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Preface

We would like to present, with great pleasure, the inaugural volume-3, Issue-11, November 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

Environmental Research:

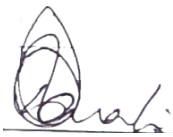
Environmental science and regulation, Ecotoxicology, Environmental health issues, Atmosphere and climate, Terrestric ecosystems, Aquatic ecosystems, Energy and environment, Marine research, Biodiversity, Pharmaceuticals in the environment, Genetically modified organisms, Biotechnology, Risk assessment, 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.

Mukesh Arora
(Editor-in Chief)



Dr. Bhagawan Bharali
(Managing Editor)

Fields of Interests

Agricultural Sciences	
Soil Science	Plant Science
Animal Science	Agricultural Economics
Agricultural Chemistry	Basic biology concepts
Sustainable Natural Resource Utilisation	Management of the Environment
Agricultural Management Practices	Agricultural Technology
Natural Resources	Basic Horticulture
Food System	Irrigation and water management
Crop Production	
Cereals or Basic Grains: Oats, Wheat, Barley, Rye, Triticale, Corn, Sorghum, Millet, Quinoa and Amaranth	Oilseeds: Canola, Rapeseed, Flax, Sunflowers, Corn and Hempseed
Pulse Crops: Peas (all types), field beans, faba beans, lentils, soybeans, peanuts and chickpeas.	Hay and Silage (Forage crop) Production
Vegetable crops or Olericulture: Crops utilized fresh or whole (wholefood crop, no or limited processing, i.e., fresh cut salad); (Lettuce, Cabbage, Carrots, Potatoes, Tomatoes, Herbs, etc.)	Tree Fruit crops: apples, oranges, stone fruit (i.e., peaches, plums, cherries)
Tree Nut crops: Hazlenuts. walnuts, almonds, cashews, pecans	Berry crops: strawberries, blueberries, raspberries
Sugar crops: sugarcane. sugar beets, sorghum	Potatoes varieties and production.
Livestock Production	
Animal husbandry	Ranch
Camel	Yak
Pigs	Sheep
Goats	Poultry
Bees	Dogs
Exotic species	Chicken Growth
Aquaculture	
Fish farm	Shrimp farm
Freshwater prawn farm	Integrated Multi-Trophic Aquaculture
Milk Production (Dairy)	
Dairy goat	Dairy cow
Dairy Sheep	Water Buffalo
Moose milk	Dairy product
Forest Products and Forest management	
Forestry/Silviculture	Agroforestry
Silvopasture	Christmas tree cultivation
Maple syrup	Forestry Growth
Mechanical	
General Farm Machinery	Tillage equipment
Harvesting equipment	Processing equipment
Hay & Silage/Forage equipment	Milking equipment
Hand tools & activities	Stock handling & control equipment
Agricultural buildings	Storage

Agricultural Input Products	
Crop Protection Chemicals	Feed supplements
Chemical based (inorganic) fertilizers	Organic fertilizers
Environmental Science	
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
Theoretical production ecology	horticulture
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Socio- Economic Factors Influencing the Probability of Market Participation among the Cattle Farmers in Adamawa State, Nigeria

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Abstract— *The study examined Socio- economic factors influencing the probability of market participation among the cattle farmers in Adamawa state, Nigeria. Multistage sampling procedure was employed to sample the respondents. Structured questionnaires were used to collect data from 400 respondents in the study area. Descriptive statistic was used to analysed the socioeconomic characteristics of the respondents and logit regression analysis was employed to estimate the determinants market participation among the market participants. The result of the findings shows that cattle market participants were averagely aged 43 years, they are predominantly male (95.04%), (83.48%) were married, while (77.96%), (57.02%) had formal education and are full-time cattle farmers respectively. the result of logit regression analysis indicates that five variables (gender, education, distance to market, prior market information and seasonality) were significantly found to influence the farmers' decision to participate in the market. The marginal effects were used to interpret the results. Recommendations were made such as to encourage more formal education among the farmers, the more the participant is educated, the better the chance of participation in the cattle market and also to encourage female and those that are unmarried to participate in cattle marketing activities. Provision of reliable market information through mass media or extension services is paramount important in improving market participation and to provide adequate pasture land and water supply so as to curb the problems of exposure to avarice of weather, creates more additional sales point at farming communities is paramount important in the intensity of cattle market participation.*

Keywords— cattle, factors, farmers, market and participation.

I. INTRODUCTION

World agriculture is facing a tremendous challenge due to rapid growth of human population. Global population is estimated to grow annually by 76 million and to exceed 9 billion by the year 2050 (UN, 2009). In developing countries food consumption, in particular animal-product consumption has rapidly increased over the past decades as a result of population and economic growth, higher disposable income and urbanization (Steinfeld *et. al.,* 2006). However, as Nigerian's make up about 50% of beef consumers in ECOWAS, It is therefore, experiencing a remarkable demographic expansion and a great change in food habits. With a population growth of about 2.8% per annum, the current domestic production is far from being able to meet up the challenging demand (Bernard *et al.,* 2011).Adesina (2013) lamented that Nigeria will increase the National Dairy Production from its current 469, 000 metric tons to 1.1 million metric tons by attaining an average milk yield per lactating cow from below 500 litres to 2,000 litres per lactation by 2015.Nigeria being the most populous country in West Africa with a population of about 167 million people (National Population Commission, 2012). Moreover, despite the availability of natural resources the country is forced to import more than (25) % of the beef consumed (Bernard *et al.,* 2010). In developing countries, it's observed that since 1980s the growth of per capita animal-product consumption exceeds that of other groups of food commodities (cereals, roots and tubers) (FAO, 2009). The global production of livestock products (meat, milk and egg) is projected to increase by 50 % by the year 2050 (Steinfeld *et. al.,* 2006). It is observed that one of the most important commodities of livestock subsector is beef cattle, it produce quality meat that has high economic value, and has more important role in public life, important social function in community, therefore it is important to developed the sector and to ensure availability of the product (Prasetyo *et. al.,* 2012).Consequently Markets and improved market access are very essential to rural poor households as a pre-requisite for enhancing agricultural productivity based by improving the competitiveness of farming enterprise, improve the standard of living of rural farmers and to meet up with the challenging global demand by the year 2050. Hence, participation of smallholder farmers in market remains very low due to a range of constraints (Ohen*et al.,* 2013). Market participation among farmers has long been on agricultural economist research programme in both developed and developing countries (Egbetokun and Omonona, 2012). But, still there is a need for more research especially on the area of livestock sector. Marketing in agriculture takes a central role in promoting the future of agricultural business, increased importance and dependence over the past decade on market as the foundation for

growth strategies put more premiums on understanding market participation among farmers. Moreover, Transformation from subsistence to more specialized and market-oriented systems of agriculture is of significance for a large number of developing countries with a high degree of dependency on agriculture for livelihoods and national income, such a transformation has enormous potential to promote inclusive growth and development, allowing millions of people to escape poverty and food insecurity (Wickramasinghe and Weinberger, 2013). In Somalia livestock are reared and marketed, in 2011, over three million sheep and goats worth over USD 200 million (39.8 billion) were exported to the Middle Eastern countries (mainly Saudi Arabia) (Wanyoike *et. al.,* 2015). However, it is observed that, limited accesses to capital, as well as the difference in livelihood strategies and motivations are the major factors hindering small -scale farmer's participation in the livestock marketing in south-Africa (Ndoro, 2014). Gani and Adeoti (2011) lamented that, in Nigeria Socioeconomic variables such as cooperative membership, family size, high output commercialization ratio and education and supportive infrastructure have been found to be important variables affecting market participation among farmers. Bellemare and Barrett, (2006) also observed that, there has been scant research on market participation especially in developing countries like Nigeria.

Objectives of the study are to;

1 Find out the socio-economic characteristics of cattle farmers in the study area;
2 Identify the factors influencing market participation among the cattle farmers in the study area;

II. MATERIALS AND METHODS

2.1 Sample and sampling Techniques

A Multi-stage, random sampling and purposive sampling techniques were employed in the selection of the respondents. In the first stage, two Local Government Areas (LGAs) were purposively selected from each zone of the four zones of Adamawa State Agricultural Development Programme (ADADP), the selection was based on their relative importance in cattle farming.

In the second stage, twenty-six (26) districts were randomly selected from forty five (45) districts of the eight (8) selected LGAs proportionately.

The third stage, involves the random selection of 400 cattle farmers proportionately from the selected districts. Information on the sampling frame was obtained from the Ministry of Livestock Productivity and Nomadic Settlement, Yola. The selection of the four hundred respondents will be based on the proportionality factor presented in equation 1, as adopted from Giroh*et al*. (2012).

$$S = {}^{r}/_{R} \times {}^{N}/_{1} \quad (1)$$

Where:

S = total number of respondents sampled in each district

r = number of cattle farmers in a particular district

R = Total number of farmers in all the selected districts

N =Sample size

The sample size of 400 cattle farmers with a total population of 6,170 respondents was obtained when use 5% margin error (confidence interval) and with 95% confidence level (MarCorr 2014, creative research systems 2012, and Didier 2013). Moreover, the Sample size will also be determined by using Taro Yamane formula as shown below: (Polonia, 2013).

$$n = \frac{N}{1+N(e)^2}$$

Where: n = Sample size

N = Population size

e = Limit of tolerance error

With a significance level of 95% the degree of tolerance level will be 5% (i.e. 0.05).

$$n = \frac{6170}{1+6170\,(.05)^2}$$

$$n = \frac{6170}{1+6170*.0025}$$

$$n = \frac{6170}{16.425}$$

n = 376 to nearest hundred = 400 Sample size.

2.2 Data analysis techniques

Descriptive statistics was used to analysed the socioeconomics characteristics of cattle farmers and binary logistic regression analysis was used to analysed socioeconomic factors influencing market participation in the study area.

2.3 Model Specification

2.4 Binary Logistic Regression

In this study binary logistic model was employed because of its comparable simplicity to probit and to bit regressions. By using the logistic regression the probability of a result being in one of two response groups (binary response) is modelled as a function of the level of one or more explanatory variables.

Thus, the probability whether or not the farmer sells cattle may be modelled as a function of the level of one or more independent variables. For this study, the response variable is 1, when the farmer sells livestock in the past twelve months and .0, when the farmer did not sell. The functional form is denoted in equation (2) (Bahta and Bauer, 2007).

$$ln\left(\frac{\emptyset_i}{1-\emptyset_i}\right) = \beta_0 + \sum_{j=1}^{k}\beta_j X_{ij} + \varepsilon_i \quad (2)$$

Where: j is the response category (1 or 0), i denotes cases (1, 2, 3, 4., n), $\emptyset$ is the conditionalprobability, β_0 is the coefficient of the constant term, β_j is the coefficient of the independent variable, X_{ij}, ε_i is the matrix of unobserved random effects, $\frac{\emptyset_i}{1-\emptyset_i}$ is "odd", and $ln\left(\frac{\emptyset_i}{1-\emptyset_i}\right)$ is the logarithm of "odds".

Equation (2) can be manipulated to give the odds ratio using equation (3):

$$\frac{\emptyset_i}{1-\emptyset_i} = \exp\left(\beta_0 + \sum_{i=1}^{k}\beta_i X_i\right) \quad (3)$$

The probability that farm households sell livestock can be calculated using equation (4):

$$\emptyset_i = \frac{exp\left(\beta_0+\sum_{i=1}^{k}\beta_j X_{ij}\right)}{1+exp\left(\beta_0+\sum_{i=1}^{k}\beta_j X_{ij}\right)} \quad (4)$$

Equation (4) is intrinsically linear since the logit is linear in X_i (Gujarati, 1988); it indicates thatprobability $\emptyset_i$lies between zero and one and vary non-linearly with X_i. The equation for calculatingpartial effects of continuous variable is denoted by:

$$\frac{\partial \emptyset_i}{\partial x_i} = \emptyset_i(1-\emptyset)\beta_j \quad (5)$$

The partial effects of the discrete variables will be calculated by taking the difference of the mean probabilities estimated for the respective discrete variable, X_i = 0 and X_i = 1

TABLE 1
EXOGENOUS VARIABLES IN THE BINARY LOGISTIC REGRESSION MODEL

Variable	Description	Measurement	Expected sign
Age	Age of the respondents	In years	±
Gender	Sex of the respondents	Binary variable (1=male, 0=female)	±
Education	Level of educational attainment	Number of years spent in school	+
Family size	Number of persons in a household	Number	±
Distance	Distance from home to the market	Kilometers	+
Market information	Information about cattle marketing	Binary variable (1=Access to information, 0= no access)	±
Seasonality	Growing season	In time	+

III. RESULT AND DISCUSSIONS

3.1 Socio-economic characteristics of the respondents

Result in Table 2 showed that the cattle market participants were averagely aged 43 years had a family size of average 10 persons, has 19 years' experience in cattle marketing and had average herd size of 49 cattle. They are predominantly male (95.04%), married (83.48, %) while (77.96%) of the households had one form of formal education or the other and (57.02%) are full-time cattle farmers.

The result shows that (95.04%), of the cattle farmers were male. It is how ever male-headed households are more likely to participate in the market as a cattle farming is considered a patriarchal activity. Female-headed households are therefore expected to have lower probability of market participation compared to their male counterparts.

The result in table 2 also indicated that more than 83% of the sampled respondents were married. It can be inferred that since majority of the respondents were married, they have social obligations to cater for at the household level and this may cause them to take their participation in cattle marketing activities very seriously in order to generate income and to meet their financial obligations.

Education level of the household head could lead to increase in the household's ability to access and utilize market information. From the findings about 78% of the households had one form of formal education or the other. This implies that they could be able to utilize information more efficiently and consequently improve their managerial skills. This finding conforms to the findings of Randela *et. al*. (2008) and Enete and Igbokwe (2009) who reported that education provides households with better production and managerial skills which could translate to increased market participation.

TABLE 2

SOCIOECONOMICS FACTORS OF THE SAMPLED CATTLE FARMERS

Attribute	Frequency	%
Age		
<20	12	3.31
20 to 34	57	15.70
35 to 49	217	59.78
50+	77	21.21
Family size		
<5	56	15.43
5 to 9	94	25.90
10 to 14	186	51.24
15+	27	7.44
Years of market experience		
<5	21	5.79
5 to 14	91	25.07
15 to 24	209	57.58
25+	42	11.57
Herd size		
<50	183	50.41
50 – 99	65	17.91
100 – 149	68	18.73
150+	47	12.95
Gender		
Male	345	95.04
Female	18	4.96
Marital status		
Single	50	13.77
Married	303	83.48
Widowed	5	1.38
Divorced	5	1.38
Education		
No formal education	80	22.04
Adult/Primary Education	83	22.87
Secondary	108	29.75
Post-secondary	92	25.34
Major occupation		
Cattle farming	207	57.02
Civil servant	92	25.34
Trading	57	15.7
Farming	7	1.93

Source: Field survey, 2016

3.2 Factors influencing cattle market participation

To determine the factors influencing the probability of participation in the cattle market in Adamawa State, a logit model was estimated. The result presented in Table 3 indicates that five variables (gender, education, distance to market, prior market information and seasonality) were significantly found to influence the farmers' decision to participate in the market. The marginal effects were used to interpret the results.

The result in table 3, reveals that gender of the household head significantly and positively influenced market participation. Being male-headed household increases the probability of participating in the cattle market by 45.71%, all other factors held constant. This suggests that the male headed households are more market oriented than female, hence they participate more in the market. The finding concurs with findings of Onoja *et. al*. (2012) who reported that the probability of participating in fish marketing was significantly determined by sex of the fish farmer/marketer in Niger Delta Region.

Educational level of the household head significantly and positively influenced market participation. One year increases in household head education, increase the probability of participating in the cattle market by 13.33%, all other factors held constant. This can be explained by the fact that as an individual access more education he/she is empowered with the marketing skill and knowledge that will spur individual to participate in the market, this suggests that higher level of education provides a greater opportunity for the farmers to participate in the cattle market. This is in consistent with Girei and Omonona (2009) who shows that education have positively influenced participation as a net seller in the cattle market in Nigeria. The more the participant is educated, the better the chance of participation in the cattle market. The implication of the results is that education assists in participation in cattle market by providing information on prices and market information systems.

Market distance is negative but significantly related to the probability of participating in the cattle markets. The partial effect of the market distance on the conditional probability for participation is -0.0022, this implies that each unit increment in the market distance will decrease the probability of participation by 0.0022. Logically it makes sense since the markets are not readily available in rural areas. Thus, the results suggest that those households which can "reach" the desired marketing distance are more likely to participate mainly due to high producer margin and low variable transaction cost. This is in line with the findings of Uchezuba et al (2009) who indicates that market distance have a negative impact on the probability of the small-scale farmers marketing their animals to formal markets in south Africa.

Market information is negatively and significantly related to the probability of participating in the cattle markets. This implies that the receivers of market information are less likely to sell more cattle than non-receivers. The results indicate that a unit increase in receiving market information has the probability chances of decreases participation in the cattle markets by 0.133. This study is in line with findings Erick *et al.* (2015) who showed that, access to market information negatively affect market participation, because Market conditions are dynamic and bound to change frequently with regards to price, potential consumers' lifestyle, taste and preference change and government regulations. But contrary to the findings of Apin *et. al* 2015, Musah *et. al.,* 2014, Ohen *et. al* 2014, Omiti *et. al.,* 2009, Terfa *et. al.*, 2012, Wanyoike *et. al*., 2015, and Zamasiya et al., 2014 who reported that access to market information boosts confidence of household who are willing to participate in the market. However, this study may not be unconnected with the fact that most of cattle buyers transport them to southern and eastern part of the country. Therefore, they buy cattle every other week; hence, if a household receives information say on favorable price this week, participation in the market the following week may not replicate same since the availability and the price of cattle in southern and eastern part of the country determined the price in the north.

Seasonality was used as proxy for drought risk. Drought risk was defined as the possibility of a danger which might affect grazing, water and other related resources due to the absence of rainfall (Montshwe, 2006). High drought risk is defined by very low rainfall and the lack of natural grazing which leads to the use of alternative methods of feeding cattle during drought times. It is hypothesized that an increase in risk will lead to increased participation in the cattle markets by small-scale cattle farmers. The result indicated that the variable season was positive and significant at 1% level. The marginal effect indicated that seasonality increases the probability of participating in cattle markets.

TABLE 3
RESULTS OF THE LOGIT REGRESSION ANALYSIS FOR FACTORS INFLUENCING MARKET PROBABILITY OF PARTICIPATION

Variables	Coefficients (B)	Standard Error	Z	Significance	Marginal effect
Age	0.006	0.014	0.43	0.656	0.0012
Gender	2.008	0.374	5.37	0.000***	0.4571
Education	0.641	0.305	2.10	0.036**	0.1333
Family size	0.003	0.023	0.13	0.898	0.0005
Distance to market	0.011	0.007	-1.74	0.083*	-0.0022
Prior market information	-0.790	0.372	-2.12	0.034**	-0.1331
Season	0.700	0.269	2.60	0.009***	0.1418
Constant	-1.593	0.753	-2.12	0.034	-
Chi-square ($\chi 2$)	48.75***				
Log likelihood	-191.205				
Number of farmers	363				

Source: Field survey, 2016
****** Significant at 1%*** ***** Significant at 5%*** **** Significant at 10%***

IV. CONCLUSION AND RECOMMENDATIONS

4.1 Conclusion

The following conclusions were drawn from this study:

i. This finding concluded that cattle farmers have good chances of driving market advantage, because majority of farmers are at their productive age.

ii. Those in cattle business were predominantly male, married. Therefore good market participation will help in raising household income.

iii. Since majority of the respondents are literate they can be able to utilize information more efficiently and consequently improve their managerial skills.

iv. Variables like proximity to market and market information decrease farmers' participation in the market while gender, education, and seasonality promote market participation in the area.

v. Inadequate feed is the cause of severe constraint on cattle business in the study area, followed by inadequate water and the least problem is theft. These observed constraints may force the cattle farmers to dispose of their cattle and hence affect the productivity of their business and market commercialization.

4.2 Policy Recommendations

The following policy recommendations are made based on the findings from the study:

i. Since majority of the respondents were of moderate age,youths should be encouraged to participate in marketing activities to expand the current marketing system and to address the challenges facing agriculture in term of food insecurity and revenue generation.

ii. Cattle-marketing is male-dominated. Therefore, there is need to encourage women to participate actively in the cattle market; and also, the unmarried should be encouraged to participate as most of the participants are married.

iii. Since market information has positively influenced the probability of participation in the cattle market. Therefore, provision of reliable market information through mass media or extension services is of paramount importance in improving market participation in the study area.

iv. The study also recommends that efforts should be made to upgrade road networks in the study area so as to reduce the transaction cost and encourage cattle market participation.

v. Since the major problems encountered by cattle farmers were inadequate feed and water during dry season and problem of insecurity, there is need to provide adequate pasture land, water supply and security for the cattle farmers so as to curb the problems of theft, pilfering as well as exposure to adverse weather.

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Acclimatization of KFeFRIM01: A Superior Clone of *Labisia pumila* var. *alata*

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***Abstract*—** *Labisia pumila or commonly known as kacip fatimah is one of the popular medicinal plant in Malaysia. The constituents of this plant have been reported to possess anti cancer, antioxidant, anti-obesity and anti-inflammatory properties. The growth and production of L. pumila is greatly influenced by the environmental condition such as shade, humidity and growing media. In this study, the survivality of L. pumila var. alata (KFeFRIM01) plantlets derived from tissue culture technique using temporary immersion system were analyzed during pre and post acclimatization process. The plantlets were pre acclimatize in different potting media namely 100% sand and 100% jiffy and grown in plastic growth chamber with different percentage of shade (0%, 50% and 70%). After a month, the plants were transferred to the nursery for post acclimatization. Three experiments were conducted to evaluate the survivality of plants placed under different percentage of shade (70% and 50%), suitable growing media and watering requirement per day. The growth of the plants such as plant height, number of leaves, leaf length and leaf width were recorded during the acclimatization process. The analysis of variance (ANOVA) was conducted to evaluate the survivality and growth of this plant during the acclimatization process. KFeFRIM01 was found to grow better in 0% shade compared to 50% shade and 70% shade. However, there were no significant difference recorded in the usage of two potting medium (100% sand and 100% jiffy) on the growth of KFeFRIM01 during pre acclimatization process. While in post acclimatization, KFeFRIM01 placed under 50% shade produce better growth in term of leaves number (8.44a ± 0.20), leaf length (6.13a ± 0.14) and leaf width (3.17a ± 0.06) compared to 70% shade. KFeFRIM01 plants planted in treatment 1-top soil: leaf compost: sand (2:3:1) gave the highest growth performance in all parameter measured. Whereas, KFeFRIM01 plants that watered twice per day (9AM & 4PM) have greater plant height (8.83a ± 0.34) and leaves number (7.53a ± 0.39) compared to plants watered once per day. The findings from this study are essential for mass production of L. pumila using tissue culture technology in future as a successful protocol for acclimatization of this plants obtained.*

***Keywords*— adaptation, environmental condition, plant growth, survivality, clonal propagation.**

I. INTRODUCTION

Labisia pumila is an herbal plant from Primulaceae family. The plant usually found in the tropical forest of Asian countries. This plant favors shady area and humus rich soil (Sunarno, 2005). *Labisia pumila* is very synonym in treating women health and due to that numerous of *L. pumila* based products are sold in the market. The most common method of *L. pumila* propagation is through cuttings (Aminah et al., 2008). However, for large scale production of planting materials, tissue culture technique using temporary immersion system (RITA™) is preferable (Syafiqah et al., 2016).

The process of tissue culture technique involved surface sterilization, culture initiation, shoots multiplication, *in vitro* or *ex vitro* rooting and acclimatization. The acclimatization process is a prerequisite in many species grown *in vitro* to ensure high plant survivality and vigorous growth when transferred to soil. This is because, in lab, tissue culture plantlets were developed within culture vessels under low level of light, high humidity, sufficient sugar and nutrients for heterotrophic growth (Preece & Sutter, 1991). Therefore, a step by step of acclimatization process is required for the plantlets to prevent transfer shock.

KFeFRIM01 is the superior clone of *L. pumila* var. *alata* discovered by Farah Fazwa et al. (2012) through clonal trial study. This clone has vigorous growth in terms of plant size and leaf number as well as high in total phenolic content (TPC) which act as antioxidant properties that essential to prevent free radical damage to our body. The clone was mass produced through *in vitro* propagation using RITA™. Thus, to ensure high survivality of the plants at *ex vitro* condition, a good acclimatization practice must be considered.

To date, there is lack of study reported on the acclimatization of *L. pumila* to *ex vitro* condition. Therefore, this study was conducted with the objective to determine the suitable requirements needed by the plants during pre and post acclimatization

process in order to increase the survivality and the growth of the plants. High survivality of the plants could ensure sustainable supply of raw materials to the industry. In future, Malaysia can be one of the main exporters of high quality *L. pumila* and reduce the dependency on imported raw materials as well as from the wild.

II. MATERIAL AND METHOD

Experiment 1- Acclimatization of plant in growth chamber (Pre-acclimatization)

In vitro rooted of KFeFRIM01 plantlets were used in this study. The plantlets with the height of 2-5 cm were taken out from RITA™ and washed under running tap water to remove the traces of medium from the plantlets surface. After that, the plantlets were dipped into Thiram solution (fungicide) for few seconds to disinfect the plants. About 120 plantlets of KFeFRIM01 were planted in two different potting medium; 100% jiffy and 100% sand within three replicates. The plantlets were kept in a transparent plastic chamber (1.5 m x 1.0 m size) with different percentage of shade (0%, 50% and 70%). The plantlets were watered once per day for 30 seconds to maintain the humidity. The survival rates and growth such as stem height, number of leaves, leaf length and leaf width of the plants were recorded before and after acclimatization process. The environmental data such as temperature and humidity in each acclimatization bed were also monitored. The pre acclimatization process for FaFaF01 was conducted in a transparent plastic chamber for a month period.

Experiment 2- Acclimatization of plant in green house (Post-acclimatization)

The plants of KFeFRIM01 were subjected for three different experiments during post acclimatization. The survival rates and growth such as stem height, number of leaves, and leaf length and leaf width of the plants were recorded once per month. The post acclimatization process took about three month period.

i) Shade percentage

The plants were placed under different percentage of shade; 70% and 50%.

ii) Growing media

Three treatments of growing media containing combination of soil, compost and sand were tested for FaFaF01.

Treatment 1- Top soil: leaf compost: sand (2:3:1)

Treatment 2 - Top soil: leaf compost: cocopeat: sand (2:2:1:1)

Treatment 3 - Top soil: cocopeat: sand (2:3:1)

iii) Watering schedule

The plants were watered daily based on three types of schedule:

Schedule 1: Twice per day at 9.00 am and 4.00 pm

Schedule 2: Once per day at 9.00 am

Schedule 3: Once per day at 4.00 pm

III. RESULTS AND DISCUSSION

3.1 Effects of different potting media on the growth of KFeFRIM01 during pre acclimatization

After one month of acclimatization in transparent plastic chamber, it was observed that plantlets in 100% jiffy had 94% survival rate while 100% sand recorded 88.3% (Figure 1). In other study, Muhammad Fuad et al. (2015) reported that the usage of 100% jiffy during acclimatization of *Eurycoma longifolia* (tongkat ali) gave 100% of survival rate while 100% sand gave 67% of survival rate. The ability of 100% jiffy in retaining moisture compared to 100% sand could be the factor of high survivality. This is because during acclimatization, minimal watering is provided in order to harden the plants. In terms of growth, analysis of variance (ANOVA) shows there was significant difference between two potting media (100% sand and 100% jiffy) except for leaf length variable. Based on Figure 2, plantlets in 100% sand had higher stem height (3.76 ± 0.07 cm) and number of leaves (5.81 ± 0.11) compared to 100% jiffy. In contrast, the leaf width of KFeFRIM01 was found greater in 100% Jiffy (1.40 ± 0.03) than 100% sand. While the leaf length of KFeFRIM01 in 100% jiffy and 100% sand was 2.21 ± 0.05 cm and 2.20 ± 0.05 cm respectively. The findings from this study, suggest that 100% jiffy was the suitable potting media for *L. pumila* var. *alata* (KFeFRIM01) during pre acclimatization.

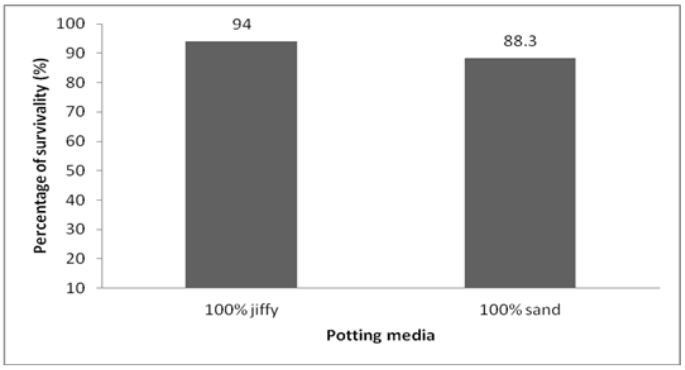

FIGURE 1: PERCENTAGE OF KFEFRIM01 SURVIVALITY IN 100% JIFFY AND 100% SAND

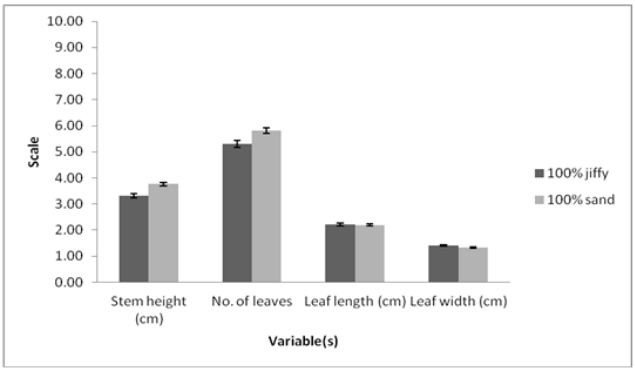

FIGURE 2: GROWTH OF KFEFRIM01 IN 100% JIFFY AND 100% SAND

3.2 Effects of different shade percentage on the growth of KFeFRIM01 during pre acclimatization

The plantlets of KFeFRIM01 were acclimatized in different percentage of shade for one month. The survivality of the plantlets was recorded 100% in 0% (no shade) followed by 50% shade (94%) and 70% shade (80%) (Figure 3). The ANOVA test was conducted to determine the effects of different shade percentage on the growth of KFeFRIM01. The result shows that there was significant difference between different percentage of shade with the growth of KFeFRIM01 (Figure 4). Plantlets of KFeFRIM01 grown in 0% (no shade) recorded the highest growth for all variables measured followed by 50% shade and 70% shade. The findings revealed that *L. pumila* var. *alata* (KFeFRIM01) can be acclimatized in transparent plastic chamber without shade (0%) and increasing the percentage of shade from 50% to 70% may contribute to mortality effect and slow gowth. This finding is in line with Ginting et al. (2015) where the higher the percentage of shades, the lower the number of tiller in upland rice plants.

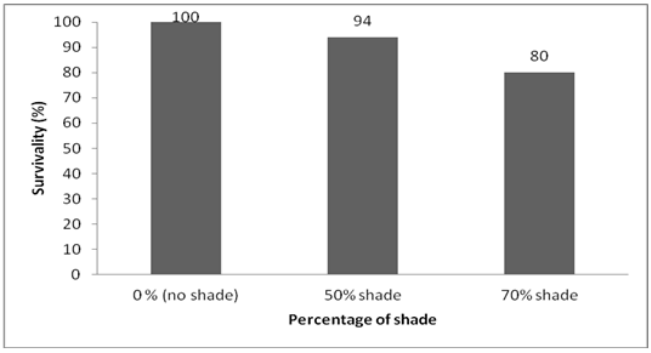

FIGURE 3: PERCENTAGE OF KFEFRIM01 SURVIVALITY IN DIFFERENT PERCENTAGE OF SHADE

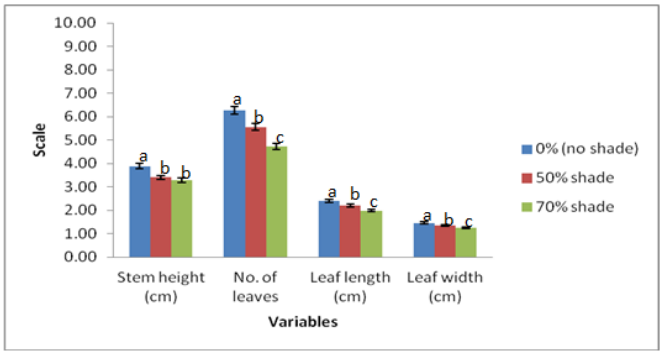

FIGURE 4: GROWTH OF KFEFRIM01 IN DIFFERENT PERCENTAGE OF SHADE

3.3 Effects of different shade percentage on the growth of KFeFRIM01 during post acclimatization stage.

During post acclimatization, the plants of KFeFRIM01 were placed at two different shade houses with 50% and 70% shade for three months. The growth performances of the plantlets were monitored monthly and presented in Table 1.

TABLE 1
EFFECTS OF DIFFERENT SHADE PERCENTAGE ON THE GROWTH OF KFEFRIM01 DURING POST ACCLIMATIZATION STAGE.

Treatment	Parameter				
	Plant height (cm)	No of leaves	Leaf length (cm)	Leaf width (cm)	Collar diameter (mm)
50 % shade	5.04b ± 0.13	8.44a ± 0.20	6.13a ± 0.14	3.17a ± 0.06	2.63b ± 0.07
70 % shade	5.51a ± 0.20	6.87b ± 0.33	5.15b ± 0.24	2.64 b ± 0.12	2.88a ± 0.07

Means with the same letters are not significantly different at 0.05 level of confidence

Based on ANOVA, the growth of KFeFRIM01 under different percentage of shade is significant at $p<0.05$. The plants placed under 50% shade produce better growth in term of leaves number (8.44a ± 0.20), leaf length (6.13a ± 0.14) and leaf width (3.17a ± 0.06) compared to 70% shade. However, plants placed under 70% shade had greater plant height (5.51a ± 0.20) and collar diameter (2.88a ± 0.07) than 50% shade. The difference in results may relate to the photosynthetically active radiation (PAR) that vary between the two shade used. Similar results were reported for red and blue colour shading net, which cause reduction in the amount of incident radiation and affecting the induction of multi-shoot plants of torch ginger (Rodrigues et al. (2015)). *L. pumila*, the leaves production is more important than other parameter since leaves contribute to the high biomass production. Therefore, it is suggested that the plants of *L. pumila* were placed under 50% shade for high production of leaves and biomass.

3.4 Effects of different growing media on the growth of KFeFRIM01.

The plants of KFeFRIM01 were transferred from jiffy to three different growing media during the post acclimatization period. Based on ANOVA, there is significant different in the growth of KFeFRM01 planted at different growing media at $p<0.05$. Plants planted in treatment 1-top soil: leaf compost: sand (2:3:1) gave higher growth performance in all parameter measured followed by treatment 2-top soil: leaf compost: cocopeat: sand (2:2:1:1) and treatment 3-top soil: cocopeat: sand (2:3:1) (Table 2). The combination of leaf compost into substrate in treatment 1 may influence the growth performances of *L. pumila.* According to Roy (1914), leaf compost is not normally considered as fertilizer as it is too low in nutrient content. However, it serves primarily as an organic amendment and a soil conditioner that improves the physical, chemical, and biological properties of soils.

TABLE 2
EFFECTS OF DIFFERENT GROWING MEDIA ON THE GROWTH OF KFEFRIM01.

Treatment	Parameter				
	Plant height (cm)	No of leaves	Leaf length (cm)	Leaf width (cm)	Collar diameter (mm)
T1- Top soil: leaf compost: sand (2:3:1)	6.58a ± 0.28	8.20a ± 0.39	10.0a ± 0.27	4.80a ± 0.13	3.47a ± 0.13
T2- Top soil: leaf compost: cocopeat: sand (2:2:1:1)	6.60a ± 0.29	7.13ab ± 0.33	9.84a ± 0.42	4.58a ± 0.19	3.17a ± 0.09
T3- Top soil: cocopeat: sand (2:3:1)	5.29b ± 0.16	6.60b ± 0.42	7.06b ± 0.22	3.50b ± 0.10	2.79b ± 0.10

Means with the same letters are not significantly different at 0.05 level of confidence

3.5 Effects of different watering schedule on the growth of KFeFRIM01

Based on ANOVA, the effects of different watering schedule were significant at $p<0.05$ for parameters; leaves number and leaf length only. Plants that watered twice per day (9AM & 4PM) have greater plant height (8.83a ± 0.34) and leaves number (7.53a ± 0.39) compared to plantlets watered once per day. Whereas, plants watered at 9AM daily had greater growth of leaf length (12.06a ± 0.38), leaf width (5.76a ± 0.16) and collar diameter (4.39a ± 0.85) compared to plants watered at 4PM and 9AM & 4PM. The plants that watered once per day may result in drought stress and disturb the growth of the plants as reported by Jaleel et al. (2008).

TABLE 3
EFFECTS OF DIFFERENT WATERING SCHEDULE ON THE GROWTH OF KFEFRIM01.

Watering schedule	Parameter				
	Plant height (cm)	No of leaves	Leaf length (cm)	Leaf width (cm)	Collar diameter (mm)
9AM, 4PM	8.83a ± 0.34	7.53a ± 0.39	11.70a ± 0.34	5.67a ± 0.16	4.11ab ± 0.16
9AM	8.65ab ± 0.42	7.40a ± 0.45	12.06a ± 0.38	5.76a ± 0.16	4.39a ± 0.85
4PM	7.60b ± 0.39	5.97b ± 0.40	10.37b ± 0.52	5.13a ± 0.29	3.90b ± 0.19

Means with the same letters are not significantly different at 0.05 level of confidence

IV. CONCLUSION

During the acclimatization process, the percentage of shade, type of substrate and water regime can significantly affect the growth, development and morphogenesis of *L. pumila*. Therefore, the finding from this study can be the reference for the key players to ensure high survivality of the plants during acclimatization stages.

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Hiperparasitism on mycotoxigenic fungus *Aspergillusochraceus* G. Wilh. By *Cladosporiumcladosporioides* (Fresen) de Vries

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***Abstract*—** *Biocontrol agents (BCAs) for the control of fungal diseases and pests has been the focus in the development of, thus, the agrochemical industry has refocused priorities on integrated control to include the use of BCAs in research and programmes for disease and pest control. The mechanism of action has often included direct antagonism between the BCA and fungal pathogen, competitive exclusion of the pathogen by niche occupation, production of secondary metabolites, hyperparasitism, and the production of volatile organic compounds (VOCs). It is considered as a characteristic of antagonist, to present more than one mechanism, because in this way, their chances of success are increased. The present study, objectived to evaluate, in vitro, competition between the fungus considered a bioprotective agent of the coffee quality Cladosporiumcladosporioides (Fresen) de Vries and the potentially toxigenic fungus Aspergillus ochraceus G. Wilh. Competition between isolates of C. cladosporioides and A. ochraceus was evaluated in vitro conditions by adding conidia aliquotas in Petri dishes containing PDA (potato, dextrose, ágar) culture medium.The antagonist relationship between isolates was observed in Olympus SZX7TR-ILA trinocular stereoscopic microscope coupled to a camera and in scanning electron microscopy ZEISS®. The fungus C. cladosporioides (Fresen) de Vries, showed its antagonism on potencialmycotoxigenic fungus A. ochraceus by of antibiosis and hyperparasitism mechanisms.*

***Keywords*— Biocontrol, sustainability, food safe.**

I. INTRODUCTION

In the coffee crop, favorable conditions for the development of fungi can contribute to the depreciation of the beverage, due to the taste produced by the fermentations, besides compromising the safety by the production of mycotoxins, being the main mycotoxigenicfungal genera Aspergillus, Penicillium and Fusarium, contaminants coffee plantations[1].OchratoxinA (OTA) is a naturally occurring foodborne mycotoxin found in a wide variety of agricultural commodities worldwide, ranging from cereal grains to dried fruits to wine and coffee. It is produced by several different fungi including *Aspergillus ochraceus*, *A. carbonarius*, *A. niger* and *Penicilliumverrucosum*[2].

Biocontrol agents (BCAs) for the control of fungal diseases and pests has been the focus in the development of, thus, the agrochemical industry has refocused priorities on integrated control to include the use of BCAs in research and programmes for disease and pest control. The mechanism of action has often included direct antagonism between the BCA and fungal pathogen, competitive exclusion of the pathogen by niche occupation, production of secondary metabolites, hyperparasitism, and the production of volatile organic compounds (VOCs)[3]. It is considered as a characteristic of antagonist, to present more than one mechanism, because in this way, their chances of success are increased[4].

Based on the antagonistic interactions and in the several studies on coffee quality, the fungus *Cladosporiumcladosporioides* (Fresen) de Vries can be considered as antagonistic agent against harmful fungi to quality beverage[5].Studies show that the microorganism acts through the mechanisms of competition antibiosis and parasitism, due to the great capacity for natural adaptation, as well as the rapid capacity to colonize the substrate[6]. In the antibiosis process, the production of cladosporol by the fungus *Cladosporium sp.* is responsible for the inhibition of the development of insects and other fungi species, and its action as a parasite has been shown to promote hyperparasitism in some fungi species[7].

The present study, objective to evaluate, *in vitro*, competition between the funguses considered a bioprotective agent of the coffee quality *Cladosporiumcladosporioides* (Fresen) de Vriesandthe potentially toxigenic fungus *Aspergillus ochraceus* G. Wilh.

II. MATERIAL AND METHODS

The tests were carried out at the Laboratory of Phytopathology and Microbiology of EPAMIG Sul in Lavras-MG and in the Laboratory of Electron Microscopy and Ultra-structural Analysis (LME / UFLA), according to the methodology proposed

by[8]. The isolates of the fungi *Cladosporiumcladosporioides* (Fresen) de Vries and *Aspergillus ochraceus* G. Wilhtested were already identified and deposited in the collection of microorganisms culture of the Department of Food Science / UFLA.

In each Petri dish containing a purified colony of each isolate, 40mL of distilled and sterilized water was added. Then the mycelium was scraped to obtain a suspension which was subsequently filtered with the aid of a sterile gauze to obtain only the spores. After filtration, an30µL aliquot of spore solution was transferred by means of an automatic pipette to Petri dishes of 6cm in diameter containing PDA (potato, dextrose, ágar) culture medium, the solution being spread by Drigalsky's handle.

After inoculation, the plates were maintained in BOD at 25 °C with a 12-hour photoperiod, and the spore germination behavior was observed by an Olympus SZX7TR-ILA trinocular stereoscopic microscope coupled to a camera and by scanning electron microscopy Zeiss® with competition observed at 3, 10 and 20 days. The fungus *C. cladosporioides* (Fresen) de Vries, showed its antagonism on potencialmycotoxigenic fungus *A. ochraceus*by of antibiosis and hyperparasitism mechanisms.

III. RESULTS AND DISCUSSION

At 20 days, the deformation of some *Aspergillus ochraceus* G. Wilh spores was observed under the scanning optical microscope in addition to the total inhibition of germination due to the presence of *Cladosporiumcladosporioides* (Fresen) de Vries, in contrast to the treatment inoculated only with fungus *A. ochraceus* G. Wilh.

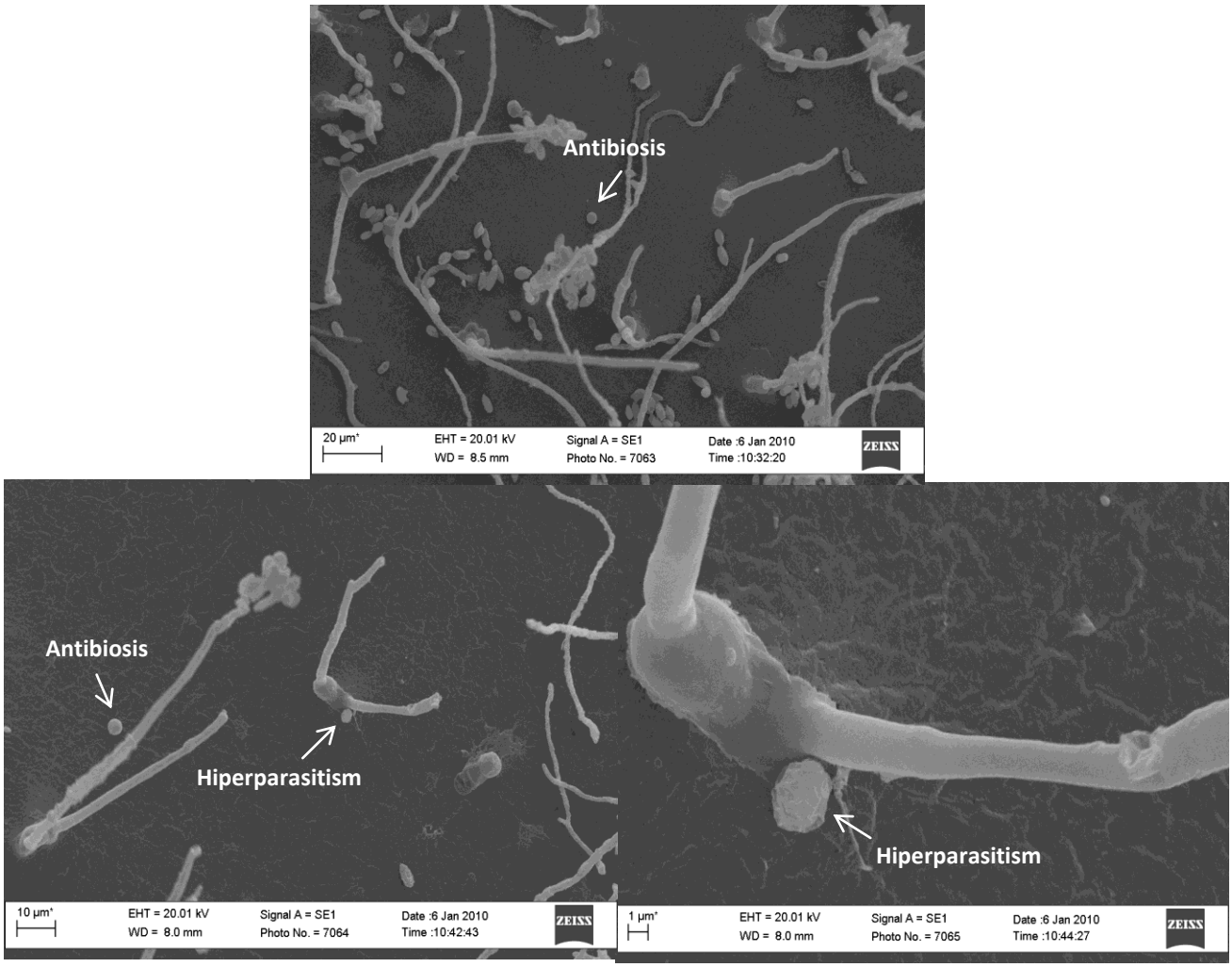

FIGURE.1: *In vitro*hyperparasitism between *C. cladosporioides* (Fresen) de Vries and *A. ochraceus*G. Wilh with 20 days of competition. (A) Structure of *C. cladosporioides* (Fresen) de Vries formed and conidia of *A. ochraceus* G. Wilh (B) Hyperparasitism of *C. cladosporioides* (Fresen) de Vries on conidia of *A. ochraceus* G. Wilh. (C) Detail of the degradation of the conidium of *A. ochraceus* G. Wilh.

The Fig. 1 demonstrates the hyperparasitism exerted by *Cladosporiumcladosporioides* (Fresen) de Vries on *Aspergillus ochraceus* G. Wilh. with 20 days of *in vitro*competition.

In the Fig. 1A, it can be observed that the structure of the *C. cladosporioides*(Fresen) de Vries is fully formed, and whereas the fungus *A. ochraceus* G. Wilhalthough presented its circular shape and smooth texture but doesn't germinated by the antibiosis effect of the *C. cladosporioides.*

Figure 1B shows hiperparasitism effect by differences in size and texture between the conidia of *A. ochraceus* G. Wilh, which was in contact with the hypha of *C. cladosporioides* (Fresen) de Vries and conidia without contact with the hypha.

In the detail of figure 1C, it is possible to better understand that the hypha of *C. cladosporioides*(Fresen) de Vries has a point of contact with the conidium of *Aspergillus ochraceus* G. Wilh, probably involving the production of extracellular lytic enzymes responsible for cellular membrane degradation and consequent disruption of the microbial cell. The pathogen suppression of several plants worldwide is due to the occurrence of naturally occurring hyperparasites[9], demonstrating appressory formation and penetration into the host by means of mechanical force[10] or enzymatic breakdown[11].

IV. CONCLUSION

The fungus *C. cladosporioides* (Fresen) de Vries, showed its antagonism on potencialmycotoxigenic fungus *A. ochraceus* by of antibiosis and hyperparasitism mechanisms

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Bacterial indicators and antibiotic resistance of *Escherichia coli* in groundwater

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Abstract— *The aim of this research was to investigate the groundwater quality in El Barreal basin (Córdoba, Argentina), through bacteriological analysis and antibiotic resistance of fecal bacteria indicators and their relationship with geochemical and land uses characteristics. Groundwater samples were collected in 36 wells and the following parameters were determined: major chemical components, heterotrophic plate counts, total and fecal coliforms, Escherichia coli and Pseudomonas aeruginosa. Antibiotic resistance of E. coli was analyzed using standard methods. The chemical and bacteriological analysis showed that more than 80 % of samples were unfit for human consumption. Bacteriological contamination was significant in 36 % of samples. The multivariate analysis between bacterial and geochemical variables explained local contamination conditions, evidenced by the arrival of bacteria and some typical indicators (NO_3^-, Cl^- y HCO_3^-) to groundwater. No significant correlation between NO_3^- and bacterial counts was observed, which let us to interpret that part of NO_3^- contents can be supplied by inorganic fertilizers. Total and fecal coliforms were linked to a local increase of Cl^- and HCO_3^- and a decrease of pH and dissolved oxygen, indicating the arrival and degradation of organic matter into groundwater. The resistance pattern of total E. coli isolates (n=12) showed that the highest percentages were observed for antibiotics of animal use (ampicillin, tetracycline and cephalothin). The analysis of results revealed the impact of land uses demonstrating that livestock activities are the main punctual contaminant sources in this sedimentary aquifer.*

Keywords— antibiotics, bacterial contamination, hydrogeochemistry, land uses.

I. INTRODUCTION

Groundwater importance lies on its ability to act as a large reservoir of freshwater that provides "buffer storage" during periods of drought [1]. As was stated by the mentioned authors, regardless of its importance, groundwater is often misused, usually poorly understood and rarely well managed. In recent decades there has been increasing concern about the final destination of numerous chemical and biological pollutants (pathogenic bacteria, pesticides, fertilizers, industrial by-products and pharmaceuticals) that have a strong impact on water resources, including groundwater. Specifically in agricultural ecosystems there are potential polluting activities like pesticide and fertilizer uses, dairy farms and concentrated animal operations (CAFOs), especially for cows, pigs and poultry. *Escherichia coli* is the best indicator of fecal contamination, however the presence in the water does not provide definitive information about its possible origin [2]. This is why phenotypic methods, such as antibiotic resistance profiles of *E. coli* have been used as a tool to elucidate the origin of the contamination in various environments [3]. In rural areas, antibiotics are used in veterinary medicine in a prophylactic way (antiparasites and antibiotics) or as growth promoters (used in subtherapeutic doses), generating selective pressure in indicators of fecal contamination. The use of these compounds, coupled with the increase of animal husbandry in increasingly smaller areas, makes the contamination in rural areas considerably increasing [4].

The groundwater of the South of the Cordoba province (Argentina), almost entirely located in sedimentary aquifers, have been studied for different contamination problems [5]. The need to measure pollution indicators in groundwater is increasingly evident as they are useful for assessing the relationship between environmental variables and the causes and consequences of environmental changes. In this way, it is possible to contribute to the improvement of water resources and environment management. In this context, and taking into account as the main hypothesis that the intensive animal breeding affects in a punctual and more concentrated way the unconfined aquifer, the objective of this research was to carry out an integral study to evaluate the groundwater quality in the Barreal basin (Córdoba, Argentina). Bacteriological parameters and antibiotic resistance of bacteria indicative of fecal contamination were analyzed and linked to hydrogeological, hydrochemical and land use characteristics.

II. MAIN CHARACTERISTICS OF THE STUDY AREA

The Barreal basin (100 km^2) is located in La Cruz-Gigena valley, between Comechingones Mountains and Las Peñas Mountains (Córdoba, Argentina). According to Matteoda [6] the geomorphological units and the lithological characteristics of the sediments, have conditioned the dynamics and quality of surface and groundwater. The unconfined aquifer, 10 to 80 m thick, is lithologically heterogeneous, constituted by loessical sediments (very fine sand and silts), that intercalate with paleochannels (sands and gravels of high permeability). The depth of the water table is very variable, between 0 and 40 m depth, conditioned mainly by the relief. This aspect and the lithology of the unsaturated zone control the probable arrival of contaminants to the aquifer and its transport, transformation and destination [6]. The climate is dry-subhumid with little to no excess water, with an average annual precipitation of 753 mm, distributed in two noticeable periods, being the wetter from November to March (70% of the total annual precipitation). The basin is a predominantly rural area in which groundwater is used for human consumption, livestock, irrigation and industrial activities. The main activity is agriculture (soybean, maize and wheat crops) while extensive stockbreeding as well as in CAFOs, is practiced. Taking into account the observed land uses, their spatial distribution and the main features (quantity of animals, effluent lagoon, permanence, etc.), the pig feedlots appears as one of the most important threats to the aquifer contamination.

III. MATERIALS AND METHODS

During the field work, land uses were surveyed and 36 water wells were sampled. The collection of groundwater samples was carried out during the wet season (December and February 2008-2009).The following parameters were measured in situ: temperature (T, digital thermometer, in ºC), electrical conductivity (EC, Hanna conductivity meter, in mS/cm) and dissolved oxygen (DO, Orion selective ion electrode, in mg/L). At each sampling site, 1 L of water was collected in clean plastic bottles for physico-chemical analysis and 500 mL in sterile containers for bacteriological analysis. The latter were kept at 4 ° C until reaching the laboratory and the determinations were performed within 24 h post-collection. The sampling activities were carried out according to techniques standardized by APHA [7]. It is important to point that each sample was taken after emptying 3 times each well to ensure the groundwater sample representativeness.

In relation to the bacteriological analysis, the determination of heterotrophic plate counts (HPC) was carried in plate count agar, incubated at 35 ºC during 24 h. The total (TC) and thermotolerant coliforms (fecal coliforms, FC) were determined through the multiple-tube fermentation (MTF) technique. Probability tables (McCrady tables) were used to determine the Most Probable Number (MPN) and to estimate the number of coliform organisms per 100 ml of water. The TC were incubated in Mac Conkey broth at 35 ºC during 24-48 h and FC in BRILA broth (Brilliant Green Bile Lactose 2% broth) at 44.5 ºC during 24 h. The presence of *E. coli* was determined in 100 ml of sample in Mac Conkey broth incubated at 35 ºC during 24-48 h. Then, an aliquot was spread onto EMB agar plates and incubated at 35°C for 24 h. Isolates were confirmed as *E. coli* by using a series of biochemical tests, including indole, Voges-Proskauer, methyl red tests and the inability to grow on citrate agar (IMViC). The presence of *Pseudomonas aeruginosa* was determined on a volume of 100 ml of sample in Asparagine broth incubated at 35 ºC during 24 - 48 h. The isolation was carried out in Cetrimide agar plates and colonies were confirmed by the following biochemical tests: oxidase, growth at 42ºC, and pigments production in agar P and F. The methodology was carried out according to Standard Methods for Examination of Water and Wastewater [7] and Argentine Food Code (AFC) [8].

After the general bacteriological analysis, the isolated and identified strains of *E. coli* were evaluated for resistance to antibiotics by the plaque diffusion method using 6 antibiotic disks [9, 10]. They correspond to the drugs most commonly used in the treatment of infections caused by Gram-negative bacilli in both humans and animals and on their use as a food additive and as growth promoters in animals. An *E. coli* inoculum was prepared in Tripticasa Soya broth of approximately $2x10^8$ cfu/ml whose turbidity corresponds to tube Number 0.5 of the McFarland scale. Then, 200 µl of this cultivation was placed in 5 ml of sterile physiological solution and the optical density (600 nm) of each mixture was adjusted to about 0.08. The bacterial suspension was inoculated onto plates with 150 mm of Mueller Hinton agar and then the commercial antibiotic discs were placed. The plates were incubated at 35 °C for 18 to 20 h. Diameters (in millimeters) of the clear areas of growth inhibition around each antibiotic disk were measured with a precision caliber. The criterion of sensitivity or resistance to each antimicrobial was determined as established by CLSI (Clinical and Laboratory Standard Institute) [11]. *E. coli* strain ATCC 25922 was used as a control.

The physico-chemical analysis included the determination of the ions HCO_3^-, $SO_4^=$, Cl^-, Na^+, K^+, Ca^{++}, Mg^{++} and NO_3^- which were analyzed following APHA [7] standard techniques. Finally, the statistical analysis was made using the SSPS v.11.5 package. The multivariate analysis was performed using the factorial method by principal components (PC) to determine

possible relationships between bacterial contents (TC and FC) and physico-chemical variables. The variable TDS (total dissolved salts) was not considered in the multivariate analysis, because it is represented by the EC.

IV. RESULTS AND DISCUSSION

The physical-chemical results indicate that 22 % of the samples were not suitable for human consumption, due to nitrate values and/or total salts established in AFC, although Matteoda et al. [6] indicate that 89% is not apt if Arsenic and Fluorine values are considered. The EC values, which explain the water salinity, were between 670 and 5.830 µS/cm. Table 1 shows the statistics of the different chemical components in groundwater. The geochemical composition allowed defining six geochemical groups of groundwater: sodium bicarbonate (38.8%), sodium sulphate (27.7%), sodium bicarbonate-sulphate (13.8%), sodium chloride-sulphate (5.5%) and calcium bicarbonate (2.7%). They are close related to the geomorphological environments in the studied basin [6].

TABLE 1
STATISTICS OF PHYSICOCHEMICAL PARAMETERS IN GROUNDWATER (mg/L, EC in µS/cm and T in ^{0}C)

Parameters	Minimum	Maximum	Average	Typical deviation
DO	2	11	7.90	1.76
pH	7.49	8.80	8.12	0.39
T	18.00	22.30	20.25	1.13
EC	670.00	5830.00	1847.86	1152.08
TDS	469.00	4081.00	1293.51	806.45
$CO_3^=$	0.00	21.80	4.03	6.77
HCO_3^-	207.50	1182.50	434.51	179.15
$SO_4^=$	19.30	2565.90	454.94	480.15
Cl^-	8.60	1028.60	86.03	175.39
Na^+	36.40	1071.80	349.04	228.50
K^+	6.20	49.20	15.53	8.72
Ca^{++}	2.80	354.40	60.60	84.38
Mg^{++}	0.10	182.40	18.60	30.60
NO_3^-	0.00	297.00	33.35	51.94

TABLE 2
BACTERIOLOGICAL GROUNDWATER QUALITY

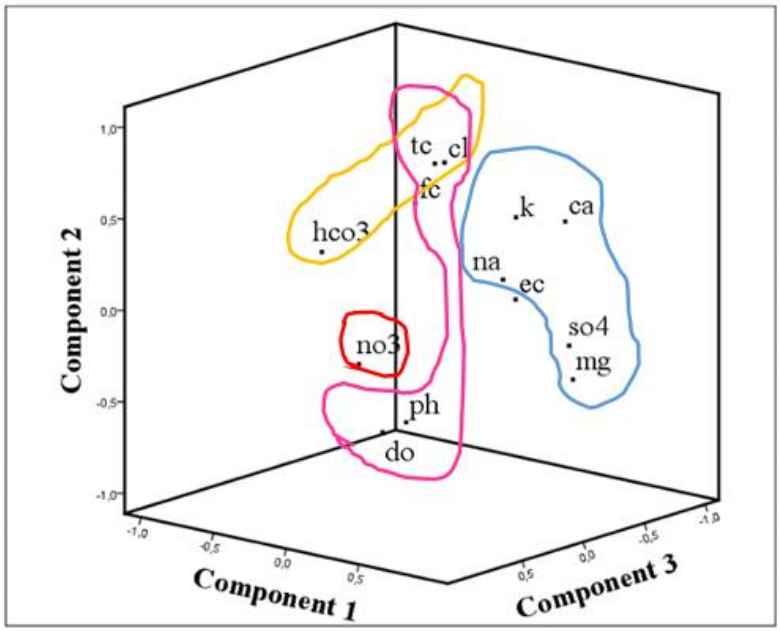
	Bacteriological parameters	Limits according to AFC	Min-Max	Samples numbers above the limit of AFC (%)
Samples (n=36)	HPC (cfu/mL)	No > 500	$0 – 2x10^4$	14 (38 %)
	TC (MPN/100 mL)	≤3	$0 – 5x10^4$	30 (83 %)
	E. coli	Absence	-	9 (25 %)
	Ps. aeruginosa	Absence	-	8 (22 %)

HPC: heterotrophic plate* counts, *TC: total coliforms, AFC: Argentine Food Code

The bacteriological analysis revealed that 83% of the samples were not suitable for human consumption according to AFC (2012) (Table 2). FC counts were observed in 5 samples (14%) and the highest value was $1x10^3$ MPN/100 mL. From the total samples, 13 (36%) were the most contaminated (SC3, SC4, B8b, B11, B15, B23, B39, B42, B43, B44, B45, B46, B63), showing TC above 4 MPN/100 mL, in some cases FC above 9 MPN/100 mL and presence of *E. coli*. These results highlight the vulnerability of the aquifer in some places, where the water table is closer to the land surface or the sediments of the unsaturated zone have high hydraulic conductivity, situations that allow the rapid transport of contaminants towards the aquifer. The high percentages of samples with a high TC count, as well as the detection of fecal contamination bacteria (fecal coliforms and *E. coli*), reveal the probable existence of pathogenic microorganisms in groundwater since the transmission of pathogenic strains of *E. coli* by means of drinking water is well documented [12].

The counts of TC, FC and presence of *E. coli* were compared with land use in each well site. For this purpose, the wells were categorized according the surrounding land uses: those adjacent to livestock activity and/or presence of septic wells (punctual contamination, PC) and those with only surrounding agricultural activities (diffuse pollution, DC). The results showed that in

the PC wells (especially surrounded by porcine and bovine pens) the TC and FC bacteria counts were highest, as well as the presence of *E. coli*. On the other hand, in DC wells, none of the indicators of fecal contamination were observed and TC counts were only found in few wells. The relationships between land uses and bacterial counts showed that the major bacterial contamination was related to punctual contamination where the higher contaminant load has allowed the bacteria to avoid the different processes of attenuation in the unsaturated zone, occurring then the percolation of contaminated water to the aquifer.



	Component			
	1	**2**	**3**	**4**
DO	-,292	-,644	-,327	,105
TC	,267	,573	,594	-,181
FC	-,055	,630	,082	,153
pH	,131	-,714	,090	,072
EC	,906	,035	,359	,126
HCO_3^-	-,053	-,007	,893	,119
$SO_4^=$	,941	-,127	-,090	-,047
Cl^-	,377	,571	,656	-,170
Na^+	,844	,042	,490	-,011
K^+	,743	,425	,374	-,094
Ca^{++}	,805	,486	,056	-,220
Mg^{++}	,838	-,175	-,394	,077
NO_3^-	,008	-,005	,020	,963

FIGURE 1: THE PRINCIPAL COMPONENTS OBTAINED FOR THE BACTERIAL AND PHYSICO-CHEMICAL VARIABLES IN THE EL BARREAL BASIN. STANDARDIZATION WITH KAISER

In relation to geochemical and bacteriological indicators relationships, it may be highlighted that the linear correlations between TC and FC vs. nitrate values were statistically non-significant (r = -0.105 and r = -0.28). The multivariate statistical analysis (Table 3 and Figure 1) showed that 81.4% of the total variance is explained by four principal components (PC) with the following constitution: The first PC, which explains 43.8% of the total variance, is formed by EC, SO_4^-, Na^+, K^+, Ca^{2+} and Mg^{2+} and it explains the mineralization of water. Specifically, it includes the variables that define the natural background of the physico-chemical groundwater composition, mainly influenced by the relief, lithology and hydrodynamic factors of the basin that control the mineral weathering and the passage of solutes to the solution [6]. The others PC, which account for a low proportion of the total variance, represent more local pollution processes. The second PC (20.1% of total variance), is formed by 2 subgroups that show negative correlation, DO and pH opposed to FC, TC and Cl^-. This component allows us to interpret that low DO and pH values, good indicators of contamination from organic matter, which consume oxygen and acidify the groundwater, are correlated with high bacteria and Cl^- values. The latter is a conservative solute in groundwater and an adequate indicator of contamination derived from urine in livestock scenarios. The third PC, which represents only 10% of the total variance, is formed by HCO_3^- and Cl^-, weakly associated with TC. It may be interpreted that this component represents local contamination situations. The increase of HCO_3^- is an indicator of the incorporation of CO_2 by biological activity and / or by the arrival of organic matter derived, for example, from excreta. In the regional aquifer, which shows an oxidizing state [5] the organic matter degrades to CO_2 and H_2O, lowering the pH. The fourth PC (7.3% of variance) was expressed only by NO_3^-. This is a good indicator of contamination since, if they found values are higher than the "natural regional background" (generally less than 10 mg/L, [13]), there is an evidence of the contamination arrival to the aquifer. NO_3^- values may be derived from livestock and human fecal residues, organic and inorganic fertilizers, among others. In this study, it was observed that the samples presented a wide range of nitrate values, surpassing the range of the natural regional background. However, when NO_3^- was related to the TC and FC variables, no correlation was observed between these indicators, which indicates that the detection of bacteria does not necessarily explain the presence of high NO_3^- values, and that this compound may has inorganic origin, for example fertilizers, as Piccone et al. [14] state. In the regional

agroecosystems, Giuliano Albo et al. [15] traced the NO_3^- origin using isotopic tools, defining that both agriculture and livestock sources can contaminate aquifers with NO_3^- although the higher values are related to cattle.

Taking into consideration the pattern of antibiotic resistance, we evaluated a total of 12 *E. coli* strains which were isolated from 9 wells. All isolates were resistant to one or more antibiotics. 25% was resistant for AMP and the unique strain resistant to TET was that isolated from the B8b well, located downstream of a pig farm in which the use of tetracycline was detected. In addition, another *E. coli* strain was isolated from the lagoon located in this pig CAFO which also showed resistance to TET. Regarding the rest of the antibiotics analyzed, 17% showed resistance for CEF, while 100%, 92% and 92% respectively, were sensitive for CIP, AMC and CHL (Table 4). The pattern of antibiotic resistance showed that the highest percentages of resistance were observed for AMP, results that coincide with those of Sapkota et al. [16]. Ampicillin is an approved antibiotic by FDA (Food and Drug Administration) and is used in pigs as a therapeutic agent. TET is the most commonly used antibiotic in veterinary medicine and is also administered as a feed additive in a non-therapeutic form as a growth promoter [17]. Interestingly, results published by Fluckey et al. [18] showed that oral TET reduced susceptibility to AMP when they studied resistance to antibiotics in *E. coli* isolated from poultry and pigs. On the other hand, the percentages of resistance for cephalothin observed in this research were relevant. All these aspects should be highlighted, since few authors have investigated the presence of resistant bacteria in groundwater [19].

TABLE 4
TESTED ANTIBIOTICS AND RESISTANCE PATTERNS

Antibiotics (abbreviation)	[a]Use		Disk concentration (µg)	Resistance pattern of *E. coli* (n=12)		
	Human	Veterinary		R	% I	S
Ampicillin (AMP)	++	++	10	25	50	25
Tetracycline (TET)	+	++	30	8	8	84
Cephalotine (CEF)	++	+	30	17	17	66
Ciprofloxacin (CIP)	++	-	5	0	0	100
Amoxicillin + clavulanic acid (AMC)	++	-	10	0	8	92
Chloramphenicol (CHL)	++	-	30	0	8	92

[a] Frequencies of use of antibiotics in human or animal medicine for Argentina are based on data provided by Laplumé (2011) report published by the X Argentine Congress of the Argentine Society of Infectious Diseases: not used (-), little used (+/-), used (+) and widely used (++).
R: resistance, I: intermediate, S: sensitive

V. CONCLUSION

The relationship between bacteriological indicators and physical-chemical parameters with hydrogeological characteristics and land uses shows that the physical and chemical composition of groundwater is partially modified by human activities, which also induce the arrival of bacteria. This research shows that a high percentage of groundwater samples in the studied basin is unfit for human consumption. The results demonstrate the arrival of different pollutants to the groundwater, including some typical contamination tracers such as NO_3^-, which show values above the regional natural background. Also, high Cl^- values were associated with bacteria, which allow to link pollution to livestock activity. The slight acidification of the medium and increase of HCO_3^- associated to coliform bacteria in sectors of livestock activity also shows the local impact of fecal residues.

The presence of indicator bacteria of fecal contamination which are resistant to ampicillin, tetracycline and cephalothin antibiotics confirmed that the bacterial contamination in water comes almost exclusively from animal waste. The bacteria arrival to the aquifer is highly variable depending on the contamination scenario and the hydrogeological characteristics of each sector of the basin, higher in areas where the water table is near the land surface.

From what has been explained, it is considered that livestock activity is the main source of punctual pollution in the sedimentary aquifer. However, there is a general diffuse chemical contamination, fundamentally represented by the varied NO_3^- values in the whole basin, which may be fundamentally associated to the extended use of fertilizers.

The isolation of *E. coli* resistant to antibiotics in the unconfined aquifer of the Barreal basin represents an important contribution in the study of antibiotic resistance, since the selection, dissemination and persistence of resistant bacteria is an increasing problem that limits the effectiveness of antibiotic therapy.

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Change of Peptides and Free -Amino Acids Contents during Nanjing Dry-Cured Duck Processing

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***Abstract*—** *In order to explore the relationship between the change of peptides and free-amino acid (FAA) and its unique flavour, Dry-cured duck samples of different processing phases were used to study the change of free-amino acid by High Performance Liquid Chromatography (HPLC) in this paper, meanwhile the trichloroacetic acid precipitation method for modeling use to establish the quantitative predicated peptides. The changes of small peptides and free amino acids in the process were studied. The results showed that the level and amount of proteolysis increased with the processing time at traditional technology, meanwhile the amount of peptides were positively correlated with FAA contents (R^2=0.86).*

***Keywords*— dry-cured duck, Free-amino acids, proteolysis, Peptides.**

I. INTRODUCTION

Special flavor of cured meat product depended on special processing technology of different countries and regions, Monica Flores [1] and other scientists studied the flavor and sensory descriptions of correlation of Serrano ham of amino acids, peptides etc by HPLC and capillary electrophoresis and sensory analysis method. the flavor material with proportional combination could produce special dry pickled flavor characteristics. Ya-jun zhang etc[2] studied the relationship between protein degradation products and ham quality in jinhua ham. The analysis showed that the essential amino acid composition of proteins in jinhua ham made were more consistent with recommendation model and produced a large number of low molecular weight proteins and peptides after processing. The Nanjing dry-cured duck was a kind of traditional Chinese meat product, which was popular with consumers. However, the study on the mechanism of protein degradation in the processing of protein has not been reported.

Based on the different stages of processing in Nanjing dry-cured duck as the research object, the change of small peptides and free amino acid were determined during product process by HPLC, which provided theoretical basis for the modern control technology for Nanjing dry-cured duck.

II. MATERIAL AND METHOD

2.1 Experimental Materials

The thigh muscle sample during seven control critical points of manufacturing process (raw material, dry salting, wet salted, fold, dried 5 d, 10 d dry, dry 15 d) were removed as test materials, three animals were selected from every process control point respectively, transferred to lab and stored at -80^{o}C for analysis. Samples provided by the Nanjing Rurun Group Jin Furun Food Co., LTD.

2.2 Reagents and Instruments

2.2.1 Reagents

acetonitrile, trifluoroacetic acid, pure chromatographic; 2 mercaptoethanol, sodium dodecyl sulfate, Tris, disodium hydrogen phosphate, sodium dihydrogen phosphate etc, analytical pure;acrylamide (Shanghai colorful), methylene double acrylamide (Promega), TEMED (Promega), ammonium persulfate (Shanghai colorful), the molecular weight protein Marker (Promega).

2.2.2 Experimental Instruments

High speed dispersion (ultra-turrax T25), high-speed centrifuge (Beckman allegra 64R), 10kDa ultrafiltration membrane, high performance liquid chromatograph (Agilent1100), electrophoresis (602S stabilized current meter)

2.3 Test Methods

2.3.1 Study on small peptide of protein in duck：

3g duck thigh muscles were accurately weighed, added 60ml0.2mol/L phosphate buffer solution (pH6.5), homogenized with high-speed disperser (6000rpm) 3min, then high-speed centrifugation (10,000×g, 4^{o}C) 20min. the supernatant 1ml took out,

added 2.5ml acetonitrile, centrifuge (15,000×g, 4°C, 20min), 2.5ml supernatant took out, vacuum drying to remove water and organic solvents, with 40μl of eluent A (0.05% Fluoroacetic acid). 20μl of sample took into the C18 column (inner diameter 4.5mm, length 250mm) for HPLC determination. Eluent A (0.05% trifluoroacetic acid), eluent B (acetonitrile: water: trifluoroacetic acid = 60: 40: 0.04) [3,4,5].Elution process: first with eluent A plus 1% eluent B washed 5min respectively, and then gradient elution, eluent B from 1% gradually was increased to 100%, elution 25min, eluent speed control with 0.9ml/min, detection wavelength of 214 nm.

2.3.2 Determination of Free amino acid (FAA)

With reference to Ventanas[4] and Cordoba[6] and other methods, some changes have been made. The specific method was as follows: after the natural thawing of the sample, the visible fat and fascia were removed , minced, about 5g (accurate to 0.001g) weighed ,deionized water 20ml added, in the ice bath with ULTRA TURRAX (IKAT18basic, German) (22,000 rpm, each 10s, interval 10s), then 10% of the sulfosalicylic acid 20ml mixed evenly, at 4℃for 17h, the medium speed filter paper, the filtrate adjusted first with 4mol/LKOH to the pH Value 6.0, and then with deionized water to 50ml, 10kDa ultrafiltration membrane ultrafiltration to remove macromolecules. The ultrafiltrate was derivatized with AccQ Fluor Reagent Kit (P/N WAT052880) and the free amino acid content in the sample was determined by HPLC.The main technical parameters of HPLC: AccQ·Tag column (Nava-PakC18, 3.9×150mm, F0.4μm), column temperature 37°C; injection volume 10μl; Waters515 double pump gradient elution, eluent A AccQ Tag Liquid A was diluted 11 times with ultrapure water. The eluent B was 60%. The elution rate was 1.0 ml·min^{-1}, the elution time was 50 min, and the Waters 2487 UV detector was used.

2.4 Statistical Analysis

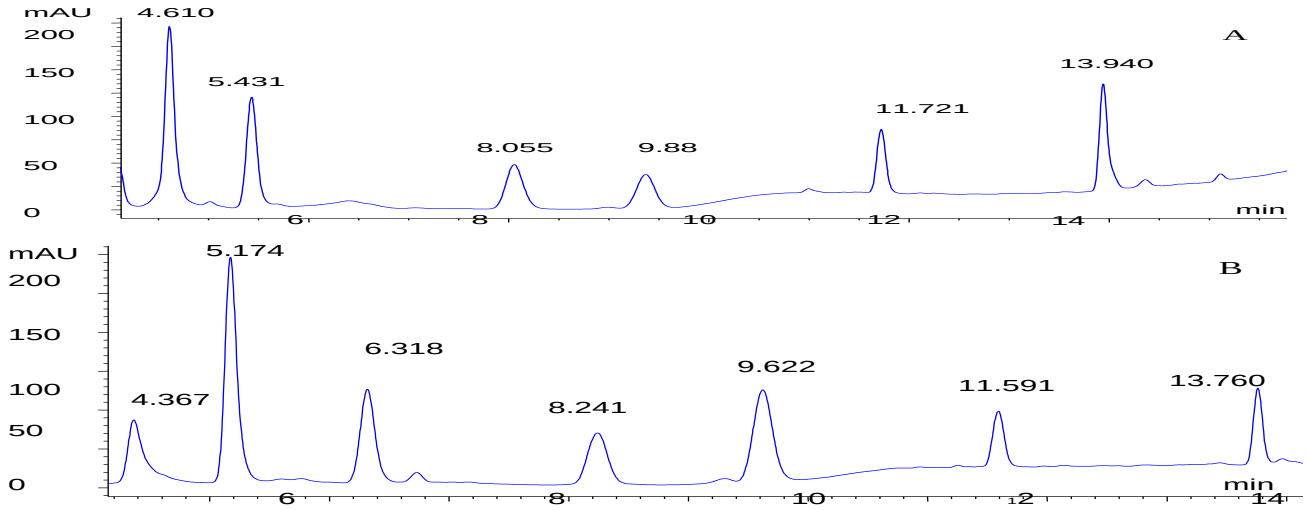
Using SASO.2 ANOVA analysis of variance, and with Duncan's multiple comparison, the difference was significant($p \leq 0.05$).

III. RESULTS AND DISCUSSION

3.1 Changes of Protein Peptides in Duck Muscle

It can be seen from Figure 1, in the processing stage of the duck, from the raw material to dry 5d retention time was short, the content of the polarity material in the dry picking stage first declined, and then increased and stabilized. In the dry stage of 10d, a new peptide peak appeared at 7.002, 12.632 and 17.429min, and the peptide peak were decreased at 6.330 and 9.664min. This may be related to the fact that "high levels of small peptides imparted flavor to the product and low levels of peptides made the product sweet" [7,8].

The main peak area of various major peptides in thigh meat was shown in Table 1. RP-HPLC analysis of small peptides showed that a total of ix significant peptides elution peaks were obtained in the ducks and the peak area of each elution peak was calculated by integral method. The number of elution peaks were consistent with that of Eugenio Rodriguez-Nune[9], and the first six peaks were concentrated in the first 14 min, indicating that the polarity of the polypeptide in the duck was mostly strong. ($P < 0.05$), which was probably due to the fact that the peptides were dissolved in the pickled liquid during the pickling stage ($P < 0.05$), and the total area of the peak increased significantly ($P < 0.05$), followed by dehydration mature process, endogenous enzyme decomposition of the protein as a short peptide



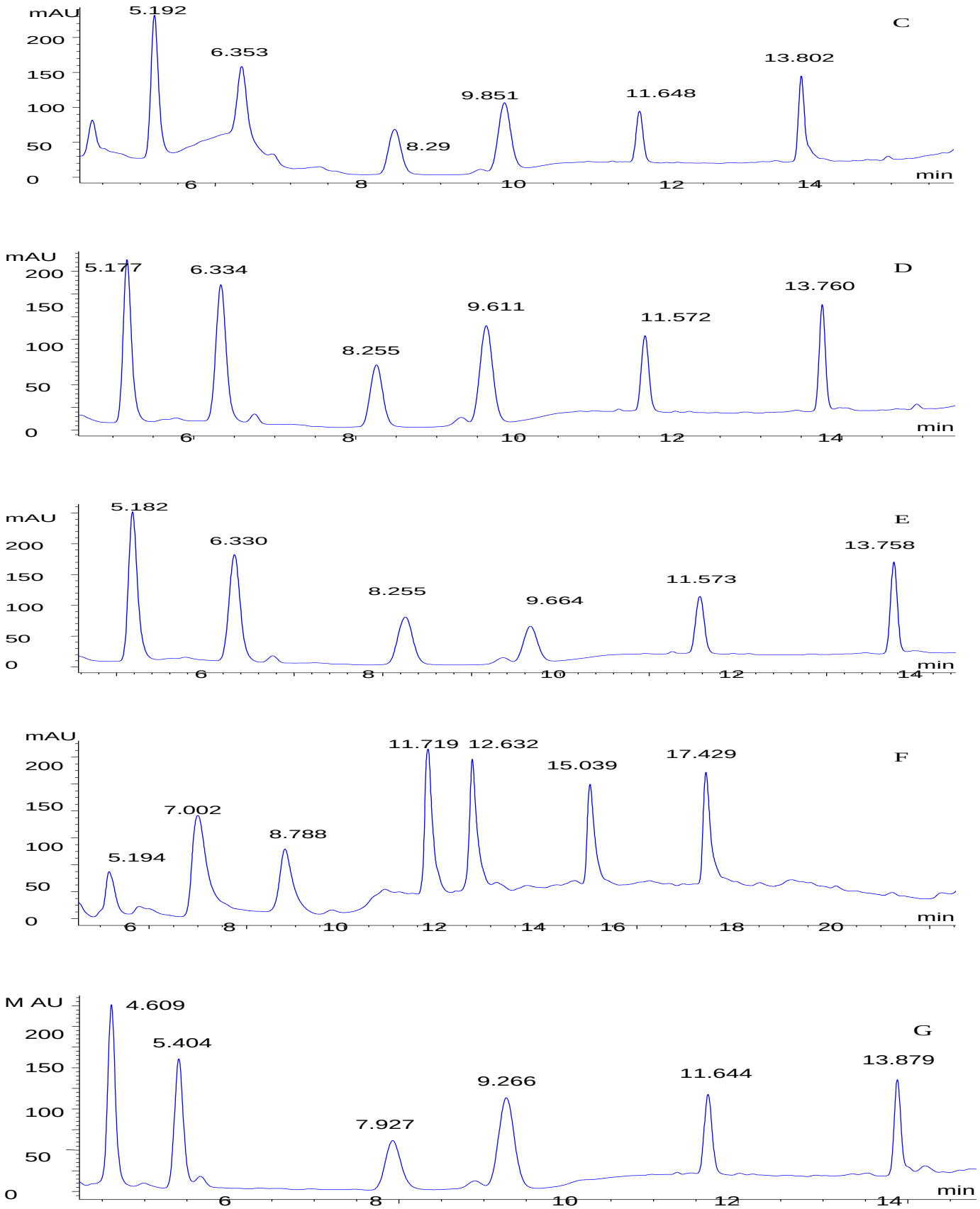


FIGURE 1: HPLC PATTERNS OF PEPTIDES IN LEG EXTRACT WITH DIFFERENT PROCESSING PERIOD

A-material ; B-cured ; C-salted ; D-overlaped ; E-drying 5d ; F-drying 10d ; G-drying 15d

TABLE 1
THE RESULT OF PEPTIDES OF HPLC IN LEG DURING PROCESSING PERIOD OF DRY-CURED DUCK [UNITS: AREA（ mAU*s）

Retain time(min)	raw material	cured	salted	overlaped	drying 5d	drying 10d	drying 15d
4.90	1284.80 ±187.71^{c}	1828.93±370 .84^{a}	406.50±15 9.50^{d}	1202.33±11 3.82^{c}	1659.17±1 85.73^{ab}	453.30 ±23.21^{d}	1332.47 ±145.04^{bc}
6.02	825.40±68.4 1^{e}	957.60±134. 86de	1436.17±9. 41^{ab}	117.77 306.39^{bcd}	1364.20±3 39.16^{abc}	1649.47± 69.36^{a}	1071.30±34. 65^{cde}
8.29	531.50±37.4 3^{c}	694.131±41. 22^{b}	739.33 ±1.55^{b}	669.37 ±72.39^{b}	878.90 ±35.21^{a}	900.83 ±24.83^{a}	783.93± 86.94^{a}
9.88	435.40±27.1 5^{c}	1152.13±295 .37^{a}	1099.53±2 3.99^{ab}	956.50 ±354.55^{ab}	758.40 ±187.79^{bc}	1217.70± 22.65^{a}	1268.57±20 4.34^{a}
11.77	403.77±67.0 4^{d}	443.90±68.6 8^{cd}	477.70±10. 16^{cd}	513.37 ±90.50^{c}	670.46 ±34.48^{b}	1056.73± 18.77^{b}	636.23± 13.10^{a}
13.99	676.13±33.0 2^{bc}	516.77± 13.70^{d}	813.30± 26.46^{a}	560.40 ±117.73^{cd}	772.27 ±31.58^{ab}	818.37± 19.37^{a}	742.13±128. 84^{ab}
total	4156.98±398 .54^{b}	5593.44±101 8.60^{a}	4972.56±1 79.12^{ab}	5077.02±10 54.88^{ab}	6103.38± 372.39^{a}	6102.42± 113.05^{a}	5934.64± 164.75^{a}

Note: Area are expressed as mean ± S.D. of triplicates; means within the same line with common superscripts are not significantly different (P>0.05).

3.2 Changes of Free Amino Acids in Duck Muscle

The determination of the major free amino acids in thigh meat was shown in table 2. In the processing of duck, in addition to arginine, threonine, cysteine little change, the other kinds of free amino acid concentration with the processing have increased to varying degrees. Compared with the pre-pickled, most of the free amino acids in the duck were increased by 1-2 times, among which the glutamic acid increased most, more than 2 times, followed by aspartic acid, serine acid and isoleucine. At the end of the processing, the higher content of free amino acids was glutamic acid, arginine, histidine and threonine. From the point of view the changes in content, aspartic acid, proline, tyrosine, valine, lysine, isoleucine and leucine were consistent, at the beginning of pickled stage were little changed, and later each stage of continuous rise($P < 0.05$). From the total amount of change, the free amino acid from the beginning of the raw materials to dry 5d continuous increase, and then a slight decline, which might be the amount of decomposition was greater than the amount produced by hydrolysis, decomposition into flavor small molecular components such as aldehydes, Alcohol compounds [10].

TABLE 2
MAIN FAA CONTENT IN LEG DURING PROCESSING PERIOD OF DRY-CURED DUCK (unit：mg/100g)

Items	material	cured	salted	drying 5d	drying 10d	drying 15d
Asp	12.25±0.81bc	12.07±1.31bc	10.16±2.96c	16.37±1.15ab	17.61±1.42ab	19.92±6.73a
Ser	13.77±1.37b	24.84±2.03a	21.55±2.68a	24.59±3.74a	27.29±1.56a	22.70±7.25a
Glu	19.91±0.59b	38.79±12.22a	36.73±3.16a	46.19±1.55a	50.21±5.90a	43.6397±15.34a
Gly	8.13±1.14c	15.02±2.09ab	11.66±1.00bc	16.47±0.67a	15.94±1.85ab	15.72±4.84ab
His	18.72±2.00c	32.43±3.79b	39.67±10.17ab	16.47±0.67a	42.16±1.39ab	35.02±8.26ab
Arg	77.57±24.24c	203.93±64.31a	189.35±43.07ab	230.64±27.22a	151.72±18.72abc	119.55±34.51bc
Thr	20.96±2.97b	47.24±16.86a	48.55±6.08a	53.35±4.74a	57.28±9.24a	38.86±14.67ab
Ala	14.58±1.85b	30.73±11.62a	29.64±6.04a	35.01±2.85a	34.95±4.16a	27.85±9.62a
Pro	10.74±0.73c	11.66±4.89c	13.54±1.12bc	16.88±0.97abc	20.14±1.99ab	21.52±6.73a
Cys	5.60±1.38a	1.36±0.73b	0.96±0.24b	0.12±0.50b	0.20±0.41b	8.81±5.00a
Tyr	12.32±1.66b	11.05±3.22b	10.99±2.84b	13.77±1.72ab	13.65±1.81ab	18.24±5.37a
Val	10.46±0.78c	11.21±2.94bc	10.74±2.45c	16.75±2.03ab	16.79±1.94ab	18.24±5.99a
Met	10.09±0.34b	6.46±1.74c	6.65±1.51c	9.00±1.25bc	8.40±1.04bc	13.07±2.61a
Lys	14.76±1.05b	19.93±5.16ab	19.15±3.63ab	28.99±2.90a	29.87±4.67a	29.87±11.98a
Ile	10.06±0.55bc	7.61±1.94bc	7.08±1.61c	11.54±1.47b	11.41±1.44b	15.61±4.40a
Leu	11.80±0.81b	15.83±4.09ab	16.31±3.13ab	22.78±3.45a	21.64±2.36a	21.86±7.03a
Phe	10.73±0.59b	8.37±2.47b	8.75±1.72b	12.55±2.44b	11.83±1.48b	17.18±4.63a
total	282.51±38.62b	468.56±134.02a	481.38±86.62a	602.09±54.92a	545.90±66.01a	487.72±154.41a

Note : FAA are expressed as mean ± S.D. of triplicates; means within the same line with common superscripts are not significantly different (P>0.05).

3.3 The Correlation between Small Peptides and Amino Acids

Using the correlation analysis of SAS8.2, the correlation equation y=1.0879x + 384.43 (R^2 = 0.86) which was obtained for the small peptide and amino acid.

IV. DISCUSSION

The processing time of the ducks was shorter (about 21 days) and the salt content of the products was higher (about 10%), which could be neglected the role of microorganisms in the formation of small peptides and free amino acids. In contrast muscle endogenous protease had strong ability to decompose muscle protein, among which cathepsin B, H, L, D and other decomposition was strong. In the process of processing, the muscle protein was first acted upon by cathepsin/calcein, decomposed into short peptides, and then decomposed by aminopeptidase to produce free amino acids. Capillary electrophoresis showed that a large number of peptides were produced by peptidase during the dry maturation of ham, especially in I and III dangks, some of which were associated with special tastes [11]. The peptide peptides obtained by RP-HPLC showed that the increase in peak area could be noted in the accumulation of free amino acids during maturation.

FAA in the cured products in the taste characteristics has been reported. Ventanas [12] studied the degradation of muscle protein in Iberian ham processing and found that muscle protein degraded in different degrees during processing, and the content of peptide, nonprotein nitrogen and free amino acid increased gradually. Zhang Yajun et al [2] studied Jinhua ham in the aspartic acid, glutamic acid on the role of flavor, phenylalanine, methionine, isoleucine, valine, histidine and other effects on bitter taste, serine, Glycine, arginine, proline and other effects on the sweet. In the processing of flakes and ducks, the FAA with flavor had a specific proportion combination and high NaCl content, which was the key factor to produce flavor characteristics.

V. CONCLUSION

During the different stages of processing in nanjing dry-cured duck, the content of small peptides showed a significant increase ($P < 0.05$) at the beginning stage, then decreased in the dry pickled stage and finally increased and stabilized. By using HPLC analysis of the free amino acids, in addition to arginine, threonine, cysteine little be changed, the other kind of free amino acids concentration with the processing time had different degrees of increase, which was a flavor of glutamic amino acid could be risen highest. From the total amount of change, the free amino acid from the beginning of the raw materials to dry 5d could be continuously increased, and then could be slightly declined.

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Effect of Temperature on Physical Properties of CNSL based Termiticides

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***Abstract*— *Cashew nut shell liquid based termiticides using neemseed oil, karanjseed oil and bhilawan shell liquid, were developed and the effect of 30, 60, 90 and 120 ^{0}C temperatures on viscosity, refractive index, specific gravity and colour was studied. It was observed that viscosity and refractive index of termiticides decreased with heating temperature from 30 to 120 ^{0}C, specific gravity found decreased with increase in temperature from 30, 60 and 90^{0}C. Colour of Neemseed oil became bright yellow and of Cashew nut shell liquid, Karanjseed oil and Bhilawan shell liquid became darker when heated at temperature from 30 to 120 ^{0}C. CNSL based termiticides were oil based therefore the properties of oils were reflected in the termiticide formulations with respect to the temperature.*

Keywords*— *Cashew nut shell, Termiticides , neemseed oil, karanjseed oil, bhilawan shell liquid.

I. INTRODUCTION

Use of chemicals for termite control is in huge quantity world over which adds in the problems of biotic and abiotic factors in the environment. Plant oils in the nature having termiticidal properties can be safer, cheaper and easily available alternative to the hazardous chemical termiticides in the market. Various plants like Cashew nut (*Anacardium occidental*), Neem (*Azadirachta indica*), Karanj (*Pongamia pinnata*) and Bhilawa or Markingnut (*Semecarpus anacardium Linn*.) have been found to have termiticidal properties. These trees are abundantly available in coastal and semi-arid regions of India and the world. Therefore, cashew nut shell liquid(CNSL), neemseed oil(NSO), karanjseed oil(KSO) and bhilawan shell liquid(BSL) were used to develop CNSL based termiticides to reduce the use of hazardous chemicals in agriculture as pesticides in general and for termite control in special. Sixteen formulations of CNSL (100, 80, 70, 60, 50%), NSO (10, 15, 20, 25, 100%), KSO (10, 15, 20, 25, 100%) and BSL (10, 15, 20, 25, 100%) were made to develop CNSL based termiticide. The influence of atmospheric temperature on physicochemical properties of oils used in the study may affect the mixing of oils, forming uniform coat on the surface of wood samples and absorption of solution by wood samples when dipped in it. Hence effect of temperature on viscosity, refractive index, specific gravity and colour of CNSL based termiticides was studied. This study was conducted in the laboratories of Dept. of Agricultural Process Engineering., Dept. of Electrical and Other Energy Sources; and Dept. of Soil Science and Agricultural Chemistry, Dr. BSKKV, Dapoli, Dept. of Petrochemical Engineering in Dr. Babasaheb Ambedkar Technical University, Lonere, Dist. Raigad and Insta Pollutech Lab, Pune.

II. MATERIALS AND METHODS

Cashew Nut Shell Liquid was procured from Shri. Uma Industries, Plot. No. D-48, Addl. MIDC Kudal, At. Po. Nerur-Waghchaudi, Dsit. Sindhudurga- 416520. Neemseed oil was procured from Biocrop Agro Industries, 594/4, Shiroli MIDC, Kolhapur- 416122. Karanjseed oil was procured from Vijaya Agro Industries, Plot No. 136, A- Section Sangamner Co-Op. Industrial Estate, Ta. Sangamner, Dist. Ahmednagar- 02425-222415. Bhilawan Shell Liquid was procured from Shri. Hanmant Einathrao Kausalle, At. Po. Chincholi, Ta. Kandhar, Dist. Nanded. These were the tree born oils obtained directly from the source of production and were used in the study.

Cashew nut shell liquid based termiticides were developed by using NSO, KSO and BSL at different levels, mixed in CNSL as shown in Table 1. Two oils each time among NSO, KSO and BSL were mixed in CNSL to make up of the total of formulation 100%. The levels of first four formulations TO_1, TO_2, TO_3 and TO_4 were 100% of CNSL, NSO, KSO and BSL, respectively. In the next twelve formulations from TO_5 to TO_{16}, the levels of CNSL were 100, 80, 70, 60, 50%; and of NSO, KSO and BSL were 10,15,20 and 25 % each.

Effect of temperature on viscosity, refractive index, specific gravity and colour of CNSL based termiticides was studied by following standard procedures and methods as shown in Table 2.

TABLE 1
CNSL BASED OIL FORMULATED TERMITICIDES

No.	Treatment	Oils			
		CNSL (%)	NSO (%)	KSO (%)	BSL (%)
1	TO_1	100	0	0	0
2	TO_2	0	100	0	0
3	TO_3	0	0	100	0
4	TO_4	0	0	0	100
5	TO_5	80	10	10	0
6	TO_6	80	10	0	10
7	TO_7	80	0	10	10
8	TO_8	70	15	15	0
9	TO_9	70	15	0	15
10	TO_{10}	70	0	15	15
11	TO_{11}	60	20	20	0
12	TO_{12}	60	20	0	20
13	TO_{13}	60	0	20	20
14	TO_{14}	50	25	25	0
15	TO_{15}	50	25	0	25
16	TO_{16}	50	0	25	25
17	TO_{17}(Control)	0	0	0	0

(TO- Treatment of oil formulation, CNSL- Cashew nut shell liquid, NSO- Neemseed oil, KSO- Karanjseed oil, BSL- Bhilawan shell liquid)

TABLE 2
METHODS USED FOR DETERMINATION OF PROPERTIES OF CNSL BASED TERMITICIDES

Sr. No.	Property	Method
1	Viscosity	ISO 2555:1989
2	Refractive Index	AOAC, 2000
3	Specific Gravity	AOAC, 2000
4	Colour	I.S. 548(Part 1) - 1964

2.1 Viscosity

Viscosity is a measure of resistance to flow of a fluid. Although molecules of a fluid are in constant random motion, the velocity in a particular direction is zero unless some force is applied to cause fluid to flow. The magnitude of the force needed to induce flow at a certain velocity is related to the viscosity of a fluid. Viscosity of CNSL based termiticides (T_1 - TO_{16}) was determined at temperatures 30, 60, 90 and 120 ^{0}C by using Brookfield Viscometer as per the 'ISO 2555:1989' in the laboratory of the Department of Agricultural Process Engineering, Dr. Annasaheb Shinde College of Agricultural Engineering & Technology, MPKV, Rahuri and results are given in the Table 3.

2.2 Refractive Index

The ratio of velocity of light in vacuum to the velocity of light in the oil or fat; more generally, it expresses the ratio between the sine of angle of incidence to the sine of angle of refraction, when a ray of light of known wave length (usually 589.3nm, the mean of D lines of sodium) passes from air into the oil or fat. Effect of temperature on refractive index of CNSL based termiticides TO_1 to $TO_{16}$was studied at temperatures 30, 60, 90 and 120 ^{0}C as per the 'AOAC 2000' in the National Agricultural Innovative Programme Laboratory, CAET, Dr. BSKKV, Dapoli by using Refractometer and results are given in the Table 4.

TABLE 3
EFFECT OF TEMPERATURE ON VISCOSITY OF CNSL BASED TERMITICIDES

Sr. No.	Treatment	Formulation CNSL:NSO:KSO:BSL	Viscosity (cP)				Response to temperature (%)
			Temperature (^{0}C)				
			30	60	90	120	
1	TO_1	100:0:0:0	562	475	448	395	29.72
2	TO_2	0:100:0:0	89	20	10	5	94.38
3	TO_3	0:0:100:0	32	12	6	4	87.50
4	TO_4	0:0:0:100	1070	890	650	422	60.56
5	TO_5	80:10:10:0	462	449	343	321	30.52
6	TO_6	80:10:0:10	673	645	489	437	35.07
7	TO_7	80:0:10:10	667	621	467	405	39.28
8	TO_8	70:15:15:0	411	385	352	318	22.63
9	TO_9	70:15:0:15	727	679	438	410	43.60
10	TO_{10}	70:0:15:15	719	688	413	391	45.62
11	TO_{11}	60:20:20:0	361	344	310	296	18.01
12	TO_{12}	60:20:0:20	783	721	474	419	46.49
13	TO_{13}	60:0:20:20	771	705	458	401	47.99
14	TO_{14}	50:25:25:0	311	293	267	234	24.76
15	TO_{15}	50:25:0:25	838	782	539	495	40.93
16	TO_{16}	50:0:25:25	824	781	516	473	42.60

(TO-Oil Formulation Treatment, CNSL- Cashew nut shell liquid, NSO- Neemseed oil, KSO- Karanjseed oil, BSL- Bhilawan shell liquid)

TABLE 4
EFFECT OF TEMPERATURE ON REFRACTIVE INDEX OF CNSL BASED TERMITICIDES

Sr. No.	Treatment	Formulation	Refractive Index				Response to temperature (%)
			Temperatures (°C)				
		CNSL : NO : KO : BSL	30	60	90	120	
1	TO_1	100:0:0:0	1.512	1.508	1.486	1.471	2.71
2	TO_2	0:100:0:0	1.471	1.462	1.448	1.443	1.90
3	TO_3	0:0:100:0	1.423	1.411	1.406	1.391	2.25
4	TO_4	0:0:0:100	1.526	1.510	1.504	1.489	2.42
5	TO_5	80:10:10:0	1.499	1.494	1.49	1.459	2.67
6	TO_6	80:10:0:10	1.510	1.504	1.499	1.469	2.72
7	TO_7	80:0:10:10	1.505	1.499	1.495	1.464	2.72
8	TO_8	70:15:15:0	1.491	1.486	1.481	1.453	2.55
9	TO_9	70:15:0:15	1.507	1.501	1.495	1.468	2.59
10	TO_{10}	70:0:15:15	1.500	1.494	1.489	1.46	2.67
11	TO_{11}	60:20:20:0	1.496	1.479	1.473	1.448	3.21
12	TO_{12}	60:20:0:20	1.506	1.499	1.493	1.467	2.59
13	TO_{13}	60:0:20:20	1.507	1.489	1.485	1.457	3.32
14	TO_{14}	50:25:25:0	1.479	1.472	1.465	1.814	3.04
15	TO_{15}	50:25:0:25	1.505	1.497	1.49	1.467	2.52
16	TO_{16}	50:0:25:25	1.492	1.483	1.479	1.454	2.55

(TO-Oil Formulation Treatment, CNSL- Cashew nut shell liquid, NSO- Neemseed oil, KSO- Karanjseed oil, BSL- Bhilawan shell liquid)

2.3 Specific Gravity

Specific gravity is the ratio of weight of termiticide at 30 ^{0}C to the weight of water at 30 ^{0}C. Specific gravity of CNSL based termiticides TO_1 to $TO_{16}$was determined at different temperatures 30, 60 and 90 ^{0}C, as per the 'AOAC, 2000 in the laboratory of Soil Science and Agricultural Chemistry, College of Agriculture, Dr. BSKKV, Dapoli by the formula given below

$$\text{Specific gravity of termiticide at } 30\ ^0\text{C} = \frac{A-B}{C-D} \quad (1)$$

Where,

A = weight in gm of specific gravity bottle with termiticide at 30 ^{0}C

B = weight in gm of specific gravity bottle at 30^{0}C

C = weight in gm of specific gravity bottle with water at 30 ^{0}C

Results of specific gravity are shown in the Table 5.

TABLE 5
EFFECT OF TEMPERATURE ON SPECIFIC GRAVITY OF CNSL BASED TERMITICIDES

Sr. No.	Treatment	Formulation	Specific Gravity			Response to temperature (%)
			Temperature (°C)			
		CNSL : NO : KO : BSL	30	60	90	
1	TO_1	100:0:0:0	0.948	0.942	0.939	0.94
2	TO_2	0:100:0:0	0.938	0.937	0.933	0.50
3	TO_3	0:0:100:0	0.933	0.932	0.932	0.03
4	TO_4	0:0:0:100	0.987	0.985	0.983	0.40
5	TO_5	80:10:10:0	0.950	0.940	0.930	1.54
6	TO_6	80:10:0:10	0.960	0.950	0.930	3.43
7	TO_7	80:0:10:10	0.950	0.930	0.910	3.51
8	TO_8	70:15:15:0	0.950	0.940	0.920	2.91
9	TO_9	70:15:0:15	0.960	0.940	0.940	2.45
10	TO_{10}	70:0:15:15	0.950	0.940	0.92	3.78
11	TO_{11}	60:20:20:0	0.950	0.930	0.91	3.51
12	TO_{12}	60:20:0:20	0.950	0.940	0.920	3.40
13	TO_{13}	60:0:20:20	0.950	0.940	0.910	4.88
14	TO_{14}	50:25:25:0	0.940	0.920	0.910	3.72
15	TO_{15}	50:25:0:25	0.970	0.950	0.930	3.21
16	TO_{16}	50:0:25:25	0.960	0.950	0.930	3.95

(TO-Oil Formulation Treatment, CNSL- Cashew nut shell liquid, NSO- Neemseed oil, KSO- Karanjseed oil, BSL- Bhilawan shell liquid)

2.4 Colour

The colour (L*, a* and b* values) of CNSL based termiticides TO_1 to TO_{16} was determined by using Colour Flex Meter. L* indicates the lightness and extends from 0 (black) to 100(white). The other two coordinates a* and b* indicate redness (+a) to greenness (-a) and yellowness (+b) to blueness (-b), respectively. The colour is expressed as the sum total of the yellow and red slides used to match the colour of the specimen oil sample. Colour of CNSL based termiticides was determined at temperatures 30, 60, 90 and 120 ^{0}C, by Colour Flex Meter in the laboratory of the Department of Agricultural Process Engineering, Dr. Annasaheb Shinde College of Agricultural Engineering & Technology, MPKV, Rahuri as per the 'I.S. 548(part 1)-1964' and results are given in the Table 6.

TABLE 6
EFFECT OF TEMPERATURE ON COLOUR OF CNSL BASED TERMITICIDES

Treatment	Formulation CNSL:NSO: KSO:BSL	Colour difference values at 30^0C			Colour difference values at 60^0C			Colour difference values at 90^0C			Colour difference values at 120^0C		
		DL*	Da*	Db*	DL*	Da*	Db*	DL*	Da*	Db*	DL*	Da*	Db*
TO_1	100:0:0:0	-72.49	0.85	-2.26	-72.40	0.72	-2.15	-72.59	0.52	-2.18	-73.14	0.91	-2.59
TO_2	0:100:0:0	-71.26	1.48	0.08	-71.34	2.52	0.15	-71.50	1.74	-0.12	-72.53	5.70	1.11
TO_3	0:0:100:0	-63.15	11.65	13.06	-60.67	10.13	17.01	-58.74	9.44	19.68	-68.61	10.75	13.47
TO_4	0:0:0:100	-72.36	0.44	-2.35	-72.79	0.46	-2.31	-72.53	0.49	-2.35	-73.09	0.59	-2.57
TO_5	80:10:10:0	-73.36	1.13	-1.94	-72.93	1.19	-2.06	-73.52	1.48	-2.04	-73.25	1.58	-2.01
TO_6	80:10:0:10	-73.39	0.84	-1.76	-73.38	0.69	-1.34	-73.35	0.68	-1.75	-73.40	1.43	-2.46
TO_7	80:0:10:10	-72.94	1.47	-2.10	-72.95	1.27	-2.26	-72.73	1.91	-2.50	-72.46	1.81	-2.70
TO_8	70:15:15:0	-72.29	0.48	-2.55	-72.42	0.45	-2.88	-72.43	0.29	-2.93	-72.60	0.86	-3.16
TO_9	70:15:0:15	-71.99	0.80	-3.33	-72.19	0.81	-3.11	-72.17	0.59	-2.80	-72.29	0.61	-2.88
TO_{10}	70:0:15:15	-72.14	0.86	-2.88	-72.03	0.61	-2.94	-72.26	0.58	-3.10	-72.19	0.35	-2.99
TO_{11}	60:20:20:0	-71.46	0.50	-2.73	-71.72	0.70	-2.79	-71.83	0.54	-2.80	-71.65	0.66	-2.50
TO_{12}	60:20:0:20	-72.02	0.66	-3.04	-72.62	0.56	-2.98	-72.19	-0.10	-2.70	-72.36	0.76	-2.97
TO_{13}	60:0:20:20	-72.15	1.13	-3.37	-72.35	0.77	-3.33	-72.34	1.09	-2.85	-72.39	0.63	-2.60
TO_{14}	50:25:25:0	-72.06	0.33	-2.56	-72.22	0.28	-2.94	-72.31	1.06	-3.13	-72.13	0.60	-2.83
TO_{15}	50:25:0:25	-72.48	0.90	-2.91	-72.27	0.27	-2.50	-72.70	0.46	-2.94	-72.76	0.40	-2.71
TO_{16}	50:0:25:25	-72.13	0.96	-3.11	-72.42	1.17	-3.07	-72.69	0.67	-3.42	-72.65	0.63	-3.10
+DL* = Whiteness, –DL*= Blackness, +Da*= Redness, -Da*= Greenness, +Db* = Yellowness, -Db*= Blueness													

(TO - Oil Formulation Treatment, CNSL- Cashew nut shell liquid, NSO- Neemseed oil, KSO- Karanjseed oil, BSL- Bhilawan shell liquid)

III. RESULTS AND DISCUSSION

3.1 Viscosity

Table 3 shows that viscosity of CNSL based termiticides TO_1, TO_2, TO_3 and TO_4 was 562, 89, 32 and 1070 cP, at 30 ^{0}C, respectively and found decreased to 395, 5, 4 and 422cP, respectively with increase in temperature from 30 to 120^{0}C. The viscosity of TO_4 (BSL 100%) was found the highest and that of TO_3 (KSO 100%) the lowest among the formulations TO_1 to TO_4 (pure oils). Viscosity of TO_2 (NSO 100%) was found decreased by 94.38% with increase in the temperature from 30 to 120^{0}C. Asogwa et al. (2007), Djibril et al. (2015), Bobade and Khyade (2012), and Lad et al. (2016) have also observed the similar results of viscosity of CNSL. NSO, KSO and BSL, respectively.

The viscosity of oils was reflected in formulations of termiticides TO_5 to TO_{16} and observed in the range of 296 to 1070 cP in the temperature range of 30 to 120^{0}C. The viscosity of formulation TO_{15} was the highest i.e. 838, 782, 539 and 495 cP and that of the TO_{14} the lowest i.e. 311, 293, 267 and 234 cP at 30, 60, 90 and 120 ^{0}C, respectively among all termiticides. All the oils melt at higher temperatures therefore the viscosity of termiticides was found decreased with increasing temperature from 30 to 120 ^{0}C.

3.2 Refractive Index

Table 4 shows that among the formulations TO_1 to TO_4 (pure oils) refractive index of termiticide TO_4 (BSL) was the highest i.e. 1.526,1.510, 1.504 and 1.489 and that of TO_3 (KSO)the lowest i.e.1.423, 1.411, 1.406 and 1.391 at the temperatures 30, 60, 90 and 120 ^{0}C, respectively. Mukhopadyaya et al. (2010), Djibril et al. (2015), Bhalerao and Sharma (2014), and Lad et al. (2016) have also observed the similar results of Refractive Index of CNSL. NSO, KSO and BSL, respectively.

Refractive index values of oils were reflected in all the termiticides from TO_5 toTO_{16}. Among termiticides from TO_5 toTO_{16}, refractive index of termiticide TO_6 was the highest, i.e. 1.510, 1.504, 1.499 and 1.469 and that of TO_{14} the lowest i.e. 1.479, 1.472, 1.465 and 1.434, at 30, 60, 90 and 120 ^{0}C temperatures, respectively. The refractive index values of all the termiticides were found the highest at 30 ^{0}C and then decreased with increase in the temperature from 30 to 120 ^{0}C.

3.3 Specific Gravity

Table 5 shows that among the formulations TO_1 to TO_4 (pure oils), the specific gravity of TO_4(BSL) was the highest i.e. 0.987, 0.985 and 0.983 and that of TO_3 (KSO) the lowest i.e. 0.933, 0.932 and 0.932 at the temperatures 30, 60 and 90 ^{0}C, respectively. Asogwa et al. (2007), Djibril et al. (2015), Bobade and Khyade (2012), and Chopra and Chopra (1956) have also observed the similar results of specific gravity of CNSL. NSO, KSO and BSL, respectively.

Among termiticides from TO_5 to TO_{16}, the specific gravity values of the TO_{15} were found the highest i.e. 0.970, 0.950, and 0.930 and that of TO_{14} the lowest i.e.0.940, 0.920 and 0.910. The specific gravity of all the formulations from TO_1 to TO_{16} was found decreased with increase in the temperature from 30 to 90 ^{0}C. With increase in temperature the molecules of oils intermiticides melted due to which its specific gravity was decreased.

3.4 Colour

Table 6 shows that the DL*(+ whiteness and –blackness) values of all the termiticides (TO_5 to TO_{16}) were negative showing colour darkness (blackness). The TO_3 (KSO) was having the least darkness values (-63.15) among all the oil formulated termiticides and it was seconded by TO_2 (NSO) (-71.26) at 30 ^{0}C. DL* value was decreased in TO_3 (KSO) initially when heated from 30 to 90 ^{0}C and then found increased at 120 ^{0}C. Darkness value of TO_1, TO_2 and TO_4 were increased with increase in temperature from 30 to 120 ^{0}C. The Da*(+ redness and – greenness) value of CNSL, NSO and BSL and all oil formulations was less green whereas that of KSO it was 11.65 i.e. reddish. This value of CNSL was observed decreased, of NSO increased and of BSL slightly increased while that of KSO it was increased with increase in temperature from 30 to 120^{0}C.

The Db* (+yellowness and – blueness) value of CNSL, NSO and BSL and all oil formulated termiticides was bluish whereas that of KSO it was (11.06) yellowish. With increase in temperature from 30 to 120 degree Celsius, the yellowness of KSO was increased (from 11.06 to 19.68), blueness of NSO slightly increased and that of CNSL and BSL blueness was increased. The reflection of colour values of oils is clearly observed in all the oil formulated termiticides with slight differences at the temperatures from 30 to 120 ^{0}C. Asogwa et al. (2007), Djibril et al. (2015), Bobade and Khyade (2012), and Chopra and Chopra (1956) have also observed the similar results of colour values of CNSL, NSO, KSO and BSL, respectively.

IV. SUMMARY AND CONCLUSIONS

Four oils: Cashew nut shell liquid (50, 60, 70, 80, 100), Neemseed oil (10, 15, 20, 25, 100), Karanjseed oil (10, 15, 20, 25, 100) and Bhilawan shell liquid (10, 15, 20, 25, 100) were used to make CNSL based termiticide. Effect of temperature on viscosity, refractive index, specific gravity and colour of termiticide formulations was studied and conclusions were drawn as below:

- Viscosity refractive index and specific gravity of CNSL based termiticide was found decreased with increase in temperature from 30 to 120 ^{0}C.
- Colour of CNSL based termiticide became darker when heated from 30 to 120 ^{0}C.
- CNSL based termiticides were oil based therefore the properties of oils were reflected in the termiticide formulations with respect to the temperature.

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Analysis of the Avian Biodiversity in Qingliang Mountain Area in spring

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Abstract**—** *The Qingliang mountain area located in the downtown of Nanjing, **t**here were plenty of botany species in Qingliang Mountain Area, such as forest, bamboos and grasslands, the environment was good habitat to avians. In this paper, avian resource of Qingliang mountain area was studied, 11 species was watched which belonging to 2 Orders and 7 Families. 2 summer breeding birds and 9 resident birds were recorded, 4 species were oriental realm birds, 2 species were palaearctic realm birds. The Shannon-Wiener Index was 1.6869, The Pielou Index was 0.3055, and The G-F Index was 0.2261. The feeding condition and safety condition affects the biodiversity of avian of Qingliang mountain area.*

Keywords**—** ***Avian Biodiversity Qingliang Mountain.***

I. INTRODUCTION

Zhang Xiulei (2006) investigated the bird composition and population number of Mamize Nature Reserve in Sichuan, and recorded a total of 105 species, 7 orders, 23 families, and 1948 of birds, including 6 species of the national protected birds, 7 species of unique birds in China [1]. Birds' areas are mainly composed of oriental components. The results of the analysis of varieties of the birds showed that the similarity index of bird community was the highest among plantations and shrub grassland habitats, and the similarity of bird community between shrub grassland and virgin forest was the lowest. The diversity index of secondary forest bird diversity was the highest, the lowest in the shrub grassland. And the number of the birds in the original forest was the biggest. The species had the highest density in the middle layer of the forest.

Xu Xiaojun (2006) conducted a census of bird resources in Shanghai World Expo Park and surrounding areas, and studied the correlation of bird quantity, species and environmental factors. The results showed that there were 67 species, 23 families, 7 orders of birds in the region, with a community diversity index of 3.46 [2]. The community structure and distribution characteristics of bird community were analyzed by probabilistic statistical method. The bird community index and the eight environmental indicators were analyzed. The results showed that the number, quantity and diversity index of the bird were significantly correlated with the area of the park, and water area, the shape index and the habitat species. The diversity and uniformity index of birds were significantly correlated with the total number of species. The number of birds was related to the location conditions in the environment.

Wu Yi (2007) investigated the diversity of birds in Yuexiu Park, Guangzhou, and recorded 43 species of birds, belonging to 8 orders and 20 families, of which 27 species of birds, two summer migratory birds, eight winter migratory birds, and 6 species of other breeding birds [3]. The characteristics of bird community in summer and winter habitats were compared and analyzed. And the diversity, density and dominance of birds in summer were higher than those in winter, while the diversity index and uniformity index were lower than those in winter. There are great differences in the species, quantity and diversity of birds in the six habitats of the lawns of Nanxiu Lake, the Jinyin Playground, Bamboo Forest, Nanxiu Grove, Nanxiu Lake and Central City. The diversity of birds is the highest. According to the diversity of birds in different environments of the park put forward the protection measures.

Qingliang Mountain, also known as Stone Mountain in the past, is squatting in the west corner of Nanjing City, located in the western of the Guangzhou Road, Nanjing. The height of Qingliang Mountain is more than 100 meters, a radius of about 4 km, has been built as Qingliang Mountain Park. The trees in the garden are lush, the terrain are steep. Qingliang Mountain Park is located in the downtown area of Nanjing, the surrounding residents

gathered, shops everywhere, from 2006 onwards, Qingliang Mountain Park free to the public, as a city of Green Island, gets more and more attention from the public, this article started from the Qingliang Mountain area Bird diversity, trying to analyze the composition of the birds to reflect the urbanization process on the impact of urban birds and the evaluation of the environment.

II. MATERIAL AND METHOD

2.1 Survey using sample method

A number of samples are selected in different habitat types, and the number and type of birds are recorded and heard by the investigators in the sample.

2.2 Using the sample line survey

In different habitat types selected in different lines, and the investigators observed on both sides of the sample line.

And record the type and number of birds within 50 meters, each sample line repeated investigation; use the repeated observed average as a statistical basis.

At the time of observation, only birds in the opposite direction of flight are counted. The survey was completed from 20 March 2008 to 10 April 2008, and the sample and sample lines are shown in Figure 1. Selected samples are more concentrated birds of woodland and bushes; sample line is taken in the park within the road.

2.3 Diversity index

Shannon-Weaver diversity index

$$H' = -\sum_{i=1}^{s} \frac{n_i}{n} \ln \frac{n_i}{n}$$

s: species number; n_i: the number of biological individuals; n: the total number of individual individuals

The statistics of the evenness index were calculated using the pielou index

$$J=H'/H_{max}$$

H_{max} = Ln S, s is the number of species

G-F index analysis:

Use the distribution of birds and mammals, and the Shannon-Wiener index based on the information measure, the species diversity of DG, the diversity DF and the standardized GF index were calculated and defined: if there is only one species in one region or only a few species distributed in different families, the definition of the region GF index is zero.

1) F index, DF:

In a particular section k:

$$D_{Fk} = \sum_{i=1}^{n} \rho i \, ln \rho i$$

pi = ski / Sk, Sk = the number of species in the k group in the directory, ski = the number of species in the family, and n = k.

F index for a region:

$$D_F = \sum_{k=1}^{m} D_{Fk}$$

m = the number of subjects in the list of birds or beasts.

2) G index, DG:

$$D_G = -\sum_{j=1}^{\rho} D_{Gi} = -\sum_{j=1}^{\rho} q_j \ln q_j$$

Where: $q_j = s_j / s$, S = the number of species in the bird or beast in the list, s_j = the number of species in the j genus of the bird or the beast, p = the number of species in the bird or the beast.

3) G-F index:

$$D_{G-F}=1-\frac{D_G}{D_F}$$

If all the families of the bird or the beast are single species, ie DF = 0, the G-F index of the area is specified to be zero, $D_{G-F} = 0$.

G-F index characteristics:

(1) The more non-single species, the higher the G-F index. Since $p_i = s_{ki} / S_k = 1$; $D_{Fk} = 0$; the contribution of single species to F index (DF) is zero, the more non-single species, the $\frac{D_G}{D_F}$ smaller, the higher the G-F index.

(2) The G-F index is a measure of 0 to 1. D_G is the diversity of the D_F subclasses, with reference to Pielou, $D_G \leq D_F$, so, generally, $0 \leq \frac{D_G}{D_F} \leq 1$.

III. RESULTS AND ANALYSIS

3.1 Results

TABLE 1
LIST OF BIRDS OBTAINED FROM THE SURVEY

Birds	Order	Family	Migration habit	Distribution	Habitat type
Turdus merula	Passerine	Muscicapidae	Residents	Oriental Region	L, D
Pycnonotus sinensis	Passerine	Pycnonotidae	Residents	Oriental Region	L
Cettia fortipes	Passerine	Sylviidae	Residents	Oriental Region	L
Garrulax perspicillatus	Passerine	Sylviidae	Residents	Widespread	L, **G**
Parus major	Passerine	Paridae	Residents	Widespread	L, G, D
Streptopelia chinensis	Columbiformes	Columbidae	Residents	Oriental Region	L
Streptopelia orientalis	Columbiformes	Columbidae	Residents	Widespread	L, D
Eophona migratoria	Passerine	Fringillidae	Migrants	Palaearctic realm	L, D
Eophona sp.	Passerine	Fringillidae	Migrants	Palaearctic realm	L
Cyanopica cyana	Passerine	Corvidae	Residents	Widespread	L
Pica pica	Passerine	Corvidae	Residents	Widespread	L, D

Description: (1) "ancient" ancient north boundary, "East" Oriental world, "wide" for the wide variety; "winter" winter migratory birds, "stay" to stay birds. (2) L-woodland, G-shrub, D-land. (3) In the column of the International Protection Agreement, the R-generation refers to the type of protection in the Sino-Japanese Migratory Bird Protection Agreement.

TABLE 2
SPECIES QUANTITY COMPOSITION

Birds	Number	Habitat
Eophona sp.	1	tree
Turdus merula	6	land
Streptopelia orientalis	7	Tree and land
Pica pica	5	tree
Pycnonotus sinensis	46	tree
Cettia fortipes	3	tree
Garrulax perspicillatus	6	Tree, land, Shrub
Parus major	6	Tree, land, Shrub
Streptopelia chinensis	4	Tree and land
Eophona migratoria	50	tree
Cyanopica cyana	1	tree

There were 11 species of spring birds in the area, which belonged to 2 orders and 7 families, accounting for 135, accounting for 81.82% of stay birds and 18.18% for winter migrants (Table 1). There were 4 species of birds in the eastern part of the region, accounting for 36.36% of the birds in the region, and 2 species of ancient north boundary birds, accounting for 18.18%, and 5 species that were widely distributed in the Oriental and Palaearland, accounting for 45.46%. The survey found only one species of protected birds in the region, belonging to the protected species of Sino-Japanese migratory birds, accounting for only 9.99% of the total number of birds. From the quantitative point of view (Table 2), *Pycnonotus sinensis* and Black-tailed Worries were the dominant species in the Qingliang Mountain area in spring, and the number of turtles, big tits, black-faced babes and blackbirds were also more, all were common species for spring.

3.2 Analysis

3.2.1 Retro birds and migratory birds and floristic analysis

The observation of the spring birds in Qingliang Mountain area showed that stay birds were accounting 81.82%, 18.18% were winter birds, and most of the birds were resident birds. There were 4 species of birds in the eastern part of the region, accounting for 36.36% of the birds in the region, and 2 species of the ancient north boundary birds, accounting 18.18% of the birds, and 5 species were widely distributed in the Oriental and Palaearland, accounting for 45.46%.

3.2.2 Analysis of the protected birds

Only one case was observed, the *Eophona migratoria*, belonging to the Sino-Japanese migratory protection agreement.

3.2.3 Analysis of ecological types

Observations of the 11 species of birds are distributed in the woodland. Blackbirds, big tits, mountain tigers, magpies and *Eophona migratoria* are in the ground activities, black noodles and large tits in the shrub activities, large tits habitat type more in the woodland, and also active in shrubs and the ground.

3.2.4 Related Statistical Analysis

The Shannon-Weaver diversity index was 1.6869 and the pielou evenness index was 0.3055.

G-F index analysis, the spring birds $D_F = 2.7728$, $D_G = 2.1458$ and the G-F index were 0.2261.

IV. DISCUSSION

Through the analysis of the diversity of birds in Qingliang Mountain, the species and quantity of birds were obtained, and the diversity analysis was carried out to evaluate the surrounding environment of Qingliang Mountain, so as to provide quantitative basis for the corresponding protection and monitoring measures for urban ecological construction provide evidence. In recent years, due to the development of economic resources in the region, human disturbance and damage are becoming increasingly serious, so the investigation of the situation of bird resources in the study area, and strengthen the protection of bird resources in the region, are very important in protect the species resources and maintain ecological balance. [4].

The oriental boundary is not obvious from the nature of the winter birds in the region. The increase of the northern part of the birds in the region is related to the flying ability of the birds themselves and the migration to the winter. The main reason may be that Jiangsu is located at the junction of the Oriental and the ancient North boundary, the fauna composition has two characteristics at the same time, and Jiangsu in Australia - Siberian bird migration line, which is the definitely road of a lot of migratory birds. So in the spring the keep birds and migratory birds can be observed at the same time.

From the quantitative point of view, the dominant birds are *Pycnonotus sinensis* and *Eophona migratoria*, *Pycnonotus sinensis* is a medium-sized (19 cm) olive *Pycnonotus sinensis*. Eyes have a white wide stretch to the neck, black head slightly feathers, mustache black, hip white. The color of the young birds' head is olive; their chest is gray and striped. This is named after the *Pycnonotus sinensis*, the call is twittering and simple and no rhythm of the call is widely distributed in most of the country, often clustered in the shrubs, mangroves and forest garden. Lively, crowded on the fruit tree activities, and sometimes from the habitat flight predation. Also known as white head or Pulsatilla. Black tail wax mouth is slightly larger (17 cm) and the pier of the birds, with a huge yellow and black mouth. Breeding male shape is very similar to a black hood, body gray, and wings near black. Female is like a male but a black head. Young birds like brown but heavier brown. The song is a series of whistles and tremolo, euphemistic. They widely distributed in the southeastern region of China, the distribution of: local common. Generally inhabit in the woodland and orchards.

Eophona migratoria is the songbird that has wonderful sounds, Nanjing people have the tradition of raising the mouthbirds, in the Nanjing local chronicles on the use of the mouthbirds for the performance of the record, the sale of mouthbirds business can often seen in the Confucius Temple flowers and birds market ,The population of the mouthbirds in Qingliang Mountain area reached more than 50, and former cooperation with the Jiangsu Provincial Department of Agriculture and Forestry, found that someone in the Qingliang Mountain area to capture the mouthbirds, thus there should be more effective protection to make in the spring Qingliang Mountain area .

Large tits are act in the woodland, shrubs and the ground, the common name "brush througher", naturally like to move, and in the branches or the ground continually to move to find food. [7] Blackbirds, mountain turtles and magpies also have activities on the ground; the main reason is that these birds need to seek grass seeds or insects on the ground. Bead neck and dove have the body color that is close to the wild and more difficult to distinguish, but the two birds of different ecological types, bead neck doves generally only in the woodland activities, and mountain turtles often in open or farmland activities, this ecological characteristics can be used as the basis for the resolution of telling the two similar birds [8].

In comparison with other people's findings, in this study, the diversity of birds was lower and the uniformity was lower in spring. This shows that the biodiversity of birds in the Qingliang Mountain area is not well protected, and there are some problems with dominant species and other species. This may be due to the spring season is the migration of birds, some species of birds will also have a distribution of the distribution area, while some migratory birds transit, will have an impact on diversity and evenness. [9] But on the whole, the Qingliang Mountain area has more frequent human activities and has a greater impact on bird activities, so the diversity of birds is not rich in the expected.

In this survey, only bird species and pigeon-shaped birds were found. The number of birds was less, only 7 families. Most of the birds belonged to the locusts. The Qingliang Mountain area belonged to the typical type of birds liked habitat. More surprisingly, in this survey, did not find Nanjing common birds, sparrows but found more *Pycnonotus sinensis*, Li Yongmin (2005) pointed out that with the socio-economic development, In the past 10 years, the urban and rural ecological environment in Wuhu City has undergone great changes, which has made great changes in the community structure of birds. Compared with historical data, the rate of sparrows in Wuhu City is reduced from 45.64 per hour to 14.403 per hour, and the meeting rate of bluish blossom increased from 2.35 per hour to 47.27 per hour, and *Pycnonotus sinensis* was replaced by tree. The sparrow became the absolute dominant species of garden birds in Wuhu. There are cities in Hangzhou and other places in the farmland habitat [tree] sparrow and swallows have a significant decline. In the rural area the housing structure changes and pesticides, fertilizers in large quantities using, there may be the cause of this phenomenon. In the summer, in the farmland roadside shrub or sponge gourd, often can see dozens to hundreds of mercerized starling cluster activities, so that the meeting rate of encounter reached 46.862 per hour, such a high density is worth discussion [10].

The spring birds in the Qingliang Mountain area, DF = 2.7728, DG = 2.1458 and the GF index was 0.2261. Among them, the F index was significantly lower, because the single species were more in the calculation, The F index, and G index is not high, because the single species are more, single family also more. At the same time, the composition of the spring birds is only a small part of the annual composition of birds in a region, the diversity of the assessment is more one-sided, but also only typical and not universal significance, therefore, it can be inferred that if the Qingliang Mountain area birds The class composition makes a systematic monitoring that will provide more comprehensive data and results.

V. CONCLUSION

5.1 Relationship between species and quantity and season

On the whole, there are few types of birds in the spring of Qingliang Mountain area, which are not high in number, low in diversity, and low in uniformity and obvious in dominant species. This may be due to the beginning of the snowstorm, causing a large number of birds died, while the disaster also affected the migration and reproduction of birds. In February 2008, the Naval Air Force Nantong Airport Bird Survey was conducted to find the phenomenon of summer breeding of summer black birds in winter. This phenomenon can be used as evidence of a low number of bird species in Qingliang Mountain area.

5.2 Relationship between species and quantity and human activity

The distribution of birds and human activities have a great relationship between the activities of birds are generally human activities or less disturbed areas, Qingliang Mountain area of human interference is large, and because of the winter bird biological characteristics and Ecological habits, resulting in the habitat of the species is small and uneven distribution, the

frequency of human activities and the distribution of birds inversely proportional to the density, in summary, the Qingliang Mountain area of birds in the place where the interference is very small, by the impact of human interference.

From the above points, it can be seen that the factors that affect the diversity of birds in the spring area of Qingliang Mountain are food, human disturbance, niche, climate, species ecological habit and biological characteristics, but the factors affecting spring bird diversity are Food and safe habitat. According to the observation and analysis, the diversity of birds in Qingliang Mountain area is not high in spring, and the number of dominant species is relatively simple. It is in line with the typical characteristics of birds in the landscape state of urban landscape. For the management of Qingliang Mountain area, the following suggestions: you can plant a variety of landscape gardens, so that birds can provide a variety of habitat, which attract more kinds of birds. Strengthen the scenic area of plant conservation management, we found in the investigation of a lot of damage to the vegetation of greening behavior, in order to make the city park green and green, for the broad masses of people to provide a harmonious environment, the need for management attention, but also need the general public with the active.

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Effect of basidiomycete fungi on the discoloration and phytotoxicity of synthetic dye and textile effluent

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***Abstract*—** *The elimination of toxic wastes from industrial activities, mainly the textile industry, has induced the researchers to seek new techniques that reduce or eliminate the toxicity of these effluents. The textile effluent has a high chemical demand of oxygen and strong coloration, requiring an especific treatment. The aim of this study was to evaluated the decolorizationRemazol Brilliant Blue R (RBBR) and textile effluent using pre-selected cultures of basidiomycete fungi: Lentinula edodes, Pleurotusostreatus and Pleutotuspulmorarius, and phytotoxicity of the dye and effluent before and after treatment with fungi. The decolorization test was realized in a liquid medium and the absorbance determined in spectrophotometer. For the dye was used to two pH values (5.0 and 9.0) and concentration (0.1 gL-1 and 0.5 gL-1). Lactuca sativa* L. *seeds were exposed to dye samples and textile effluents and the parameters evaluated were the germination rate and root lenght. The fungus Pleurotuspulmonarius was the one with the best result on the decolorization of dye RBBR on the both values: pH and concentration. As the textile effluent there was no significant difference among the treatments. In some treatments with the dye germination rate decreased indicating toxicity after decolorization. However there was an increase in root growth in the presence of the dye treated with P.pulmonarius.*

***Keywords*— *Pleurotuspulmonarius; Remazol Brilliant Blue R.; Decolorization; Toxicity*.**

I. INTRODUCTION

In recent years, studies on urban environmental problems have been gaining prominence, mainly because the evolution of urban centers and continued population growth have led to the emergence of some forms of pollution that have reached catastrophic dimensions, which can be observed through changes quality of soil, air and water [1].

The textile sector is one of the activities that contribute most to the environmental contamination, because it uses in its processes large volumes of water, besides several substances during the staining, such as caustic soda, gums, detergents, defoamers, chlorine, formols, emulsions, oils and resins, among others. This leads to the production of a complex, toxic and recalcitrant residue [2].

Approximately 8,000 different dyes and pigments are used industrially, representing an annual consumption of around 800 000 tonnes worldwide, of which approximately 10-15% is discharged into the environment [3].

The textile effluent has high chemical oxygen demand and coloration due to the presence of dyes, pigments and auxiliary chemicals, which causes that the effluent needs a specific treatment [4].

When not properly treated and released into water bodies, the effluents from the textile fiber dyeing process can modify the ecosystem, reducing the transparency of the water and the penetration of solar radiation, which can modify the photosynthetic activity and the solubility regime of gases [5].

With regard to the removal of textile dyes present in industrial effluents, new technologies have been sought to minimize the environmental damages they may cause. Although there are physical and chemical forms of treatment of textile effluents, microorganisms have been intensively studied for this purpose [6].

Studies indicate that lignin-degrading basidiomycete fungi are efficient in the degradation of several compounds, including dyes, presenting high action potential in the recovery of contaminated environments [7].

The extracellular enzymes present in these fungi (lignin peroxidase, manganese peroxidase and laccase) are an effective means of degradation of pollutants, presenting specific mechanisms of degradation of recalcitrant compounds. However, there is a demand for biological agents not only able to degrade these effluents more efficiently, but also to reduce their toxicity [8].

It is in this context, from the constant search for alternative methods of treatment of effluents, that the need arises to study new fungal species that have degradation capacity of dyes and textile effluents, aiming at the optimization of traditional treatment processes.

The present work was carried out with the objective of evaluating the discoloration of Remazol Brilliant Blue R dye and textile effluent using pre-selected basidiomycete fungi cultures and verify the phytotoxicity of the dye and effluent before and after treatment with fungi, with *Lactuca sativa* L.

II. MATERIAL AND METHODS

The dye used was the Remazol Brilliant Blue R ,Sigma - Aldrich®, being an anthraquinone type dye. Its empirical formula is C22H16N2Na2O11S3 and its molecular weight, 626.54 g / mol.

The effluent was collected from the receiving tank of the effluent treatment plant (ETE) of the TrelConfecções Ltda. Textile industry, located in the city of Lavras, MG. The industry manufactures garments, and RBBR (Remazol Brilliant Blue R) textile dye is routinely used.

The sample was collected in previously sterilized flasks, totaling 1,000 mL, which were then taken to the Laboratory of Microbiology of the Agricultural Research Institute of Minas Gerais (EPAMIG), located at the Federal University of Lavras Campus in Lavras, MG. .

For the discoloration treatments with the RBBR textile dye and the textile effluent, basidiomycete fungi Lentinula edodes (INCQS - FIOCRUZ 40220), Pleurotusostreatus and Pleurotuspulmonarius were used. The first one was donated by the Oswaldo Cruz Foundation of Rio de Janeiro and the others by the Laboratory of Edible Mushrooms of the Federal University of Lavras.

The basidiomycetes fungi used were initially inoculated into Petri dishes with AM medium. After seven days of incubation at 25 ° C, three 5 mm diameter disks containing the fungal samples were removed from the colonies of each fungus under study and transferred to 250 mL Erlenmeyers containing 50 mL of malt extract (EM) 2 % supplemented with the RBBR dye [7].

Two concentrations of the dye (0.1 and 0.5 g L-1) and two pH values (5.0 and 9.0) were used. These values of pH and dye concentration were analyzed, since they are the most found in the treatment tanks of the effluent treatment plants (ETE) of the textile industries.

The flasks were incubated at 28 ° C (mean treatment tank temperature) in the dark and under agitation of 150 rpm. After 15 days, aliquots of 5 ml of the supernatant were filtered in 0.45 μm membrane (Whatman PVDF) for spectrophotometric analysis (BEL PHOTONICS - SP 1105). The wavelength in which the dye had the highest absorbance was used. Each test presented as a control flasks containing 2% culture medium and dye, without the fungal sample. When necessary, dilutions of the samples were made. A standard curve was drawn from the initial solution (0.5 g L-1 of RBBR) and different dilutions in 2% MS with their respective absorbance at the same wavelength used to monitor the behavior of the dye.

Disks containing the fungi samples were transferred to flasks containing 50 mL of the untreated textile effluent, supplemented with 0.5% MS. The same wavelength used for the RBBR dye was used and, as a control, flasks only with the textile effluent, without the fungal sample.

Seeds of the same batch of lettuce (*Lactuca sativa* L.) cv. Summer Wonder (Topseed), purchased locally. The evaluated parameters were germination rate (TG) and root length (CR). The biological tests were performed according to Rodríguez et al. (2006), with modifications. The bioassays were conducted at room temperature for seven days, when observations and calculations of germination rate and root length were performed.

All experiments were performed in triplicate, both for the samples and for the controls. Data from all discoloration and phytotoxicity tests were submitted to analysis of variance (ANOVA) and the means were compared by the Scott-Knott test at 5% significance.

III. RESULTS AND DISCUSSION

The discoloration capacity of RBBR dye and textile effluent was tested by the three fungi cultures, *Lentinula edodes*, *Pleurotusostreatus* and *Pleurotuspulmonarius*. The results, after 15 days of treatment with the RBBR dye in the different values of pH and concentration and with the textile effluent, can be seen in Figs. 1I, 1II and 1III.

Lentinula edodes showed better discoloration in the treatment with RBBR dye at pH 5.0 and 0.5 gL-1 (Fig.1I) and *Pleurotusostreatus fungus*, in treatments at pH 5.0 and 0.5 gL-1 and in pH 9.0 and 0.5 gL-1 (Fig.1 II). However, the fungus *Pleurotuspulmonarius* showed a significant difference for all treatments with the dye (Fig.1III). Regarding the treatments with the textile effluent, there was no significant difference for any of the fungi tested.

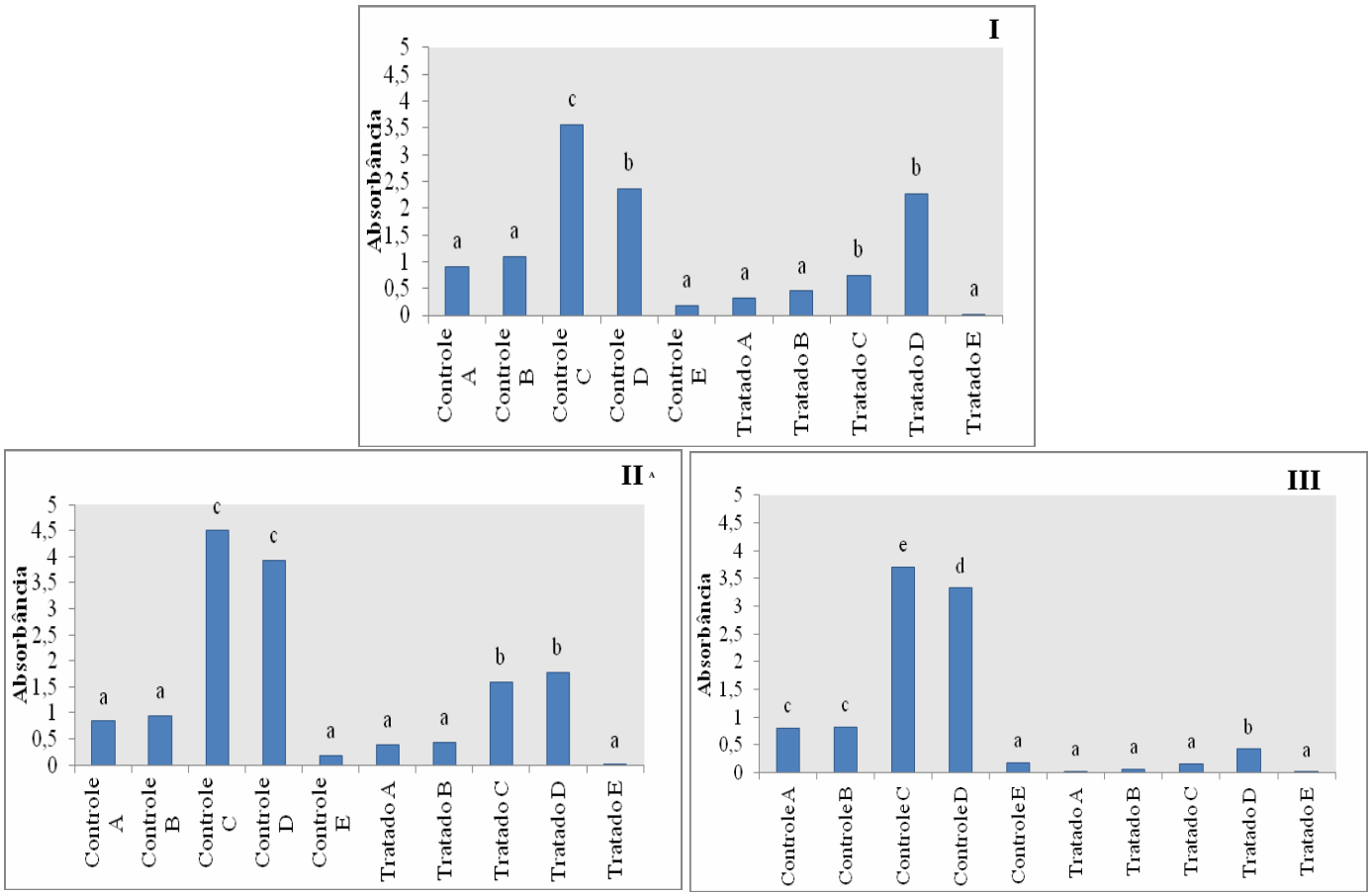

FIG. 1: Treatment with the fungi *Lentinula edodes* (I), *Pleurotusostreatus* (II) and *Pleurotuspulmonarius* (III). (A) RBBR dye at pH 5.0 and 0.1 gL-1; (B) RBBR dye at pH 9.0 and 0.1 gL-1; (C) RBBR dye at pH 5.0 and 0.5 gL-1; (D) RBBR dye at pH 9.0 and 0.5 gL-1; (E) textile effluent. In controls there was no presence of inoculum. CV = 56.45%. Means followed by the same letter do not differ by Scott-Knott test at 5% significance.

Some treatments using pH 9.0 were efficient in dye discoloration, however, most discolored better at pH 5.0. [9] also performed discoloration experiments of the RBBR dye in order to evaluate the most appropriate pH. They used a pH range between 3.0 and 7.0, with the best discoloration results being those at pH in the range of 4.0 and 5.0.

The discoloration activity of the RBBR dye is an indicative method of a multienzyme system and may become a useful tool when used in biodegradation studies of xenobiotics, as well as indicate the physiological conditions of the basidiomycete fungus during the bioremediation process [10].

Considering that discoloration is not always an indication of successful detoxification, toxicity tests with the dye and with the textile effluent before and after discoloration were performed.

There was a variation in the germination rate (%) in the presence of the RBBR dye after treatment. In some treatments, this rate decreased, but in the majority, the germination rate remained the same as the control (96.67%) (Table 1).

In the presence of the untreated and treated textile effluent a decrease of this rate was observed when compared to that of the control. When untreated and treated textile effluents were compared, no change in germination rate was observed (Table 2).

TABLE 1
COMPARISON OF GERMINATION RATE (%) AND ROOT LENGTH (cm) **OF *L. SATIVA* L., IN THE PRESENCE OF RBBR TEXTILE DYE**

Treatments	GR (%)	RL (cm)
Control	96,67 a	3,92 c
RBBR 0,1gL-1 pH5,0	90,00 a	3,21 d
RBBR 0,1gL-1 pH9,0	88,33 a	2,63 e
RBBR 0,5gL-1 pH5,0	96,67 a	3,50 d
RBBR 0,5gL-1 pH9,0	86,67 a	2,83 e
RBBR 0,1gL-1 pH5,0 and (*L. edodes*)	78,33 b	3,35 d
RBBR 0,1gL-1 pH9,0 and (*L. edodes*)	83,33 a	3,29 d
RBBR 0,5gL-1 pH5,0 and (*L. edodes*)	81,67 a	2,32 e
RBBR 0,5gL-1 pH9,0 and (L. edodes)	91,67 a	3,23 d
RBBR 0,1gL-1 pH5,0 and (*P. ostreatus*)	85,00 a	3,68 c
RBBR 0,1gL-1 pH9,0 and (*P. ostreatus*)	88,33 a	3,70 c
RBBR 0,5gL-1 pH5,0 and (*P. ostreatus*)	91,67 a	3,35 d
RBBR 0,5gL-1 pH9,0 and (*P. ostreatus*)	85,00 a	4,07 c
RBBR 0,1gL-1 pH5,0 and (*P. pulmonarius*)	63,33 b	3,97 c
RBBR 0,1gL-1 pH9,0 and (*P. pulmonarius*)	68,33 b	3,92 c
RBBR 0,5gL-1 pH5,0 and (*P. pulmonarius*)	65,00 b	4,09 c
RBBR 0,5gL-1 pH9,0 and (*P. pulmonarius*)	76,67 a	3,97 c

CV (germination rate) = 10,74%; CV (root length) = 8,07%. Means followed by the same letter in the columns do not differ by Scott-Knott's test at 5% significance.

TABLE 2
COMPARISON OF GERMINATION RATE (%) AND ROOT LENGTH (cm) OF *L. SATIVA* L. IN THE PRESENCE OF TEXTILE EFFLUENT

Treatments	GR (%)	RL (cm)
Control	96,67 a	3,92 c
Textile effluent not treated	66,67 b	5,05 a
Textile effluent and (*L. edodes*)	75,00 b	4,99 a
Textile effluent and(*P. ostreatus*)	75,00 b	4,46 b
Textile effluent and (*P. pulmonarius*)	73,33 b	4,49 b

CV (germination rate) = 10.74%; CV (root length) = 8.07%. Means followed by the same letter in the columns do not differ by Scott-Knott's test at 5% significance

As shown in Table 1, in some treatments with the dye, after discoloration by fungi, there was a decrease in seed germination, which can be attributed to the production of intermediate metabolites more toxic than the original dye, which makes it necessary to perform of more studies [11].

When analyzed the growth of the root (cm), this presented a great variation between the different treatments. In the presence of untreated dye, a decrease in root growth was observed when compared to control (3.92 cm) for all pH and concentration values (Table 1).

All treatments with the dye treated by the fungus *Pleurotuspulmonarius* showed an increase in root growth when compared to the untreated dye, demonstrating a decrease in toxicity to this parameter after treatment. In their studies with decolorization and detoxification of industrial reactive dyes [12], they found a significant reduction of toxicity after biological treatment.

In the treatments in the presence of untreated and treated textile effluent there was also a root growth promoter effect when compared to the control (Table 2).

There was also a relationship between lower germination rate and root length in treatments with the textile effluent (Table 2). This may have occurred because of the characteristic of plants competing for light, water and nutrients, revealing constant competition. This competition contributes to the survival of the species and, therefore, some have developed defense mechanisms, by which, during their growth, they release products of the secondary metabolism, thus preventing the germination and development of other plants relatively nearby, a process called allelopathy [13].

There are few ecotoxicological studies using dyes and industrial effluents before and after their treatment with fungi isolates, which is why the need for complementary studies to better understand this process (MALACHOVÁ et al., 2006 [14]).

IV. CONCLUSIONS

The fungus *Pleuriticpulmonarius* was able to decolorize the RBBR dye, both at pH 5.0 and at pH 9.0 and at the two concentrations used, 0.1 gL-1 and 0.5 gL-1;

Lentinula edodes obtained better discoloration in the treatment with the RBBR dye at pH 5.0 and 0.5 gL-1 and the fungus *Pleurotusostreatus*, in the treatments at pH 5.0 and 0.5 gL-1 and at pH 9.0 and 0.5 gL-1;

The dye showed a phytotoxic effect after the discoloration, reducing the germination rate in some treatments. However, after treatment with the fungus *Pleurotuspulmonarius*, it induced root growth, which did not present a toxic effect for this parameter;

The treatments with the textile effluent showed lower germination rates and, in contrast, higher promoter effect on root growth.

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Isolation and characterization of plant growth promoting bacteria (PGPB) from anaerobic digestate and their effect on common wheat (*Triticum aestivum*) seedling growth

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Abstract— *The use of anaerobic digestate as fertilizer is considered beneficial since it provides plant nutrients and organic matter to soils. However, there is limited information about plant growth promoting bacteria (PGPB) in digestate. In this study, we isolated Bacillus and Pseudomonas from two types of anaerobic digestates, and selected three different plant growth promoting traits and antifungal activity to screen 200 bacteria isolated from each digestate. Then 6 isolates based on plant growth promoting traits were selected and inoculated with common wheat seeds to evaluate their plant growth promoting activity. Cultivable population of Bacillus and Pseudomonas were 2.20 × 10^6 and 6.98 × 10^4 CFU g^{-1} dry matter in mesophilic digestate, while were 6.86 × 10^5 and 5.65 × 10^4 CFU g^{-1} dry matter in thermophilic digestate. Twenty-five bacterial isolates from mesophilic digestate and 12 bacterial isolates from thermophilic digestate showed positive plant growth promoting traits or antifungal activity. In plant growth promoting assay, all isolates significantly promoted growth of wheat seedlings ($p < 0.05$). Seedlings stem length was increased from 28.5% to 38.6% by bacteria inoculation. In addition, bacteria inoculation increased seedlings stem weight from 113.3% to 214.2% and root weight from 108.6% to 207.2% as compared to un-inoculated control. The results showed that anaerobic digestate was a potential source for isolation of PGPB, and PGPB in digestate would be beneficial for plant growth with fertilizer application.*

Keywords— Anaerobic digestate, Plant growth promoting bacteria (PGPB), Bacillus, Pseudomonas, Common wheat (Triticum aestivum).

I. INTRODUCTION

Anaerobic digestion of organic wastes produces biogas and a nutrient-rich digestate. Digestate contains partially-degraded organic matter, inorganic plant nutrients and microbial biomass, therefore it can be used as soil conditioner or fertilizer on agricultural field (Alburquerque et al., 2012). The use of digestate as a fertilizer is considered eco-friendly since it recycles plant nutrients in the organic waste and thus reduces large scale use of chemical fertilizers. Furthermore, plant nutrients are present in inorganic plant-available forms in digestate at a markedly higher level compared to undigested organic wastes, because of the mineralization of organic nutrients during anaerobic digestion process (Umetsu et al., 2002). Previous researches have documented the beneficial effects of digestate as organic fertilizer on plant growth and nutrients uptake, and soil structure and microbial activity (Muscolo et al., 2017; Risberg et al., 2017; Solé-Bundó et al., 2017; Tampio et al., 2016).

Plant growth promoting bacteria (PGPB) represent a wide variety of bacteria, which occupy the rhizosphere of many plant species and promote host plant growth directly by solubilizing minerals such as phosphorus, producing siderophores that chelate iron and producing phytohormones (Grobelak et al., 2015). Phosphorus (P) is one of the major macronutrients required for growth and development of plant. Generally, soils have large reserves of total P, but the amount available to plants is low as majority of soil P is found in insoluble forms (Ahemad and Kibret, 2014; Vessey, 2003). PGPB could make phosphorus available to plants by solubilizing and mineralizing inorganic and organic phosphorus in soils (Ahemad and Kibret, 2014). Iron is also an essential nutrient plant growth. However, iron exists mainly as Fe^{3+} in aerobic environment and is likely to form insoluble hydroxides and oxyhydroxides which are not unavailable to plants (Rajkumar et al., 2010). The siderophores, which are low-molecular mass iron chelators, secreted by some PGPB could solubilize iron from minerals or organic compounds under conditions of iron limitation to make iron accessible to plants (Indiragandhi et al., 2008). Indole-3-acetic acid (IAA) is the primary phytohormone produced by RGPB and has various effects on plant growth promotion such as cell division and elongation, stimulation of seed germination, and increase root development (Ahemad and Kibret, 2014). PGPB can also stimulate plant growth indirectly by suppressing phytopathogens in forms of producing antibiotics, siderophores, and fungal cell wall-lysing enzymes (Ji et al., 2014). The largest groups of PGPB are *Pseudomonas*, *Bacillus*, *Enterobacter*, and *Erwinia* (Grobelak et al., 2015). Majority of researched PGPB are isolated from rhizosphere and they are

generally known as plant growth promoting rhizobacteria (PGPR) (Khalid et al., 2004). However, anaerobic digestates are host to numerous PGPB and little attention has been focused on the isolation and characterization of PGPB from anaerobic digestate.

In the present study, two groups of PGPB: *Bacillus* and *Pseudomonas* isolated from two types of anaerobic digestate were screened on plant growth promoting traits including phosphate solubilization, siderophore production and phytohormone production, as well as antifungal activity. Selected bacterial isolates were further evaluated for their growth promoting activity on common wheat (*Triticum aestivum*).

II. MATERIAL AND METHOD

2.1 Anaerobic digestate samples collection

Anaerobic digestate samples were collected from two continuously stirred tank reactors (CSTR) (Yamashiro et al., 2013) operated at mesophilic (37°C) and thermophilic (55°C) temperatures. Mesophilic and thermophilic digesters were fed daily with dairy manure. To ensure homogeneity of samples, digesters were thoroughly stirred before digestate samples were collected. Mesophilic and thermophilic digestates collected from the digesters were thereafter referred to as MAD and TAD, respectively. Digestate samples were immediately kept at 4°C and isolation of bacteria was done within 24 h.

2.2 Isolation of bacteria from anaerobic digestate samples

Bacillus and *Pseudomonas* were isolated by the spread plate method. Samples were diluted 10-fold with phosphate buffered saline (pH 7.4), and 100 µl of diluent was spread on BD BBL™ MYP (BD Falcon™, Franklin Lakes, NJ, USA) plates to isolate *Bacillus*, and Difco™ Cetrimide Agar Base (Becton, Dickinson and Company, Sparks, MD, USA) plates to isolate *Pseudomonas*, respectively. After incubation, typical colonies were counted and calculated as colony forming units per gram of dry matter (CFU g^{-1} dry matter). Then one-hundred *Bacillus* isolates and one-hundred *Pseudomonas* isolates of each digesate sample were selected randomly and maintained on the LB agar plates for further analyses.

2.3 Screening of bacterial isolates for plant growth promoting traits and antifungal activity

Phosphate solubilization ability of bacterial isolates was determined with a Pikovskaya's agar plate (HiMedia Laboratories Ltd, Mumbai, India). Bacterial strains were spotted on Pikovskaya's agar plate and incubated at 28°C for 3 days. The isolates which produced a halo zone around the colony was determined as having ability to solubilize phosphate.

Chrome Azurol Sulphonate (CAS) assay was used to detect siderophore production of bacterial isolates. The CAS agar plate was made according to method described by Lakshmanan et al. (2015). Bacterial isolates were spotted on CAS agar and incubated at 28°C for 3 days. Formation of orange halo around the colonies confirmed the production of siderophore.

IAA (indole-3-acetic acid) production of bacterial isolates was determined according to the method previously described by Ji et al. (2014). Bacterial strains were inoculated into 5 ml LB broth with 0.1% (w/v) L-tryptophan and incubated on a rotary shaker at 150 rpm for 3 days at 30°C. The cultures were centrifuged at 10,000 rpm for 10 min at 4°C to obtain a supernatant. The supernatant (2 ml) was mixed with 4ml of Salkowski's reagent (2 ml 0.5 M $FeCl_3$ and 98 ml 35% perchloric acids) and incubated for 25-30 min in the dark at room temperature. The development of a pink color indicates IAA production, and optical density of mixtures was read at 530 nm with a spectrophotometer (NanoDrop2000c, Thermo Scientific). The concentrations of IAA produced per milliliter of culture (µg ml^{-1}) were estimated by comparison with a standard curve of IAA in the range of 0.5-100 µg ml^{-1}.

Antifungal activity of bacterial isolates was tested using the dual culture method with Potato Dextrose Agar (PDA, Becton, Dickinson and Company, Sparks, MD, USA). In this study, the fungal strain *Fusarium nivale* f. sp. g*raminicola* (MAFF 235153) purchased from National Institute of Agrobiological Sciences, Japan (NIAS; Tsukuba, Japan) was used. The fungal mycelia were inoculated in the center of a PDA agar plate and incubated for 24 h at 25°C followed by inoculation of the isolates 3 cm away from the center of the PDA plate. The fungal mycelium alone was inoculated as a control. After incubation at 28°C for 7 days, the antifungal activity was measured by the percent of inhibition of growth (PGI): PGI = (1 – R/Rc) × 100%, where R represents the radius of the fungal mycelia in the plate inoculated with bacteria isolates, and Rc represents the radius of the fungal mycelia in the control plate.

2.4 Identification of bacteria isolates

For identification of bacterial isolates, Bruker microflex mass spectrometer system (microflex LT/SH, Bruker Daltonics, Kanagawa, Japan) was used. Two methods, direct smear method and on-plate extraction method were used in this study. For

the former method, bacterial colony was directly smeared onto a spot on polished steel MALDI target plates using sterile toothpicks. Thin spots of bacteria were then dried in a safety cabinet, and subsequently overlaid with 1µl of the matrix solution, comprising a HCCA (α-Cyano-4-hydroxycinnamic acid) matrix (Bruker Daltonik) for 5 min. For the on-plate extraction method, an extraction step by 1µl of 70% formic acid (Wako Pure Chemical Industries, Osaka, Japan) was introduced before cocrystallization with the matrix. *Escherichia coli* (K-12, laboratory stock) was used as a positive and quality control, and formic acid and the matrix was used as negative control at each run. The Bacterial Test Standards (Bruker Daltonics) was used for instruments calibration with each run. The samples prepared by each method were subjected to the microflex mass spectrometer, and results were analyzed by MALDI Biotyper 3.0 software (Bruker Daltonics).

2.5 Plant growth promoting assay with common wheat (*Triticum aestivum*)

Plant growth promoting assay with common wheat was conducted as described by Grobelak et al. (2015). The seeds of common wheat (*Triticum aestivum*) were surface sterilized with 1.5% (v/v) sodium hypochlorite for 10 min and washed with sterile water for 3 times. Subsequently, sterilized seeds were planted in plastic pots filled with 100g of commercial soil which was sterilized by autoclave. Bacterial isolates were incubated in LB broth at 30°C for 3 days and 150 rpm in a rotary shaker. Then, the bacterial cultures were centrifuged at 6000 rpm for 10 min, cell pellets were suspended in sterile water and densities were adjusted to 1×10^8 CFU ml^{-1}. The bacterial suspensions were applied immediately after seeding with 1 ml pot^{-1}. Only sterile water was applied as control. Pots were maintained at room temperature (26-28°C) for 4 weeks with five replicates, and then stems and roots of the plants were weighed for biomass determination and length of the plants was also measured.

2.6 Statistical analysis

Results are expressed as mean values ± standard deviation. Data from plant growth promoting assay were statistically analyzed by analysis of variance (ANOVA) with treatment means separated by Tukey test at $p < 0.05$ using SAS Statistical Software version 9.4 (SAS Institute Inc., USA).

III. RESULTS AND DISCUSSION

3.1 Isolation and characterization of bacteria for plant growth promoting traits and antifungal activity

Cultivable population of *Bacillus* and *Pseudomonas* were 2.20×10^6 and 6.98×10^4 CFU g^{-1} dry matter in MAD, which were higher than 6.86×10^5 and 5.65×10^4 CFU g^{-1} dry matter in TAD. Then 100 *Bacillus* isolates and 100 *Pseudomonas* isolates were selected from each digestate sample and screened for plant growth promoting traits and antifungal activity. The results are presented in Table 1. Twelve *Bacillus* isolates (12%) from the MAD showed siderophores production and antifungal activity, in which 5 isolates also showed IAA production. Thirteen *Pseudomonas* isolates (13%) showed siderophores and IAA production, in which only one isolate showed phosphate solubilization. For *Bacillus* isolates from TAD, only 5 isolates (5%) were positive for plant growth promoting traits or antifungal activity, and 7 *Pseudomonas* isolates (7%) produced IAA in which 6 isolates also showed siderophores production.

It is known that anaerobic digestion process inactivates bacteria in feedstock due to many factors, such as reactor temperature, feedstock retention time, and digestate pH (Smith et al., 2005; Wagner et al., 2008). Thermophilic temperature causes greater inactivation of bacteria than mesophilic temperature (Iwasaki et al., 2011), which explains higher cultivable bacteria and percent of PGPB observed in MAD than in TAD.

TABLE 1
NUMBER OF BACTERIAL ISOLATES SHOWED PLANT GROWTH PROMOTING TRAITS AND ANTIFUNGAL ACTIVITY FROM ANAEROBIC DIGESTATES

Sample	Bacterial genus	Phosphate solubilization	Siderophores production	IAA production	Antifungal activity
MAD	*Bacillus*	0	12	5	12
	Pseudomonas	1	13	13	0
TAD	*Bacillus*	0	4	3	5
	Pseudomonas	0	6	7	0

MAD: Mesophilic anaerobic digestate; TAD: Thermophilic anaerobic digestate; IAA: Indole-3 acetic acid.

For plant growth promoting assay, 6 bacterial isolates were selected and their plant growth promoting traits and antifungal activity are presented in Table 2. Bacteria capable of phosphate solubilization are known to promote plant growth by increasing phosphorous uptake. The phosphate solubilizing isolate (MAD-21) was identified as *Pseudomonas putida*. Similarly, phosphate solubilizing ability of *Pseudomonas putida* has been reported in previous studies (Malboobi et al., 2009; Pandey et al., 2006). Fluorescent pseudomonads are considered to be one of the most promising groups of PGPB (Bhattacharyya and Jha, 2012). In this study, fluorescent pseudomonads isolate (MAD-17) showed siderophores production and IAA production of 17.3 μg ml^{-1}, similar plant growth promoting traits of fluorescent pseudomonads were reported by Saber et al. (2015).

TABLE 2
BACTERIAL ISOLATES SELECTED FOR PLANT GROWTH PROMOTING ASSAY

Bacterial isolate no.	Phosphates solubilization	Siderophores production	IAA production (μg ml^{-1})	Antifungal activity (PGI%)	Identification
MAD-05	-	+	1.06 ± 0.03	62.75 ± 2.45	*Bacillus subtilis*
MAD-17	-	+	17.3 ± 1.47	-	fluorescent pseudomonads
MAD-21	+	+	18.43 ± 1.0	-	*Pseudomonas putida*
TAD-05	-	-	11.7 ± 1.18	43.53 ± 2.35	*Bacillus licheniformis*
TAD-11	-	+	11.59 ± 0.28	-	*Pseudomonas* spp.
TAD-12	-	-	24.54 ± 1.24	-	*Pseudomonas aeruginosa*

MAD: Mesophilic anaerobic digestate; TAD: Thermophilic anaerobic digestate.
Phosphate solubilization (+); non phosphate solubilization (-). Siderophores production (+); non siderophores production (-). IAA: Indole-3 acetic acid; values are expressed as means ± standard deviation. PGI: percent of growth inhibition; values are expressed as means ± standard deviation; non growth inhibition (-).

The production of phytohorrnones by bacteria is one of the most important factors of plant growth promotion (Ahemad and Kibret, 2014). Khalid et al. (2004) have categorized IAA-producing bacteria into three principal groups: lower producers (1 to 10 μg ml^{-1}), medium producers (11 to 20 μg ml^{-1}) and higher producers (21 to 30 μg ml^{-1}). Among 6 isolates for plant growth promoting assay, MAD-05 (*Bacillus subtilis*) was lower IAA producer (1.06 μg ml^{-1}), and TAD-12 (*Pseudomonas aeruginosa*) produced highest amount of IAA (24.54 μg ml^{-1}), which was higher producer. The rest of isolates were medium producers (Table 2).

Biological control, or biocontrol means to control plant diseases by application of microorganisms, which is an environmental-friendly and efficient disease management approach (Ahemad and Kibret, 2014). In this study, *Bacillus* isolates (MAD-05, *Bacillus subtilis* and TAD-05, *Bacillus licheniformis*) showed high antifungal activity of 62.75% and 43.53% PGI, which may contribute to the competition for space and nutrients and secretion of antifungal compounds (Yang et al., 2015). Similarly, *Bacillus* species have been widely reported to have antifungal activity against a wide variety of phytopathogens (Ji et al., 2014; Kumar et al., 2012; Liu et al., 2016). Therefore, further application of *Bacillus* isolates as biocontrol agents could be expected.

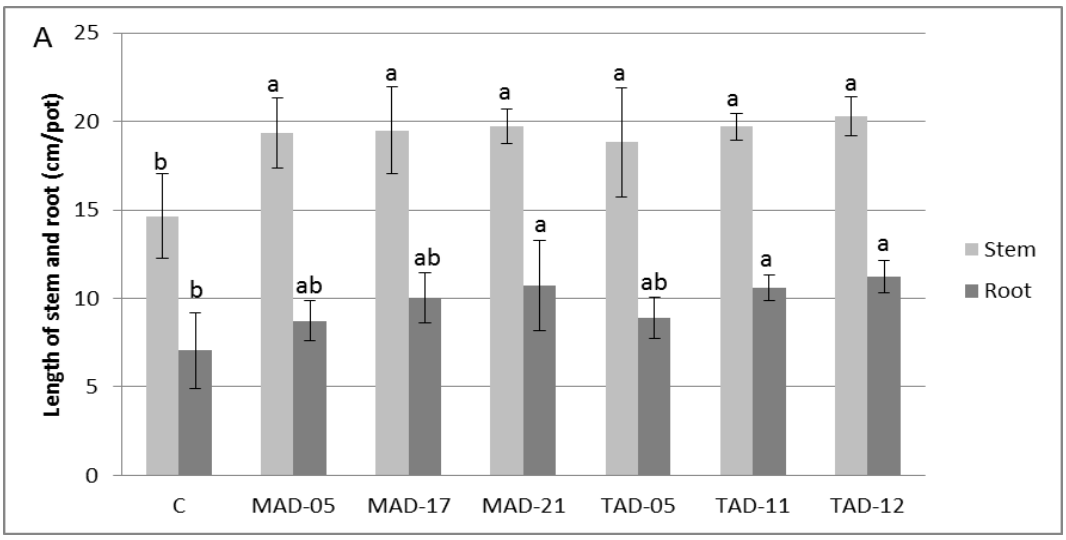
3.2 Effect of bacteria inoculation on plant growth of common wheat (*Triticum aestivum*)

The effects of selected bacterial isolates inoculation on plant growth were evaluated with common wheat (Fig. 1 and 2). Stem length of the seedlings inoculated with bacterial isolates (Fig. 2A) significantly increased from 28.5% to 38.6% compared to those of un-inoculated control ($p < 0.05$), and the differences between each treatments were non-significant ($p > 0.05$). Inoculation with MAD-21 (*Pseudomonas putida*), TAD-11 (*Pseudomonas* spp.) and TAD-12 (*Pseudomonas aeruginosa*) also significantly ($p < 0.05$) increased 51.8%, 50.1% and 59.21% of root length (Fig. 2A). The bacterial isolates inoculation further increased biomass of seedlings stem and root (Fig. 2B). Inoculation with TAD-12 (*Pseudomonas aeruginosa*) showed the highest increases in stem and root weight (214.2% and 207.2%, respectively) of the seedlings. After the TAD-12, other 5 bacterial isolates inoculation increased stem weight from 113.3% to 163.6%, and root weight from 108.6% to 160.1% compared to un-inoculated control ($p < 0.05$).

FIG.1. PLANT GROWTH PROMOTING ASSAY WITH COMMON WHEAT. C: UNTREATED CONTROL; MAD-05: *BACILLUS SUBTILIS*; MAD-17: FLUORESCENT PSEUDOMONADS; MAD-21: *PSEUDOMONAS PUTIDA*; TAD-05: *BACILLUS LICHENIFORMIS*; TAD-11: *PSEUDOMONAS* SPP.; TAD-21: *PSEUDOMONAS AERUGINOSA*.

The inoculation of plants with PGPB increased plants length of stem and root, these results were agreement with observation of Balseiro-Romero et al. (2017) and Grobelak et al. (2015). It is well-known that inoculation with IAA-producing bacteria increases plant growth by promoting root growth and length, resulting in greater root surface area which enables the plant to absorb more nutrients from soils (Vessey, 2003). Inoculation with TAD-12 (*Pseudomonas aeruginosa*) showed the highest promotion in stem and root weight, which can be related with the highest production of IAA observed in the isolates (Table 3). Similarly, several researches have demonstrated that *Bacillus* and *Pseudomonas* strains produced IAA and are able to regulate root development (Ji et al., 2014; Kumar et al., 2012; Scagliola et al., 2016; Son et al., 2014).

It has been suggested that the performance of PGPB could be enhanced through the use of PGPB mixtures, and Dary et al. (2010) and Malboobi et al. (2009) have demonstrated that inoculation with mixed PGPB can promote plant growth more than a single strain. Although the effects of mixed PGPB inoculant were not investigated in this study, it could be expected that digestate is an inoculant of PGPB mixtures and promote plant growth more effective than single bacterial strain inoculant.



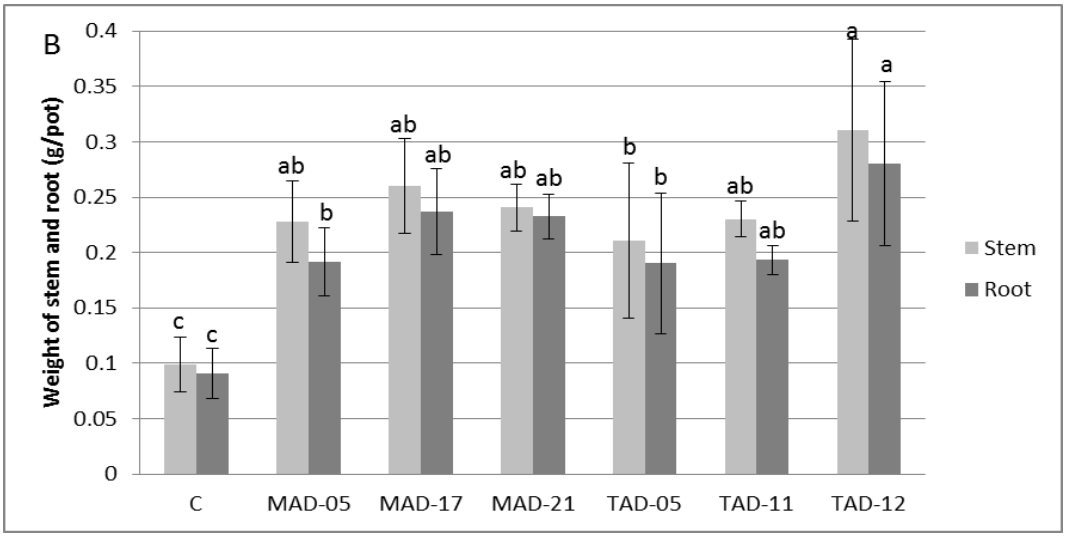


FIG.2. STEM AND ROOT LENGTH (A) AND WEIGHT (B) OF COMMON WHEAT SEEDLINGS INOCULATED WITH BACTERIAL ISOLATES IN PLANT GROWTH PROMOTING ASSAY. C: UNTREATED CONTROL; MAD-05: *BACILLUS SUBTILIS*; MAD-17: FLUORESCENT PSEUDOMONADS; MAD-21: *PSEUDOMONAS PUTIDA*; TAD-05: *BACILLUS LICHENIFORMIS*; TAD-11: *PSEUDOMONAS* SPP.; TAD-21: *PSEUDOMONAS AERUGINOSA*.

IV. CONCLUSION

In conclusion, anaerobic digestate is a large reservoir of bacteria capable of promoting plant growth. In this study, plant growth promoting *Bacillus* and *Pseudomonas* were isolated and characterized from mesophilic and thermophilic digestates. Two types of digesates contained different cultivable bacteria and percent of PGPB which may be attributed to the different operation temperature of digesters. Bacterial isolates showed plant growth promoting traits including phosphate solubilization, siderophores production and IAA production. The selected bacterial isolates significantly promoted plant growth, which is most probably due to their ability to produce IAA. These isolates can be applied as inoculants for improving plant growth. *Bacillus* isolates from digestates showed antifungal activity, therefore, it will be important to perform further studies investigating their antifungal activity in field experiments.

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Incidence and Level of Mistletoe Infestation in Tree Species at Botswana University of Agriculture and Natural Resources' Sebele Content Farm Campus, Botswana

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Abstract**—** *A survey was conducted at Botswana University of Agriculture and natural Resources at Sebele content farm to determine the intensity and level of mistletoe infestation in tree species. The intensity and the total number of trees per tree species infected by the parasitic weed were determined. Acacia eriobola, Acacia mellifera, Terminalia sericea, Ziziphus mucronata, Schinus molle, Acacia tortilis, Acacia erubesens and Acacia flekii hosted the highest number of mistletoe. Results revealed significant variation in level of mistletoe infestation between tree species. A 100% infestation was recorded in some tree species such as Acacia eriobola and Ziziphus mucronata. Severely infested indigenous tree species were dying, whereas most of the exotic tree species were either not infested or have very little number of mistletoe on them. The variation observed could be due to the fact that indigenous species the frequently visited by dispensers looking for food and shelter than exotic species. In addition, there is a possibility that the mistletoe species co-evolved with the indigenous tree species and the vector may be well established on the host tree species than on exotic species.*

Keywords**— Avian-dispersers, Botswana, Infestation, Mistletoe, Tree species.**

I. INTRODUCTION

Mistletoe is a name originally referring only to the European species *Viscum album*, but is now used as a general term for woody shoot parasites in several plant families especially Loranthaceae and Viscaceae which belong to the order Santalales. Loranthaceae (Parker and Riches, 1993). There are about 400 species in several genera of Viscaceae, of which at least five can be of economic importance (Barlow, 1993). In Botswana the common mistletoes are *Viscum album*, *Tapinanthus oleifolius*, *Plicosepalus kalachariensis* and *Erianthemum ngamicom* and all these species are well represented on Botswana University of Agriculture and natural Resources Sebele content farm where this study was conducted.

Mistletoes (excluding *Arceuthobium* spp.) are leafy and forms spherical, bush like growths reaching as far as 1.0 m and are particularly eye catching in winter when exposed by the loss of leaves of their host tree (Butin, 1995). The seeds are distributed by birds and germinate if they reach the bark of a suitable host plant, each initially forming a sticky adhesive disc at the tip of the radicle (Parker and Riches, 1993). Most species of Loranthaceae and Viscaceae have sound established photosynthetic capacity from an early stage and should not need to rely on the host as a source of carbon, however, completely dependent on the host for both water and minerals (Liddy, 1993). Mistletoes tend to grow on isolated trees, on the edge of the forest and in the higher branches of the trees (Parker and Riches, 1993;Buen *et al*., 2002).

Like other parasitic organisms, mistletoes show specialization on host species due to a number of factors. Some mistletoe species are specialized in living on different host due to frequent encounters between mistletoe seeds and commonest plants (Fadini, 2011) and in others, non-random perch preferences of seed dispensers play an important role in determining host specificity (Monteiro *et al.,* 1992). Host tree preference (Fadini, 2011) and host tree height (Rahmad *et al*., 2014) are also reported to influence the concentration of mistletoe seeds on a host.

It has been observed that several trees species were dying at Botswana University of Agriculture and Natural Resources, Sebele content farm and in different localities across the country because of mistletoe infection. However, there has never been a comprehensive attempt to establish an inventory of the tree host species of mistletoes and the level of mistletoe infestation in the country. The aim of the present study is therefore to establish a baseline inventory of the known host tree species and the level of susceptibility of each host tomistletoes. It is hoped that this paper will stimulate a systematic documentation of the host species of these parasitic plants and the level of susceptibility of each host species to mistletoe infestation.

II. MATERIALS AND METHODS

2.1 The Study area

The study was conducted at Botswana University of Agriculture and Natural Resources at Sebele content farm campus. The campus is located 24°33'S, 25°54'E, and 994 m above sea level (Bekker and De Wit, 1991). The climate in Sebele is semi-arid with average rainfall of 538 mm. Most rainfall occurs in summer, which generally starts in the late October and continues to March/April (Persaud *et al*., 1992). Prolonged dry spell during rainy seasons are common and rainfall tends to be localized.

2.2 Data collection

Both exotic and indigenous tree species (+20 cm stem diameter and +3 m plant height) on campus were identified to their species level and recorded. The abundance of each tree species was determined by counting the number of tree of each species. The number of infested trees for each species was also counted to determine the incidence and infestation percentage of each species. Mistletoe incidence per species was obtained by dividing the proportion of infested trees by the total number of trees of each host species in the campus, and this value was translated into a percentage. The level of infestation per species was determined by calculating the average number of mistletoe plants per tree.

2.3 Data analysis

Data collected was subjected to descriptive statistical analysis. Percentage of infested trees was calculated as a proportion of the total number of trees in each species.

III. RESULTS

A total of 243trees belonging to 26 species from 15 families of plants were recorded at Botswana University of Agriculture and Natural Resources, Sebele content farm campus (Table 1). Of these 17 species belonging to 10 families were indigenous to Africa and the remaining 9 species belonged to 6 families were exotic. Indigenous trees dominated in abundance as compared to the exotic constituting 72.4% of the tree stands in the campus. Individual tree species abundance in the campus varied from a minimum of a single tree to a maximum of 37 trees per species with an average 9.3 trees per species.

TABLE 1
COMMON NAME AND SCIENTIFIC CLASSIFICATION OF THE TREE SPECIES RECORDED IN BOTSWANA UNIVERSITY OF AGRICULTURE AND NATURAL RESOURCES, SEBELE CONTENT FARM CAMPUSDURING THE SURVEY OF MISTLETOE.

Common Name	Scientific Name	Family Name	Origin
Pepere/California pepper tree	*Schinus molle*	Anacardiaceae	Exotic
Morolwana/China berry	*Melia azedarach*	Meliaceae	Indigenous
Mosetlha	*Peltophorum africanum*	Caelsapiniaceae	Indigenous
Eucalyptus	*Eucalyptus globulus*	Myrtaceae	Exotic
Flamboyant	*Delonix regia*	Fabaceae	Exotic
Mosokaphala	*Bolusanthus speciosus*	Papilionoideae	Indigenous
Tipu tree	*Tipuana tipu*	Fabaceae	Exotic
Morula	*Sclerocarya birrea*	Anacardiaceae	Indigenous
Mongana	*Acacia mellifera*	Mimosaceae	Indigenous
Mogonono	*Terminalia sericea*	Combretaceae	Indigenous
Mohudiri	*Combretum apiculantum*	Combretaceae	Indigenous
Mosu	*Acacia tortilis*	Mimosaceae	Indigenous
Mokgalo	*Ziziphus mucronata*	Rubiaceae	Indigenous
Mothono	*Maytenus senegalensis*	Celastraceae	Indigenous
Motlopi	*Boscia albutrunca*	Caparaceae	Indigenous
Morojwa	*Azanza garkeana*	Malvaceae	Indigenous
Moloto	*Acacia erubesens*	Mimosaceae	Indigenous
Mohahu	*Acacia flekii*	Mimosaceae	Indigenous
Moduba	*Combretum zeyheri*	Combretaceae	Indigenous
Motswiri	*Combretum imberbe*	Combretaceae	Indigenous
Mogotlho	*Acacia eriobola*	Mimosaceae	Indigenous
Brazilian pepper tree	*Schinus terebinthifolius*	Anacardiaceae	Exotic
Australian fig	*Ficus australis*	Moraceae	Exotic
Jacaranda	*Jacaranda mimosifolia*	Bignoniaceae	Exotic
Silver oak	*Grevillea robusta*	Proteaceae	Exotic
Weeping bottle bush	*Callistemon viminalis*	Myrtaceae	Exotic

The tree species infested by mistletoes, the incidence of mistletoe infestation per tree species and the percentage incidence of mistletoe in host tree species in the campus are indicated in Table 2. Of the 26 tree species recorded in the campus 15 tree species, 11 indigenous and 4 exotic, were found infested by mistletoe parasitic weed. Abundance and percentage incidence varied across host tree species (Table 2).The two rare host tree species in the campus, *Ziziphus mucronata* and *Acacia eriobola* were found to be the highly preferred hosts registering the highest level (100%) of incidence (Table 2). *Acacia erubesens* and *Terminalia sericea*, the two dominant tree species in the campus, were the other preferred tree species with a percent incidence of 78.3 and 88.2 respectively. The least preferred host tree species was *Combretum apiculatum* with 18.8% (Table 2). In total, 108 out of 182 of the host trees (59.3%) in the campus were infested by mistletoe. The highest percentage incidence recorded on an exotic tree species was on *Schinus molle* (77.8%) and the lowest was 25% on *Jacaranda mimosifolia* (Table 2).

TABLE 2
INCIDENCE OF MISTLETOE IN HOST TREE SPECIES IN RECORDED IN BOTSWANA UNIVERSITY OF AGRICULTURE AND NATURAL RESOURCES, SEBELE CONTENT FARM CAMPUS

Tree species	Total number of trees per species	Number of infested trees per species	Percentage incidence in infested tree species	Incidence as percentage of the total host trees
Acacia erubesens	37	29	78.3	15.9
Terminalia sericea	34	30	88.2	16.5
Acacia tortilis	25	12	48.0	6.6
Combretum apiculatum	16	3	18.8	1.6
Melia azedarach	12	3	25.0	1.6
Acacia mellifera	9	6	66.7	3.3
Schinus molle	9	7	77.8	3.8
Acacia flekii	8	5	62.5	2.7
Jacaranda mimosifolia	8	2	25.0	1.1
Delonix regia	6	3	50.0	1.6
Tipuana tipu	6	2	33.3	1.1
Sclerocarya birrea	6	2	33.3	1.1
Combretum zeyheri	3	1	33.3	0.5
Ziziphus mucronata	2	2	100	1.1
Acacia eriobola	1	1	100	0.5

The number of mistletoe plants on a host tree varied from a maximum of 57 on *Terminalia sericea* to a minimum of one mistletoe per host tree (Table 3). Some of the host trees were free of the parasitic weed. Of the three *Combretum zeyheri* in the campus only one was infested with single mistletoe. The highest average mistletoe per tree species of 19% was recorded in *Acacia eriobola* (Table 3). The number of mistletoe plants on exotic tree species was generally low with the maximum average mistletoe per tree of 7% registered in *Schinus molle* (Table 3).

TABLE 3
LEVEL OF MISTLETOE INFESTATION IN TREE SPECIES AT BOTSWANA UNIVERSITY OF AGRICULTURE AND NATURAL RESOURCES, SEBELE CONTENT FARM CAMPUS DURING THE SURVEY OF MISTLETOE

Tree species	Average number of mistletoe per tree	Range of mistletoe per plant
Acacia eriobola	19.0	19
Acacia mellifera	14.0	1-48
Terminalia sericea	12.35	1-57
Ziziphus mucronata	7.0	5-9
Schinus molle	7.0	1-29
Acacia tortilis	6.32	1-32
Acacia erubesens	5.92	1-27
Acacia flekii	5.63	2-22
Melia azedarach	4.58	5-45
Delonix regia	1.33	1-4
Jacaranda mimosifolia	1.25	4-6
Combretum apiculatum	0.75	1-7
Sclerocarya birrea	0.5	1-2
Tipuana tipu	0.33	0-1
Combretum zeyheri	0.33	0-1

The number of trees infested and level of mistletoe infestation on indigenous tree species was very high. *Acacia eriobola*, *Acacia mellifera* and *Terminalia sericea* wereheavily infested with the parasitic (Figure 1) and some of trees in these species were killed due to heavy mistletoe infestation (Figure 2).

FIGURE 1: MISTLETOE ON ACACIA TREE AT BUAN CAMPUS. FIFTEEN MISTLETOE PLANTS WERE IDENTIFIED ON THIS TREE

FIGURE 2: *TERMINALIA SERICEA* TREE KILLED BY MISTLETOE PARASITIC PLANTS AT BUAN CAMPUS

IV. DISCUSSION

This study catalogues the diversity of plant species which can be parasitized by mistletoe and the variation in the level of mistletoe infestation among host tree species. The compilation of the current lists will serve as a baseline data for future study on host-specificity and host- parasite-disperser interaction. This will be a significant contribution to our understanding of the interactions between parasitic angiosperms and their host species that trails behind that of other plant symbiotic associations (Musselman and Press, 1995).Host plants documented were mostly dicotyledonous angiosperms with the genus *Acacia* contributing most of the host species recorded. Several acacia tree species were reported to be preferred hosts of mistletoe parasitic weeds (Downey, 1998). Acacia tree species are known to have a wide, big and open canopy. The branches spread out at the top with bi-pinnate leaves to form this huge canopy. As observed in this survey, almost all Acacia tree species were infested by mistletoe and had the highest intensity of mistletoe than all the other species. These results are in agreement with Lopez de Buen and Ornelas (1999) and Buen *et al*.(2002), who stated that the survival of mistletoe seedlings on its host species is affected by the canopy opening above the branch, and better seedling survival on host trees with more open canopies of Acacia species. Norton and Reid (1997) and Rahmad *et al*. (2014) also reported that host compatibility and habitat are important regulators of mistletoe abundance and distribution. Other factors such as host resistance, seedling establishment, changes in the surrounding environment and individual tree factors such as branch size, age and height, and seed predation also influence the abundance and distribution of mistletoe (Lopez de Buen and Ornelas, 1999; Buen *et al*., 2002). This is in line with our result that shows higher intensity of mistletoe infestation on the Acacia tree species.

Terminalia sericea was the other preferred host tree species that was highly infested by mistletoes. Some of the *Terminalia sericea* trees were already dying due to high intensity of mistletoe. The trees produced considerable witches broom and some of the mistletoe brooms were drying or already dried because of higher mistletoe infestation that resulted in the death of the host plant. Resources available to parasitic plant are often dependent on resources available to hosts. If a resource is limiting to parasites because it is limiting to hosts, supplementing that resource for hosts should affect parasites (Spurrier and Smith, 2005). According to Fisher (1993) water potential gradient between the host and mistletoe provides the mechanism by which water and dissolved solutes flow towards the parasite. The gradient is maintained through a combination of high parasite transpiration rates and high resistance in the hydraulic pathway between the host and parasite, especially at the haustorium interface (Whittington and Sinclair, 1988; Davidson and Pate, 1992). A significant negative relationship between mistletoe volume and host survival was also observed in this study. Tennakoon and Pate (1996) found that mistletoe infestation cause gradual death of the host branch tissue distal to the point of mistletoe attachment and increased growth of branches proximal to the mistletoe, relative to the branches supporting a similar area of host foliage. In another similar research by Ehleringer *et al*. (1986), mistletoe infection reported to cause declines in leaf water potential, stomatal conductance, photosynthesis and leaf nitrogen content in its host.

One of the interesting observations in this study was the absence of mistletoes in most of the exotic tree species and some indigenous trees. The host plant preference by the parasitic organisms observed in this study due to a number of factors.

Some mistletoe species are specialized in living on different host due to frequent encounters between mistletoe seeds and commonest plants (Fadini, 2011). Mistletoe seeds in the campus may have a better and easy access to acacia and Terminalia tree species which are the dominant ones in the area dominant ones in the area (Rahmad *et al*., 2014). The height and canopy of tree species in these two genera may also contributed to the preference and high level of infestation observed. These are some of the tree species which usually grow tall and dense with good nesting structures and fruits so are mostly preferred by birds which are known to disperse mistletoe seeds. According to Overton (1994), tall trees could attract more seed dispersal birds, and therefore receive more mistletoe seeds.

V. CONCLUSION

The study identified that indigenous tree species are preferred hosts of mistletoe and carry higher level of infestation compared to exotic species. The frequency of infection and density of mistletoe in each host results from interspecific differences in growth habit and abundance. The process of colonization of a tree likely depends on visit by dispersers, mistletoe seed deposition and establishment. Although the study touched upon only two factors that impinge on parasitism frequency, it highlighted a significant aspect of the interaction between mistletoes, hosts, and avian-seed dispersers. Further detailed research on the tri-trophic interaction and the effect on the ecosystem is recommended.

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Possible New Species of Araecerus (Coleoptera: Anthribidae) associated with *Mastixiodendronpachyclados* (Rubiaceae) of Papua New Guinea

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***Abstract*—** *Araecerus is genus of beetles of the Anthribidae family which are important economic pests of various crops including coffee (Rubiaceae), with A.fasciculatus (Degeer) being the common pest (weevil) of coffee beans. This paper presents a study in which five undescribed species of genus Araecerus were reared predominantly from the seeds of M.pachyclados (Rubiceae), a native tree of Papua New Guinea (PNG). Fruits of M. pachyclados were regularly sampled and insects attacking them were reared, preserved and identified. Fruits were hand collected, photographed, weighed and reared. Insects emerging from the fruits were captured and preserved in 99% ethanol. All the specimens were identified into morphospecies at the laboratory. The five new species discovered were designated as A. sp.1, A. sp.2, A. sp.3, A. sp.4 and A.sp.5. This was accorded based on differences in body length; scutellum color, size, hair-scales and visibility; length of first and second segments of fore tarsus; apical and subapical teeth-size (mandible and maxillary palpi); declivity of dorsal abdomen; basal-anterior eye markings; lateral eye markings; absence of eye markings; and shape of pygidium. We discovered A. sp.1 has yellowish gold marking inside the base of the eye, A. sp.2 with pygidium almost vertically-flat at abdominal apex, A. sp.3 has eyes without yellowish gold marking and generally dark in color, A. sp.4 with distinct yellowish gold interior-lateral marking in its eye, and A. sp.5 with pygidium pointed at abdominal apex.*

Keywords*— *Araecerus, coleoptera, anthribidae, Mastixiodendronpachyclados, PNG.

I. INTRODUCTION

Araecerus is a genus of beetles belonging to the family Anthribidae. They are important storage pests of cash crops such as coffee (Rubiaceae) and cocoa (Malvaceae). The species *A. fasciculatus* (DeGeer) is a common storage pest of coffee beans (coffee bean weevil) (Barrera 2008). *A. fasciculatus*is also a common pest infesting cocoa beans in storage (Eduku2014). Duke (1993) mentioned that *A. fasciculatus* was one of the pest of Nutmeg. *A. fasciculatus* was reported by Abo and Ja (2014) as major pests of yam and cassava flour. *Araeceruslevipennis* Jordan is a pest of leguminous plant *Leucaenaglauca* (L.) that has high protein content that is mainly used as a valuable foraging crop in ranches (Sherman and Tamashiro1955).

Early entomologists like Pascoe (1860) and Jordan (1894) extensively described about one hundred genera and one thousand species under Anthribidae family in which *A.*Schönherr, (Valentine 2005) was categorized under Subfamily Choraginae and Tribe *Araecerini*. Choraginae subfamily consisted of 3 species in South America in comparison with 14 species in Central America (Blackwelder1947). Jordan (1907) described ten species while Valentine (1999; 2002) contributed to the revision of the species. At least 98 species of Choraginae existed in the Old World (Mermudes and Lesche, 2014). The first species of the tribe Araecerini is *A.fasciculatus* (DeGeer1775), a pluralistic species of the genus *A.*Schoenherr, 1823 that comprises of about 70 described species in Indo Pacific (Mermudes2015).

Ctvrtecka *et. al.* (2014) obtained 1200 specimens of Anthribidae as a part of Curculionoidea in the same location where our research was carried out, but they could not be reliably identified to species. Seven species were found but had to be excluded also because most of the specimens remain unsorted (Ctvrtecka *et. al.*2014). Morimoto (1972) described *A.Schoenherr* as having tarsal segment 3 bilobed, nearly as broad as segment 2; tarsi slender, segments 1 and 2 much longer than wide; front tibiae simple at least in female; front tarsi normal; lateral prothoracic carinae reaching the middle; front tarsal segment 1 longer than the remaining segments added together; and eyes oval, less prominent.

This study was carried out to discover host-specific new species of *Araecerus* associated with *M.pachyclados* (Rubiaceae) using its "fallen fruits".

II. MATERIALS AND METHODS

2.1 Field Sampling

The study was conducted in relatively a primary forest at Wanang Conservation Area (5°13 ′S, 145°04 ′E), Madang, PNG between 15th January to 31st May, 2015. The vegetation of the study area is of mix evergreen rainforest on latosol with a humid climate (Laidlaw *et al.*2007; Paijmans 1976; Whitfeld *et. al.*2012). The annual rainfall is 3600 mm, and experiences a mild dry season from July to September, with annual temperature of 26°C (McAlpine *et. al*. 1983; Ctvrtecka*et al.*2014).

Mastixiodendronpachyclados is a locally abundant fruit bearing tree species, therefore was selected for this study. Fruits were collected (sampled) systematically following rows from within an existing 50 ha forest dynamics plant plot and few were sampled outside the plot. Sampling was carried out in different areas of the forest, including areas of both low and high abundance of *M. pachyclados*. The densities of fruits on the ground were measured and the sampling covered areas of both high and low density of fruits. Fruits of each tree species were separately placed into plastic bags and given an unique tree number code for identification. A fruit from each tree was sliced in half, and photographed together with unsliced fruits.

The fruits were then separated into plastic rearing containers (plastic boxes) and weighed on an electronic balance. Three fruits from each tree were sliced and measured (length, width, height, seed length, seed width and seed height). The rearing containers were closely monitored on a daily basis for hatched insects. The insects which hatched were collected by opening the side of the plastic lid, put in a pre-labeled test tube and preserved in 99% ethanol.

2.2 Identification

All the wet specimens were taken to New Guinea Binatang Research Center (NGBRC) in Madang, PNG to be identified. Identification was carried out with the aid of reference text books, online insect databases (www.buglife.com), and insect database and reference collections of NGBRC. The specimens were initially sorted into morpho-species and given codes based on distinct morphological features. Identification was done to genus level and species were divided into *A.* sp.1, *A.* sp.2, *A.* sp.3, *A.* sp.4 and *A.*sp.5, according to their differences in morphology.

The morphological features used for identification of the 'possible new species' were: body length, tarsal segment, eye markings, apical teeth, scutellum and pygidium. Descriptions of these distinctive morphological features were analyzed using Dichotomous Key System.

III. RESULTS

The identification keys of each species and pictures of their specific body parts are presented below.

- Length of body equal to or more than 4mm (Figure 1a) and length of body equal to or less than 4mm (Figure 1b)

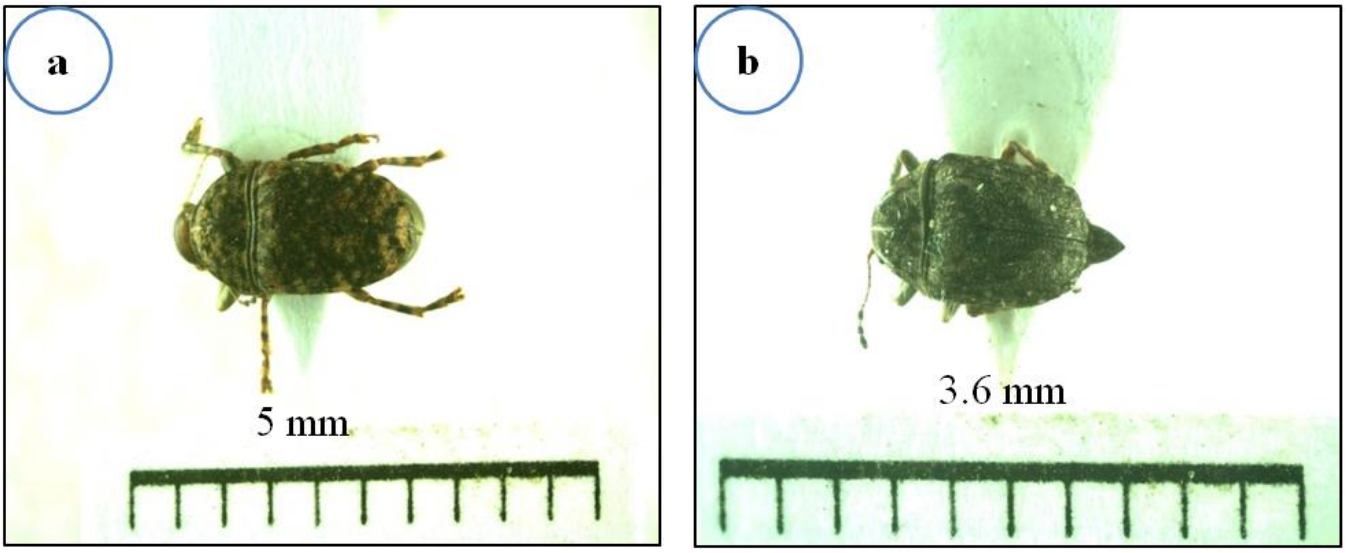

FIG. 1.BODY LENGTH OF THE SPECIES

- Scutellum very small, poorly visible or dark in color (Figure 2a) and scutellum small and densely covered with yellowish brown hair-like scales (Figure 2b).

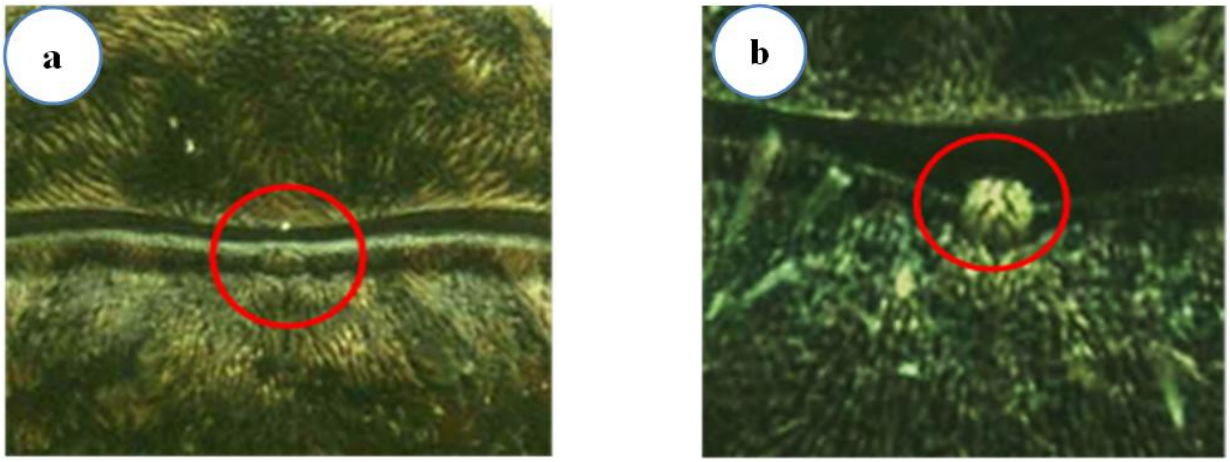

FIG.2.SCUTELLUM SIZE AND COLOR OF THE SPECIES

- First segment of fore tarsus longer than second segment (Figure 3a) and first segment of fore tarsus equal to or shorter than second segment (Figure 3b).

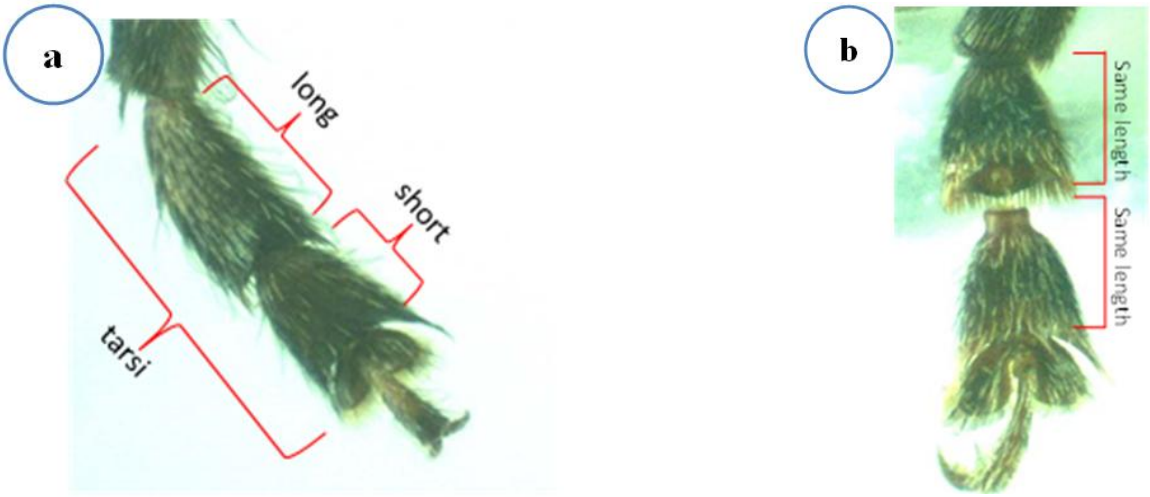

FIG.3.FORE AND SECOND SEGMENT OF TARSUS

- Unequal-sized apical and subapical teeth (mandible and maxillary palpi) (Figure 4a) and almost equal-sized apical subapical teeth (mandible and maxillary palpi) (Figure 4b).

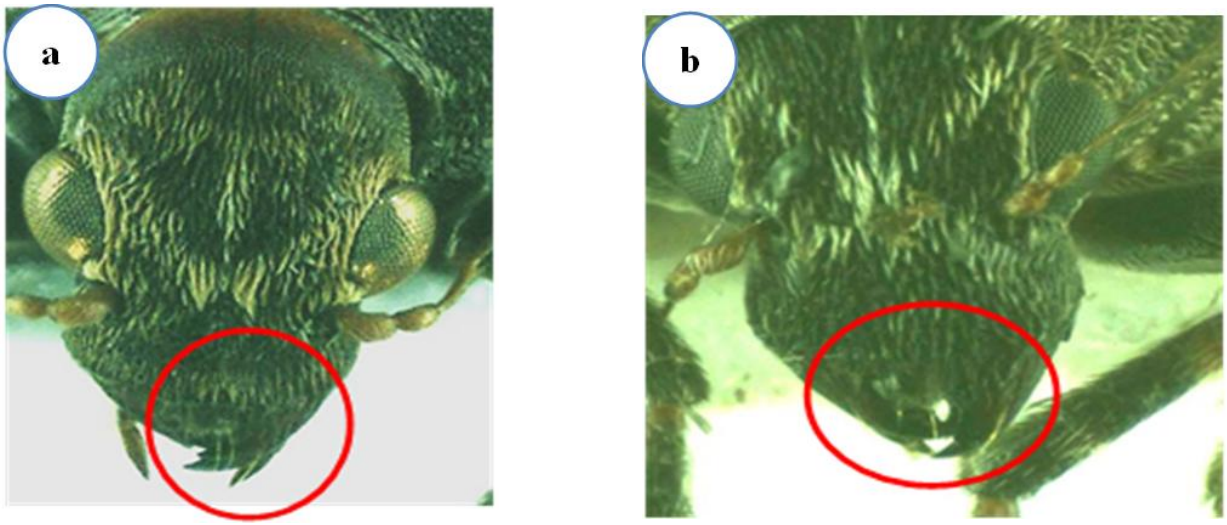

FIG.4.MANDIBLE AND MAXILLARY PALPI OF THE SPECIES

- High declivity towards the abdominal apex (Figure 5a) and low declivity towards the abdominal apex (Figure 5b).

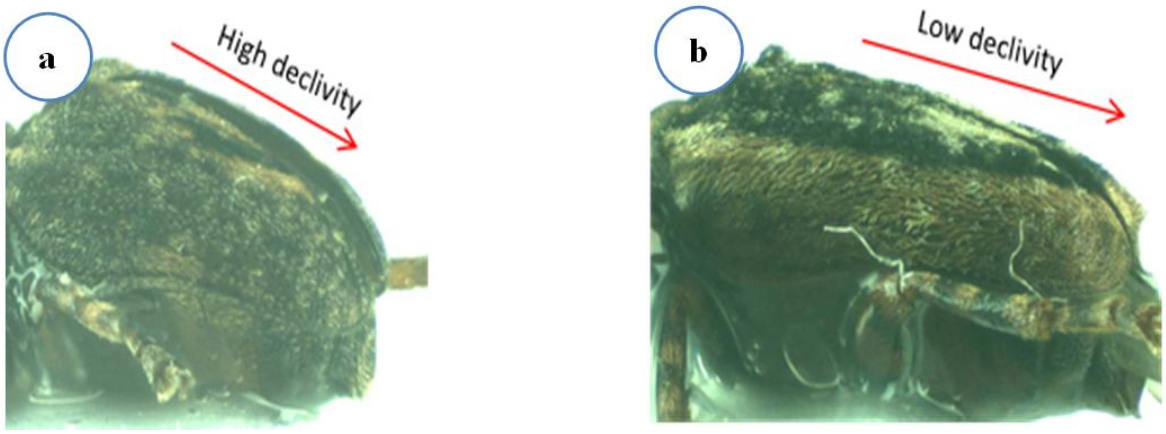

FIG.5. DECLIVITY OF THE SPECIES

- Yellowish gold marking inside the base of eye on A. sp. 1(Figure 6a) and eyes without yellowish gold marking inside the base of eye (Figure 6b).

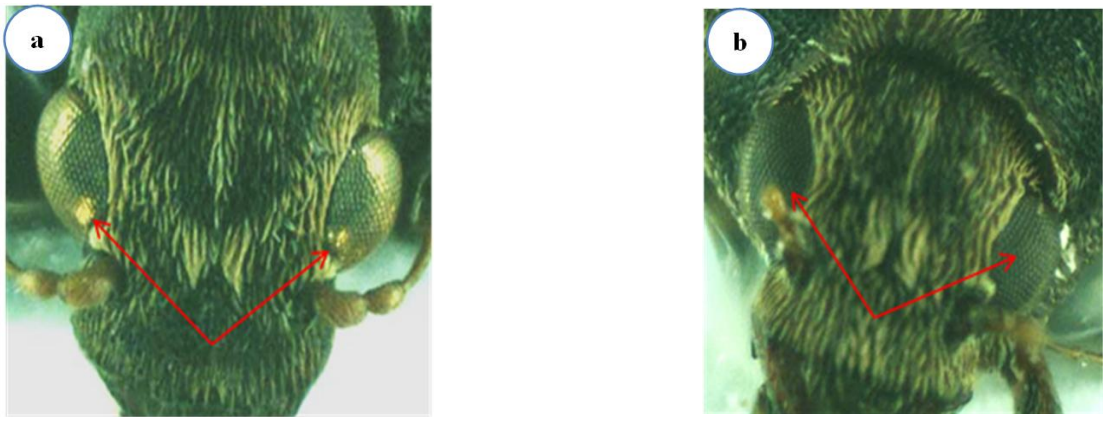

FIG. 6.COLORATION INSIDE THE BASES (6A) SPECIES 1 AND (6B) SPECIES

- Pygidium pointed at abdominal apex in A. sp. 5 (Figure 7a) and pygidium almost vertically-flat at abdominal apex in A. sp. 2 (Figure 7b).

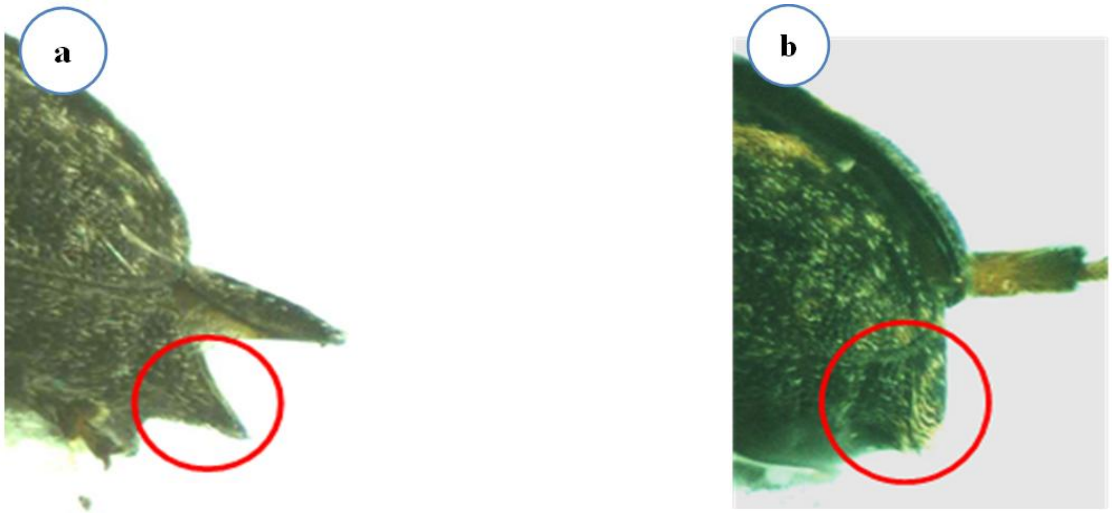

FIG.7.PYGIDIUM ORIENTATION (7A) SPECIES 5 AND (7B) SPECIES 2

- Eye with yellowish gold interior-lateral marking in A.sp. 4 (Figure 8a) and eye without marking and generally dark in color of A.sp. 3 (Figure 8b).

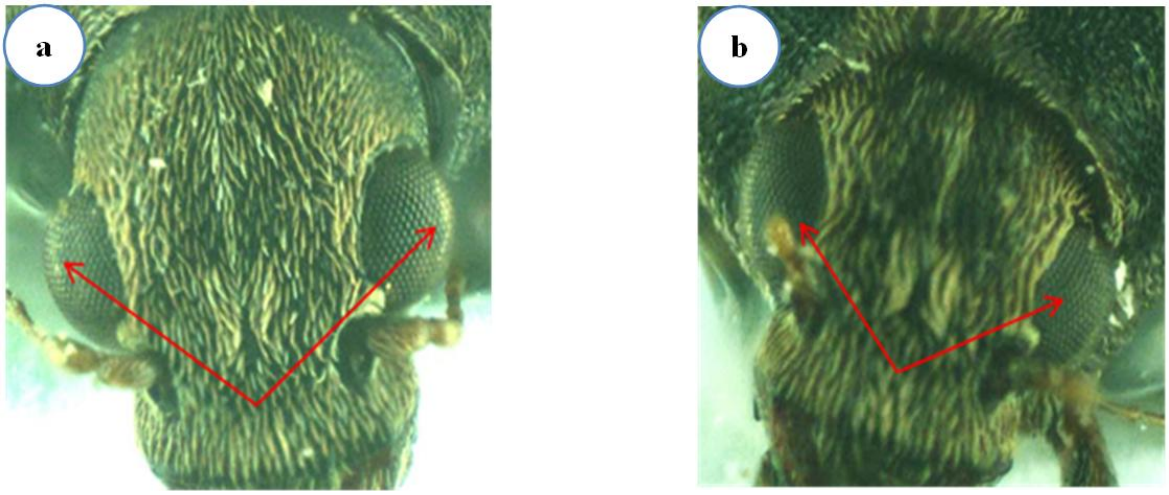

FIG.8.INTERIOR LATERAL MARKING (8A) SPECIES 4 AND (8B) SPECIES 3

IV. DISCUSSION

Araecerus belonging to the Anthribidae family has the prominent club shaped antenna; adults are elongated and slightly oval; have a short beak and straight antennae; elytra with distinct rows of striae absent; and pronotum with a transverse ridge towards the base. As per Holloway (1982), the genus *Araecerus* has (i) second segment of tarsi forming lobes around the third segment; (ii) elytra with areas of differently colored scale-like hairs; (iii) antenna attached on the top of the head; (iv) base of antennal segment and groove is visible from above; (v) first segment of the tarsi is three times longer than wide; (vi) pronotum without a transverse ridge but with the base itself slightly raised; and (vii) base of pronotum gently curved with mid part converging towards scutellum.

The five species identified differ from their close relative *A. fasciculatus* by certain morphological features. *A. fasciculatus* possess antennal club, and the entire eye and the elytra without distinct striation (Bright 1993). Body length of an adult beetle is about 3–5 mm (El-sayed 1935; Bright 1993; Robinson 2005). All the five species identified fall in the same body length range; *A.*sp.1 (5mm), *A.* sp.2 (3.8 mm), *A.* sp.3 (4mm), *A.* sp.4 (4.5mm) and *A.*sp.5 (3.6mm). In comparison to the five species, *A. fasciculatus* has paler elytra with hairs denser and irregular so that there are darker and lighter areas and the first segment of the front tarsi is as long as the rest of the segments combined (including the claws). The five species have dense hairs, regular markings on the elytra and first segment of the front tarsi shorter than the rest of the segments combined (including the claws).

Interestingly, all species identified are host-specific to *M.pachyclados* and predominantly reared from seeds rather than the mesocarp. Since coffee (*Coffeaarabica & C. robusta*) belongs to the same family as *M. pachyclados*, this unique feeding guild thus categorizes the five "new possible species"as seed feeders. Species under *Araecerus*,e.g. *A.*fasciculatus, mostly are reported as seed feeds of important crops such as coffee (Barrera 2008), cocoa (Eduku 2014), nutmeg (Duke 1993), leguminous plant *Leucaenaglauca* L.) (Sherman and Tamashiro 1955) and on pesticide plant, *Meliaazedarach* L. (Ardakani and Nasserzadeh 2014). These indicate that the outcome of this study has implications for management of the species identified.

The fact that the insects were reared from *M.pachyclados* in a tropical rainforest habitat demonstrates wide host range of *Araecerus* beetles, and a range of forest trees as alternate host for breeding, oviposition, migration and food resources. We propose additional studies on molecular characterization and analysis for confirmation of these species.

V. CONCLUSION

The genus *Araecerus* is a major pest of important cash crops (e.g. coffee and cocoa) and identification of new pests and quantification of their abundance are prerequisites to crop protection. It is known now that *A. fasciculatus* was found feeding on pesticide plant *Meliaazedarach* L., which may support their ability to tolerate application of botanical pesticides. In such

case, more suitable and effective control measures should be used to overcome such problems. Since these five species have not been described, it is presumed that they are possibly new species. Their morphological descriptions can therefore be used for nomenclatural system, and thus contributes to overall insect taxonomy.

ACKNOWLEDGEMENT

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Short Term Effects of Dietary Boron on Mineral Status in Dairy Cows*

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Abstract— *Aim of this study was to obtain knowledge on boron supplemented diet to mineral status of body fluids and feces in short term nutrition of dairy cattle. A total of 24 healthy Holstein dairy cows were used. The animals were fed with standard ration, and boron at three different doses was added to experimental' diets as boron compound: borax, for 10 days. Boron and other macro and trace minerals were determined in serum, milk, urine and feces samples taken on 0 and 11st days. In this study, there were no overt signs of toxicosis, and a pivotal knowledge was obtained in dairy cattle fed with boron supplemented diet on boron absorption, excretion, and its interaction with other minerals. Boron could not completely absorb from gastrointestinal tract. Urine was the most important excretion way of boron. More less boron was also eliminated by milk. Boron levels in body fluids (serum and milk, p<0.000) were increasingly changed based on the dose. Boron, among minerals, provided a striking increase for Ca (p<0.003) and Mg (p<0.028) levels in serum by increasing absorption of these minerals. This topic is worth evaluating as an alternative approach in the prevention of hypocalcemia in transition cows.*

Keywords— Boron, Macro minerals, Micro minerals, Cattle.

I. INTRODUCTION

Today, most nutritionists do not consider a trace element essential unless it has a defined biochemical function in higher animals or humans (Nielsen, 2014a). Trace minerals have critical roles in the key interrelated systems of immune function, oxidative metabolism, and energy metabolism in ruminants. To date, the primary trace elements of interest in diets for dairy cattle have included Zn, Cu, Mn, and Se although data also support potentially important roles of Cr, Co, and Fe in diets (Overton and Yasui, 2014). In vitro, animal, and human experiments have shown that boron is a bioactive element in nutritional amounts whereas boron has received little attention in ruminant nutrition (Nielsen, 2014b; Fry *et al.*, 2011). Boron appears to be of relatively low toxicity to animals. As in previous studies (Owen, 1944; Green and Weeth, 1977), inorganic boron is of low toxicity to cattle. There are many studies on energy, mineral and vitamin supplementation in transition cows in order to prevent production diseases. The objective of the study presented was to determine how dietary boron at different doses affects the distribution of macro and trace minerals in body fluids of dairy cattle.

II. MATERIAL AND METHOD

The experimental design was approved by the Committee on Use of Animals in Research of the Selcuk University, Faculty of Veterinary Medicine. A total of 24 pregnant, multiparous Holstein cows in late lactation were enrolled; mean age was 3.5 years, mean 305-day milk production was 6,500 kg, and mean body weight was 650 kg at the start of the experiment. Cows were fed individually and had free access to tap water.

The cows were randomly allocated to 4 groups:1 control and 3 experimental, each 6 animals which were offered drinking water (0.032 mg B/L) and a basal diet (0.55 mg B/kg). Ingredient and daily consumption ratios were corn silage (12 kg), sugar beet pulp (10 kg), wheat straw (4.5 kg) and concentrate (8.5 kg). The concentrate consisted of 35% barley, 19.85% wheat, 15%, wheat bran, 25% cotton seed meal, 3% limestone, 0.3% salt, and 0.35% vitamin-mineral mixture. It contained 21.5% crude protein and 2,850 kcal/kg metabolizable energy. Three experimental groups received 5g, 10g and 15g of borax (567, 1134 and 1701 mg of boron), respectively as an oral bolus. The bolus administration schedule was chosen to fit within the normal management activities of large dairies, and it required cows to be restrained in headlocks only once daily.

Blood, urine, milk and feces samples were collected in just before and after the 10-day period. Blood samples were taken from the coccygeal vein. Serum was harvested within an hour by centrifugation for 15 minutes at 3,000 rpm. All the samples were stored at -20°C till analysis. They were analyzed for macro and micro minerals (B, Ca, P, Mg, Na, K, Mo, Mn, Cr, Cu, Fe, Ni, S, Zn) by ICP-AES (VARIAN VISTA AX CCD) using Reference Material 8414 (National Institute of Standards and Technology) on day 0 and day 11^{th}. Only boron levels were measured on three consecutive days after dietary boron exposure.

All laboratory data were presented as the mean ± SD. Statistical significance between groups' first and last samples was determined with Paired *t* test. One Way Anova Posthoc Tukey was applied for statistical significance between groups' last samples' values. Values of $P < 0.05$ were considered significant. SPSS 21.0 program was used for statistical analyses.

III. RESULTS

All animals appeared healthy during the boron-diet treatment period. Feed and water consumption were unaffected by treatments. There were no overt signs of toxicosis. Increased boron ingestion increased serum, urine, milk and feces boron concentrations (Table 2). The percentage of boron was increased with the 60, 120 and 180 mg of B/kg of diet treatments.

Apart from boron Ca, P, Mg, Na, K, Mo, Mn, Cr, Cu, Fe, Ni, S, Zn concentrations were also measured in all the samples. Compared to the samples in the beginning of the boron exposure, some changes in macro and trace minerals' level at the end of the experiment were as following: for Ca ($p<0.003$) and Mg ($p<0.028$) concentrations significant increases in serum and significant decreases (Ca: $p<0.001$, Mg: $p<0.001$) in feces were observed in association with boron intake. This data indicates that boron increases Ca and Mg absorption. The same thing may be expressed for Na mineral, because Na was increased in serum ($p<0.007$) and decreased ($p<0.000$) in feces. It was also decreased in urine. P, K and S concentrations tended to be decreased in serum samples. Ca and K concentrations, and Mg and P concentrations (only in experimental 3) were significantly decreased in milk samples (Tables 3, 4, 5 and 6). Although these changes are outstanding, it seems to be difficult interpreting them properly.

TABLE 1
BASAL DIET ANALYSIS

TSBM[f, %]	**61,39**
SE-1X, Mcal/kg[f]	2,78
ME-3X, Mcal/kg[f]	2,12
NEL-3X, Mcal/kg[f]	1,30
NEL-4X, Mcal/kg[f]	1,22
NEM-3X, Mcal/kg[f]	1,30
NEG-3X, Mcal/kg[f]	0,71
Dry matter, %	60,96
Crude Protein, %	12,82
NDICP, %	1,36
ADICP, %	0,70
Crude fat, %	4,63
NDF, %	37,31
ADF, %	23,42
NFC, %	31,93
Lignin, %	5,31
Crude ash, %	13,30
N fraction A, % HP	17,62
N fraction B, % HP	76,94
N fraction C, % HP	5,44
RUP, CP %, %2, (Bypass Protein) [f]	33,45
RUP, CP%, %4, (Bypass Protein) [f]	37,64
RUP Sind, % [f]	67
[f]:calculated by formula	

TABLE 2

BORON LEVELS (mg/L).BETWEEN GROUPS' IN THE BEGINNING AND AT THE END OF THE EXPERIMENT

Body fluids	Experimental 1			Experimental 2			Experimental 3		
	Beginning Mean±SD	End Mean±SD	P	Beginning Mean±SD	End Mean±SD	P	Beginning Mean±SD	End Mean±SD	P
Serum	0,052 ± 0,033	0,114 ± 0,017 [b]	0.018	0,061 ± 0,025	0,136 ± 0,064 [ab]	0.068	0,08 ± 0,038	0,23 ± 0,080 [a]	0.008
Milk	0,051 ± 0,011	0,131 ± 0,037	0.001	0,038 ± 0,012	0,114 ± 0,021	0.000	0,003 ± 0,025	0,089 ± 0,042	0.001
Urine	0,689 ± 0,272	2,081 ± 1,543	0.015	0,893 ± 0,347	2,132 ± 1,840	0.032	0,494 ± 0,225	2,721 ± 1,034	0.000
Feces	0,085 ± 0,021	0,144 ± 0,049 [b]	0.014	0,086 ± 0,019	0,155 ± 0,027 [b]	0.000	0,093 ± 0,010	0,208 ± 0,018 [a]	0.000
Percentage rate of boron (%) between groups' at the end of the experiment (mg/L).									
Body fluids	Experimental 1			Experimental 2			Experimental 3		
	Beginning Mean	End Mean	Percentage rate %	Beginning Mean	End Mean	Percentage rate %	Beginning Mean	End Mean	Percentage rate %
Serum	0,052	0,105	101,9	0,061	0,136[b]	121.8	0,07	0,214	202.1
Milk	0.051	0,131	161,2	0,038	0,114	184,7	0,003	0,089	206,4
Urine	0,689	2,681	276,7	0,893	3,132	286,0	0,494	2,721	450,6
Feces	0.085	0.144	71.4	0.086	0.155	81.9	0.093	0.208 [a]	118.2

Data are expressed as the mean±SD.

TABLE 3

MINERAL LEVELS (PPM) IN SERUM SAMPLES IN THE BEGINNING AND AT THE END OF THE EXPERIMENT

Minerals	Experimental 1			Experimental 2			Experimental 3		
	Beginning Mean±SD	End Mean±SD	P	Beginning Mean±SD	End Mean±SD	P	Beginning Mean±SD	End Mean±SD	P
Co	0.000±0.000	0.005±0.002	0.008	0.000±0.000	0.005±0.004	0.014	0.000±0.000	0.004±0.003	0.024
Mo	0.001±0.001	0.002±0.001	0.233	0.001±0.001	0.002±.002	0.683	0.002±0.001	0.002±0.002	0.960
Ca	5.751±1.372	13.810±1.700[a]	0.013	6.991±2.626	13.525±1.556[a]	0.003	8.205±3.904	18.859±2.787[a]	0.003
Cr	0.000±0.000	0.003±0.001 [a]	0.000	0.000±0.000	0.003±0.002 [a]	0.002	0.000±0.000	0.003±0.001 [a]	0.002
Cu	0.033±0.023	0.031±0.011	0.832	0.036±0.016	0.025±0.007	0.164	0.040±0.019	0.042±0.013	0.591
Fe	0.225±0.181	0.116±0.034	0.228	0.147±0.048	0.098±0.044	0.139	0.139±0.011	0.122±0.038	0.264
K	12.91±11.53	12.43±3.18	0.930	19.68±8.55	11.62±4.93[a]	0.131	22.60±10.48	19.26±6.87	0.412
Mg	1.116±1.168	1.867±.433 [a]	0.245	1.581±.812	1.742±0.557	0.739	1.815±1.099	2.992±1.105[b]	0.028
Mn	0.002±0.002	0.000±0.000	0.072	0.001±0.000	0.000±0.000	0.000	0.000±0.000	0.000±0.000	0.005
Na	71.43±23.01	109.84±8.29[a]	0.022	82.34±19.91	107.40±12.06[a]	0.053	84.79±12.49	124.57±14.70[a]	0.007
Ni	0.000±0.000	0.005±0.002	0.013	0.000±0.000	0.005±0.003	0.002	0.000±0.000	0.005±0.002	0.006
P	6.998±6.212	6.530±1.708	0.865	8.918±3.684	6.205±2.661	0.249	11.374±6.014	10.978±4.596	0.855
S	55.454±40.016	48.310±13.113	0.697	68.891±24.963	47.461±15.207	0.174	84.572±37.287	73.812±23.209	0.419
Zn	0.042±0.038	0.056±0.021	0.436	0.063±0.037	0.050±0.024	0.488	0.0631±0.034	0.114±0.040	0.115

Data are expressed as the mean±SD

TABLE 4

MINERAL LEVELS (PPM) IN URINE SAMPLES IN THE BEGINNING AND AT THE END OF THE EXPERIMENT.

Minerals	Experimental 1			Experimental 2			Experimental 3		
	Beginning Mean±SD	End Mean±SD	P	Beginning Mean±SD	End Mean±SD	P	Beginning Mean±SD	End Mean±SD	P
Co	0.000±0.000	0.005±0.002	0.001	0.000±0.000	0.007±0.003	0.003	0.000±0.000	0.005±0.003	0.001
Mo	0.002±0.001	0.004±0.004[ab]	0.264	0.001±0.001	0.009±0.006 [a]	0.022	0.002±0.001	0.003±0.002[b]	0.312
Ca	4.498±5.136	10.294±.903[a]	0.020	5.210±5.649	12.173±3.021 [a]	0.022	1.716±0.967	10.939±1.988[a]	0.000
Cr	0.000±0.000	0.003±0.001[a]	0.000	0.000±0.000	0.003±0.001 [a]	0.000	0.000±0.000	0.002±0.001[b]	0.002
Cu	0.002±0.005	0.000±0.000	0.291	0.001±0.001	0.000±0.000	0.048	0.002±0.003	0.000±0.000	0.039
Fe	0.025±0.020	0.070±0.046	0.076	0.0321±0.008	0.083±0.055	0.083	0.083±0.130	0.049±0.022	0.413
K	188.38±88.56	155.51±62.50[a]	0.377	247.51±85.5	184.41±77.89 [a]	0.201	116.12±50.42	106.87±48.52[b]	0.095
Mg	21.374±10.632	27.069±19.618 [a]	0.494	30.182±13.290	32.042±17.395	0.853	15.630±7.598	23.362±7.327 [a]	0.055
Mn	0.000±0.000	0.000±0.000	0.000	0.001±0.001	0.000±0.000	0.012	0.005±0.007	0.000±0.000	0.090
Na	4.584±5.240	15.56±15.214 [a]	0.105	14.826±12.229	17.190±7.652 [a]	0.167	6.647±6.057	18.680±33.566[b]	0.324
Ni	0.000±0.000	0.007±0.003	0.001	0.000±0.000	0.004±0.002	0.005	0.000±0.000	0.005±0.002	0.000
P	0.352±0.122	0.336±0.210	0.847	0.481±0.119	0.456±0.264	0.793	0.333±0.134	0.428±0.300	0.453
S	14.399±6.76	25.200±15.074 [a]	0.067	19.045±6.047	36.810±28.097[a]	0.232	9.296±3.913	24.523±8.859 [a]	0.001
Zn	0.000±0.000	0.007±0.007	0.029	0.007±0.013	0.016±0.011	0.179	0.047±0.084	0.039±0.042	0.796

Data are expressed as the mean±SD.

TABLE 5
MINERAL LEVELS (PPM) IN MILK SAMPLES IN THE BEGINNING AND AT THE END OF THE EXPERIMENT.

Minerals	Experimental 1			Experimental 2			Experimental 3		
	Beginning Mean±SD	End Mean±SD	P	Beginning Mean±SD	End Mean±SD	P	Beginning Mean±SD	End Mean±SD	P
Co	0.000±0.000	0.005±0.002	0.000	0.000±0.000	0.005±0.002	0.000	0.000±0.000	0.006±0.001	0.000
Mo	0.003±0.001	0.002±0.001	0.014	0.002±0.001	0.003±0.001	0.438	0.003±0.001	0.002±0.002	0.512
Ca	27.323±4.443	26.156±8.266	0.739	37.580±14.53	29.804±9.546[a]	0.229	55.575±16.500	25.502±5.692[b]	0.001
Cr	0.003±0.001	0.006±0.002[a]	0.001	0.003±0.001	0.006±0.001[a]	0.003	0.004±0.002	0.006±0.002	0.011
Cu	0.022±0.003	0.028±0.007	0.084	0.021±0.004	0.031±0.009	0.039	0.025±0.009	0.022±0.003	0.369
Fe	0.195±0.022	0.298±0.066[ab]	0.005	0.220±0.102	0.346±0.081[a]	0.004	0.224±0.057	0.254±0.059	0.274
K	48.096±14.91	39.034±17.514[a]	0.309	60.171±18.19	49.086±14.540[a]	0.266	92.119±27.64	36.795±12.61[b]	0.001
Mg	2.468±0.562	2.667±1.133	0.673	3.579±1.584	3.462±1.444	0.885	4.960±1.306	2.648±.908[a]	0.001
Mn	0.002±0.001	0.000±0.000	0.000	0.012±0.020	0.000±0.000	0.140	0.004±0.002	0.001±0.002	0.032
Na	12.955±4.241	12.303±7.286	0.839	16.785±8.310	15.631±5.349	0.764	19.754±3.146	11.166±4.382	0.004
Ni	0.000±0.001	0.007±0.003	0.000	0.003±0.002	0.014±0.014	0.058	0.003±0.002	0.014±0.015	0.157
P	19.110±4.033	18.179±8.396	0.773	24.149±8.525	23.327±10.249	0.872	36.206±9.626	16.834±6.500[a]	0.001
S	7.427±1.558	7.878±3.208	0.753	9.207±2.959	9.649±2.555	0.751	12.184±2.836	7.437±2.483[a]	0.004
Zn	0.114±0.015	0.129±0.033	0.336	0.148±0.055	0.146±0.044	0.948	0.198±0.062	0.184±0.137	0.715

Data are expressed as the mean±SD

TABLE 6
MINERAL LEVELS (PPM) IN *FECES SAMPLES* IN THE BEGINNING AND AT THE END OF THE EXPERIMENT

Minerals	Experimental 1			Experimental 2			Experimental 3		
	Beginning Mean±SD	End Mean±SD	p	Beginning Mean±SD	End Mean±SD	p	Beginning Mean±SD	End Mean±SD	p
Co	0.000±0.000	0.005±0.002[a]	0.000	0.000±0.000	0.003±0.002[b]	0.007	0.000±0.000	0.005±0.002[ab]	0.000
Mo	0.005±0.001	0.005±0.001	0.656	0.005±.002	0.006±0.002	0.508	0.006±0.001	0.007±0.003	0.266
Ca	103.35±18.35	54.456±17.27[a]	0.001	107.18±25.5	48.285±10.092[a]	0.000	114.74±12.77	71.58±27.65[a]	0.010
Cr	0.010±0.007	0.012±0.002	0.445	0.008±.0037	0.013±0.003	0.000	0.007±0.002	0.017±0.007	0.008
Cu	0.069±0.010	0.074±0.012	0.465	0.069±.0095	0.069±0.016	0.901	0.077±0.011	0.080±0.022	0.705
Fe	2.156±0.323	2.316±0.689	0.544	2.219±.433	2.143±0.558	0.573	2.349±0.394	3.142±1.008	0.097
K	4.465±2.311	14.155±7.141	0.001	6.584±1.938	12.361±4.925	0.008	7.566±2.101	9.833±4.904	0.243
Mg	46.125±3.837	23.859±7.430[ab]	0.000	46.852±5.839	21.442±5.152[b]	0.000	48.683±3.249	30.768±4.990[a]	0.000
Mn	0.233±0.044	0.279±0.102	0.301	0.241±.0.536	0.237±0.046	0.845	0.270±0.025	0.385±0.154	0.082
Na	2.454±2.077	4.666±2.919	0.015	7.101±1.436	4.615±3.166	0.069	8.827±2.831	3.544±1.551	0.000
Ni	0.017±0.005	0.017±0.007	0.990	0.016±0.004	0.021±0.008	0.074	0.018±0.001	0.022±0.007	0.123
P	7.430±1.625	8.332±3.834	0.586	8.344±1.892	7.176±2.217	0.058	8.145±1.102	9.133±4.308	0.572
S	5.784±0.614	6.877±1.529	0.115	6.749±1.913	6.410±1.258	0.307	7.313±1.660	7.667±1.905	0.693
Zn	0.223±0.037	0.286±0.096	0.127	0.244±0.056	0.276±0.063	0.079	0.254±0.029	0.356±0.120	0.064

Data are expressed as the mean±SD

IV. DISCUSSION

This is the first study evaluating dietary boron supplementation at different doses on body fluid distribution of boron, and other macro and trace minerals.

The biochemical function of boron is still speculative. Lactating cows fed with approximately 620 mg of B/100 kg of body weight/day without toxic effects. Daily intakes of about 765 mg of B/100 kg of body weight (as borax) have deleterious effects (Owen, 1944; Green and Weeth, 1977). The signs of excessive boron ingestion are rather nonspecific (Brook and Boggs 1951). Exposure to large amounts of boron (about 30 g of boric acid) over short periods of time can affect the stomach, intestines, liver, kidney, and brain and can eventually lead to death in humans. Studies of dogs, rats, and mice indicate that the male reproductive organs, especially the testes, are affected if large amounts of boron are ingested for short or long periods of time. The doses that produced these effects in animals are more than 1,800 times higher than the average daily intake of boron in food by adults in the U.S. population (ARSDR, 2010). One outstanding feature in persons is an erythematous skin rash (Polson and Tattersal, 1969). This rash appears to the redness and edema seen in the legs and around the dew claws of cattle (Green and Weeth, 1977). In this presented study here, adverse effects were not seen with daily

intakes of 450, 900 and 1350 mg of boron in these 10 days of boron exposure. In other similar study (Weeth *et al.*, 1981) where beef heifers were given drinking water to which 0, 15, 30, 60 or 120 mg of B/L of water was added for 10 days period, it was found that plasma and urine boron levels were significant increased. Similarly to this, in the present study boron levels increased based on the doses in body fluids. Greatest increase of boron was in urine samples, and lowest in faces samples. Serum and milk boron levels were similar. This indicated dietary boron has been much more eliminated by urine than milk and feces.

Macro and trace minerals are essential components of a feeding program for dairy herds and (Swecker, 2014) have important roles in immune function and may affect health in transition dairy cows (Speer and Weiss, 2008). Deficiencies of calcium, phosphorus, and magnesium result in improper skeletal formation. Deficiencies in other macro minerals (e.g., sodium, chloride, potassium) and trace minerals (e.g., copper, zinc, manganese, cobalt, iron) cause biochemical dysfunctions that lead to inefficient metabolism and growth (Smith, 2015).

Boron seems to affect the metabolism of P as well as Ca in animal and human models (Hunt, 1988). A significant change in serum Zn concentration was reported by Kurtoğlu *et al.* (2005) when chicks were given 5 mg/kg boron. Chromium levels' increases in serum and urine samples, and sulfur levels' increases in only urine samples at the end of the experiment became outstanding in the present study. Studies indicated that Cr supplementation may affect health and immune response in ruminants (Spears, 2000). In our previous study (Basoglu *et al.*, 2010), boron (in solution of 1%) 10, 30 and 50 mg/kg body weight/day, given to rabbits by oral gavage at 96 h interval for 7 months, any changes were not observed in serum Ca, P, Mg, Na, K and Cl levels. Dietary combination with boron and phytase did not create a synergism with regard to growth performance and bioavailability of the minerals (Cinat *et al.*, 2015). Boron affects blood P and Mg in humans; serum P concentrations are lower in boron-supplemented subjects than in subjects receiving placebos, and are lower at the end of the study period than during baseline analysis (Meacham *et al.*, 1994).

Effective transition management requires an integrated approach to nutritional and environmental management to provide cows with freedom from rumen disruption, mineral deficiencies, immunosuppression, disorders of lipid metabolism, and other forms of stress (eg, toxic feeds, social disruption) (Sundram, 2015). Clinical milk fever is one of the most recognized diseases of dairy cattle (Oetzel, 2013). Hypocalcaemia around calving is a risk factor for many of these diseases and is an indirect risk factor for increased culling (Goff, 2014). The incidence of clinical hypocalcaemia (milk fever) in the field generally ranges from 0–10%, but may exceed 25% of cows calving (DeGaris and Lean, 2008). In the present study, boron could not be completely absorbed, a part of dietary boron was eliminated by feces. While Ca and Mg levels decreased in feces, their levels in serum increased, associated with boron intake.

V. CONCLUSION

Boron increases Ca and Mg absorption. This topic is worth evaluating as an alternative approach in the prevention of hypocalcemia in transition cows.

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Interspecific Interaction Between *Phytoseiulus macropilis* and *Neoseiulus californicus* (Acari: Phytoseiidae) Preying on *Tetranychus urticae* (Acari: Tetranychidae) on Rosebush Growing in Greenhouse

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***Abstract*—** *Greenhouses provide favorable conditions to grow roses (Rosa spp.) but also for the pest mite Tetranychus urticae Koch (Acari: Tetranychidae). Although chemical control continues to be used against this pest, consumer demand has encouraged research on less aggressive agricultural practices and biological control is now a viable option. The objective of this study was to investigate biological control of the two-spotted mite, in rosebush growing in greenhouse, by Phytoseiulus macropilis (Banks) and Neoseiulus californicus (McGregor) (Acari: Phytoseiidae), individually or in combination. Potted rosebushes (Rosa alba L., Rosaceae) were used in five treatments: 1 - control group, rosebushes only with T. urticae; 2 - rosebushes with T. urticae and N. californicus; 3 - rosebushes with T. urticae and P. macropilis; 4 - rosebushes with T. urticae, N. californicus, and, after 4 weeks, P. macropilis and 5 - rosebushes with T. urticae, N. californicus, and P. macropilis. Biological control was evaluated by counting live mites. The results showed that both predatory mites were efficient in the control of the two-spotted mite on rosebushes cultivated in the greenhouse, either alone or in combination, but, when only P. macropilis was released, this predaceous mite was more efficient than N. californicus, at either low or high prey density, but the N. californicus can survive for longer in the absence of prey or in its low presence, for being a generalist predatory mite.*

***Keywords*— Biological control, Rosa spp., Two-spotted spider mite, Protected crop, Agricultural acarology.**

I. INTRODUCTION

The flower trade in Brazil is growing and emerging as a highly profitable business sector. In 2014, the sector gained approximately US$1.42 billion, and 8% growth is estimated for 2015 [1].

The state of Minas Gerais, Brazil, is a leader in this sector, particularly for the production of rosebushes (*Rosa* spp.) and other conventional cutting flowers. Although up-to-date data for this activity in Minas Gerais are not available, the primary municipalities for flower production in this state are Barbacena, Andradas, Araxá, and Munhoz [2].

The Asian rosebush grows well in Brazil and is cultivated in various Brazilian regions, with much of the production in greenhouses. Although the greenhouse environment provides better conditions for the control of pests and diseases, the environment is also more favorable for pests and diseases to develop. The two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae), is a pest mite that deserves special attention as one of the major problems to affect rosebushes in protected cultivation systems [3].

Pest control is one of the challenges for the cultivation of flowers and ornamental plants, because any damage caused by insects and other arthropods to flowers and foliage makes them unacceptable to consumers, and therefore depreciates the final product to be marketed [4].

Although producers and consumers strongly demand reduced use of agrochemicals [5], the spraying of chemical pesticides remains the primary tactic for the control of pests and diseases. Spraying is often used for prevention [6], which causes ecological disequilibrium and environmental contamination in many cases [7], particularly when these products are misused, such as erroneous doses of plant protection products, unnecessary spraying beyond recommended, use of unregistered products for the crop etc.

The concerns of consumers for human health and environmental preservation have encouraged researchers to investigate the use of less aggressive agricultural practices that promote sustainability in agroecosystems [6]. Therefore, in the ecological management of pests, practices that preserve natural enemies in the environment are essential, and the use of selective products that minimize harmful effects to non-target fauna and maintain the ecological balance of the agroecosystem is indispensable [8].

Biological control is an important alternative for pest management. In addition to providing efficient control, biological control can add value to an agricultural product when well-planned and implemented, because an image can be established for consumers of a product that is produced ecologically [9].

The predatory mites *Neoseiulus californicus* (McGregor) and *Phytoseiulus macropilis* (Banks) (Acari: Phytoseiidae), among others, are used for the biological control of two-spotted spider mites on fruit trees, ornamental plants, and other crops. This method of biological control reduces the amount of chemical pesticides used for cultivation and includes the other benefits of reduced exposure of workers to chemicals and fewer residues in the environment [10].

The predatory mites can be classified into 4 types, and Type I were subdivided into subtypes to highlight the specificity in relation to the pest mite. In this group is *P. macropilis* being a mite of subtype I-a, that is, specialized in predating the genus *Tetranychus*. Type II mites are those that are selective of Tetranychidae mites but can also be fed from other genera or group of mites such as Tydeoidea, Eriophyidae, Tarsonemidae or even pollen. Although some authors question that *N. californicus* should be classified as Type III of predatory mites, they are maintained as Type II because they are associated with mites that produce large amounts of webs such as *T. urticae*. Type III of predatory mites does not get along with mites that produce webs because they can often get stuck in them. These Type III are those that are generalists and have also been subdivided into subtypes according to their habitat. Type IV of predatory mites are those for which pollen is an important part of the diet [11].

In Europe and the United States of North America, the cost of biological control methods may be similar to the cost of chemical control, particularly when the number of predators released is based on an estimation of the pest density, which makes biological control an attractive alternative for pest management [12] [13].

However, for biological control with predatory mites to be a viable solution in the productive sector, the biological characteristics and efficiency of these predaceous mites for pest control must be determined, and therefore investigations are required of the interactions between predatory mites.

Therefore, the objective of this study was to investigate the biological control of the two-spotted spider mite, *T. urticae*, on rosebushes growing in a greenhouse with the individual or joint release of the predatory mites, *P. macropilis* and *N. californicus*.

II. MATERIAL AND METHODS

The study was conducted at the Acarology Laboratory of the *Empresa de Pesquisa Agropecuária de Minas Gerais* - EPAMIG-Sul de Minas - *Centro de Pesquisa em Manejo Ecológico de Pragas e Doenças de Plantas* - EcoCentro, Lavras, MG, Brazil, under the controlled conditions of 25 ± 2 °C, 70% ± 10% RH, and a 14 h photophase and, in a greenhouse at the Experimental Station of EPAMIG, also in Lavras.

Both predatory mites, *P. macropilis* and *N. californicus*, and the pest mite *T. urticae* were obtained from the *Instituto Federal de Educação, Ciência e Tecnologia do Sul de Minas - Campus de Inconfidentes*, Minas Gerais, Brazil.

2.1 Rearing of the two-spotted spider mite

Uncovered petri dishes (15 cm in diameter) were used with 1 cm thick foam occupying the entire bottom surface of each dish. The foam was kept moist with distilled water. A jack bean leaflet [*Canavalia ensiformis* L. (DC), Fabaceae] was placed on top of the foam and, to prevent the mites from escaping and better preserve the leaflet, the leaflet was surrounded by strips of cotton that were also in contact with the damp foam. Pest mite, *T. urticae*, were placed on top of the jack bean leaflets, which were replaced weekly [14].

2.2 Rearing of predatory mites

Arenas of black flexible PVC plastic sheets (26 × 22 cm) were placed on Styrofoam of equal size, and these were placed on water in plastic trays (32 × 26.5 × 5.5 cm). Cotton in contact with water from the tray was placed around the Styrofoam and the arena and was used to prevent the escape of mites, in addition to preserving the jack bean leaves. Leaves were placed in

the arenas with the petiole under the damp cotton and were infested with *T. urticae* to provide food for the predatory mites; as the leaves withered other new leaves infested with *T. urticae* were placed on the old leaves [14].

2.3 Biological control assay

The experiment investigating biological control of the two-spotted spider mite, *T. urticae*, was conducted between September and November 2012 in a greenhouse with a transparent plastic cover and fine mesh fabric on the sides. White rosebushes (*Rosa alba* L., Rosaceae) with table grafting and approximately 4 years old in 22 liter pots were used. A mixture of ravine soil, manure, and sand was used as the substrate. During the growing cycle, the necessary cleaning and pruning were conducted to avoid affecting the population of predatory mites during release periods.

The experiment consisted of five treatments with five replicates each of five plants/treatment in an experimental design of randomized blocks, to avoid any variation between treatments: 1 - control group, rosebushes only with *T. urticae*, 2 - rosebushes with *T. urticae* and *N. californicus*, 3 - rosebushes with *T. urticae* and *P. macropilis*, 4 - rosebushes with *T. urticae*, *N. californicus*, and, after 4 weeks, *P. macropilis*, and 5 - rosebushes with *T. urticae*, *N. californicus*, and *P. macropilis*. To prevent dispersal of pest mites and predatory mites, rosebushes were placed individually, isolated from each other, in wood cages, 0.60 × 0.60 × 1.50 m, covered with *voil* cloth fabric.

First, rosebushes were infested with 20 female two-spotted spider mites per plant. The first release of predatory mites was conducted nine days after the pest mite infestation [15].

In treatment 1 (control group), predatory mites were not released; and, in treatments 2 (*N. californicus* vs. *T. urticae*) and 3 (*P. macropilis* vs. *T. urticae*), a total of 16 female predatory mites were released at two per week for eight weeks. In treatment 4 (*N. californicus* vs. *T. urticae*, and, after 4 weeks, *P. macropilis*), initially, only two *N. californicus* were released per week during the first month of evaluation for a total of eight female predatory mites, assuming that infestation by *T. urticae* would still be low, and then, after this period, only two *P. macropilis* were released per week for a total of eight predatory mites of this species, assuming that this infestation by *T. urticae* would already be higher; thus a total of 16 female predatory mites were released, with eight of each species. In treatment 5 (*N. californicus* + *P. macropilis* vs. *T. urticae*), two predatory mites were released weekly, one of each species.

Females of the predatory mites were transferred with a fine-tipped brush to leaves with signs of pest mite infestation between 8:00 a.m. and 12:00 p.m. The releases were conducted over two months, for a total of eight releases.

Rosebush leaflets were collected weekly beginning a week after the two-spotted spider mite infestation until the end of the experiment. A total of six leaflets per plant were collected each week, with two randomly selected from each part of the plant (apical, middle, and basal). Leaflets collected from each part were identified, separated, and transferred immediately to the laboratory properly packaged in paper bags, wrapped with polyethylene bags, and placed in a Styrofoam cooler containing ice. A stereomicroscope was used to count the eggs and any type of active stage of the pest mite on the leaflets.

2.4 Statistical analyses of the biological control assay

Because the data for analysis were mite counts (eggs and any other type of active stage of the pest mite), a negative binomial regression model, which accounts for the effect of over dispersion, was considered a logarithmic linkage function with the systematic components given by the following: $\eta_i = \beta_0 + \beta_1 x_{1i} + \beta_2 x_{2i}^2 + \beta_3 z_{1i} + \beta_4 z_{2i} + \beta_5 z_{3i} + \beta_6 z_{4i}$, where x_{1i} is the temporal effect of the week, x_{2i}^2 is the square of the temporal effect of the week, and z_{ji} *(j = 1, 2, 3, 4)* are the categorical variables related to treatments one (control group), two, three, four, and five, respectively. Therefore, the control treatment was used as a reference when *z = (0, 0, 0, 0)*. The parameters β_j *(j = 1,..., 6)* represented the associated effects with each variable, and the log-linear linkage function $\log \mu_i = h_i$, where μ_i represents the response variable average y_i in the week x_i and in the treatment z_j. Therefore, the model that related the average number of prey with the *x* and *z* variables is given by the following equation (1), with ε_i the experimental error associated with the ith portion:

$$\mu_i = e^{\beta_0 + \beta_1 x_{1i} + \beta_2 x_{2i}^2 + \beta_3 z_{1i} + \beta_4 z_{2i} + \beta_5 z_{3i} + \beta_6 z_{4i}} + \varepsilon_i \quad (1)$$

The parameters of the model were estimated using the maximum likelihood method [16], and the validation was conducted using the simulated envelope graphic [17].

Percentage rates, indicated as ψ, were calculated with the objective of measuring the effect of a treatment in comparison with the control group, considering the reference model obtained when $z = (0, 0, 0, 0)$ and the model for a particular z_j. Percentage rates (or odds ratios) among the models are given by equation (2) [18].

$$\psi = e^{\beta j} \tag{2}$$

Given these specifications, based on the model proposed in equation (1), scenarios were organized with the following treatments: scenario 1, comparison between the control group and the other treatments; scenario 2, predator *N. californicus* released individually or released with *P. macropilis*; scenario 3, predator *P. macropilis* released individually or released with *N. californicus*; and scenario 4, comparison between the predatory mites *N. californicus* and *P. macropilis*, both individually released. Statistical analyses were all conducted with the R statistical software package [19].

III. RESULTS AND DISCUSSION

3.1 Biological control assay

For further clarification and interpretation of results, the odds ratios and parameters of significance are described in Tables 1 - 5, with the estimations of parameters of the adjusted models as the control group. Therefore, results that corroborated the validation of the model initially allowed interpretation of all adjusted models for the scenarios 1 through 4, as previously described, as presenting satisfactory adjustment quality (Fig. 1).

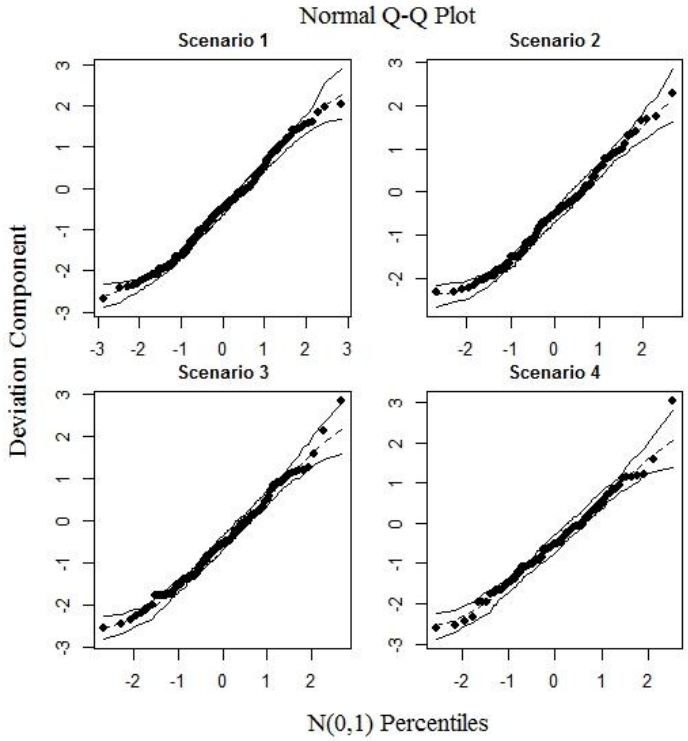

FIGURE 1. SIMULATED ENVELOPES BASED ON THE NEGATIVE BINOMINAL LOG-LINEAR MODEL, ADJUSTED TO THE DATA OF THE NUMBER OF PREY *T. URTICAE* PREDATED ON SIX LEAFLETS FOR THE FOLLOWING: SCENARIO 1 (CONTROL GROUP VS. OTHER TREATMENTS); SCENARIO 2 (PREDATORY MITE *N. CALIFORNICUS* RELEASED INDIVIDUALLY OR RELEASED WITH *P. MACROPILIS*); SCENARIO 3 (PREDATORY MITE *P. MACROPILIS* RELEASED INDIVIDUALLY OR RELEASED WITH *N. CALIFORNICUS*), AND SCENARIO 4 (COMPARISON BETWEEN PREDATORY MITES *N. CALIFORNICUS* AND *P. MACROPILIS*, BOTH INDIVIDUALLY RELEASED)

In the comparison with treatment 1 (control group, only *T. urticae*), the release of the two predatory mites individually or in combination significantly reduced the number of pest mites (Table 1; Fig. 2, Scenario 1). Notably, in treatments 3 (*P. macropilis* vs. *T. urticae*) and 5 (*N. californicus* + *P. macropilis* vs. *T. urticae*), both with the predatory mite *P. macropilis* from the beginning of the experiment, the expected number of pest mites was reduced by approximately 93% and 92%, respectively, compared with the control group (Table 1).

TABLE 1
PARAMETER ESTIMATIONS OF THE NEGATIVE BINOMINAL REGRESSION MODEL, RESPECTIVE STANDARD ERRORS AND PERCENTAGE RATES ψ AMONG THE TREATMENTS J (J = 2, 3, 4, 5), AND THE REFERENCE (TREAT 1= T. URTICAE ONLY) FOR THE NUMBER OF T. URTICAE ON SIX LEAFLETS

Variable	Parameter	Estimation	Standard error	*p-value*	ψ	1-ψ (%)
Intercept	β_0	4.2277	0.4409	<0.0001	-	-
Week	β_1	1.5246	0.1797	<0.0001	-	-
Week2	β_2	-0.1631	0.0175	<0.0001	-	-
Treat2[1]	β_3	-1.9508	0.3230	<0.0001	0.1422	85.78
Treat3	β_4	-2.6620	0.3251	<0.0001	0.0698	93.02
Treat4	β_5	-1.7084	0.3229	<0.0001	0.1812	81.88
Treat5	β_6	-2.5081	0.3234	<0.0001	0.0814	91.86
Dispersion	ϕ	0.4276	0.0379	-	-	-

[1] **Treat2 = *T. urticae* + *N. californicus*, Treat3 = *T. urticae* + *P. macropilis*, Treat4 = *T. urticae* +*N. californicus* and, after 4 weeks, release of *P. macropilis*, Treat5 = *T. urticae* + *N. californicus* + *P. macropilis***

With treatment 1 as the control group (only *T. urticae*), the incidence of the pest was higher near the fifth week (4.7) compared with the other treatments (Table 2, Scenario 1).

TABLE 2
EXPECTED MAXIMUM NUMBERS OF *T. URTICAE* ON SIX LEAFLETS OBTAINED FROM THE ADJUSTED MODELS FOR SCENARIOS 1 TO 4

	Scenarios	Treatment[1]	Maximum	Week
1	Comparison between the control group and all other treatments	Treat1	2,418.90	4.70
		Treat2	343.90	4.70
		Treat3	168.90	4.70
		Treat4	438.20	4.70
		Treat5	196.90	4.70
2	Predatory mite *N. californicus* released individually or released with *P. macropilis*	Treat2	394.21	4.60
		Treat4	573.53	4.60
		Treat5	217.08	4.60
3	Predatory mite *P. macropilis* released individually or released with *N. californicus*	Treat3	150.26	4.20
		Treat4	789.18	4.20
		Treat5	185.76	4.20
4	Comparison between predatory mites *N. californicus* and *P. macropilis*, both released individually	Treat2	585.86	4.12
		Treat3	176.35	4.12

[1] ***Treat1 = Control (T. urticae), Treat2 = T. urticae + N. californicus, Treat3 = T. urticae + P. macropilis, Treat4 = T. urticae +N. californicus and, after 4 weeks, release of P. macropilis, Treat5 = T. urticae + N. californicus + P. macropilis***

Specifically regarding treatment 2 in which the predatory mite *N. californicus* was released individually, and using this treatment as the control group, no significant difference was observed between the treatments when the predatory mite *P. macropilis* was released 4 weeks after the release of *N. californicus* (treatment 4) (Table 3; Fig. 2, Scenario 2).

TABLE 3
PARAMETER ESTIMATIONS OF THE NEGATIVE BINOMINAL REGRESSION MODEL, RESPECTIVE STANDARD ERRORS AND PERCENTAGE RATES ψ AMONG TREATMENTS J (J = 4, 5), AND THE CONTROL GROUP (TREAT 2 = *T. URTICAE* + *N. CALIFORNICUS*) FOR THE NUMBER OF *T. URTICAE* ON SIX LEAFLETS

Variable	Parameter	Estimation	Standard error	*p-value*	ψ	1-ψ (%)
Intercept	β_0	1.9028	0.5698	0.0008	-	-
Week	β_1	1.7563	0.2455	<0.0001	-	-
Week2	β_2	-0.1893	0.0240	<0.0001	-	-
Treat4[1]	β_5	0.3749	0.3420	0.2729	1.4549	-
Treat5	β_6	-0.5966	0.3427	0.0817	0.5507	44.93
Dispersion	ϕ	0.3824	0.0447	-	-	-

[1] **Treat4 = *T. urticae* +*N. californicus* and, after 4 weeks, release of *P. macropilis*, Treat5 = *T. urticae* + *N. californicus* + *P. macropilis***

With treatment 3 (*P. macropilis* vs. *T. urticae*) as the control group, when *P. macropilis* was released only after the fourth week in treatment four (*N. californicus* and, after 4 weeks, the release of *P. macropilis* vs. *T. urticae*), the average number of pest mites was approximately 5-fold greater than that in the control (Table 4; Fig. 2, Scenario 3). Compared with the control (treatment 3), such a large difference in prey was not observed in treatment 5 (*P. macropilis* + *N. californicus* vs. *T. urticae*), with both predators released in combination from the beginning of the experiment (Table 4; Fig. 2, Scenario 3). Therefore, the predatory mite *P. macropilis* was highly efficient in the predation of the two-spotted spider mite, at either low or high prey density.

TABLE 4
PARAMETER ESTIMATIONS OF THE NEGATIVE BINOMINAL REGRESSION MODEL, RESPECTIVE STANDARD ERRORS AND PERCENTAGES RATES ψ AMONG TREATMENTS J (J = 4, 5), AND THE CONTROL GROUP (TREAT 3 = *T. URTICAE* + *P. MACROPILIS*) FOR THE NUMBER OF *T. URTICAE* ON SIX LEAFLETS

Variable	Parameter	Estimation	Standard error	*p-value*	ψ	1-ψ (%)
Intercept	β0	1.9002	0.5355	0.0004	-	-
Week	β1	1.4908	0.2311	<0.0001	-	-
Week2	β2	-0.1785	0.0227	<0.0001	-	-
Treat4[1]	β5	1.6586	0.3217	<0.0001	5.2519	-
Treat5	β6	0.2121	0.3231	0.5116	1.2362	-
Dispersion	ϕ	0.4367	0.0519	-	-	-

[1] **Treat4 = *T. urticae* +*N. californicus* and, after 4 weeks, release of *P. macropilis*, Treat5 = *T. urticae* + *N. californicus* + *P. macropilis***

With treatment 2 (*N. californicus* vs. *T. urticae*) as the control group, in the comparison of the two predators in treatments 2 and 3 (*P. macropilis* vs. *T. urticae*), prey numbers were lower with the predator *P. macropilis* in treatment 3.

Thus, similar to in Fig.2 Scenario 3, the predatory mite *P. macropilis* performed better in reducing prey mite numbers than the predatory mite *N. californicus* (Table 5; Fig. 2, Scenario 4), and, compared with *N. californicus*, the expected number of two-spotted spider mites was reduced by approximately 70% in the fourth week of the evaluation (Table 5).

TABLE 5
PARAMETER ESTIMATIONS OF THE NEGATIVE BINOMINAL REGRESSION MODEL, RESPECTIVE STANDARD ERRORS AND PERCENTAGES RATES ψ FOR TREATMENT J (J = 3), AND THE CONTROL GROUP (TREAT 2 = *T. URTICAE* + *N. CALIFORNICUS*) FOR THE NUMBER OF *T. URTICAE* ON SIX LEAFLETS

Variable	Parameter	Estimation	Standard error	*p-value*	ψ	1-ψ (%)
Intercept	β0	2.7234	0.6194	<0.0001	-	-
Week	β1	1.7652	0.2784	<0.0001	-	-
Week2	β2	-0.2134	0.0275	<0.0001	-	-
Treat3[1]	β3	-1.2006	0.3158	<0.0001	0.3010	69.90
Dispersion	ϕ	0.4603	0.0665	-	-	-

[1] ***Treat3 = T. urticae + P. macropilis***

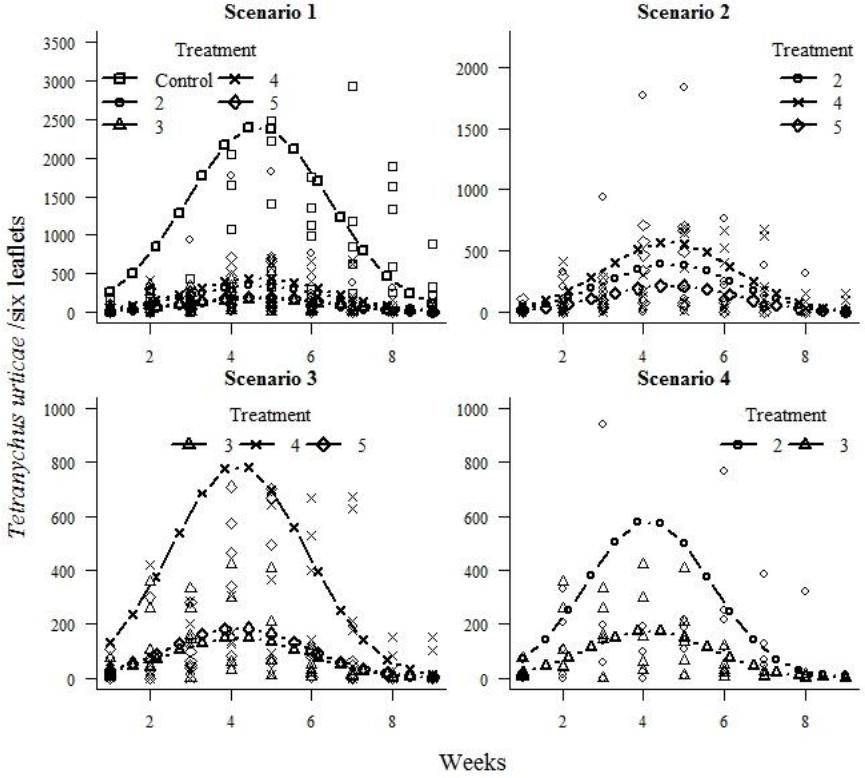

FIGURE 2. ADJUSTED MODELS FOR SCENARIOS 1, 2, 3, AND 4. SCENARIO 1, COMPARISON BETWEEN THE CONTROL GROUP AND ALL OTHER TREATMENTS; SCENARIO 2, PREDATORY MITE *N. CALIFORNICUS* RELEASED INDIVIDUALLY OR RELEASED WITH *P. MACROPILIS*; SCENARIO 3, PREDATORY MITE *P. MACROPILIS* RELEASED INDIVIDUALLY OR RELEASED WITH *N. CALIFORNICUS*; SCENARIO 4, COMPARISON BETWEEN PREDATORY MITES *N. CALIFORNICUS* AND *P. MACROPILIS*, BOTH RELEASED INDIVIDUALLY.
Treatment 1 Control Group, Rosebushes Only With T. urticae; TREATMENT 2 ROSEBUSHES WITH T. urticae AND N. californicus; TREATMENT 3 ROSEBUSHES WITH T. urticae AND P. macropilis; TREATMENT 4 ROSEBUSHES WITH T. urticae, N. californicus, AND, AFTER 4 WEEKS, P. macropilis AND TREATMENT 5 ROSEBUSHES WITH T. urticae, N. californicus AND P. macropilis.

As illustrated in Tables 1 and 2 and Fig. 2, after the fourth week of the release of the predatory mites, all treatments were significantly different from the control group, with predatory mites reducing the populations of *T. urticae* to very low levels at the end of the experiment. These results are similar to those found for strawberry plants (*Fragaria* spp., Rosaceae) [20] on which a population of two-spotted spider mite began to decrease 20 days after the release of *P. macropilis*.

In a comparison of different releases of *N. californicus* for the control of the European red mite *Panonychus ulmi* (Koch) (Acari: Tetranychidae) in apple trees [*Malus domestica* (Borkh.) Borkh., Rosaceae], the release of 15 mites per m^2 provided the most efficient control, and therefore the most predatory mites provided the greatest control of the pest mite [21]. A similar result was observed in the biological control of the two-spotted spider mite on rosebushes with *N. californicus* 1 month following the release of the predatory mite at densities that varied from 0 to 28, and, with the increase in predatory mite density, the decrease in the number of two-spotted spider mites on the leaflets was greater [14].

A study on the potential of *N. californicus* to control *T. urticae* on rosebushes found that an initial density of ten predatory mites/m^2 was not sufficient to control the pest, however, when 20 predatory mites/m^2 were used, although the infestation of *T. urticae* was larger, the pest mite was controlled rapidly [22].

Predatory mite/prey ratios of 1:5, 1:7.5, and 1:10 for *N. californicus* provided good control of *T. urticae* on strawberry and prevented the pest from causing damage that exceeded the economic threshold, which was 50 mites/leaflet [23].

Additionally, when *N. californicus* was released at predatory mite/prey ratios between 1:5 and 1:10, this predatory mite was an effective biological control agent of *T. urticae* on strawberry plants in the field and in greenhouse conditions and maintained the pest population at low levels for long periods [24].

For control of the two-spotted spider mite on geranium (*Geranium* spp., Geraniaceae), a week after release, the predatory mite *P. persimilis* at predatory mite/prey ratios of 1:4 and 1:20 caused a significant decrease in numbers of *T. urticae* and maintained low levels of the pest, and the plants suffered less damage [25].

In the present study, the results show as the number of predatory mites released on the plants increased, the number of pest mites in the treatments decreased. However, the number of pest mites also decreased in the control group, which was unexpected in the absence of predatory mites. As a possible explanation, when the number of pest mites increases significantly, these mites have a tendency to disperse and search for healthier plants [26], because the control treatment plants were very weak when compared to plants whose treatments had predatory mites. This phenomenon was observed in the control group, and, in an attempt to disperse, the mites concentrated on the leaves at the top of plants and on the floral buds. Thus, although the density of pest mites on the plants in control treatment was high, the pest density was likely underestimated because leaves were collected from all parts of the plants and not only from the most affected regions. After the release of *N. californicus*, *P. persimilis*, and *N. californicus* + *P. persimilis* on gerbera (*Gerbera* spp., Asteraceae) growing in a greenhouse, each species of predatory mite individually decreased the population of *Tetranychus cinnabarinus* (Boisduval, 1867) (Acari: Tetranychidae) to zero [27]. In the present study, a similar result was also observed for *N. californicus* and *P. macropilis* on *T. urticae* on rosebushes. When generalist and specialist predatory mites are released in combination, intraspecific and interspecific competition occurs, leading researchers to conclude that only one predatory mite species must be released at a time, with each predatory mite considered separately before selection [27]. However, in the present study, releasing both predatory mite species in combination was as viable as the individual release of only one of the species.

Although the individual release of *P. persimilis* is a favorable strategy for the immediate control of *T. cinnabarinus* in the greenhouse [27] [28], the long-term control of pest mites was improved by the sequential or combined release of *N. californicus* and *P. persimilis*, which is also valid for the control of *T. urticae* [29]. When an alternative food is available, e.g., pollen, *N. californicus* can be released to persist before the occurrence of pest mites or, in the absence of an alternative food, can be released as soon as the pest is detected. As soon as the densities of the pest increase and *N. californicus* can no longer control pest mites, *P. persimilis* can be released [27].

A similar approach could be used with *P. macropilis* and *N. californicus* for control of the two-spotted spider mite on rosebushes. *Phytoseiulus macropilis* has a high rate of predation, and this predatory mite species can be used to locate *T. urticae* at long distances in infested strawberry plants through volatile substances induced by the herbivory of *T. urticae* [20]. By contrast, the predator *N. californicus* can function as a stabilizer, because this predator survives better under conditions of low prey densities and may be more resistant to chemical products [30].

For the conditions of this study, both species of predatory mites, *P. macropilis* and *N. californicus*, were efficient in the control of the two-spotted spider mite, *T. urticae*, on rosebushes growing in the greenhouse. Thus, in situations close to these, both the release of *N. californicus* and the release of *P. macropilis* alone could be recommended without the need for joint release. However, *P. macropilis* would be a more viable option, since it reduced a greater number of pests when compared to *N. californicus*. However, one should not forget to analyze each case and situation according to their need for control.

IV. CONCLUSIONS

The predatory mites *N. californicus* and *P. macropilis* were effective biological control agents of the two-spotted spider mite, *T. urticae*, on rosebushes growing in the greenhouse, released individually or in combination.

When the predatory mites were released as a single species, *P. macropilis* was more efficient in the biological control of *T. urticae* than *N. californicus*, at either low or high prey density, but the *N. californicus* can survive for longer in the absence of prey or in its low presence, for being a generalist predatory mite.

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Evaluation of biological and physicochemical risk of hospital liquid waste in Morocco

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Abstract— *Hospitals could be the source of pollution that must be taken into account in a general approach to health and environmental risk assessment. This study is part of the characterization of hospital liquid waste in a region in Morocco, whose objective is to assess their physicochemical, bacteriological and toxicological quality.*

The physicochemical analyzes carried out on 144 samples taken during one year showed a great fluctuation of some parameters such as: BOD5, COD, MES, PH, T°, as well as an important bacteriological load: Total coliforms, E coli, faecal Streptococci, pathogenic germs such as: Salmonella, Staphylococcus, 20% multi-resistant, virulent germs of emerging nature such as: Pseudomonas aeroginosa, and trace metal elements such as: mercury and copper that exceed the threshold of acceptability. At the end of this study, it turned out that hospital effluents are heavily loaded with pollutants. We therefore hope, in the light of the results provided by this study, to have generated a real awareness of the competent authorities on strengthening and improving the treatment of hospital liquid effluents such as developed countries, as the impact of these effluents on human and environmental health is harmful and can spread emerging diseases.

Keywords— Wastewater, Liquid effluent, physicochemical pollution, bacteriological, trace elements.

I. INTRODUCTION

All human activity generates solid and liquid waste. The numerous studies carried out in recent years on the sectors and methods of disposal of solid waste have demonstrated the interest that health and administrative professionals in hospitals and the public authorities attach to this subject in particular, and to the protection of the environment and public health in general. This awareness appeared concomitantly with new micro-organisms (viruses, multi-resistant bacteria and prions) and evolved with the progress of medical techniques and the application of aseptic rules associated with the extension the use of unique uses.

Hospital centers whose size corresponds to small or medium-sized agglomerations use for their activities and hygiene, large volumes of water which are then rejected, loaded with microorganisms, some of which are multi-resistant and often toxic chemicals and sometimes radioactive. This study shows that health care institutions must master the management and treatment of their liquid waste. [1],[2],[3],[4],[5],[6],[7],[8],[9],[10].

II. MATERIALS AND METHODS

As sampling sites for this study were selected specific manifolds in two major hospitals: The collectors of the hospital 1:- The kitchen collector;- The manifold that connects the emergency services and traumatology; the manifold that connects lingerie and laboratory;- The collector of motherhood. The collectors of the hospital 2- The main collector; - Emergency collector. Sampling of liquid effluent two hospitals under study was performed six times a season and three times a day at the outlets of the collectors of sewage chosen over a period of a year, for a total of 144 samples. The samples were held in the flow area of the sewer where water movement is most active. Samples of releases have been made according to Moroccan standards. The analyzes were performed at the National Institute of Hygiene. The desired parameters: Physicochemical: T, PH, Cond, COD, BOD5, TSS, ... Metallic trace elements: Hg, Cd, Cr, Cu, Pb;Microbiological: contamination germs Fecal (coliforms,

E. coli ...) germs pathogens (Salmonella, Vibrio, ...), germs of hospital origin (Pseudomonas, Staphylococcus aureus, Enterococci intestinal, ...)Microbiological analyzes were performed by the membrane filtration method according Moroccan standards. The analyzes of trace metals were made by atomic adsorption spectrophotometry electrothermal furnace with graphite VARIAN.240 Type (GF-AAS) for the lead, copper, chromium, and cadmium. The temperature, pH and conductivity

were measured on site using a mobile ph-meter HANNA type of direct readout and type conductivity CONSORT 535 expressed in microseconds / cm.

III. RESULTS

3.1 Physicochemical parameters:

3.1.1 Electrical conductivity

The mean value of the conductivity is 1500 microseconds / cm in the rejection of the maternity hospital1.This is due to the misuse of detergents and ionizing injections [11]. This class value release to category 3 (release quality grid); [14].

TABLE 1
TEMPORAL VARIATION OF ELECTRICAL CONDUCTIVITY AS A FUNCTION OF TIME

		Hospital 1				Hospital 2	
Conductivité électrique (µs/cm)		Emergency+Traumatology	Maternity	Laboratory+Lingerie	Kitchen	Mainrejection	Rejection of emergencies
	Winter	767,67	**1546,33**	1323,67	1323 ,66	926,33	1249,33
	Spring	767,67	1521	1328	1387,67	928,33	1251,33
	Summer	759	1511,33	1348	1381	923,66	1247,66
	Autumn	762,33	1535,33	1348,67	1385,33	928	1252,33
	Mean	763	1536,19	1337,085	1385	926,58	1250,1625
	STDEV	3,571	18,097	13,113	2,881	2,134	2,083

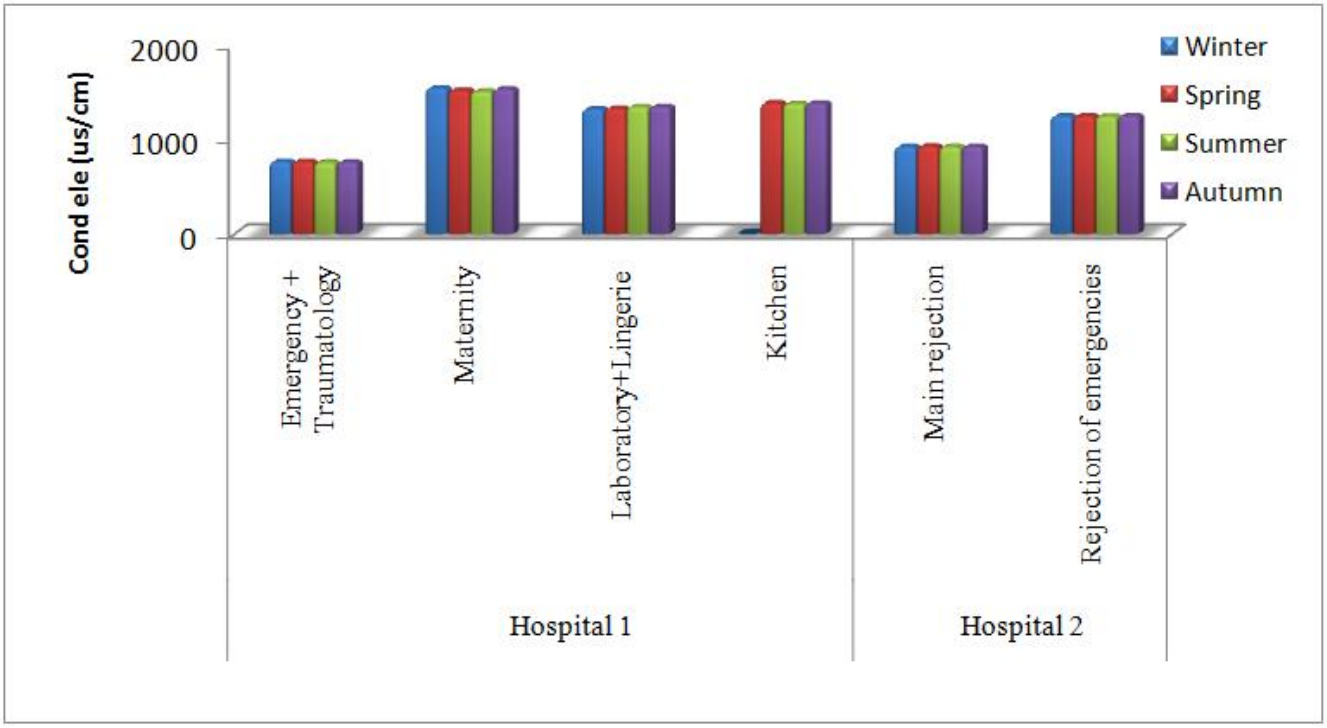

FIG 1: CHANGE IN ELECTRICAL CONDUCTIVITY DEPENDING ON THE SEASON

3.1.2 Turbidity:

The measurement of turbidity makes it possible to specify the visual information on the water. Turbidity indicates the presence of particles suspended in water (organic debris, clays, microscopic organisms, etc.).

TABLE 2
MEASUREMENT OF THE TURBIDITY OF HOSPITAL EFFLUENTS IN THE HYDROLOGY LABORATORY - INH – RABAT

		Hospital 1				Hospital 2	
		Emergency + Traumatology	Maternity	Laboratory+Lingerie	Kitchen	Main rejection	Rejection of emergencies
Turbidity(NTU)	Winter	18,82	178,19	194,12	13	46,36	86,12
	Spring	18,82	170,9	193,93	12,63	46,07	178,33
	Summer	18,91	177,07	192,8	12,22	47,33	70,73
	Autumn	18,9	177,47	192,83	12,46	47	70,5
	Mean	18,86	175,91	193,42	12,57	46,69	101,42
	STDEV	0,049	3,37	0,703	0,328	0,577	51,792

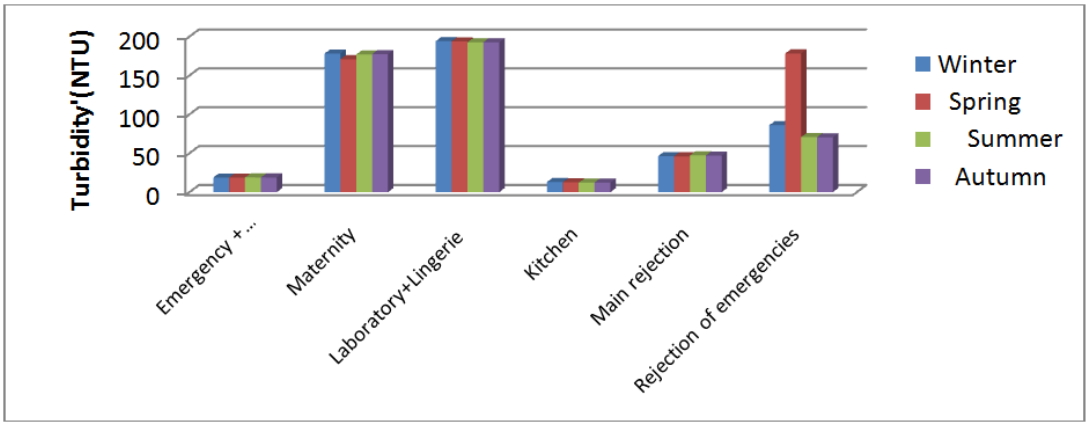

FIG 2: CHANGE IN TURBIDITY BY SEASON

The average value of turbidity is between 18 and 194 NTU (maximum value observed in laboratory collectors and lingerie to the hospital1).This prevents the propagation of light; therefore the hospital sewage clarification is necessary.

3.1.3 COD / BOD5:

TABLE: 3
REPORT EVALUATIONS FOLLOW SAMPLING SITES

		Hospital 1				Hospital 2	
		Emergency + Traumatology	Maternity	Laboratory+Lingerie	Kitchen	Main rejection	Rejection of emergencies
DCO/DBO5	9 H 30	7,75	3,98	7,98	7,02	8,02	8,09
	11 H 40	7,19	4,01	7,89	6,81	8	7,99
	13 H 30	6,27	3,97	7,9	6,77	7,98	7,92
	Moyenne	7,07	3,99	7,92	6,87	8	8

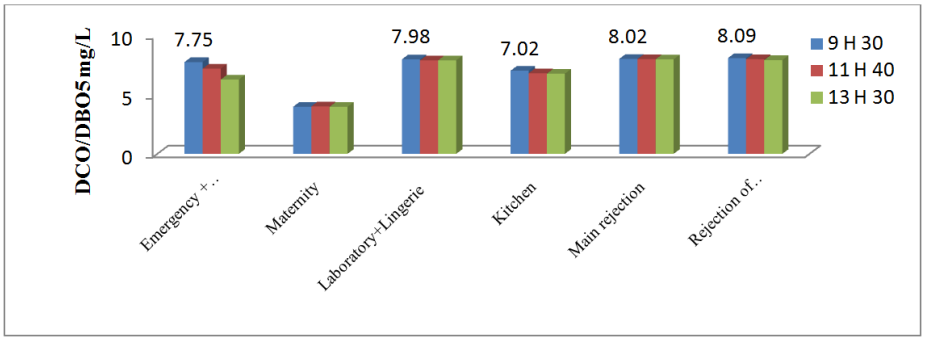

FIGURE 3: EVALUATION OF THE BIODEGRADABILITY OF HOSPITAL DISCHARGES ACCORDING TO THE DIFFERENT SITES

The average values for the period of samples for the six releases of the two hospitals are between 6.5 and 8.21mg/L, indicating poor biodegradability due to the chemical nature of hospital discharges

3.2 Metalic trace elements:

- Mercury (Hg): Mercury concentrations are ten times the threshold value (0.05 mg / l) .This is due to the frequent use of mercury thermometers at the hospital and the use of mercury-based detergents.
- Copper (Cu): Copper reached 531.02 mg / l during the fall season for six of the two hospitals sampling sites; the concentration increase is mainly due to the use of reagents and chemicals in hospitals.

3.3 Bacteriological parameters:

3.3.1 Microbiological indicators of faecal contamination

The maximum value of E. coli was noticed during the period of high activity of hospital services, this indicates recent faecal contamination.

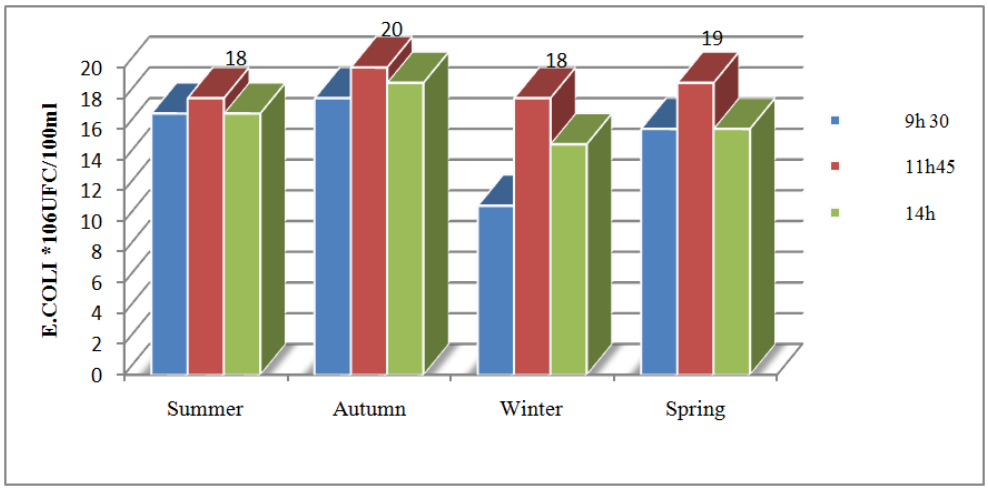

FIG 4: TEMPORAL VARIATION IN ESCHERICHIA COLI

3.3.2 Pathogens

The burden of Staphylococcus aureus increases during the summer, especially during the period of high activity (11h 45min). The load reaches 4.5 10^6 CFU / 100 ml.

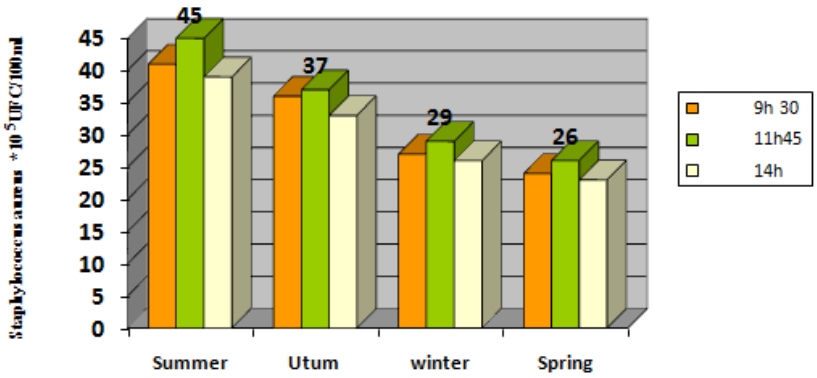

FIG 5: TEMPORAL VARIATION IN STAPHYLOCOCCUS AUREUS

3.4 Virulent germs

Eight Salmonella have been identified in the main collectors and emergency hospital 2: Salmonella Chester, entertidis (MDR) and 2 Salmonella (MDR) in the collectors of the hospital1. and 2 Vibrio parahaemolyticus were isolated in the releases of the two hospitals for specific CHROMagar Vibrio and poly monovalent and serology.

IV. CONCLUSION

The results of the analyzes we have done during one year showed that the hospital effluents have a high rate of pollution both physicochemical, biological and toxicological; Indeed test results exceeded the thresholds set by national standards of direct and indirect discharges [14] , and could have a detrimental effect on the health of the population of the study area and its direct environment. Indeed , the concentration of BOD5 (259.55 mg / l) classifies liquid discharges from two hospitals as standards (quality Grid) in the category of poor quality .The results of toxicology tests showed values above the threshold allowed by legislation and national and international standards, such as: Mercury: 0.9mg/l in the rejection of Traumatology and Emergency + 0.8mg / l in maternity, This is due to the misuse of mercury thermometers that are banned in France referring to Jehanin and the use of some mercury-based detergents; Copper: 500 times exceeds the normative limit indirect discharges These two heavy metals have a harmful effect on health and the environment: Mercury is a cumulative poison that causes neurological disorders, gingivitis and stomatitis, as well as kidney damage. In the environment, it turns into organomercury (CH3Hg +) that pass the placental barrier causing teratogenic and fetotoxic; Copper causes an alteration of fish gills, and a greening effect oysters, it becomes toxic when complexed with the organic matter. Other physico-chemical analyzes of wastewater two hospitals showed that the values of PH obtained belong to the favorable PH area development of most microorganisms. The pH value varies between 6.02 and 7, 43, and the temperature is below 30°C. This explains the indicators of fecal contamination obtained show an increasing trend throughout the study. Indeed, the maximum concentration of the medium during the CT seasons is 43.10^7UFC / 100ml and that E. coli is 19.10^6UFC / 100ml. Moreover, the most contaminated sites in the most pathogenic bacteria such as Salmonella are, emergency trauma +, the main rejection and motherhood. These bacteria are highly virulent and have a high impact on the health of the population and the spread of epidemic diseases.

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Ectopic Expression of Hawthorn *SND1* Gene in Tobacco

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***Abstract*—** *NAC proteins are plant-specific transcription factors (TFs) and have been shown to function in plant development processes and abiotic and/or biotic stress responses. SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN1 (SND1) is one type of NAC TFs, which is a key regulator in the regulation network for secondary wall synthesis. In this study, the SND1 gene, named CpSND1 because it has a conservative N-terminal DNA-binding domain with AtSND1, was isolated from hawthorn (Crataegus pinnatifida). The full-length CDS of this gene was 1,203 bp, encoding 400 amino acids. The CpSND1 gene was transferred into tobacco (Nicotiana tobacum) by the Agrobacterium-mediated transformation method, and 20 transgenic lines were obtained. Tobacco plants overexpressing CpSND1 had typical phenotypes, including inhibited growth, upward-curling leaves. Our results provided functional information of CpSND1 for future genetic engineering.*

***Keywords*— hawthorn, transcription factor, SND1, ectopic expression.**

I. INTRODUCTION

Secondary cell walls are largely composed of three main polymers: cellulose, hemicellulose, and lignin. These polymers are normally required to enable vascular plants not only to build strong xylem conduits for the transport of water and minerals but also to attain strong mechanical support for the plant body (Raven et al., 1999). The formation of the secondary cell wall is mainly regulated at the transcriptional level, and most of what is known about the regulation comes from the model herbaceous plant *Arabidopsis thaliana*. NAC (NAM-ATAF1, 2-CUC2) and MYB act as the key master switches that control secondary cell wall deposition (Taylor-Teeples et al., 2015).

NAC proteins are plant-specific transcription factors (TFs) and have been shown to function in plant development processes and abiotic and/or biotic stress responses. In Arabidopsis, there are two types NAC TFs acting as on-off switches that take part in regulating secondary wall formation in vascular cells and fibre cells. The first type of NAC TFs (VASCULAR-RELATED NAC-DOMAIN) VND6 and VND7 contribute to both secondary wall biosynthesis and programmed cell death of the vessels in both root and shoot tissues (Kubo et al., 2005; Yamaguchi et al., 2008). The second type of NAC TFs consists of NST3 (NAC secondary wall thickening promoting factor 3)/SND1, NST1 and NST2, which participate in thickening the secondary wall both in vascular fibre cells and secondary xylem fibre cells (Zhong and Ye, 2014). Arabidopsis NST3/SND1 is specifically expressed in vascular fibres and xylem fibres, and expressing *SND1* under the control of the cauliflower mosaic virus 35S (CaMV35S) promoter can cause ectopic secondary wall deposition in non-sclerenchyma cells.

Protein binding assays have demonstrated that SND1 can bind to a DNA section with the sequence (T/A)NN(C/T)(T/C/G)TNNNNNNNA(A/C)GN(A/C/T)(A/T), named SNBE (secondary wall NAC binding element) (Zhong et al., 2008). Among the SND1-regulated transcription factors, MYB46 and MYB83 have been shown to be direct targets (Zhong et al., 2007). In particular, MYB46 and MYB86, functioning as another level of molecular switches, redundantly turn on the entire secondary wall biosynthetic programme (Zhong and Ye, 2015).

From early transcriptome data of soft-endocarp and hard-endocarp hawthorns (Dai et al., 2013), we found four NAC family transcription factors that were strongly down-regulated in the fruits of soft-endocarp hawthorn compared to the fruits of hard-endocarp hawthorn. We suspected that they might participate in the biosynthesis of lignin or secondary cell walls. In this study, transgenic tobacco plants with overexpressing *CpSND1* driven by the CaMV35S promoter presented growth inhibition, upward-curling leaves, which are similar to the phenotypes observed when the *SND1* gene of Arabidopsis was overexpressed. Our results indicate that the *SND1* function is conserved in different plants.

II. MATERIAL AND METHOD

2.1 Plant materials and growth conditions

Trees of *Crataegus pinnatifida* accession H8 (hard-endocarp hawthorn) were maintained in the National Hawthorn Germplasm Repository at Shenyang. The material samples were frozen in liquid N_2 for RNA extraction. Tobacco seeds

(*Nictiana tabacum*) were surface-sterilized and sown on MS medium with 16 h light/8 h dark period at 25 °C. One month old sterile tobacco plants were used for transformation.

2.2 Nucleic acid extraction

Both genomic DNA and Total RNA were extracted from plant material using the modified CTAB method (Chang et al., 2007). And the RNA samples were treated with DNase I (TaKaRa, Dalian, China) for 4 h.

2.3 Isolation and sequence analysis of *CpSND1*

The first-strand cDNA was synthesized with PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). According to the assembled contig sequence of transcriptome data, specific primers were designed to obtain the full length of hawthorn *SND1*. The primers used to amplify *SND1* were 5'-CGCCATATGTCTGATGATCAAAT-3' and 5'-CGGGTACCTTACACCGACAAGTGGC-3'. The PCR cycle profile was: an initial denaturation of 95°C for 5 min; 34 cycles of 95°C for 45 s, 58°C for 30 s and 72°C for 1 min; a final extension of 72°C for 10 min. The cDNA product was cloned into pMD18-T vector (TaKaRa, Dalian, China) and sequenced.

2.4 Overexpression vector construction

To overexpress the *CpSND1* gene in plants, coding sequence fragment of *CpSND1* was insert into plant expression vector pRI101-GFP, contain a cauliflower mosaic virus 35S (CaMV35S) promoter and the nopaline synthase terminator. The reconstructed plasmid pRI101-CpSND1 was introduced into *Agrobacterium tumefaciens* strain EHA105 using the freeze-thaw transformation method.

2.5 *Agrobacterium*-mediated transformation

To generate transgenic tobacco plants, leaf discs of tobacco were transformed by the *A. tumefaciens* EHA105(pRI101-CpSND1) mediated transformation method described by Horsch et al., 1985. The putative transgenic tobacco plants selected with kanamycin (30 mg/L) were further identified by PCR and RT-PCR analysis and then were chosen for further experiments.

2.6 Identification of transgenic plants by PCR

The polymerase chain reaction (PCR) analysis was performed with isolated genomic DNA to check for the presence of the transgene in the putative transformed plants. The primers were 35S-F: 5'-ATGACGCACAATCCCACTATCCT-3' and RV: 5'-CAGGAAACAGCTATGAC-3'. The PCR cycle profile was: an initial denaturation of 95°C for 5 min; 34 cycles of 95°C for 45 s, 58°C for 30 s and 72°C for 1 min; a final extension of 72°C for 10 min. The amplified products were analyzed byelectrophoresis in 1.2% (w/v) agarose gels.

2.7 qRT-PCR

First-strand cDNA was synthesized using the PrimeScript RT Reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Dalian, China). The reverse transcription products of cDNA, diluted four times, were used as the template for quantitative PCR. Reactions were set up with SYBR Green Fast qPCR Mix (TaKaRa, Dalian, China) according to the manufacturer's instructions in a total volume of 20 μl with each primer at 0.2 μM. The amplification programme was as follows: one cycle of 30 s at 95°C followed by 40 cycles of 5 s at 95°C and 10 s at 60°C. All reactions were run in triplicate, and average values were calculated. Relative expression levels of target genes and SD values were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

III. RESULTS AND DISCUSSION

3.1 Isolation and sequence characteristics of *CpSND1* gene

In our early study (Dai et al., 2013), we found four NAC family transcription factors that were strongly down-regulated in the fruits of soft-endocarp hawthorn compared to the fruits of hard-endocarp hawthorn. According to the conservative NAC domain of these TFs, we aligned NAC TFs in other species, which all contained a similar NAC DNA-binding domain, by the NCBI and PLAZA web blast. Because *AtSND1* was the first key switch involved in secondary wall formation, and we also found that the 8_Unigene_BMK.37276 [log2(S7/H8) = -6.31] gene was the most homologous to *AtSND1*, so we named the 8_Unigene_BMK.37276 gene as *CpSND1*. The full-length CDS of *CpSND1* gene was 1,203 bp, encoding 400 amino acids (Figure1). The amino acid sequence alignment showed that CpSND1 had a highly conserved NAC DNA-binding domain (Figure 2). These bioinformatic results revealed that CpSND1 may have a similar function as AtSND1.

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ATGTCTGATGATCAAATGAGTCTATCAATAAATGGTCAATCTCAAGTTCCCCCAGGTTTCCGGTTCCATCCAACCGAAGAGGAGCTTCTT
 M  S  D  D  Q  M  S  L  S  I  N  G  Q  S  Q  V  P  P  G  F  R  F  H  P  T  E  E  E  L  L
CACTACTATCTTAGGAAGAAAGTTGCCTTTGAGAGGATTGATCTTGATGTAATTCGAGAAGTTGATCTTAATAAGCTTGAGCCATGGGAC
 H  Y  Y  L  R  K  K  V  A  F  E  R  I  D  L  D  V  I  R  E  V  D  L  N  K  L  E  P  W  D
ATTCAAGAGAAGTGCAAAATAGGTTCCACTCCACAAAATGATTGGTACTTCTTCAGTCACAAGGACAAGAAATACCCAACCGGAACTCGA
 I  Q  E  K  C  K  I  G  S  T  P  Q  N  D  W  Y  F  F  S  H  K  D  K  K  Y  P  T  G  T  R
ACCAATCGGGCAACCACTGCTGGGTTTTGGAAGGCCACCGGGCGGGACAAGATCATCTATAGCGGCTTCAAAAGAATTGGATTGAGGAAG
 T  N  R  A  T  T  A  G  F  W  K  A  T  G  R  D  K  I  I  Y  S  G  F  K  R  I  G  L  R  K
ACACTTGTGTTTTATAAGGGTCGAGCTCCTCATGGACAAAAGTCAGATTGGATCATGCATGAATATAGGCTTGATGAAAGCAACACTCAT
 T  L  V  F  Y  K  G  R  A  P  H  G  Q  K  S  D  W  I  M  H  E  Y  R  L  D  E  S  N  T  H
GAAACCACCGTTTCTAGCTCAATGGGGGAGTCGATGACCGAAGACGGGTGGGTGGTCTGCCGGGTATTCAAGAAGAAGAATTATCAGAAA
 E  T  T  V  S  S  S  M  G  E  S  M  T  E  D  G  W  V  V  C  R  V  F  K  K  K  N  Y  Q  K
GCTTTAGAGAGCCCTAAGGCCTCCTTCTCCATGGACTCATCAAACAACCAAATGCATGGTTCAAGAAGCGATGGTGTTCTTGATCAAATA
 A  L  E  S  P  K  A  S  F  S  M  D  S  S  N  N  Q  M  H  G  S  R  S  D  G  V  L  D  Q  I
CTGATGTACATGGGAAGGACTACTTGCAAGCTGGAAAATCAAGATCAATCCTTAACCATGAATAACATCTCAGAAAGATTTATGCATCTG
 L  M  Y  M  G  R  T  T  C  K  L  E  N  Q  D  Q  S  L  T  M  N  N  I  S  E  R  F  M  H  L
CCAAGGCTTGAGAGCCCAACTCTTCCAAACCTTCCCGCTTTCGACCAGGAACGTAGCTTCAAAGCGTGCTATTCGGCCATTGATGACATG
 P  R  L  E  S  P  T  L  P  N  L  P  A  F  D  Q  E  R  S  F  K  A  C  Y  S  A  I  D  D  M
TTCATAGAAACTGAGCCTTCTTCAACAAACCAACCAAGCAATGGTTGTGACAATAATGACCTAGTCGATGATCATGAGTACCCCAAAACA
 F  I  E  T  E  P  S  S  T  N  Q  P  S  N  G  C  D  N  N  D  L  V  D  D  H  E  Y  P  K  T
AGGCTAAATGACTGGGCTACCCTTGATAGGCTTGTGGCATCCCAACTAGGTCAACTCAATGGCCAAGATCAAGAGACACCAAAACACTTG
 R  L  N  D  W  A  T  L  D  R  L  V  A  S  Q  L  G  Q  L  N  G  Q  D  Q  E  T  P  K  H  L
TCTTGCTTTGGCGATCCAAACATGGCCTTTTGTTCTTCTCCTCATCCTAATGATCAAGACAATGACGTACAACTATCATATCCATACCTA
 S  C  F  G  D  P  N  M  A  F  C  S  S  P  H  P  N  D  Q  D  N  D  V  Q  L  S  Y  P  Y  L
CGTACGAGTAGATCATCCGATCATCAATCCGAAGTATACAACAACGAGAATGATCTGTGGAACTTCACCAAATCGTCGTCATCACCGTCA
 R  T  S  R  S  S  D  H  Q  S  E  V  Y  N  N  E  N  D  L  W  N  F  T  K  S  S  S  S  P  S
TCATCGGACCCGCTTTGCCACTTGTCGGTGTAA
 S  S  D  P  L  C  H  L  S  V  *
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FIGURE 1 NUCLEOTIDE SEQUENCE OF CDS AND AMINO ACID OF SND1 IN HAWTHORN

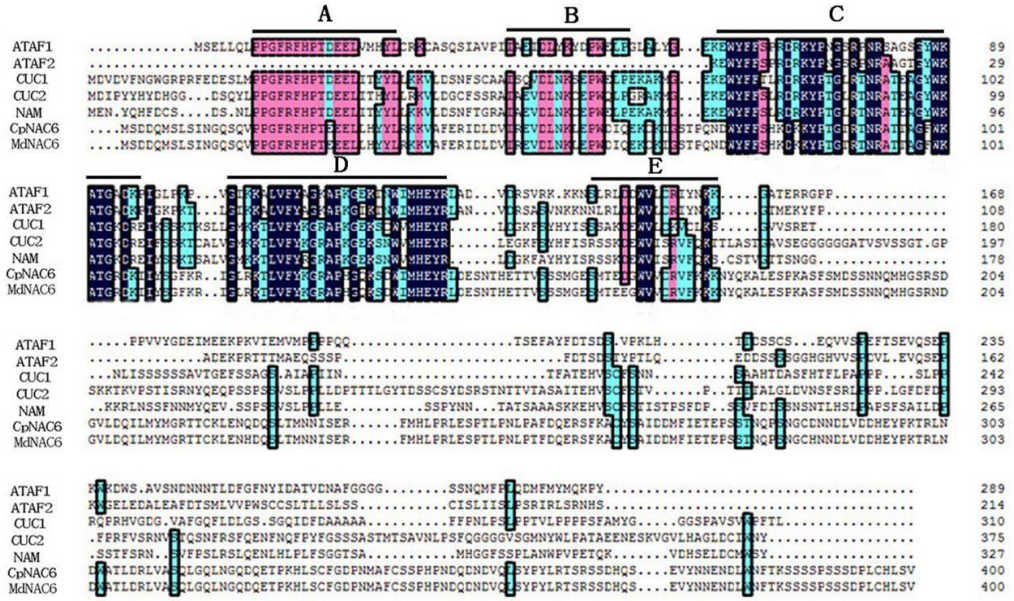

FIGURE 2 MULTIPLE SEQUENCE ALIGNMENT ANALYSIS OF HAWTHORN SND1 AND NAC DOMAIN PROTEINS FROM OTHER PLANTS

3.2 Transformation of tobacco with *CpSND1* gene

To elucidate the biological function of the *CpSND1* gene, an over-expression vector containing full CDS of *CpSND1* gene was constructed and introduced into *A. tumefaciens* strain EHA105. Transgenic lines were obtained by *Agrobacterium*-mediated transformation with leaf discs as explants (Figure 3).

FIGURE 3: TRANSGENIC PLANTS REGENERATION FROM LEAF DISCS OF TOBACCO

To confirm the presence of the *CpSND1* gene, genomic DNAs of the kanamycin-resistant plants and non-transformed control plants were isolated. PCR analysis resulted in the expected size for the amplified fragment. No amplified product was detected in the samples containing DNA isolated from non-transformed control plants (Figure 4). Twenty transgenic lines were identified.

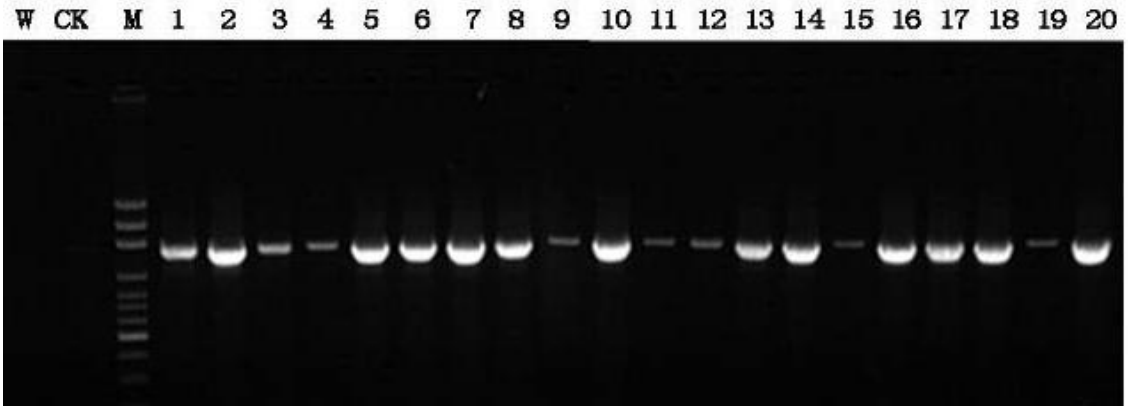

FIGURE 4: PCR ANALYSIS OF TRANSGENIC PLANTS OF TOBACCO. W: WATER; CK: NON-TRANSGENIC PLANT; M: MARKER, LINE 1~20: TRANSGENIC PLANTS

3.3 Ectopic expression of CpSND1 induced curled leaves and stunted growth in tobacco

The transgenic plants and non-transgenic plants were transplanted into pots in greenhouse at the same time. Forty days after transplantation, the phenotypes were investigated. Compared with the non-transgenic plants, the transgenic plants were smaller and exhibited stunted growth. The leaves of transgenic plants were upward-curled. These phenotypes induced by *CpSND1* were similar to those induced by *AtSND1* in Arabidopsis (Zhong et al., 2006). Ninety days after transplantation, the difference on phenotypes was more obvious (Figure 5).

To check the relationship of *CpSND1* expression level with phenotype, the transcription level of *CpSND1* gene in tobacco were analyzed by qRT-PCR. Six transgenic lines were selected, as showed in Figure 6, the expressions of *CpSND1* were detected in leaves of all transgenic plants with different levels, while it could not detected in non-transgenic plants.

FIGURE 5 THE PHENOTYPES OF TRANSGENIC PLANTS AND NON-TRANSGENIC CONTROL (LEFT) OF TOBACCO

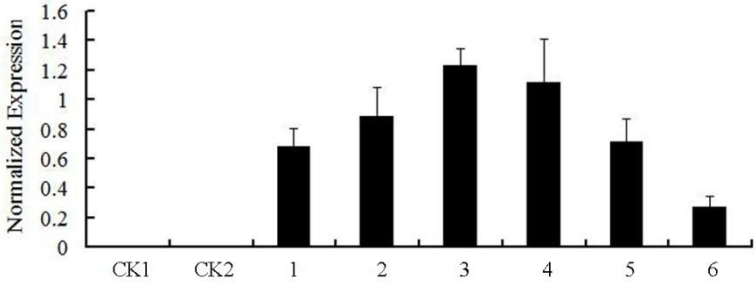

FIGURE 6 THE EXPRESSION LEVELS OF CPSND1 IN TRANSGENIC TOBACCO PLANTS. CK1 AND CK2: NON-TRANSGENIC PLANTS, LINE 1~6: TRANSGENIC PLANTS

IV. CONCLUSION

SND1 gene was isolated from hawthorn (*Crataegus pinnatifida*) and transferred into tobacco. Tobacco plants overexpressing *CpSND1* gene had typical phenotypes, including inhibited growth, upward-curling leaves.

ACKNOWLEDGEMENTS

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Study on Physicochemical Properties and Microstructure of Taro Starch in Taizhou

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Abstract— *In order to compare the physical and chemical properties of taro in Taizhou area, taro starch was extracted through the method of spray drying , the blue values, solubility, swelling force, amino acid of amylose and amylopectin and taro starch granules were studied. The results showed that the blue values of amylose were1.16, 0.97, 1.1 in Taixing, jingjiang and xinghua taro respectively, the blue values of amylopectin were 0.19, 0.14, 0.13 in Taixing, jingjiang and xinghua taro respectively. In the comparison of solubility, taixing xianghe taro had a wide distribution, the following sequence was observed: jingjiang xiangsha taro >xinghua longxiang taro. Analyses of amino acids were 3.03mg/100g in taixing xianghe taro starch which was more than the other two taros. The nutritional assessment of amino acids suggested that the taixing xianghe and xinghua longxiang taro contained delicious amino acids which of EAA were 38% and 44% respectively. Taixing xianghe taro contained the highest content of phosphorus and potassium, which were 1419 mg/kg and 8084 mg/kg separately. Granule morphology showed that starch particles of taixing xianghe taro were smaller than those of taros and the surface was smooth, uniformly. Particle shape was spherical. Xinghua longxiang taro particles with irregular diamond, Jingjiang xiangsha taro starch particles was irregular sphere, and spherical surface was uneven. Taizhou taro was delicate, delicious, which of the taixing xianghe taro was with better quality on the domestic market.*

Keywords— taro, extraction of starch, quality, analysis.

I. INTRODUCTION

Taizhou is acres of water around the city, rich in water sandy loam, especially suitable for the growth of taro. Taixing xianghe taro which is rich in protein, minerals and vitamins, saponins and other ingredients has a unique flavor, beneficial to stomach of high-quality alkaline food. At present, there are few studies on taro in other areas. Jiang Shaotong and Wang Hongpu used to separate and purify polysaccharides from raw taro, studied the chemical characteristics of each component and the effect of immune cells on mice in vitro. Three kinds of polysaccharide fractions were isolated and purified by ion exchange column and gel column [1]. Sun Zhongwei studied the extraction of taro starch by water extraction and alcohol precipitation, enzymatic auxiliaries, dialysis, freezing and drying of taro polysaccharides and the nature of taro starch [2]. YANG Ying-ying studied the optimized fermentation conditions of purple sweet potato yoghurt, the texture and flavor components were simultaneously studied by using texture technology and headspace solid-phase microextraction and gas chromatography [3] , Yu Xin and Xu Wenxing studied the effects of the ratio of raw materials, the content of dough moisture and edible oil on the quality of taro crust in the process of non-fried taro crust, and the study of taro varieties concentrated in Zhejiang Fenghua, Guangxi Lipu and Fujian and other places.

Taizhou taro really took up in people's view in 2012 in the "tongue of China" reported xinghua longxiang taro, so that hundreds of millions of visitors tasted the delicious longxiang taro, but nutrition and starch properties of the Taizhou taro was not reported so far.

In order to effectively play a greater role in the development of taro farmers and the development of taro, it was necessary to study the nutritious composition and related properties of taro in taizhou area. According to the relevant report[2], in order to ensure the quality of starch under water to extract, with ascorbic acid and sodium sulfite to protect color. The basic nutritious contents of Jingjiang xiangsha taro, taixing xianghe and xinghua longxiang were studied. The nutritious composition, solubility, swelling power, transmittance and water absorption of taro starch were studied in order to study the development and utilization of taro in the future.

II. MATERIALS AND METHODS

2.1 Materials and Instruments

Taro: The buds of taixing xianghe taro are red; the leaves of xinghua longxiang taro are dark green, petiole are green and long, leaves and petiole connected with purple halo, the mother taro near spherical, white meat, powder and incense, less

taro, oval, sticky fleshy; Jingjiang xiangsha taro smooth entrance, sweet, strong sandy, bought in Taizhou every origin; ascorbic acid and n-Butanol purchased from Sinopharm Group (Shanghai) Chemical Reagent Co., Ltd.; sodium sulfite purchased from suzhou china Airlines chemical Technology Co., Ltd.; The above reagents are analytical pure.

DS-1 high-speed tissue crusher were from Shanghai specimen model plant; GYB series of high-pressure homogenizer were from Shanghai Donghua high pressure homogenizer; B-290 small spray dryer were from Beijing Lai Hang Electronics Co., Ltd.; 754 UV spectroscopy Photometer were from Shanghai Jinghua Technology Instrument Co., Ltd.; TD5A-WS-type centrifuge were from Jintan Jinnan Instrument Manufacturing Co., Ltd.

2.2 Experimental methods

2.2.1 The step of taro starch

marketing taros, which were hypertrophy bulb, upright shape, nattiness and uniform, tissue enrichment, no dry shrinkage and hardening phenomenon, no damage and no metamorphic decay.

The rough surface of taro was removed, washed, cut into thin, and then washed again. The blocks of cut taro were covered with the ratio of material to water 1:2 and pH 7, and then the blocks were placed in a high-speed tissue crusher to a uniform consistency and no lumpy particles. In the same time the particles were ground, 0.01% ascorbic acid and 0.02% sodium sulfite [1] solution was added to soak for 70min, the homogenate extraction was filtrated with 100 mesh sieve, filtrate and filter residue was collected, which was filtered three times with a small amount of water rinse, the filtrate was collected. The filtrate was added to a high pressure homogenizer and homogenized at a pressure of 20-30 MPa. Spray drying, the inlet temperature was 135^{o}C, the outlet temperature was 75 ^{o}C. Exhaust fan 100%, peristaltic pump 20%. The starch was collected after the spray drying and stored in a constant temperature oven (40 ^{o}C) and collected.

2.2.2 Crude separation of amylose and amylopectin

5.0 g sample of taro starch were placed in a 500 mL beaker, added a small amount of anhydrous ethanol, so that the sample was fully moist, then added 200 mL 0.5 mol/L NaOH solutions. The mixing solution was heated in a boiling water bath with constant stirring for 30 min until complete dispersed. The resulting solution was cooled and centrifuged (4000 r/min, 20 min), then the undispersed residue (precipitate fraction) was removed. The centrifuged solution was neutralized with 2 mol/L HCl and added 100 mL of n-butanol-isoamyl alcohol in a ratio of 3: 1(v/v), and then heated and stirred in the boiling water bath for 20 min. At the same time, the solution was transparent and cooled to room temperature, transferred into 2~4 ^{o}C refrigerator for 24h, removed the centrifuge (4000 r/min, 20 min), the supernatant was crude amylopectin, the precipitate was crude amylose, the sample were collected for later analysis[5].

2.3 Purification of amylose and amylopectin

The precipitate (crude amylose) was transferred to a saturated n-butanol solution (120 mL). The mixture was then placed in a boiling water bath and stirred until the solution was transparent. The solution was gradually cooled to room temperature and transferred to a refrigerator (2-4 ^{o}C) for 24 h, centrifuged (4000 r/min, 20 min), and repeated the above steps 6 times, and then the precipitate was immersed in ethanol for 24 h, washed with anhydrous ethanol several times, And finally the precipitate was dried in a drying oven to obtain a pure amylose, collected for analysis[2]. The lower solution was added with 40 mL of n-butanol-isoamyl alcohol (volume ratio of 1: 1) mixture, heated and stirred in a boiling water bath until the solution was dispersed and transparent, cooled to room temperature, transferred to the refrigerator at 2~4 ^{o}C for 48h, centrifuged (4000r/ min, 20 min), removed the pellet and repeat the above steps 4 times with the supernatant. The resulting supernatant was concentrated under reduced pressure to half of the original volume, added 2 times the volume of ethanol, precipitated, centrifuged, the precipitate was dissolved in 200 mL 0.5 mol/L hotting NaOH solution, centrifuged to precipitate, the above precipitated solution was added 2 times volumes of absolute ethanol, dissolved the precipitate in 200 mL of distilled water, reprecipitated with 2 times of absolute ethanol, washed with anhydrous ethanol several times, finally, precipitated in a drying oven, amylopectin 100% pure product was obtained collected for analysis[5].

2.4 Determination of blue value

Blue value was an indicator of the binding properties of starch and iodine. The absorbance of the formed complex was measured at a wavelength, 0.5 mg of purified amylose and amylopectin was separately added to 50mL beaker, added 1mL of distilled water and 0.5mL of 1 mol / L sodium hydroxide solution, then heated in boiling water for 3min and then cooled, added 0.5mL of 1mol / L hydrochloric acid solution, added 0.1 g of sodium bitartrate, added water to 45mL, and added

0.5mL of iodine solution (mixed solution of potassium iodide and iodine, wherein the content of iodine 0.2% and the content of potassium iodide 2%), the solution was added to 50mL, mixed, placed at room temperature for 20 min, then measured with a spectrophotometer at 680nm wavelength absorbance. With the same concentration of iodine solution as a reference solution. Calculating the blue value according to the following formula: Blue value = OD_{680nm}×4/the concentration of the sample (mg/100mL)[5].

2.5 Determination of Solubility and Swelling Power of Different Taro Starch

Different starch samples was weighed, added 100mL distilled water, heated and stirred at 80°C for 30 min, and stirred to make starch solute to reach concentration of 0.4%, 0.8%, 1.2%, 1.6% and 2%, Centrifuged with speed 3000r/min for 30min. The supernatant was evaporated in a water bath to near drying, and then placed in a constant temperature oven drying, temperature 105°C to constant weight. The amount of drying material obtained was m, the solubility was calculated in the following formula[6,7].

$$Solubility = \frac{m}{M} \times 100$$

$$Swelling\ \ force = \frac{W \times 100}{100 - S} \times M$$

Formula: m-the supernatant evaporated to dryness weight (unit:g)

M-the weight of starch samples (unit:g)

W-the weight of sediment after centrifugation (unit:g)

2.6 Scanning electron microscopy

After the powdery sample was dried, the sample was glued it to holder with conductive glue. The sample holder was placed in the ion sputter and plated a 10～20 nm thick platinum film on the surface. Observation of electron microscopy under different magnification and taking pictures.

2.7 Determination of drainage rate of different taro starch solution

Different starch samples was weighed, added 100 mL of distilled water, heated and stirred at 80°C for 30 min, stirred to make starch solution to reach the concentration of 0.4%, 0.8%, 1.2%, 1.6% and 2%, placed in centrifuge tube, placed in the freezer, removed after 24h. After its natural thawing, the phenomenon was observed. Then the samples were placed in the freezer refrigerator, frozen repeatedly, centrifuged after drainage, the weight of the precipitate was brought into the equation given below to calculate the starch drainage rate[8].

$$Drainage\ rate = \frac{M - m}{M} \times 100$$

2.8 Determination of basic nutritional content in taro

Phosphorus and potassium elements were determined according to GB/T5009.91-2003 (Determination of minerals in food);

Ash content was determined according to GB 5009.4-2010;

Fat was determined according to GB/T 5009.6-2003 using the first method of soxhlet extraction;

Starch was determined according to GB/T5009.9-2008 using the second method of acid hydrolysis;

Amino acids were determined according to reference GB/T8314-2013 (Determination of total amino acids in food).

The above indicators were measured 3 times, taking the average.

2.9 Data processing

Using SAS8.2 software

III. RESULTS AND DISCUSSION

3.1 Nutritional content of taro

TABLE 1
THE NUTRITIONAL COMPOSITION OF SOME DIFFERENT TYPE OF TARO (mg/kg)

items	taixing xianghe taro	Jingjiang xiangsha taro	xinghua longxiang taro
amino acids	30.86±0.96	27.2±0.44	27.28±0.38
phosphorus	1419±12.34	1263±13.68	1036±11.54
potassium	8084±46.54	7621±49.74	7055±48.68
crude fat	2200±62.54	2540±44.58	2580±52.58
starch	91200±414.56	90240±420.48	120400±410.54
ash	9240±64.64	9140±62.36	9360±63.42

Phosphorus was a component of DNA and RNA, which was beneficial to memory and necessary to maintain bones and teeth. We can see from table 1, the content of phosphorus and potassium in Taixing xianghe taro was the highest among the three taro samples, which were 1419 mg/kg and 8084 mg/kg respectively. However, the content of potassium in xinghua longxiang taro was the lowest. The highest content of amino acid was 3.086 mg/100g in Taixing xianghe taro comparable to other two taros. The main component of dry matter was starch, the content was 9~12g/100g, of which starch content in xinghua longxiang taro was the highest 12.04 g/100g, more suitable as a processing starch varieties. From table 1 comprehensive point of view, the nutritional content of Taixing xianghe taro contrast to other two taros starch can reach the highest value

3.2 Composition and content of amino acids in taro

TABLE 2
COMPOSITION AND CONTENT OF AMINO ACIDS OF TARO (g/100g)

amino acid	taixing xianghe taro	Jingjiang xiangsha taro	xinghua longxiang taro
aspartic acid$^{\triangle}$	17.10±1.96	16.90±1.34	18.63±1.26
glutamic acid$^{\triangle}$	12.30±1.34	11.89±1.28	14.56±1.42
alanine$^{\triangle}$	4.10±0.13	4.05±0.14	5.36±0.12
glycine$^{\triangle}$	4.51±0.14	4.32±0.12	5.45±0.15
leucine	8.62±0.22	8.42±0.26	8.86±0.23
phenylalanine	6.82±0.16	6.76±0.14	6.92±0.12
valine	5.06±0.14	5.42±0.12	5.74±0.11
lysine	5.01±0.12	5.21±0.10	5.32±0.13
tyrosine	6.51±0.20	6.23±0.15	6.64±0.12
isoleucine	3.65±0.10	3.42±0.06	3.68±0.05
threonine	2.62±0.09	2.44±0.08	2.68±0.04
cysteine	0.84±0.02	0.96±0.04	0.76±0.02
methionine	0.75±0.03	0.82±0.04	0.78±0.03

Note: $^{\triangle}$delicious amino acids.

As we can see from table 2, four kinds of umami amino acids and nine kinds of essential amino acids are obtained in taizhou taro after hydrolysis, and the types of amino acid are relatively complete and the proportions are relatively balanced. The proportion of umami amino acids with the total amino acid in taixing xianghe taro and xinghua longxiang taro was 38% and 44% respectively, the content of umami amino acids was much higher than that of general food, which may be caused by taizhou taro unique incense and glutinous taste with high nutritional value. In addition, as the main limiting factor of lysine content in three taizhou taro was also rich in content, this can complement the lack of lysine.

3.3 Determination of blue value in taro starch

TABLE 3
THE BLUE VALUES OF THE DIFFERENT TYPE OF TARO

taro varieties	amylose	amylopectin
taixing xianghe taro	1.16±0.12^{a}	0.19±0.04^{a}
jingjiang xiangsha taro	0.97±0.05^{b}	0.14±0.06^{a}
xinghua longxiang taro	1.10±0.08^{a}	0.13±0.02^{a}

Note: n=3; those with the common superscript in the same column mean no significant difference (P> 0.05).

The blue value reflects the ability to bind iodine, and was related to the chain length of amylose or the side chain length of amylopectin. The longer the chain, the higher the blue value. The measured blue values of amylose and amylopectin were different due to their differences in molecular structure and linearity. Because of its high degree of linear polymerization of amylose, the blue value of amylose was generally 0.8 to 1.2; because of its short side chain of amylopectin, the general blue value of amylopectin 0.08~0.22[4]. The blue values of amylose of taixing xianghe taro, jingjiang xiangsha taro and xinghua longxiang taro were 1.16, 0.97 and 1.10, respectively, and the blue values of amylopectin were 0.19, 0.14 and 0.13 respectively. The blue value of taixing xianghe taro and xinghua longxiang taro was significantly higher than that of jingjiang xiangsha taro ($P<0.05$) in amylose, The purity of amylose and amylopectin reached the requirements.

3.4 Effects of different concentration on solubility and swelling force of taro starch

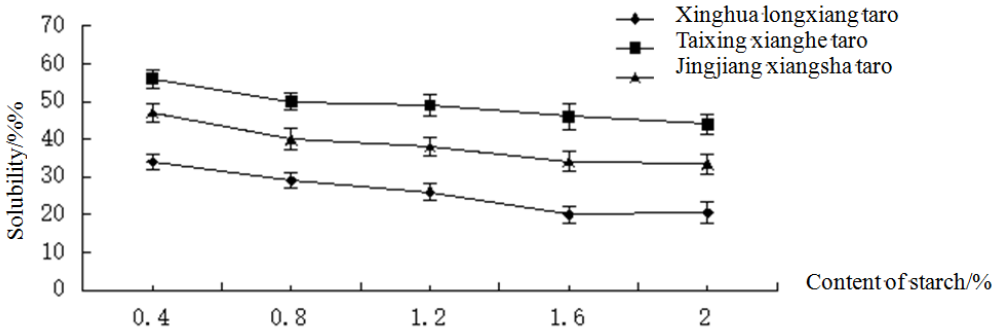


FIG.1 EFFECTS OF CONTENT ON THE SOLUBILITY OF TARO STARCH

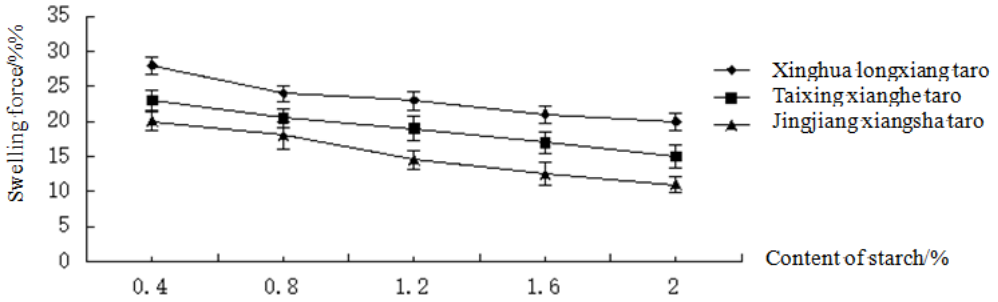


FIG.2: EFFECTS OF CONTENT ON SWELLING POWER OF TARO STARCH

The starch swelling force reflects the characteristics of amylose, and the dissolution of starch was mainly that amylose escaping from the swelling granules, the swelling force indicated the strength of the bonding force inside the starch granules [9]. From Fig. 1 and Fig. 2, we can see that the solubility of taixing xianghe taro was the largest, followed by jingjiang xiangsha and xinghua longxiang taro. As which can be seen from figure 1, with the increasing contention of starch, the corresponding decrease of solubility, swelling force and solubility of starch affect its application in food.

3.5 Coagulation and sedimentation of taro starch paste

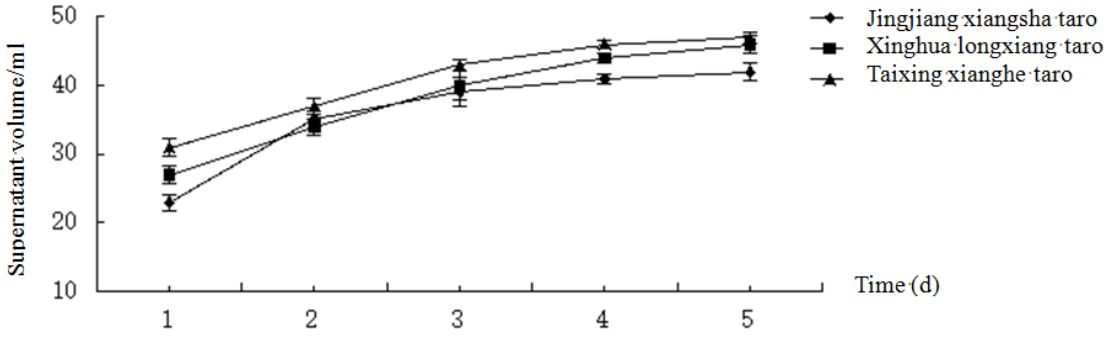
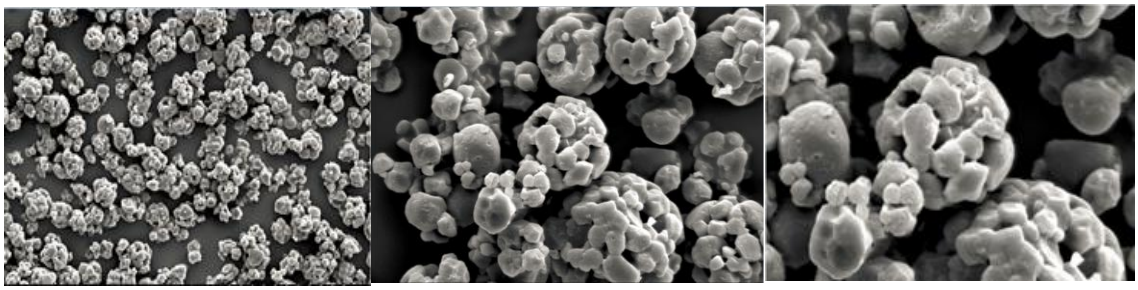


FIG.3: COAGULATION AND SEDIMENTATION OF TARO STARCH PASTE

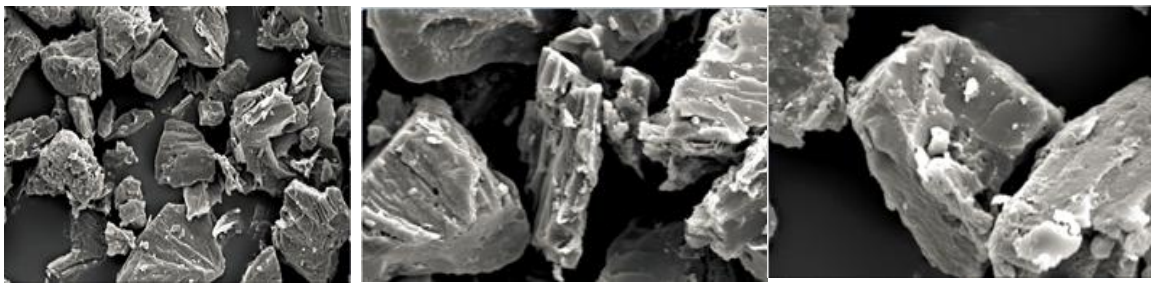
The speed of coagulation and sedimentation was an indicator of starch properties. As can be seen from Figure 3, in the initial stage, the speed of coagulation and sedimentation was very fast, but after 2d the speed became slow. In the order of the speed according to coagulation and sedimentation: taixing xianghe taro>xinghua longxiang taro>jingjiang xiangsha taro. The faster of coagulation and sedimentation in taixing xianghe taro, indicating that the content of starch and the rate of starch extraction were higher.

3.6 Observations on the morphology of taro granules



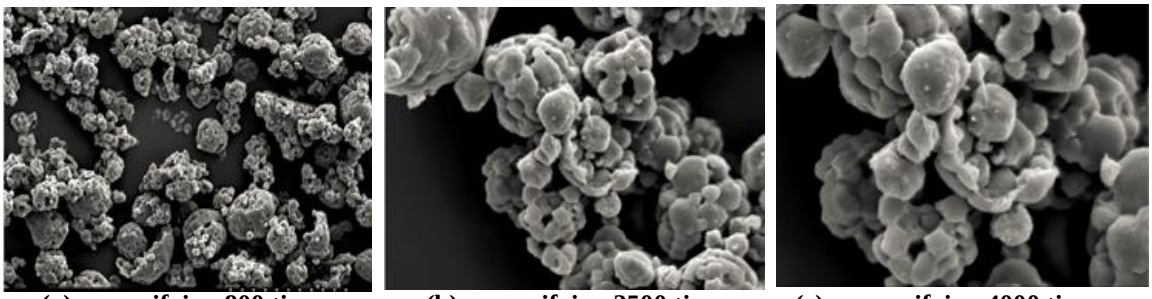
(a) magnifying 800 times (b) magnifying 2500 times (c) magnifying 4000 times

FIG.4: ELECTRON MICROGRAPHS OF TAIXING TARO STARCH



(a) magnifying 800 times (b) magnifying 2500 times (c) magnifying 4000 times

FIG.5 ELECTRON MICROGRAPHS OF XINGHUA TARO STARCH



(a) magnifying 800 times (b) magnifying 2500 times (c) magnifying 4000 times

FIG.6 ELECTRON MICROGRAPHS OF JINGJIANG TARO STARCH

The results of scanning electron microscopy on different taizhou taro were showed from figure 4~figure 6. Starch granules of different taro cultivars have different characteristics in particle size and ultrastructure. From the particle morphology point of view, Taixing taro starch granules smaller and smooth surface, uniform. Particle shape was spherical. Xinghua taro particles were irregular diamond. Jingjiang taro starch granules were irregular spherical, uneven spherical surface. Taro starch granules were small, cooked, the taste smooth, delicate, and hot and cold starch paste stability.

3.7 Effects of content on drainage rate of taro starch

As can be seen from figure 7, the drainage rate of jingjiang xiangsha taro was highest, followed by xinghua longxiang taro, the curve of drainage rate in jingjiang xiangsha taro almost coincided with that of xinghua longxiang taro, the drainage rate of taixing xianghe taro was smaller than jingjiang xiangsha taro and xinghua longxiang taro.Meanwhile, it can be seen that with the increasing concentration of starch, the drainage rate of starch decreased. It can be inferred by figure 7 that the content of amylose in taizhou taro was as follows: jingjiang xiangsha taro> xinghua longxiang taro> taixing xianghe taro. If the freeze-thawing of starch paste was not stable, which would make the free water drain, thus undermining the original texture of food, so that food quality was declined.

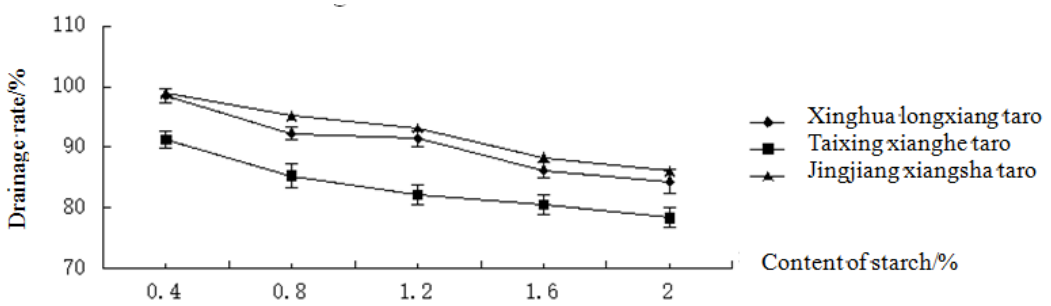


FIG.7 EFFECTS OF CONTENT ON DRAINAGE RATE OF TARO STARCH

IV. CONCLUSION

The study found that the nutritional ingredients and starch properties on the three kinds of taizhou taro, the study found that the highest total amino acid content of taixing xianghe taro was 3.03 mg/100g; the content of xinghua longxiang taro starch was highest, which was suitable as a food processing starch varieties. The proportion of umami amino acids with the total amino acid in taixing xianghe taro was highest in the comparison of taro varieties, which could be used as raw materials to develop some cooking foods. The starch particles of taixing taro were relatively smaller, which has very good applied prospects on the stability.

ACKNOWLEDGEMENTS

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The effects of explant rotation, medium types, JA and GA_3 additions on *in vitro* microtuber production from potato (*Solanum tuberosum* L.)

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Abstract— *This study investigates the effects on the in vitro microtuber formations of Solanum tuberosum L. cv. Marfona species, effected by the direction of planting the explants (horizontal or vertical), the type of medium (solid or two-phase), adding Jasmonic acid (JA) (0.0, 10 ng/L, 1 µg/L and 0.2 mg/L) and Gibberellic acid (GA_3) (0.0 and 0.2 mg/L). The cultures were incubated in a climate chamber at 22-25 ^oC, and were subjected to a light intensity of 145 µmol m^{-2} s^{-1} for 8 hours in light and 16 hours in dark photoperiods (short day) for 4-6 weeks. Microtuber production was inhibited when GA_3 was added. The maximum number of microtubers was observed when the explants were planted vertically and were grown in two-phase medium which did not contain GA_3 and had 10 ng/L JA present. It was determined that two-phase medium with 0.2 mg/L JA but without GA_3 was the most favourable medium for tuber growth (for both height and width). The best microtuber formation on single node explants were observed to occur in the short day photoperiod (8 hours light/16 hours dark) in a two-phase medium that contained 0.2 mg/L JA without the addition of GA_3. The results shows that the effect caused by JA works antagonistically with that of GA_3 thus causing the resulting microtuber formation observed.*

Keywords— Potato, In vitro Microtuberization, Two-Phase Medium, Jasmonic Acid, GA_3.

I. INTRODUCTION

The potato (*Solanum tuberosum* L.) is the fourth most important vegetable after rice, wheat, and corn in food and industry amongst the food sources for the world's population [1, 2]. When potato tubers are grown by vegetative propagation using conventional methods, the viruses on the main plant are easily transferred on to the new vegetative organs and tubers and causes serious damage to potato production. Due to seed tuber growth being significantly affected by environmental conditions, and that the possibility of disease becomes high in addition to high costs, microtubers are emphasized as an alternative seed source [3]. The microtubers that are produced from seedlings with no viral infections that were obtained from meristem cultures and propagated *in vitro* are considered to have significant advantages in potato seed production.

The success of *in vitro* microtuber production in the studies conducted in this field is based on a series of factors. Amongst these, nutrient media solutions, the chemicals used (like activated charcoal), sucrose concentration, growth regulators (such as Gibberellic acid, Jasmonic acid) and temperature, environmental factors like light intensity and genotype factors can be included [4-12]. In literature, it is emphasized that due to the plant growth regulators Gibberellic acid (GA_3), Abscisic acid (ABA), Ethylene, (2-chloroethyl) trimethylamonium chloride (CCC), and Jasmonic acid (JA) acting on each other and on environmental factors they are effective on the formation and development of vegetative storage organs like tubers [13-16]. The physiological mechanisms and their related hormones that determine potato tuber growth and development are still not fully known.

The gibberellins that are effective in many physiological processes, including plant stem development, seed germination, breaking of bud dormancy, and fruit growth, also effect the development of potato tubers. Environmental factors, like photoperiod and temperature regulate gibberellin biosynthesis [15].

In recent years, the effect of Jasmonic acid and methyl esters on tuber stimulation has been the focus of studies [17-19]. In addition to acting as a signal molecule for activating an immune response in case of pathogen invasions, JA also acts as a regulator for many physiological and developmental (such as root development, tuberization, ageing, and pollen

development) processes. Jasmonates are also potential stimulators for the expression of vegetative storage protein genes [20]. It is also reported that JA's are effective during *in vitro* microtuberization in addition to playing an important role in the development of vegetative storage organs [16, 19, 21-24]. However, JA's influence on microtuber development and its relationship with other growth regulators are still not fully elucidated. In the studies conducted, the time taken for microtuber development, the medium conditions for growing microtubers and which hormones, chemicals etc. need to be applied in what doses could not be fully determined [18, 19, 22, 25-27]. To include JA when commercially producing microtubers, more information is needed on the effect of this substance on tuberization and its relationship with other hormones.

In this study, the relationship between the planting of explants (either horizontally or vertically), the type of medium (either solid or two-phase), the addition of Jasmonic acid (0.0, 10 ng/L, 1 µg/L and 0.2 mg/L), and Gibberellic acid (0.0 and 0.2 mg/L) into the media and how they affect the development of microtubers growing *in vitro* on the economically valuable *Solanum tuberosum* L. cv. Marfona is investigated.

II. MATERIALS AND METHODS

The study was conducted in four stages: The *S. tuberosum* tuber shoots *in vivo, in vitro* shoot tip cultures, micropropagation, and microtuberization. For the purpose of shoot development, the potato tubers belonging to the Marfona variety are first washed under tap water, then they are placed in a 15% commercial sodium hypochloride (NaOCl) solution and left there for 20 minutes. They are then washed with distilled water 3 times [28] so that surface sterilization can occur. The tubers that had there surfaces disinfected are then planted into a pot filled with perlite and left to grow in a climate chamber that has a temperature of 30-35 ^{o}C, 60-65% humidity, a light intensity of 145 µmol $m^{-2}s^{-1}$, and left for photoperiods of 16 hours in light and 8 hours in dark [29]. After 4 weeks, successfully grown shoots were obtained (Figure 1).

(a) **(b)**

FIGURE 1. SHOOT DEVELOPMENT ON TUBERS a) AFTER 1-2 WEEKS, b) AFTER 3-4 WEEKS

To produce a shoot tip culture, the shoot tips obtained from potato tubers were placed in a 70% alcohol solution and shaken for 1 minute. Two drops of Tween-20 were then added and left to wait in a 10% NaOCl solution for 7-8 minutes, after which the shoot tips were washed with distilled water 3 times for sterilization [18-28]. Shoot tip explants obtained from sterilized shoots were then planted in a modified Murashige and Skoog (MS) medium [30]. Different references [31, 32] were used to produce the nutrient media and the final solution consisted of 30 g/L sucrose, 7g/L agar, 0.2 mg/L GA_3, 0.2 mg/L Kinetin, 0.2 mg/L Indole Acetic Acid (IAA), and 100 mg/L Myo-inositol. The solution was adjusted to have a 5.8 pH. Shoot tips were placed in tubes with dimensions of 15x2.5 cm and cultured in 10 mL of Modified MS (MMS) in each tube. The cultures were then incubated for 2-2.5 months in a climate chamber that had a temperature of 20-22 ^{o}C, and were subjected to a light intensity of 145 µmol $m^{-2}s^{-1}$ with 16 hours of light and 8 hours of dark photoperiods [33]. After this period, *in vitro* potato plantlets were obtained (Figure 2).

Single node explants taken from plantlets obtained from shoot tip cultures were used for micropropagation. The single nodes were planted into tubes of dimensions 15x2.5 cm that contained 1.0 mg/L IAA and 1.0 mg/L in addition to the MMS nutrient media. The MMS nutrient media was adjusted to have a 5.8 pH and contained 7 g/L agar, 30 g/L sucrose, 60 mg/L myo-inositol, 0,4 mg/L thiamine, 1 g/L pyridoxine [34]. The cultures were incubated for 4-7 weeks in a controlled climate chamber of 22 ^{o}C with 16 hours of light and 8 hours of dark photoperiods and a light intensity of 145 µmol$^{-2}s^{-1}$ [31-33].

(a) (b)

FIGURE 2. PLANTS OBTAINED FROM SHOOT TIP CULTURES a) 3-4 WEEKS AFTER b) 6 WEEKS AFTER

To determine the effects of the medium solution, the planting position (either horizontally or vertically), and type of medium (whether in a solid or two-phase medium), during microtuberization on the development of microtubers, single node explants obtained from plants that were grown *in vitro* were planted either horizontally or vertically into glass jars of dimensions 6.5x7.5 that had 50 mL each of either the solid phasic or two-phase MMS medium (Figure 3). The MMS medium used was made up of JA (0, 10 ng/L, 1 µg/L and 0.2 mg/L), GA_3 (0 and 0.2 mg/L), and the combination of these in addition to 80 g/L sucrose and 7 g/L agar. The MMS medium was also adjusted to have a 5.7 pH [9, 26, 34]. Ten explants were planted into each of the 5 jars used and each experiment was repeated 5 times. The cultures were then incubated in a climate chamber for 4-6 weeks at a temperature of 22-25^{o}C and a light intensity of 145 µmol $m^{-2}s^{-1}$ during an 8 hours light and 16 hours dark photoperiod (short day) [36]. At the end of the incubation period, the number of single nodes obtained from explants, the number of explants from which microtubers were obtained, explant yield (number of explants from which microtubers were obtianed/total number of explants), and the microtuber yield (number of microtubers/total number of explants) were determined for each group. The data was shown as percentages (%) in a chart.

The statistical analysis of the microtuber numbers and weights were conducted by using the SPSS packet programme. According to variance analysis and statistical test results, each variable was calculated to have a least significant difference (LSD) value between the significant 1% and 5% percentage value range. To compare of the groups, Oneway-ANOVA were used variance analysis and were followed by the Duncan's Multiple Range Test for comparing data in individual groups as well as between groups [37].

III. RESULTS AND DISCUSSION

The results of the effect of the direction of explant planting (either horizontally or vertically) (Figure 3), type of medium (solid and two-phase) (Figure 3), medium composition [JA (0, 10 ng/L, 1 µg/L and 0.2 mg/L) and GA_3 (0 and 0.2 mg/L)] on microtuber development from single-node potato explants are summarized in Table 1. It was observed that the effect and relationships of the type of medium, the direction in which the explants were planted, and medium composition (JA and GA_3) were significant ($p<0.05$).

Table 1 shows that in the control group and those that had JA in their medium microtuber development had occurred, while no development was observed when GA_3 was present in the medium. Therefore, it was concluded that GA_3 should not be present in media prepared for microtuber development. It was observed that the relationship between explant yield (number of explants from which microtubers were obtained/total number of explants) and microtuber yield (number of microtubers/total number of explants) generally increased in a proportional way. Accordingly, it was shown that the two-phase groups [two-phase–0.2 mg/L JA (36.53%), two-phase–1 µg/L JA (45.28%), and two-phase–10 ng/L JA (44.4%)] which gave a high microtuber percentage yield also gave a high percentage yield of the explants (30.76%, 28.3%, and 38.09% respectively) which the microtubers were obtained from. It was also observed that two-phase–control (9.09%), Horizontal–10 ng/L JA + 0.2 mg/L GA_3 (6.84%), and Vertical–10 ng/L JA + 0.2 mg/L GA_3 (10.76%) medium groups which resulted in low microtuber yields additionally generated low explant percentage yields (7.27%, 5.47%, 6.15% respectively).

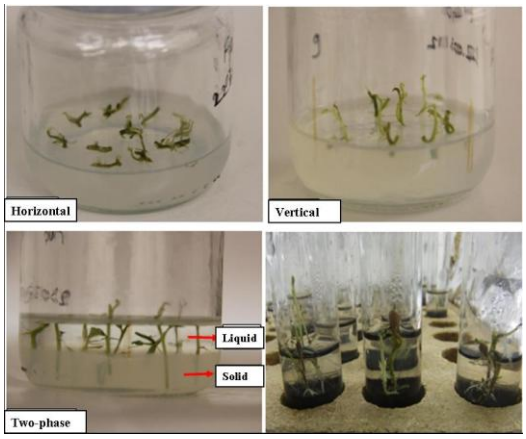

FIGURE 3. THE PLANTING DIRECTION OF SINGLE NODE EXPLANTS

Highest tuber yields were observed in two-phase–1 µg/L JA, two-phase–10 ng/L JA, and two-phase–0.2 mg/L JA media groups with percentage yield results being 45.28%, 44.4%, and 36.53% respectively. Therefore, it was concluded that two-phase media were more suitable for microtuber development.

When the microtuber weights in Table 1 are analyzed, it was observed that microtuber weight, medium composition, and direction of planting of explants had a significant relationship ($p<0.05$). The highest values obtained with respect to microtuber weight were 176 mg, 118 mg, and 97 mg for two-phase–control, two-phase–0.2 mg/L JA, and vertical–0.2 mg/L JA media groups and planting directions respectively. The effects of horizontal and vertical planting directions on microtuber weight and microtuber yield was not significant.

TABLE 1
DATA SHOWING THE EFFECTS OF MEDIA COMPOSITION AND PLANTING DIRECTION OF SEEDLINGS ON MICROTUBER DEVELOPMENT

Planting Direction	JA	GA_3 (0.2 mg/L)	Total explant number	The number of explants from which microtubers were obtained and explant yield (%)	The number of microtubers and microtuber yield (%)	Average Microtuber weight (mg)
Horizontal	- - -	-	66	0	0	0
Vertical		-	70	0	0	0
Two-phase		-	55	4 (7.27%)	5 (9,09%)	176±57 **c***
Horizontal	0,2 mg/L	-	64	12 (18,75%)	13(20,31%)	37±9 **a**
		+	80	0	0	0
Vertical		-	68	16 (23,52%)	18(26,47%)	97±26 **ab**
		+	93	0	0	0
Two-phase		-	52	16 (30,76%)	19(36,53%)	118±26 **bc**
		+	0	0	0	0
Horizontal	1 µg/L	-	48	12 (25%)	15(31,25%)	35±8 **a**
		+	72	0	0	0
Vertical		-	40	6 (13,04%)	8(20%)	45±7 **a**
		+	64	0	0	0
Two-phase		-	53	15 (28,3%)	24(45,28%)	55±14 **ab**
		+	30	0	0	0
Horizontal	10 ng/L	-	69	0	0	0
		+	73	4 (5,47%)	5(6,84%)	24±5 **a**
Vertical		-	69	0	0	0
		+	65	4 (6,15%)	7(10,76%)	32±9 **a**
Two-phase		-	63	24 (38,09%)	28(44,4%)	38±8 **a**
		+	41	0	0	0
Horizontal	-	+	66	0	0	0
Vertical		+	70	0	0	0
Two-phase		+	41	0	0	0
TOTAL			1412	114 (8,07%)	142 (10,05%)	49±5

Means followed by the different letter in the same column are significantly different by Duncan's test (P ≤ 0.05).

In the experiment in which the effects of explant direction when planting, the type of medium, and the JA–GA_3 effects on microtuber development were investigated, it was found that the highest microtuber yield (45.28%) resulted from the two-phase–1 µg/L JA group. The highest value for microtuber weight was found to be approximately 176 mg in the KC (two-phase–control) group. However, when all the data, converted to a common denominator, were analyzed in addition to considering the criteria for using microtubers as potato seeds, it was deduced that the AOC (two-phase–0.2 mg/L JA) application resulted in high values in regard to microtuber yield and microtuber weight (Microtuber yield=36.53% and Average Microtuber weight=118 mg) and thus could be more suitable (Figure 4).

FIGURE 4. MICROTUBERS OBTAINED FROM DIFFERENT MEDIUM. MICROTUBERS OBTAINED FROM THE a) TWO-PHASE MEDIUM CONTAINS 0.2 mg/L JA , b) TWO-PHASE MEDIUM CONTAINS 1 µg/L JA , AND c) TWO-PHASE CONTROL MEDIUM

In this study, the economically valuable *Solanum tuberosum* L. *cv*. Marfona was used to develop shoots *in vivo*, after which *in vitro* shoot tip cultures were developed. The effects of explant planting direction (horizontal or vertical), media type (solid phase or two-phase), and the addition of Jasmonic acid (0.0, 10 ng/L, 1 µg/L, and 0.2 mg/L), Gibberellic acid (0.0 and 0.2 mg/L) were added in to the media, on microtuber developments from the micropropagated plantlets (the shoot tip cultures) were then investigated.

Similarly Gopal et al. [32]'s results, the single nodes taken from plantlets obtained from shoot tip cultures were micropropagated in a MMS medium with 1.0 mg/L IAA and 1.0 mg/L BAP with 7g/L agar, 30g/L sugar, 60 mg/L myo-inositol, 0.4 mg/L thiamine, 1 g/L pyridoxine with a 5.8 pH. They were incubated in a climate chamber with a temperature of 22 ^{o}C and a light intensity of 145 µmol $m^{-2}s^{-1}$ for 16 hours of light and 8 hours of dark photoperiods.

In our study, whether it were shoot development from *in vivo* tubers or shoot and plant development *in vitro*, all were subjected to 16 hours of light and 8 hours of dark (long day) photoperiods. On the other hand, it was determined that during microtuberization, the highest yield of microtuber development occurred when the single node potato explants were subjected to a short day (8 hours of light and 16 hours of dark) photoperiod in a two-phase nutrient medium containing 0.2 mg/L JA.

Potato tuber development depends on hormonal and environmental factors. Similarly, many relationships between *in vitro* growth conditions during microtuberization effect microtuber development. Some important factors effecting *in vitro* microtuberization include; glucose concentration in the medium, the dose of growth regulators added to the medium, type of medium, culture type, and the conditions of the environment in which the potato cultures are incubated in (e.g. temperature, photoperiod) [34, 38-40].

Momena et al. [41] observed the effect of different combination with sucrose and some growth regulators (Kinetin and BAP) on four different potato cultivars and as a result reported that treatment T3 (8% Sucrose+4 mg/L Kinetin + 1 mg/L BAP) was the best combination for the investigated cultivars. In another study [42], researchers purposed that to find out the suitable combination of sucrose, growth regulators (benzyladenine and paclobutrazol) and medium type. At the end of the study they

reported that the best microtuberization rate was obtained in MS/2 liquid medium which include 80 g/L sucrose and 0.5 mg/L IBA.

It was deduced that the carbohydrate source, gibberellins, and anti-gibberellin-like substances and their interactions with each other all effected and regulated potato microtuberization. Harmey [43] determined that gibberellic acid strongly inhibited tuber development but that indole acetic acid and maleic hydrazide induced tuberization. It was also determined that when stem component carbohydrate sources were limited in media with less glucose concentrations, the addition of growth regulators did not induce tuberization.

In another study [44] researchers concluded that GA was an important regulator in tuber development, that ABA conversely stimulated tuber developments, and that sucrose regulated tuber development by controlling GA levels. Vreugdenhil and Sergeeva [45] deduced that internal gibberellin levels were high during condition where tuber development did not occur, and were low during conditions where tuber development did occur. When gibberellin biosynthesis inhibitors (CCC, paclobutrazol, etc.) are used the opposite effect was observed, while the addition of gibberellins in to the medium inhibited tuber development. They also found that gibberellin played a role in cellular mechanisms like cell division, cell widening, and in the lodging of microtubers.

In another study, gibberellin-photoperiod interaction effecting the regulation of microtuber development in potatoes was investigated [46]. It was concluded that *S. tuberosum* ssp. *andigena* plants needed short day photoperiods for tuber development and that this process was controlled by gibberellins.

It is indicated that jasmonic acid is carried as a signal for tuber induction and that it plays a role in tuber growth and development [47]. It was observed that when JA is found in the medium, the inhibition effect of GA_3 was removed. The reason for this was because it was thought that JA worked antagonistically against the effects of GAs [39, 48].

Abdala et al. [49] determined in their study that the highest concentrations of JA was found in leaves, while during tuber development the highest concentration was found to occur in the roots. During the process of tuber development, the JA concentrations in stolons were found to be drastically reduced.

In our study it was observed that, in regards to medium type and direction of planting the best microtuber yields and microtuber weight was obtained in the two-phase (solid+liquid) nutrient medium. Similarly, it was observed that in various microtuber production methods, the highest yield resulted from two-phase solid propagating-liquid induction systems [9]. In another study [50], the development of microtubers in liquid and solid media were compared, and it was observed that cultures incubated in a liquid medium resulted in microtubers with more weight than those incubated in a solid medium. In a study conducted by Pelacho et al. [51], however, it was found that a half-solid medium resulted in higher tuberization rates and higher tuber weight values than those obtained from liquid media. A reason for the good results, in regards to tuberization, obtained in our study when a two-phase (solid+liquid) medium was used, might be due to the molecules needed for tuberization found in the medium being able to adsorb more readily on to the plant.

It was deduced in our study that the reason for tuber development in media when JA was present while no development was seen with GA_3 and JA together, was because of the antagonistic effect of GA_3 on JA.

In a similar study, it was observed that when gibberellins were added externally, it had a negative effect on tuber development, although internal GA_3 supported tuber development [45]. Tuber formation and its subsequent development, is a result of cell division and cell growth. Internal GA_3 is thought to play a role in these cell divisions [44]. As GA_3 is an inhibitor of the glycoprotein patatin, which is associated with microtuberization, adding GA_3 to the external environment makes the concentration levels too high for the inhibition to be tolerated and thus microtuber formation is blocked [52]. Additionally, Jasik and Mantell [26] observed the effect of JA on three types of yam (*Dioscorea*) and as a results confirmed that JA supported microtuberization. In another study [46], researchers determined that gibberellins participated in the 8 hours light–16 hours dark photoperiod that encouraged potato tuberization, and that their role was as a regulator, showing gibberellins to have a relationship to tuber formation in a negative way.

In our study, the results of the microtuberization experiments showed that when looking at the effects of explant planting direction, medium type, and JA–GA_3 interactions, the highest microtuber yield (45.28%) was obtained from the two-phase, 1 µg/L JA containing medium. In regards to microtuber weight, the highest weight value (176 mg/tuber) resulted from the two-phase, control medium. In similar studies [16, 53] the researchers obtained a higher number of microtubers with greater

weight, when JA was added to the medium. In potatoes, tuberization starts with the widening of the sub-apical meristem of stolons and jasmonates, by preventing the longitudinal growth of stolons, stimulate the widening of the sub-apical meristem.

When the criteria for microtubers that will be used as potato seeds was taken into consideration it was deduced from the results obtained in our study that the medium which gave the highest microtuber yield and weight (Microtuber yield=36.53% and Avg. Microtuber weight=118mg) was the 0.2 mg/L JA two-phase medium (Figure 4).

IV. CONCLUSION

To summarize the results:

- The best medium type for microtuber formation was a two-phase medium,
- GA_3 addition to the medium inhibited microtuber formation,
- JA addition to the medium resulted in positive effects on microtuber formation and this effect resulted from working antagonistically with GA_3,
- In regards to JA-GA_3 interaction and medium type, a two-phase medium with 0.2 mg/L JA but without any GA_3 was found to be the optimal medium.

In future studies, different substances that encourage tuberization will be beneficial for researching about other and their relationship with photoperiods in developing mechanisms in the production of microtubers.

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