulfillment was used to measure the level of disparity of a region. The public infrastructure consist of public facilities such as the road, the bridge, the sewerage system, the clean water supply system, the airport and the public buildings. [4]	• Water Supply	• Water Supply Service User.
According to Urban Planning Dirjen Cipta Karya's Dictionary, "The components of a region's infrastructure are divided into 3 groups; transportation infrastructure, health infrastructure and energy and communication infrastructure.	• Energy Infrastructure	• The level of electricity's service.
According to Williamson (1940s), some critics toward the stability and balance concepts can cause the occurrence of the income's disparity concept between regions. There are two important variables; the amount of the population and the Gross Domestic Regional Product (PDRB). [5]	• The amount of the population. • PDRB	• The amount of the population • PDRB's score in the last 5 years.
The measurement of the income's imbalance level of a country was obtained by counting the sector's ratio which located in between the diagonal line and Lorenz curve divided by the half area of where the Lorenz curve is located. This ratio is also known as Gini Concentration Ratio or Gini Coefficient. [6]	• Income/ outcome	• Outcome

In the Figure 1, Gini coefficient is shown in the comparison of region A (the area with perfect equality line and Lorenz curve) with triangle BCD. The name of Gini coefficient is taken from statistic expert named C. Gini. He was the first person who found the formula in 1912.

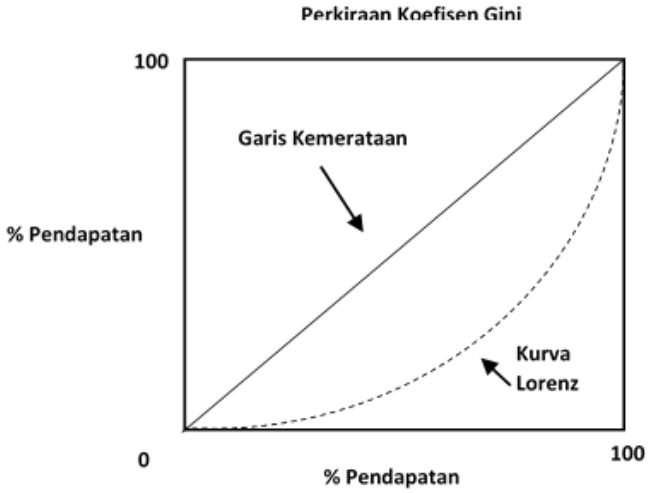


FIGURE 1. GINI COEFFICIENT ESTIMATION

MS. Excel was used to calculate Gini coefficient, with the following steps:

1. **Input data**

   Input data are population (amount and density), sosial facility (education, health and religious) and accessibility (the area, the distance to the capital, and the length of the road) and mean outcome per capita (Rp/kpita/month) based on the group of the outcome per capita a month.

2. **Calculation Process**

   Step 1. Calculate the cumulative proportion of population (Xk) with formula, add % the lowest cumulative population with % the second lowest cumulative population, divided by the total population

   Step 2. Calculate the cumulative income portion (Yk) with the formula % the lowest cumulative income plus total income divided by total income.

   Step 3. Calculate Xk – Xk – 1 (% Cumulative population)

   Step 4. Calculate Yk + Yk -1 (% Cumulative income)

   Step 5. Calculate (Xk – Xk – 1) (Yk + Yk – 1)

   Step 6. Calculate G1

3. **The Result**

   Interpretation of the result of calculation with Gini coefficient:

TABLE 2
COEFFICIENT VALUE OF GINI COEFFICIENT

Coefficient Value	Income's distribution
GI = 0	Perfect equality
0 < x < 0,4	Low inequality level
0,4 < x < 0,5	Medium inequality level
0,5 < x < 1	High inequality level
GI = 1	Inequality
	(controlled bye one person)

2.2.2 Williamson Index

Williamson index method was used to analyze how big the disparity was between regions in West Papua. Unlike the Gini Ratio that usually used to calculate the income distribution, Williamson index used the (PDRB) per capita as a fundamental data. The Williamson formula index is:

$$cv_w = \frac{\sqrt{\sum(Yi - \bar{y})^2 fi/n}}{\bar{y}} \quad (2.1)$$

Explanation:

CVw = Williamson Index

fi = the amount of population in the district

n = =The amount of West Papua's populatioon

Yi = PDRB per capita district/capital (Rupiah)

$\bar{y}$ = PDRB per capita mean West Papua (Rupiah)

III. FINDING AND ARGUMENTS

3.1 Region's Characteristic

The result of identification towards the region's characteristic showed several conditions such as:

a. Social Facilities

The largest numbers of educational facilities were located in Manokwari District with 346 units. Whereas, South Manokwari District has only 69 units. The largest numbers of health facilities were located in Manokwari District with 470 units. Whereas, Pegunungan Arfak District had the fewest health facilities with 41 units. The largest numbers of religious facilities were located in Manokwari District with 1.499 units. Whereas Pegunungan Arfak District had no religious facility.

b. Accesibility

West Papua's Total area is 9.967.163 Ha, Teluk Bintuni District has the largest area with 2.084.083 Ha or 21% of the total area of West Papua, Whereas the smallest area is Sorong City with 65.664 Ha or 1% of the total area of West Papua. The longest distance between cities are Kaimana-Manokwari, whereas the shortest distance between cities are Ransiki-Manokwati. Based on the road's pavement, the road in West Papua could be consist of one of these: asphald, gravel, soil, etc. In 2012, the lenght of the road with asphald was 1.817.824m, while in 2016, it got longer to 2.467.949 m. There was a significant enhancement of the road with asphald from 2012 to 2016, spesifically 650.126 m.

c. Infrastructure

The use of electricity especially State Electricity Company (PLN) haven't reach to some regions yet. The regions that havent got to use the State Electricity Company are: South Sorong District, Sorong District, Tambrauw District, Maybrat District, South Manokwari District and Pegunungan Arfak District. The telecommunication's network in West Papua was evolving rapidly through telephone provider that developed the network at least in the capital of every district/city. The amount of water supply service user in districts of West Papua in 2012 was 42.202 user , and in 2016, it was increased to 64.957 user.

d. Populations

The populations in West Papua was in total 194.050 individuals. Sorong City was the most populous city in West Papua with 225.588 individuals. Whereas, on 2016, the least populous city/district was Tambrauw District with 13.615 individuals.

e. Outcome

In 2012-2016, Sorong City had the largest outcome per capita in West Papua with Rp.1.448.834,00. Whereas, Tambrauw District had the smallest outcome per capita with Rp.381.247,00.

f. Gross Domestic Regional Product (PDRB)

The highest PDRB Based on Constant Price according to field of works in West Papua was Teluk Bintuni District with Rp.22.738.912,98, whereas the lowest PDRB was Pegunungan Arfak District with Rp.112.343,7.

3.2 Region's Disparity Level

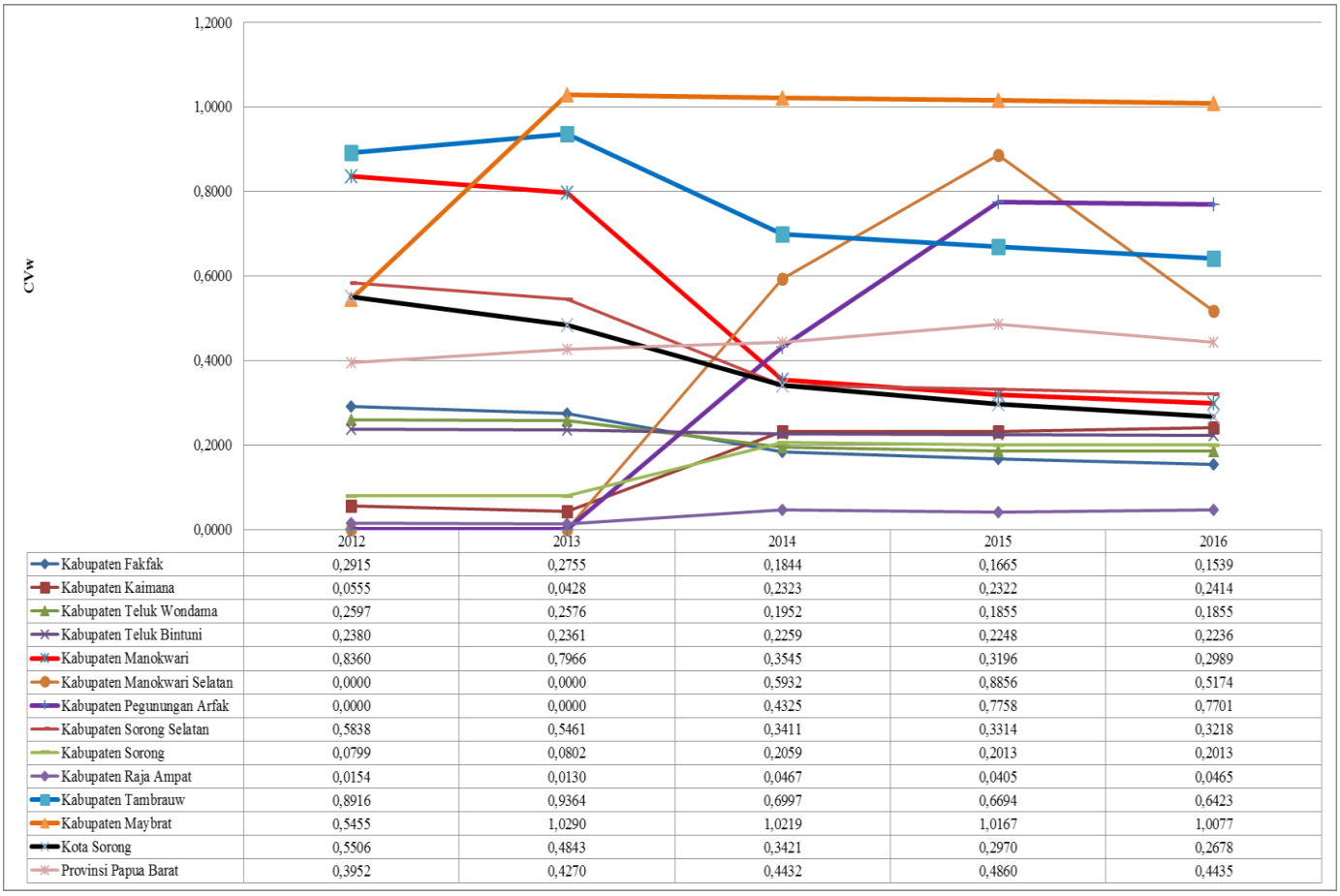
The disparity level of regions in West Papua can be determined by some aspects, i.e: Physic, Social and economic using analysis method Williamson index and Gini index.

3.2.1 Williamson Index

The analysis result of disparity in districts/cities of West Papua according to Williamson index was divided in 3 category; district/city with low disparity level (Williamson index's score is <0.3), district/city with medium disparity level (Williamson index's score is 0.3-0.7) and district/city with high disparity level (Williamson index's score is >0.7).

TABLE 3
WILLIAMSON INDEX ANALYSIS IN WEST PAPUA

No.	District/City	PDRB per capita				
		2012	2013	2014	2015	2016
1	Fakfak District	0,29	0,28	0,18	0,17	0,15
2	Kaimana District	0,06	0,04	0,23	0,23	0,24
3	Teluk Wondama District	0,26	0,26	0,20	0,19	0,19
4	Teluk Bintuni District	0,24	0,24	0,23	0,22	0,22
5	Manokwari District	0,84	0,80	0,35	0,32	0,30
6	South Manokwari District	-	-	0,59	0,89	0,52
7	Pegunungan Arfak District	-	-	0,43	0,78	0,77
8	South Sorong District	0,58	0,55	0,34	0,33	0,32
9	Sorong District	0,08	0,08	0,21	0,20	0,20
10	Raja Ampat District	0,02	0,01	0,05	0,04	0,05
11	Tambrauw District	0,89	0,94	0,70	0,67	0,64
12	Maybrat District	0,55	1,03	1,02	1,02	1,01
13	Sorong City	0,55	0,48	0,34	0,30	0,27
TOTAL	**West Papua**	0,40	0,43	0,44	0,49	0,44



	2012	2013	2014	2015	2016
Kabupaten Fakfak	0,2915	0,2755	0,1844	0,1665	0,1539
Kabupaten Kaimana	0,0555	0,0428	0,2323	0,2322	0,2414
Kabupaten Teluk Wondama	0,2597	0,2576	0,1952	0,1855	0,1855
Kabupaten Teluk Bintuni	0,2380	0,2361	0,2259	0,2248	0,2236
Kabupaten Manokwari	0,8360	0,7966	0,3545	0,3196	0,2989
Kabupaten Manokwari Selatan	0,0000	0,0000	0,5932	0,8856	0,5174
Kabupaten Pegunungan Arfak	0,0000	0,0000	0,4325	0,7758	0,7701
Kabupaten Sorong Selatan	0,5838	0,5461	0,3411	0,3314	0,3218
Kabupaten Sorong	0,0799	0,0802	0,2059	0,2013	0,2013
Kabupaten Raja Ampat	0,0154	0,0130	0,0467	0,0405	0,0465
Kabupaten Tambrauw	0,8916	0,9364	0,6997	0,6694	0,6423
Kabupaten Maybrat	0,5455	1,0290	1,0219	1,0167	1,0077
Kota Sorong	0,5506	0,4843	0,3421	0,2970	0,2678
Provinsi Papua Barat	0,3952	0,4270	0,4432	0,4860	0,4435

FIGURE 2: CVW FROM PDRB PER CAPITA OF CITIES/DISTRICTS IN WEST PAPUA AT 2012-2016

In 2012-2016, the results of Williamson index for the disparity of construction's scale of West Papua showed that West Papua's level was medium and the mean was 0.4. Whereas, the disparity of construction between districts/cities in West Papua (table 1) showed that the highest construction's disparity level Were Pegunungan Arfak District and Maybrat District which mean that the construction in those regions weren't equal. The districts with medium disparity level were Manokwari District, South Manokwari District, Tambraw District and South Sorong District. Whereas, the lowest construction's disparity level was Fakfak District, Kaimana District,Teluk Wondama District, Teluk Bintuni District and Sorong District which mean that the construction in those districts/cities were equal. The conclusion is, in 2012-2016, the development of disparities between cities/districts decreased.

3.2.2 Gini Index

The analysis results of the disparity in districts/cities of West Papua according to the population,outcome, social facilities and physic variables can be seen on table 2 and figure 2.

TABLE 4
GINI INDEX ANALYSIS IN WEST PAPUA

Variable	2012	2016
Population	0,52	0,39
Outcome	0,22	0,26
Educational	0,32	0,26
Health	0,26	0,29
Religious	0,47	0,48
Electricity	0,49	0,43
Water supply	0,76	0,47
Accessibility	0,52	0,43

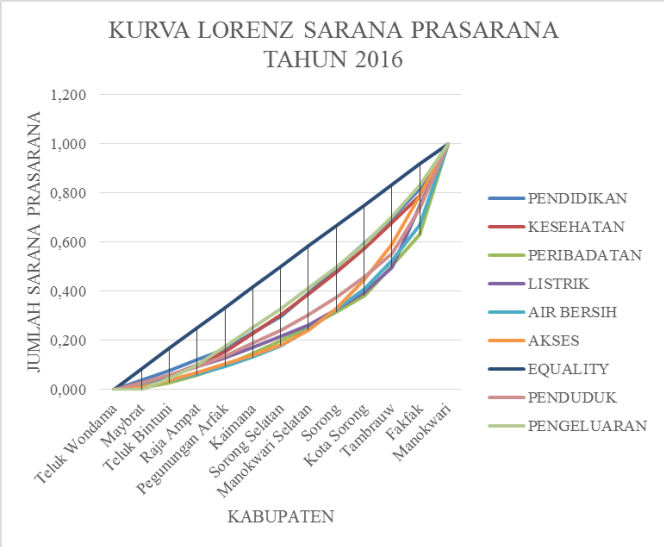


FIGURE 2 LORENZ CURVE

IV. CONCLUSION AND RECOMMENDATION

4.1 Summary

The reseach's conclusion were described as below:

a. The disparity level of West Papua in 2012-2016 was decreased. It showed that West Papua was putting an effort to make a construction's equity in every regions. The results showed that the regional disparities on the scale of a province was medium level.

b. On District scale, the results showed Pegunungan Arfak District was included in high level of disparity. Whereas the low level of disparity was concluded on Fakfak District, Kaimana District, Teluk Wondama District, Teluk Bintuni District, Manokwari Selatan District, Sorong District, and Raja Ampat District.

c. As per sectors, the disparity level of West Papua were concluded, i.e.:

- On 2012-2016, the disparity level of outcome per capita in West Papua was low. It means that the distribution of the outcome per capita was equal.
- The disparity level of population's distribution decreased, it means that the population's distribution of West Papua started to be equal.
- The disparity level of educational and health facilities was low. It means that the distribution level of educational and health facilities was equal. Whereas, the disparity level of religious facilities is medium. It means that the level of distribution of religious facilities was not equal yet.
- On 2012-2016, the disparity level of electricity infrastructure was medium. It means that the distribution's level of electricity infrastructure was equal but did not increase. Whereas, the disparity level of water supply service and accesibility decreased from the high level to medium level. It means that the distribution of water supply service and accesibility was equal and increased.

4.2 Recommendation

Based on the research results, recommendation given to the West Papua Province Government, as the main stakeholders in the effort to lessen the disparities between districts/cities, is to push the equity of economic development, increase water supply services so that it could be distributed evenly thorough the regions, and increase the educational facilities. Also, recommendation for districts government to push the equity of area's development, especially addressed to the government of Pegunungan Arfak District and Maybrat District.

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A measure of marketing price transmission in the Red Onion Market of Sri Lanka

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Abstract— *In the past there were several protectionist trade policies to safeguard the local onion production. This study examines their impact by means of nominal and effective protection rates and competiveness in resource utilization by competitiveness coefficient. There is a long-run co-integration relationship between the farm and the retail prices marketing margin resulting from this long-run relationship cause asymmetric short-run dynamic adjustments between the farm and the retail prices Welfare distribution among stakeholders is measured by classical welfare analysis. The analysis indicates that both big and red onion producers are noticeably protected by the trade policies and receive returns greater than they would get under a free market condition. Trade policies benefit consumers over producers. Gains to the nation are substantial.*

Keywords— Sri Lanka, Red Onion market, Transmission rate, onion production, farm.

I. INTRODUCTION

Sri Lanka imports annually about 34,000 metric tons (mt) (DCS, 2009) of large onion valued at approximately Rs.300 million. If the country is to be self sufficient in onions, local production must be increased by about 400 percent. Cultivation of the crop, which has specific environmental requirements, is presently restricted to a few agro-ecological regions of the dry zone. It should be possible, however to cultivate adapted varieties, fertilizer and water management.

Onion is a vital spice crop in Sri Lankan economy. Two types of onion are grown and consumed in Sri Lanka: the big onion *(Allium cepa L.)* and shallot called red onion (*A. cepa var. ascalonium),* which are close substitutes in local cuisine. Sri Lanka is near self-sufficient in red onion, importing only 10 % of the requirement. However, about 70 % of big onion requirement in imported at a cost of around Rs. 6 billion. Government, is therefore striving to achieve self-sufficiency in big onion by year 2015. Thus, onion production is under continuous surveillance and, protectionist trade policies are continuously modified and implemented to promote and safeguard the local cultivation. Sri Lanka grows big onions only seasonally and harvests 90% of its local production in the months August to October, before the October rains. Therefore, in order to help the local onion producer, the government slaps an import levy in usual practice to avoid oversupply.

Big onion is an important cash crop cultivated in Sri Lanka. Local production of big onion, which is approximately 81,707 MT per year, is not sufficient to meet the annual demand of big onion approximately 203,993 MT per year (DCS, 2009). Unavailability of good quality seeds of recommended varieties in adequate quantities is considered as the main constraint for increasing production of big onion in Sri Lanka (Mettananda, 2006). Furthermore, the quality of the imported big onion true seeds is not up to standard as they reach the country through illegal routes due to export restrictions in India (Edirimanna, 2003). Poor germination and blubbing, high thick neck percentage and low yield are characteristic to such seeds (Edirimanna and Rajapakshe, 2003)

II. MODEL AND METHODS

A TVECM is a Vector Auto Regression (VAR) model with a well-specified long run relationship and in which regime changes introduce non-linearities. In the linear VECMs even the smallest deviation from the long run already leads to an adjustment toward the long run relationship, whereas in the TVECMs the adjustment is assumed to be costly. The adjustment takes place only if the benefits of changing the price exceed the costs or the marketing margin.

In the literature above it is noted that changes in the marketing margin may cause an asymmetric price transmission. In this case, the use of the VAR models or the vector error correction linear models (VECM) would be inappropriate. This paper therefore applies a two-regime TVECM (nonlinear model) based on Von Carmon Taubadel (1998) and Meyer (2004). The error correction term serves as the threshold variable, separating the transaction cost variation into two regimes to create a nonlinear threshold model. In the following, we first briefly introduce the threshold model and then discuss our empirical model.

III. RESEARCH METHODOLOGY

The threshold autoregressive model developed by Tong (1978) and Tong and Lim (1980) uses an optimal threshold value to divide the short-run dynamic status of one economic indicator into two regimes. When there are multiple (two) regimes, the threshold model could be transformed as:

$$Z_t = (A_1 + \phi_1\ z_{t-i})I(q_{t-d} > \gamma) + \left(A_2 + \phi_{2,i}Z_{t-i}\right)\left(1 - I(q_{t-d} > \gamma)\right) + \varepsilon \quad (1)$$

$$Z_t = \begin{bmatrix} g_t \\ i_t \end{bmatrix}_{2\times1}, A_1 = \begin{bmatrix} \alpha_{10} \\ \beta_{10} \end{bmatrix}_{2\times1}, A_2 = \begin{bmatrix} \alpha_{20} \\ \beta_{20} \end{bmatrix}_{2\times1}$$

$$\phi_1 = \begin{bmatrix} \alpha_{1,11\ldots}\ \alpha_{1,1p}, \alpha_{1,21\ldots}\alpha_{1,2p} \\ \beta_{1,11\ldots}\beta_{1,1p}, \beta_{1,21\ldots}\beta_{1,2p} \end{bmatrix}_{2\times2p} \qquad \phi_1 = \begin{bmatrix} \alpha_{2,11\ldots}\ \alpha_{2,1p}, \alpha_{2,21\ldots}\alpha_{2,2p} \\ \beta_{2,11\ldots}\beta_{2,1p}, \beta_{2,21\ldots}\beta_{2,2p} \end{bmatrix}_{2\times2p}$$

where p is the lag length; q_{t-d} is the threshold variable, and d is the delay parameter; γ is the threshold value; and the error term $\boldsymbol{\varepsilon}$ has the properties such that $\varepsilon = (\varepsilon_1 * \varepsilon_2 *) \sim iid$, $E(\varepsilon_t | \Omega_{t-1}) = 0$, and $E(\varepsilon_1^2\ t\ | \Omega_{t-1}) = \sigma^2$ where Ω_{t-1} is the information set in period $t-1$; $I(\cdot)$ are the indicator functions of regimes, and it is assumed that $I(q_{t-d} > \gamma) = 1$ if there exist regimes and $I(q_{t-d} \leq \gamma) = 0$ otherwise.

We must examine the existence of the threshold effect in equation (1) before estimating the threshold model. We follow the approach of Tsay (1998) to test the linearity of the model. The null hypothesis is that the model is a linear model—and the alternative hypothesis is that the model is a nonlinear model. Tsay (1998) employs the recursive least squares method (RLS) to obtain the predictive residual of the arranged auto-regression (ARR) to build the test statistic based on the standardized predictive residual. For detailed discussion of the Tsay linearity test, please refer to Tsay (1998).

If the null hypothesis is rejected, which indicates that the model is nonlinear, then the next step is to find the values of the two parameters, the delay parameter d and the threshold value γ. Suppose that p, q, and the regimes are known. The threshold variable z_{t-d} determines the appearance of the model in two regimes.

$$y_t = \begin{cases} X_t^y \phi_1 + \sum_1^{1/2} a_t & If\ z_{t-d} > \gamma \\ X_t^y \phi_2 + \sum_2^{1/2} a_t & If\ z_{t-d} \leq \gamma \end{cases} \quad (2)$$

If γ and d are given, then the above equation can be viewed as having two independent linear regressive models, where Φ_i and Σ are obtained as follows:

$$\phi_i(\gamma, d) = \left(\sum_t^{(i)} X_t\, X_t^F\right)^{-1}\left(\sum_t^{(i)} X_t\, y_t^F\right), \hat{\Sigma}_{\mathrm{i}}\,(\gamma, \mathrm{d}) = \sum_{\mathrm{t}}^{(\mathrm{i})}\left(\mathrm{y_t} - \mathrm{X_t'}\hat{\Phi}_{\mathrm{i}}^{*}\right)\left(\mathrm{y_t} - \mathrm{X_t^F}\ \hat{\Phi}_{\mathrm{i}}^{*}\right)^{\mathrm{F}}/(\mathrm{n}; -\mathrm{k}) \quad (3)$$

where $(\ ,\ )$ $\Phi^*_i = \Phi i(\gamma\ d)$; n_i denotes the observations in regime i, $i=1, 2$; and k indicates the dimension of X_t and $k < n$. The residual sum of squares is:

$$S(\gamma, d) = S_1(\gamma, d) + S_2(\gamma, d), \quad S_i(\gamma, d) = trace\left[(n_i - k)\,\hat{\Sigma}_{\mathrm{I}}\,(\Gamma, \mathrm{D})\right] \quad (4)$$

Where γ and d are obtained from the following equation:

$$\arg min_{\gamma,d}\ S(\gamma, d), 1 \leq d \leq d_0\ and\ \gamma \in R_0$$

After attaining the optimal threshold value (γ) and the delay parameter (d), the best fit threshold model can be built.

IV. EMPIRICAL MODEL

Assume that the long-run relationship between the farm and the retail market prices of rice is expressed by the equation $p^R{}_t = a + bp^F{}_t + \varepsilon_t$. p^F is the logarithm of the farm price of rice, p^R the logarithm of the retail price. a represents the price difference, and b the cross elasticity between the two prices. Generally, $b > 1$, meaning that the variation in the retail price will be greater than the variation in the farm price.

According to von Carmon-Taubadel (1998) and Meyer (2004), the error correction term

$ECT_t = p^R{}_t - a + bp^F{}_t$ reflects the marketing margin between the farm and the retail markets. We therefore take into account a delay d and calculate ECT_{t-d} as the threshold variable in the threshold error correction model. The causal relationship between changes in the farm price Δp^F and the retail price Δp^R is examined in the context of various changes in the marketing margin to understand market responses and the impact of government policies 5. Unlike Meyer (2004), who uses a delay of one and

ECT_{t-1}as his threshold variable, we use a strict statistical method to extract the correct delay d from the data itself, taking into account the particular features of the rice production cycle.

The threshold model is divided into two regimes. In one regime price adjustments are determined by the marketing margin (or rather, transaction cost deviations from the long-run equilibrium) that exceeds the threshold γ (regime 1); in the other, adjustments are determined by the marketing margin below (and equal to) the threshold γ (regime 2). The specification for the 2-regime TVECM is given below:

Regime 1 (high marketing margin)

$$\begin{bmatrix}\Delta p^F_t \\ \Delta p^R_t\end{bmatrix} = \begin{bmatrix}\propto_1 \\ \propto_2\end{bmatrix} + \sum_{i=1}^{k} \begin{bmatrix}\beta^{F,F}_i & \beta^{F,R}_i \\ \beta^{R,F}_i & \beta^{R,R}_i\end{bmatrix} \begin{bmatrix}\Delta p^F_{t-i} \\ \Delta p^R_{t-i}\end{bmatrix} + \begin{bmatrix}\emptyset^F_1 \\ \emptyset^R_1\end{bmatrix} [ECT_{t-1}] + \begin{bmatrix}\varepsilon^F_t \\ \varepsilon^P_t\end{bmatrix}, if\ ECT_{t-d} > \gamma \quad (5)$$

Regime 2 (low marketing margin)

$$\begin{bmatrix}\Delta p^F_t \\ \Delta p^R_t\end{bmatrix} = \begin{bmatrix}\propto_1 \\ \propto_2\end{bmatrix} + \sum_{i=1}^{k} \begin{bmatrix}\beta^{F,F}_i & \beta^{F,R}_i \\ \beta^{R,F}_i & \beta^{R,R}_i\end{bmatrix} \begin{bmatrix}\Delta p^F_{t-i} \\ \Delta p^R_{t-i}\end{bmatrix} + \begin{bmatrix}\emptyset^F_2 \\ \emptyset^R_2\end{bmatrix} [ECT_{t-1}] + \begin{bmatrix}u^F_t \\ u^R_t\end{bmatrix}, if\ ECT_{t-d} \leq \gamma \quad (6)$$

Following Tsay (1998), we first assess a linear model in order to be certain that the data do contain thresholds, and that a threshold model is the most appropriate. Thus our null hypothesis is a linear VECM model, and the alternative hypothesis is a nonlinear TVECM mode

Finally, we tested for the Granger causality between the two variables in the short run within regime 1; this tests the effects of the marketing margin on the price transmission from the farm to the retailer. The Granger causality is tested using the Wald statistic, which is also known as the strong exogeneity test. The null hypothesis for the causality in regime 1 (regime 2) isH_0:$\beta^{F,R}i = 0, i = 1,..., k$ (H_0:$\delta^{F,R}i = 0, i = 1,..., k$). The null hypothesis states there is no causal relationship between Δp^R_t and Δp^F_t; rejecting this null hypothesis implies that changes in the retail price do affect changes in the farm price. In the other direction, the null hypothesis is

H_0:$\beta^{R,F}i = 0, i = 1,..., k$ (H_0:$\delta^{R,F}i = 0, i = 1,..., k$), stating that Δp^F_t does not affect Δp^R_t. Rejecting the null hypothesis implies that changes in the farm price do affect changes in the retail price.

We can use the adjustment coefficients, ϕ^F_1 and ϕ^F_2, on the ECTs at different intervals to determine whether the retail price is weakly exogenous with respect to the farm price; $\phi^R_1$and ϕ^R_2 can be used to determine whether the farm price is weakly exogenous with respect to the retail price. This assessment enables us to judge whether corrections emerge in response to short-run imbalances between the farm and the retail prices.

V. EMPIRICAL RESULTS

The variables in this model are the farm and the retail prices of the red onion. The monthly retail price and producer price data were collected from January 2005 to December 2014, a total of 120 observations. Table 1 reports the basic statistics of logarithmic farm and retail prices. The means of the two variables indicates that the fluctuation of the retail price is larger than that of the farm price. Figure 1 illustrates the time trends of the two prices and it is very obvious that the retail price is higher than the farm price. These two phenomena implies that there is the marketing margin (or transaction cost) between the farm and retail prices. The standard deviations of Table 1 could evaluate the price risk of the red onion prices. The numbers in Table 1 indicate that the farm price is riskier than the retail price is, which indicates that the red onion market that Sri Lankan farmers face is a low-return and high-risk one.

TABLE 1
THE BASIC STATISTICS OF THE PRICE VARIABLES

	PP	RP
Mean	4.125939	4.584649
Std.Dev	.3587974	.3509778
Skewness	.6242245	.6097364
Kurotis	3.204735	3.405052
observations	120	120

Note: Variables are all in natural logarithms. Variable PP the farm price per kilo (in Rupees) of red onion, RP is the retail price per kilo (in Rupees) of red onion.

When conducting the tests, we first applied two unit root tests: the Augmented Dickey-Fuller and the Phillips-Perron tests, to establish that the variables were not stationary. The unit root tests compare constant and time-trend models. The results are shown in Table 2. They indicate that the two price series are I(1) processes, i.e. the first difference of the two series are stationary. Using these results, we can test for co integration between the farm and the retail rice prices.

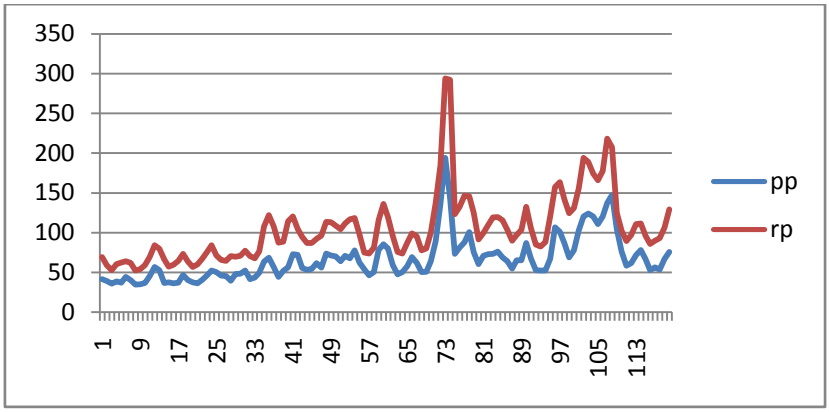

FIGURE 1: FARM AND RETAIL PRICES OF RED ONION

TABLE 2
UNIT ROOT TESTS

	ADF	
Variable	**Constant**	**Constant + trend**
Level value		
RP	-1.797 (10)	3.93 (2)
PP	-2.114 (3)	-3.75 (7)
First difference		
RP	-9.14 (2) ***	-5.632 (9) ***
PP	-11.02 (2) ***	-10.994 (2) ***

Notes: Variables are natural logarithms. A maximum of 15 lags were used in the unit root tests. The numbers in brackets [ ] are the appropriate lag lengths selected by AIC. *** indicate significance at the 1 percent level. The critical value for ADF test with constant and constant plus time trend is 4.04 and 3.51 respectively. Critical values from MacKinnon (1996).

The Johansen co integration test (Johansen, 1991; 1995) was applied to determine whether or not there is a stable long-run relationship between the farm gate price and retail price of red onion The results of the unit root tests showed no trend, implying that the *I(1)* process was not caused by a time trend. We therefore did not consider time trends in the cointegration test. Table 3 shows the results of the cointegration test: the farm and the retail prices are cointegrated. The long-run relationship between the farm and the retail prices is $p^{R}_{1,t}$ = 29.45 + -1.238247 $p^{F}_{1,t}$. The parameter value of a shows that there exists a fixed mark-up effect; the value of b shows that the cross-elasticity between the farm and the retail prices is higher than 1. This implies that variation in the retail price of red onion is larger than the variations in the farm price.

TABLE 3
CO-INTEGRATING TEST

Unrestricted Cointegration Rank Test (Trace)				
Hypothesized		Trace	0.05	
No. of CE(s)	Eigenvalue	Statistic	Critical Value	Prob.**
None *	0.177504	31.88568	25.87211	0.0079
At most 1	0.097498	10.97658	12.51798	0.0892
Unrestricted Cointegration Rank Test (Maximum Eigenvalue)				
Hypothesized		Max-Eigen	0.05	
No. of CE(s)	Eigenvalue	Statistic	Critical Value	Prob.**
None *	0.177504	20.90911	19.38704	0.0299
At most 1	0.097498	10.97658	12.51798	0.0892

Before defining the nonlinear threshold model, it is necessary to confirm that the variables are not in fact linear. We follow Hansen and seo (2002) in testing for linearity: This is the test of Test of linear cointegration vs threshold cointegration. The test statistic value is 39.2 which is significant at 1% level. We follow seo (2006) to test cointegration. This is the test of Test of no cointegration vs threshold cointegration. The test statistic value is 25.3151 which is significant at 5% level. The error term (ECT_{t-d}), taken to be a measure of the marketing margin is the threshold variable. We first selected the 4 lag period (p) which gave the best fit for the data as measured by the Akaike information criterion (AIC)..

The threshold variable for the red onion was therefore ECT_{t-2};. The threshold value for red onion model is 0.081; therefore when $ECT_{t-1} > -0.148$, the regime relating to the high marketing margin (regime 1) holds. When $ECT_{t-1} < -0.148$, the marketing margin is low (regime 2). The economic interpretation of these findings is that when the marketing margin is lower than the threshold value, the market operates freely: there is feedback between the farm and the retail prices. When the marketing margin is higher than the threshold value, there is no significant causal relationship between the two prices. We suggest that the reason for this difference is that the government makes necessary interventions in the market to stabilize the retail price when there is a large rise in the retail price. That rise may originally have been caused by changes in the farm price, but after the government intervention, a causal relationship between the two prices no longer exists. This is consistent with normal practice of the agriculture authorities in Sri Lanka.

VI. CONCLUSION

The purpose of this study is to examine the relationship between the farm and the retail prices in the Sri Lankan red onion market. We established three hypotheses and obtained several important empirical findings. Firstly, there is a long-run cointegration relationship between the farm and the retail prices. Secondly, the marketing margin resulting from this long-run relationship may cause short-run dynamic adjustments between the farm and the retail prices, which results in the asymmetric causality.

This implies that the marketing margin is an important factor when analyzing the causality in the farm and the retail markets. Because of this, we constructed a nonlinear threshold model to fully understand the effect of the marketing margin. Thirdly, when the marketing margin is low, the market operates freely; when the marketing margin is high, the government makes necessary interventions in the market to prevent excessive rises in the rice prices. When intervention occurs, the market system no longer operates.

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Protecting Human Health from Airborne Biological Hazardous Material by an Automatic Image Acquisition and Interpretation System

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Abstract— *Human beings are exposed every day to bio-aerosols in their personal and/or professional life. The European Commission has issued regulations for protecting employees in the workplace from biological hazards. Airborne fungi can be detected and identified by an image-acquisition and interpretation system. In this paper we present recent results on the development of an automated image acquisition, sample handling and image-interpretation system for airborne fungi identification. We explain the application domain and describe the development issues. The development strategy and the architecture of the system are described and results are presented.*

Keywords— Health Monitoring, Microscopic image acquisition, microbiological sample handling, image analysis, image interpretation, case-based object recognition, case-based reasoning.

I. INTRODUCTION

Airborne microorganisms are ubiquitously present in various indoor and outdoor environments. The potential implication of fungal contaminants in bio-aerosols on occupational health has been recognized as a problem in several working environments. The exposure of workers to bio-aerosols is a concern especially in composting facilities, in agriculture, and in municipal waste treatment. The European Commission has therefore issued guidelines protecting employees in the workplace from airborne biological hazards. In fact, the number of incidents of building-related sickness, especially in offices and residential buildings, is increasing. Some of these problems are attributed to biological agents, especially to airborne fungal spores. However, the knowledge of health effects of indoor fungal contaminants is still limited. One of the reasons for this limitation is that appropriate methods for rapid and long-time monitoring of airborne microorganisms are not available.

In addition to the detection of parameters relevant to occupational and public health, in many controlled environments the number of airborne microorganisms has to be kept below the permissible or recommended values, e.g. in clean rooms, in operating theaters, and in domains of the food and pharmaceutical industry. Consequently, the continuous monitoring of airborne biological agents is a necessity for the detection of risks to human health as well as for the flawless operation of technological processes.

At present a variety of methods are used for the detection of fungal spores. The culture-based methods depend on the growth of spores on an agar plate and on counting of colony-forming units [1]. Culture-independent methods are based on the enumeration of spores under a microscope, the use of a polymerase chain reaction or on DNA hybridization for the detection of fungi [1]. However, all these methods are limited by time-consuming procedures of sample preparation in the laboratory. This paper describes the development and the realization of an automated image-acquisition and sample handling unit of biologically dangerous substances and the automated analysis and interpretation of microscope images of these substances.

In the system described here, contaminated air containing bio-aerosols is collected in a defined volume via a carrier agent. Bio-aerosols are recorded by an image-acquisition unit, counted, and classified. Their nature is determined by means of an automated image-analysis and interpretation system. Air samples are automatically acquired, prepared and transferred by a multi-axis servo-system to an image-acquisition unit comprised of a standard optical microscope with a digital color camera. This part of the system is described in Section 2. To obtain a sufficient image quality, special requirements have to be fulfilled by the image-acquisition unit which will be described in Section 3.

The variability of the biological objects is very broad. Given the constraints of the image acquisition, this variability is found in the appearance of the objects as well. There are no general features allowing one to discern the type of the detected fungi. In the system employed here, images are stored, and a more generalized description for the different appearances of the same

objects is used. We will describe this novel case-based reasoning approach for the image analysis and its interpretation in Section 4. Finally, we will summarize our work in Section 5.

II. GENERAL COMMENTS ON THE IMAGE ANALYSIS OF MICROORGANISM

Classification of airborne fungal spores from environmental samples presents the image analyst with inherent difficulties. Most of these difficulties concern the automatic identification of microorganism in general [2]. For example, the types and numbers of objects (different fungal species) that may be present in any one air sample are both unknown and effectively unlimited. Also, intra-species variation of characteristics (such as size, color or texture of spores) can be large and may depend on several factors. Furthermore, the bulk size of two targeted species may be an order of magnitude or more apart, making it difficult to decide e.g. on an optical magnification setting. The dynamic and variable nature of the microorganism thus presents a formidable challenge in regard to the design of a robust image interpretation system with the ideal characteristics of high analysis accuracy but wide generalization ability. The difficulties can be summarized as follow:

- **Intra-species variation due to natural phenomenon, i.e., life-cycle, environmental effects**

The dynamic nature of living organisms results in properties such as size or color of the microorganism being statistically variable. Different growth condition of microorganism may result in uncharacteristically large or small specimens – resulting in data outliers. Ultimately, under these circumstances the classification accuracy of an image interpretation system will rely on the training database capturing as much of this variability as possible.

- **Intra-species variation due to predation, fragmentation etc.**

Often atypical characteristics occur due to predation, environmental factors, or aging.

- **To stain or not to stain?**

Many species appear clear/opaque at the resolutions used, making imaging and analysis very difficult. Staining can help to increase the resolution of the fungal material and to distinguish between viable and non-viable organisms. Depending on the application different stains have to be used. At present 10-20 different stains are frequently used for staining fungal spores. They include "all-purpose"-stains such as lactophenol cotton blue which stains fungal elements blue. The staining procedure takes only 1 to 2 minutes. The application of fluorescence stains allows discriminating between living and dead cells. However the use of epifluorescence microscopy in an automated system is more expensive and requires additional hardware. While it is common to stain specimen samples prior to analysis, staining puts special demands on an automated sample handling, image acquisition system and image interpretation system.

- **Choosing an appropriate optical resolution for imaging specimens**

The wide variation of the size of targeted species necessitates a choice of optical magnification that may not be optimal for any species. For example, to analyse the fine internal structures of species such as Wallemia sebi, a 1000x magnification would be required. Fusarium spores are the largest spores among the spores considered in this study. They would require only a 200x magnification instead of a 1000x magnification.

- **Imaging 3-dimensional objects**

The spore is a 3-dimensional object. Imagine a spore which has an ellipsoid shape. Depending on its position, the object can appear as a round object or as an elongated object in a 2-D image. Many species have a significant length in the third dimension - often greater than the depth-of-field of the imaging device - making their representation as a 2-D image difficult. As such, significant areas of the specimen will be out of focus. If only one kind of specimen appears in an image focusing may not be so difficult. However, in a real air sample different specimen can appear. In this case, a single focus level may not be sufficient. Different levels of focus may be necessary which will result in more than one digital image for one sample.

- **How to get a clean sample from the air sample?**

Samples of bioaerosols will contain a wide range of objects (organic and inorganic particles). Filters will be needed to remove particles larger than the objects of interest. But this will generally not prevent the image from containing non-targeted species. Non-targeted species/objects will generally need to be classified. Normally the sample should be covered by

water and a cover glass. To realize this in an automated handling system is not easy since handling glass by means of handling devices is difficult.

III. RELATED WORKS

Several case studies have been done on identifying fungi or other microorganism. In [3], an image analysis method was described for the identification of colonies of nine different Penicillium species as seen after growth on a standard medium. In [4], a study of image analysis based on fluorescence microscopy images was described for the improvement of the exposure assessment of airborne microorganism. Semiautomatic image analysis techniques were applied to segment the contour of fungal hyphae in [5]. Yeast cells were analyzed by image analysis techniques in [6]. Different *Fusarium* species macroconidia were analyzed in [7]. The work aimed at designing an automated procedure for collecting and documenting microscopic pictures of *Fusarium* conidia, determining various morphological parameters and statistically evaluating the effectiveness of those characteristics in differentiating the most important pathogenic *Fusarium* species occurring on wheat in Germany.

The work which is most closely related to our work is that described in [8]. The ability of an image analysis routine to differentiate between spores of eleven allergenic fungal genera was tested using image analysis based on seven basic and up to 17 more complex features, extracted from digitized images. Fungal spores of Alternaria, Cladosporium, Fusarium, Aspergillus, Botrytis, Penicillium, Epicoccum, Exserohilum, Ustilago, Coprinus and Psilocybe were examined in a series of experiments designed to differentiate between spores at the genus and species level. No specific algorithm for image enhancement and image segmentation is described in this work. It appears that only the feature measurement has been automated. The object area was labelled interactively. From the fungal spores seven basic features including length, width, width/length ratio, area, form factor (circularity), perimeter and roundness, and 17 more complex features including equivalent circular diameter, compactness, box area, radius, modification ratio, sphericity, convex hull area, convex hull perimeter, solidity, concavity, convexity, fibre length, fibre width were extracted. Linear and quadratic discriminant analyses were used for classification. It is interesting to note that the authors created a sufficiently large database of fungi spores for their analysis. The number of spores used for this study ranges from 200 to 1000 samples. The classification accuracy according to a particular class ranged from 56% to 93% for genera comparison and from 26% to 97% for species comparison. The results showed that not for all classes the right features for classification were selected. Rather, it appeared that all common features that are known in pattern recognition for the description of 2-D objects were applied to the images. No specific features have been developed that describe the properties of the different fungi genera and species. For example, considering specie Fusarium, the septation is a highly discriminating features but no such description was included in the feature list [8].

A number of successful case studies have been conducted to automate the identification of fungi and microorganism in general. In these studies, imaging methods for microorganism, automatic focussing methods, image analysis, feature description and classification have been developed. Most of these studies used 500x to 1,500x magnification for image acquisition. The most used feature descriptors are the area size and the shape factor of circularity. The color information was used only in [3], and was neglected in all other studies. Not all publications included microscopic images of the microorganism; therefore, we cannot evaluate the quality of the images. In most of the cases, the digitized images were not highly structured. The objects and the background appeared more or less homogenous allowing applying a simple thresholding technique for image segmentation. In general, these studies are characterized by applying standard image analysis and feature extraction procedures to the images. Neither a specific feature set for fungi identification has been developed nor has a good feature set for the description of microorganism been found yet as evidenced by [7] and [8].

The difference to our work is that in most of these studies images are created for only one specie and not for a variety of different species, except for the work in [8]. The creation of digitized images for a variety of different species is much harder since the species differ in size and dimension and, therefore, the selection of an optical resolution that will show the image details of the different species in sufficient resolution is not easy. Also, the image analysis is much more difficult since for all the different objects a sufficient image quality should be reached after image segmentation.

IV. DEVELOPMENT ISSUES

We decided to start the development of our system based on a data set of fungi spore images taken in the laboratory under optimal conditions and constant climate conditions. The data set should represent the prototypical appearance of the different kind of fungi strains and serve as gold standard.

The objects in the images are good representatives of the different kinds of fungal spores cultured under optimal conditions and constant climate conditions. However, as it can be seen from the images of Alternaria alternata and Ulocladium botrytis none of the objects in the image looks like another. There is no clear prototypical object. We can see a high biological variability and also younger and older representatives of the fungal strains. Depending on the image acquisition conditions we see objects from the side and from the top and this influence the appearance of the objects. Generalization about the objects cannot be done manually; rather, each case that appears in practice should be stored in the system and the system should learn more generalized descriptions for the different appearance of the same objects over time. All this suggests that a case-based reasoning approach for the image interpretation [9] should be taken rather than a generalized approach. Case-Based Reasoning [10] is used when generalized knowledge is lacking. The method works on a set of cases previously processed and stored in a case base. A new case is interpreted by searching for similar cases in the case base. Among this set of similar cases the closest case with its associated result is selected and presented on a display.

For the kind of images created in the laboratory we have to develop an image analysis procedure. It is then necessary to describe the images by image features and to develop a feature extraction procedure which can automatically extract the features from the images. The features and the feature values extracted from the images together with the name of the fungal spores make up an initial description of the data. We do not know if all image features are indeed necessary. However, we extract as many image features as possible from the images that appear meaningful in some way to ensure that we can mine the right case description from this database. From this initial description of the data we need to identify good representative descriptions for the cases by using case mining methods [10]. Based on this information we will generate the case-based reasoning system.

After reaching sufficient classification accuracy we will start to include real air samples into the system by adapting the prototypical representations of fungi spores to the real ones.

V. SYSTEM REQUIREMENTS

The system to be developed should allow to collect dust and biological aerosols in well-defined volumes over microscope slides, deposit them there, image them with an appropriate method and count and classify them with an automated image analysis and interpretation method, in order to determine the following parameters from the images:

- Total number of airborne particles
- Classification of all particles according to their size and shape
- Classification of biological particles according to their size and shape, e.g. spores, fragments of fungal mycelia, and fragments of insects
- Number of respirable particles
- Total number of airborne particles of biological origin
- Number of dead particles of biological origin
- Number of viable and augmentable particles of biological origin
- Identification of species or genera exploiting the characteristic shapes of spores and pollen
- Proportion of airborne abiotic and biotic particles
- Proportion of dead and viable airborne microorganisms.

At the beginning of the project the following requirements concerning the optical and the mechanical system were defined:

- Color images should be produced in order to facilitate the separation of dead and living objects.
- It should be possible to generate images in at least three defined depths of field.
- A marker liquid like lactophenol should be used to further enhance the separation of dead and living objects (blue color for living objects). For this purpose a cover slip is necessary in order to uniformly distribute the marker drop on the object slide.

The object slide should be covered with an adhesive in order to fix the airborne germs.

TABLE 1
STRAINS OF EMPLOYED FUNGI AND SELECTED PROPERTIES OF SPORES

Species	Strain no.	Spore shape	Spore color	Spore size [µm]
Alternaria alternata	J 37 (A^1)	Septated, clavate to ellipsoidal	Pale brown	18 – 83 × 7-18
Aspergillus niger	i400 (B^2)	Spherical, ornamented with warts and spines	Brown	Ø 3.5 - 5
Rhizopus stolonifer	J 07 (A)	Irregular in shape, often ovoid to elliptical, striate	Pale brown	7-15 × 6-8
Scopulariopsis brevicaulis	J26 (A)	Spherical to ovoid	Rose-brown	5-8 × 5-7
Ulocladium botrytis	i171(B)	Septated, ellipsoidal	Olive-brown	18-38 × 11-20
Wallemia sebi	J 35 (A)	Cubic to globose	Pale-brown	Ø 2.5 – 3.5

1(A): from culture collection of JenaBios GmbH, Jena, Germany
2(B): from the fungal stock collection of the Institute of Microbiology, University of Jena, Jena, Germany

Six fungal strains representing species with different spore types were identified as important species in different environments (Tab. 1) by our industrial project partner JenaBios GmbH. A database of images from the spores of these species was produced and was the basis of our development. The number of imaged spore per species was about 30-50. Since no commercial system was known fulfilling all requirements, a corresponding system was developed which is described in what follows.

VI. THE AUTOMATED IMAGING SYSTEM

6.1 The microscopic image-acquisition system

Following the specifications given in Section 2 we developed an automated sample-handling and digital image-acquisition system for taking microbiological material from air samples. An existing optical Leitz microscope was upgraded and its hardware expanded. A lens from Olympus with a magnification of 60X and a numerical aperture of 0.7 was used. Its focal length of 1.7 mm provided sufficient clearance between the lens and the object slide including the cover glass to avoid collisions due to their variability in thickness. The lens was inserted in an auto-focusing device from Physik Instrumente (PI, Karlsruhe, Germany) which was mounted on the lens revolver. A motorized xy-table from Märzhäuser (Wetzlar, Germany) with a controller was used to arbitrarily shift the object slide in both x and y direction. For the digital image acquisition a 1.4 Mpixel color digital camera from Soft Imaging System (SIS, Münster, Germany) was used. Our estimates showed that a pixel number larger than 1.4 Mpixel is sufficient for the given magnification. Fig. 1 demonstrates that the optical resolution is sufficient to recognize details in spores like Ulocladium

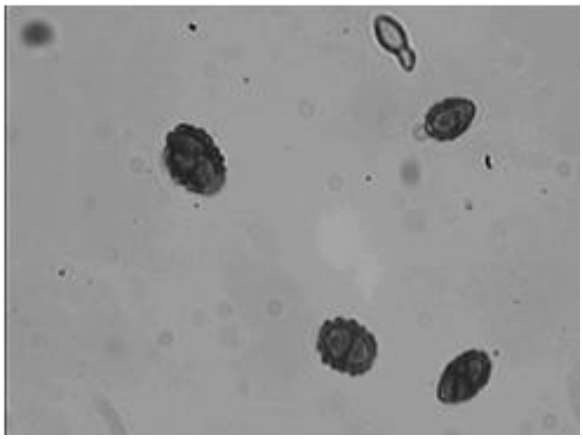


FIG. 1. IMAGE DEMONSTRATING THE RESOLUTION OF THE OPTICAL MICROSCOPE USED. THE MICROSCOPICAL IMAGE DISPLAYS SPORES OF ULOCLADIUM. THE FIELD OF VIEW IS 134×100 µm². THE SAMPLE WAS PREPARED BY AUA/JENABIOS, LENS OLYMPUS 60X/0.70. THE RESOLUTION IN THIS IMAGE IS 5 µm

The functions of image acquisition and image storage, movement of the specimen in x and y direction, and auto-focusing in z-direction are controlled by the AnalySIS Pro software from SIS. A pattern of images at any image position can be freely programmed and stored in a macro-code. This holds true also for the number of images to be captured. If necessary it is possible to capture automatically images at different depths of focus around the optimum position. By the automatic shading correction, the effect of an inhomogeneous illumination of the object can be removed.

6.2 The automatic sample-acquisition and handling system

The following chapter describes the main units and functions of the demonstration set-up realized in the course of the project. A stock of special object slides covered with a sticky layer and obtained from Umweltanalytik Holbach [11], (Fig. 2) is kept in slide storage. A sliding gripper takes the lowest slide in the storage and transports it into the slit impactor obtained from Umweltanalytik Holbach (Fig. 3). The object slides are separated by distance holders with a corresponding recess, in order to avoid sticking between the slides. The distance holder is removed by the same gripper, now moving in opposite direction and depositing the distance holder into a box. The distance holders can be used again when the slide deposit is reloaded.

In the slit impactor (Fig. 3), the air, potentially containing airborne germs, is guided onto the sticky area of the object slide by the air stream generated by an air pump. After a few tens of seconds adjustable appropriately, the pump is switched off and the object slide is transported to the pipetting unit driven by the dosing pump (Cavro XL 3000 obtained from Tecan Systems San Jose, Ca, USA). To achieve this, the object slide has to change its transporting axis and thus its direction of movement. From a thin nozzle one drop of lactophenol is deposited on the sticky area of the object slide. The object slide is afterwards transported through the coordinate origin to the cover-slip gripper unit. This gripper acts as a low-pressure sucker and takes one cover glass from the deposit and places it with one edge first on the object slide. Then the cover glass is allowed to drop down on the object slide and flattens the drop so that it will be distributed all over the sticky area forming a thin layer. In this way the airborne germs collected on the sticky layer are immersed in the lactophenol. In lactophenol living germs take on a blue color. The object slide is then transported back to the coordinate origin where it again changes its direction of movement by 90° and is transported to the xy-table of the microscope where the slide is received and directly transported into a position underneath the lens. To this end, an additional module was integrated into the AnalySIS Pro software. It controls the manual or automated shift of the xy-table between the image-acquisition position under the lens and the loading position, where the object slide is shifted from the object-slide preparation unit to the xy-table. After the object slide has reached the image acquisition position, the microscope camera then takes the images at the programmed slide positions after auto-focusing of the microscope lens at each position. The cycle of shifting the xy-table to the defined positions, auto focusing, image acquisition and storage is programmable in a macro-code integrated into the AnalySis Pro software. This can also be done for other procedures like shading correction or image acquisition at different z-positions. After having finished the imaging sequence, the slide is transported away from the xy-table with a special arm and drops into a box. While the image grabbing procedure by the microscope unit is still under way, the object-slide preparation unit already starts with the preparation of a new object slide.

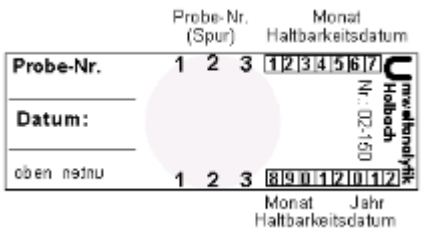


FIG. 2. OBJECT SLIDE OF STANDARD SIZE 76×26×1 mm³ WITH A CENTRAL STICKY LAYER [11]; IMAGE OBTAINED FROM UMWELTANALYTIK HOLBACH.

FIG. 3. SLIT IMPACTOR FOR COLLECTION OF AIRBORNE PARTICLES [11]; IMAGE OBTAINED FROM UMWELTANALYTIK HOLBACH.

The object-slide preparation and manipulation is performed by a hardware controller and by custom software written in C++. The transfer from the AnalySIS Pro software to the C++ software and vice versa is controlled by a communication protocol as interface between both software units. Altogether six different mechanical axes have to be handled, not counting the axes of the xy-table (Fig. 4). The unit for object-slide preparation and the expanded microscope are shown in Fig. 5.

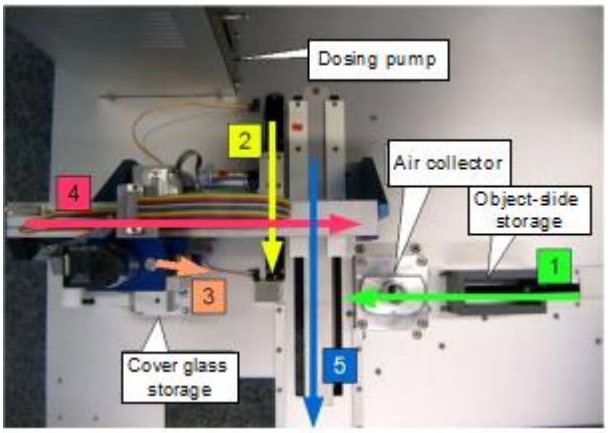


FIG. 4. TOP VIEW OF THE MECHANICAL UNIT FOR MOVING OBJECT SLIDES, INDICATING ALSO THE POSITION OF THE COVER-GLASS STORAGE, THE DOSING PUMP FOR LACTOPHENOL, THE SLIT IMPACTOR OR AIR COLLECTOR, AND THE STORAGE FOR THE OBJECT SLIDES. THE NUMERALS 1 – 5 INDICATE THE SEQUENCES OF THE MOVEMENTS; AXIS NO. 6 IS NOT SHOWN.

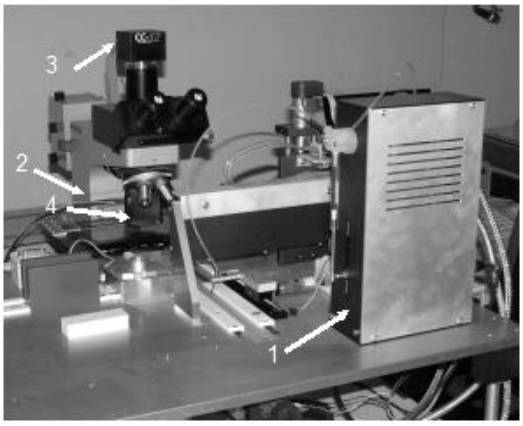


FIG. 5. PROTOTYPE SET-UP SHOWING THE DOSING PUMP (ARROW 1), SEVERAL AXES, THE OPTICAL MICROSCOPE WITH XY-TABLE (ARROW 2), AND THE DIGITAL CAMERA (CC-12, ARROW 3). THE AUTO-FOCUSING UNIT HOLDS THE LENS (ARROW 4).

VII. IMAGE ANALYSIS

Once an image has been taken it is transferred to the image-analysis unit for further processing. We will describe the overall architecture of the system [13] and its single components in the next sections.

7.1 The architecture

The architecture of the system is shown in Figure 6. Objects are recognized in the microscopic image by a case-based object-recognition unit [14]. This unit has a case-base of shapes (case base_1) for fungi spores and determines on a similarity-based inference if there are objects in the image that have a similar shape as the ones stored in the case base. In this case the objects are labeled and transferred for further processing to the feature-extraction unit. To ensure proper performance of this unit, the general appearance of the shapes of the fungi spores must be learned. To this end we have developed a semi-automated procedure [14] that allows acquisition of the shape information from the raw image data and learning of groups of shape-cases and general shape-cases. A more detailed description of the case-based object-matching unit can be found in Section 4.2.

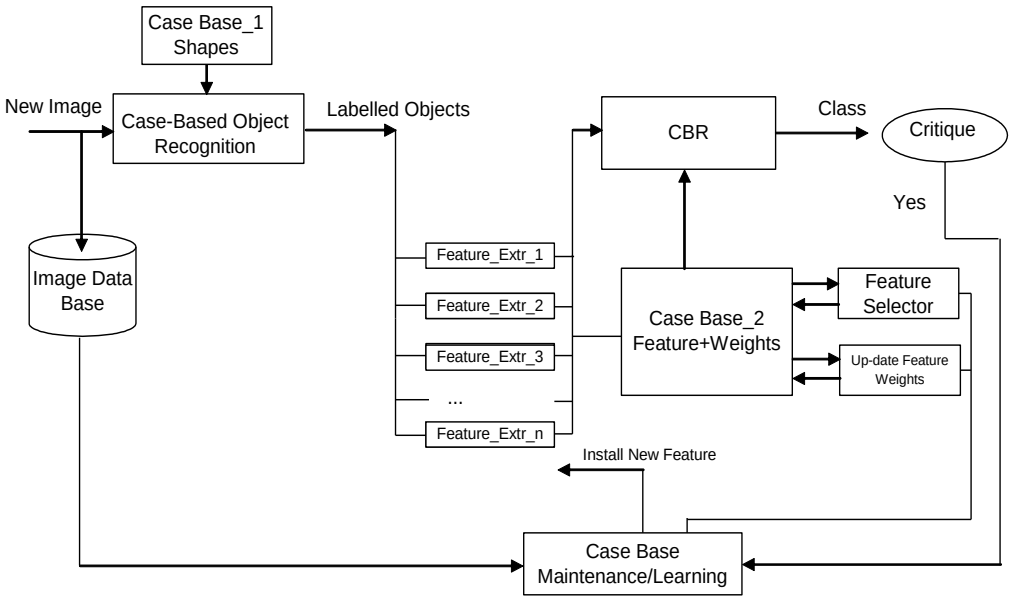


FIG. 6. SYSTEM ARCHITECTURE.

The feature-extraction procedures are based on the knowledge of an expert. Note that a particular application requires special feature descriptors. Therefore not all possible feature-extraction procedures can be implemented in such a system from the beginning. Our aim was to develop a special vocabulary and the associated feature-extraction procedures for application on fungi identification, as described in Section 4.3.

Based on the feature description, the second case-based reasoning unit determines the type of the fungi spore. This unit employs a prototype-based classifier [21]. It initially works based on prototypical cases that were selected or created by the expert. It can learn with time the different appearances of the fungi spores. The special features of this unit ensure its proper performance. It can learn the relevant prototypes from the subjectively selected set of prototypes, as well as create new prototypes. It can also learn the importance of the features of the cases. The final result of the system will be the identification of the fungi spores that appear in the image and the number of these spores. The result is shown on the display of the system and saved in a file, together with the date and time of data acquisition.

Suppose that fungi species are wrongly identified by the system. Then a case-based maintenance process will start. First the system developer must check whether new features have to be acquired for each case, or whether the whole case representation should be updated based on the learning procedures. The feature weights are learnt, as well as a subset of relevant features (see Section 4.4). To acquire new features means that necessary feature-extraction procedures have to be developed and that for all cases the new features have to be calculated and input into the existing case description. Therefore, the digital images acquired so far are retained in the image-data base. Then, the case representation as well as the index structure must be updated. This ensures that we can generate step-by-step a system that can describe the variability of the different biological objects that may appear.

7.2 Case-based object recognition

The objects in the image are highly structured. Our study has shown that the images specified in Table 1 cannot be segmented by thresholding. The objects in the image may be occluded, touching, or overlapping. It can also happen that only part of the objects appears in the image. Therefore we decided to use a case-based object recognition procedure [14] for the detection of objects in the image.

A case-based object-recognition method uses cases that generalize the original objects and compares them with the objects of the image. During this procedure a score is calculated that describes the quality of the fit between the object and the case. The case can be an object model which describes the inner appearance of the object as well as its contour. In our case the appearance of the objects as a whole can be very diverse. The shape seems to be the feature that generalizes the objects. Therefore, we decided to use contour models. We do not use the gray values of the model, but instead the object's edges. For determining the score of the match between the contour of the object and the case, we use a similarity measure based on the scalar product that measures the average angle between the vectors of the template and the object.

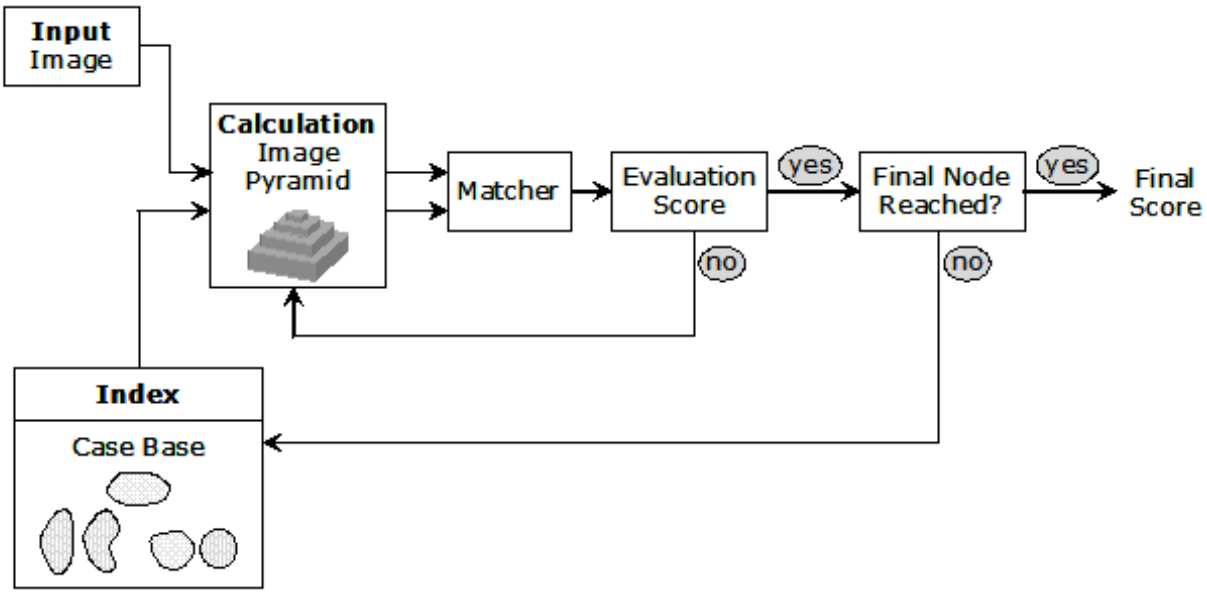


FIG. 7. PRINCIPLE OF CASE-BASED OBJECT-RECOGNITION ARCHITECTURE.

7.2.1 Case-base generation

The acquisition of the case is done semi-automatically. Prototypical images are shown to an expert. The expert manually traces the contour of the object by means of the cursor of the computer. Afterwards the number of contour points is reduced for data-reduction purposes by interpolating the marked contour by a first-order polynom. The marked object shapes are then aligned by the Procrustes Algorithm [15]. From the sample points the direction vector is calculated. From a set of shapes, general groups of shapes are learnt by conceptual clustering which is a hierarchical incremental clustering method [16]. The prototype of each cluster is calculated by estimating the mean shape [16] of the set of shapes in the cluster and is taken as a case model.

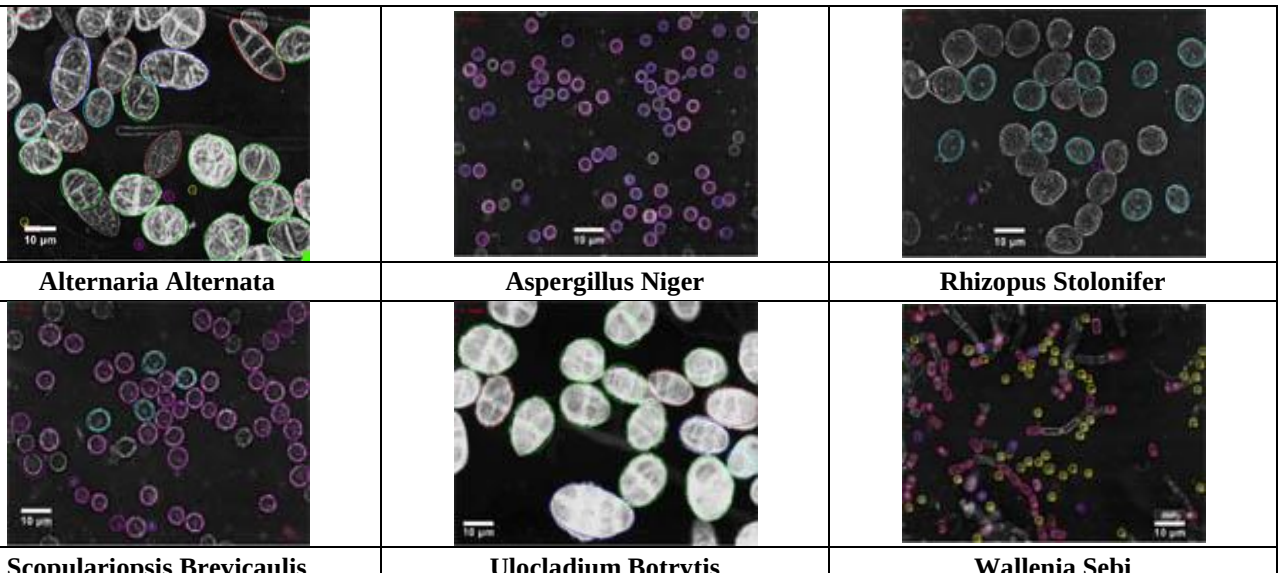
7.2.2 Results for case-based object recognition

We had a total of 10 images for each class at our disposal. From this set of images two images were selected for case generation. In these two images there were approx. 60 objects. These objects were labeled and used for the case generation according to the procedure as described in Section 4.2.1. The result was a data base of cases. These cases were applied to the image for the particular class.

The threshold for the score was set to 0.8. We calculated the recognition rate as the number of objects that were recognized in the image to the total number of objects in the images. Note that the recognition rate can be higher than 100 %, since our matching procedure also fires in image regions where no objects are present due to background noise. The aim is to configure the case-based object-recognition unit in such a way that the number of false alarms is low. The results of the matching process are shown in Figs. 8 and 9. The highest recognition rate can be achieved for the objects Aspergillus niger and Scopularioupsi, since the shape of these objects does not vary much. This is also expressed by the number of models, see Table 2. These classes have the lowest number of cases. For those classes where the variation of the shape of the objects is high, the number of the cases is also high. The recognition rate shows that we did not have enough cases to recognize the classes with a good recognition rate (see Ulocladium botrytis and Alternaria alternata). Therefore, we needed to increase the number of cases. For this task we developed an incremental procedure for the case acquisition in our tool. Objects that have not been recognized well will be displayed automatically for tracing and then the similarity to all other shapes will be calculated. The clustering will be done in an incremental fashion as well [16]. This procedure will ensure that we can learn the natural variation of the shape during the usage of the system.

7.3 Case description and feature extraction

We choose an attribute-value pair-representation for the case description. The case consists of the solution, i.e., the type of fungi spores and the features describing the visual properties of the object (see Figure 9). From each recognized object a set of features is extracted. One feature is the case number which represents the shape of the object, the similarity score between the actual shape and the shape in the case base, the size of the object, various gray-scale features, and the texture inside the object. For the description of the texture we use our texture descriptor based on random sets described in [17].



Alternaria Alternata	Aspergillus Niger	Rhizopus Stolonifer
Scopulariopsis Brevicaulis	**Ulocladium Botrytis**	**Wallenia Sebi**

FIG. 8. RECOGNIZED OBJECTS IN THE IMAGE.

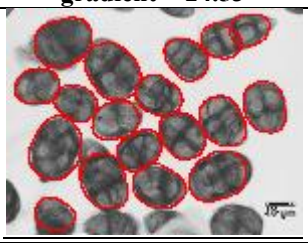
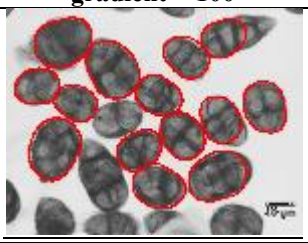
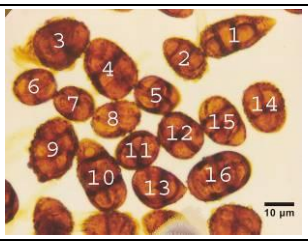
(b) Threshold for the minimal gradient = 24.53	(c) Threshold for the minimal gradient = 100	(d) Test image including the object numbers
		
Recognition Rate:112.5%	**Recognition Rate:87.5%**	

FIG. 9. COMPARISON OF MATCHED OBJECTS BY APPLYING DIFFERENT THRESHOLDS FOR THE MINIMAL GRADIENT.

TABLE 2
RESULTS OF MATCHING

Classes	Number of models	Recognition rate
Alternaria alternata	34	65.9
Aspergillus niger	5	95.2
Rhizopus stolonifer	22	87.7
Scopularioupsi	8	94.5
Ulocladium botrytis	30	77.2
Wallenia sebi	10	90.3

7.4 Classification

Our case-based reasoning procedure to recognize spores relies on prototype-based classification schemes [21]. Usually such schemes are generalized from a set of single cases. Here, we have prototypical cases represented as images that were selected by humans. This means that, when building our system, we start from the top and have to collect more information about the specific class during usage of the system. Since a human has selected the prototypical images, his decision on the importance of an image might be biased; moreover selecting only one image might be difficult for a human. He can have stored more than one image as prototypical images. Therefore, we need to check the redundancy *of the many prototypes for one class* before taking them all into the case base. According to this consideration, our system must fulfill the following functions:

- Classification based on the nearest neighbor rule
- Prototype selection by a redundancy-reduction algorithm; Feature weighting to determine the importance of the features for the prototypes
- Feature-subset to select the relevant features from the whole set of the respective domain.

The classification method is based on the nearest-neighbor rule. Since the prototypes are available at the same time, we choose a decremental redundancy-reduction algorithm proposed by Chang [**18**] that deletes prototypes as long as the classification accuracy does not decrease. The feature-subset selection is based on the wrapper approach [19] and an empirical feature-weighting learning method [20] is used. Furthermore, cross validation is used to estimate the classification accuracy. The prototype selection, the feature selection, and the feature-weighting steps are performed during each run of the cross-validation process. This rule classifies x in the category of its nearest neighbor [21]. More precisely, we call $x'_n \in \{x_1, x_2, ..., x_i, ..., x_n\}$ a nearest neighbor to x if $\min d(x_i, x) = d(x'_n, x)$, where i = 1, 2, …n. The nearest neighbor rule classifies x into category C_n where x'_n the nearest neighbor to is x and x'_n belongs to class C_n. For the k-nearest neighbor we require k-samples of the same class to satisfy the decision rule. As a distance measure, we use the Euclidean distance. The recognition rate was evaluated on a data base of 50 samples for each class based on cross-validation. The result is shown in Table 3. Based on this result, we can conclude that the classification accuracy is higher than the recognition rate for some classes. This means that it is more difficult to recognize the objects that are most likely to be fungi spores than to classify them based on the extracted features.

TABLE 3
CLASSIFICATION ACCURACY

Classes	Classification accuracy
Alternaria Alternata	90.4
Aspergillus Niger	95.0
Rhizopus stolonifer	92.0
Scopularioupsi	96.0
Ulocladium botrytis	94.0
Wallenia sebi	92.0

FIG. 10. SCREENSHOT OF THE FINAL SYSTEM

A print-out of a result obtained by the system described in this paper is shown in Fig. 10. In the display the operator will find the acquired image in one window and in the other window the determined fungi spores and their total number. The system called Fungi PAD correctly identified the name of the fungi spores and their number.

VIII. CONCLUSIONS

In this paper a system for an automated image acquisition and analysis of hazardous biological material in air is described. It consists of an image-acquisition unit, its sample-handling hardware, and the image-interpretation system. The sample-handling and image-acquisition unit collects the airborne germs, deposits them on an object slide, disperses them with a marker fluid, and takes digital images of the germs in a programmable pattern. The stored images are analyzed in order to identify the germs based on a novel case-based object-recognition method. The case generation is done semi-automatically by manually tracing the contour of the object, by automated shape alignment and by shape clustering, and eventually by prototype calculation. Based on the acquired shape cases, the object-recognition unit identifies objects in the image that are likely to be fungi spores. The further examination of labeled objects is done by calculating more distinct object features, from which a prototype-based classifier determines the kind of fungi spores. After all objects have been classified by their type, the number of one type of fungi spores is calculated and displayed for the operator on the computer screen.

The recognition rate is good enough for on-line monitoring of environments. The final information can be used to determine contamination of environments with biological hazardous material. It can be used for health monitoring as well as for process control.

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