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Preface

We would like to present, with great pleasure, the inaugural volume-3, Issue-4, April 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.

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Sustainable Natural Resource Utilisation	Management of the Environment
Agricultural Management Practices	Agricultural Technology
Natural Resources	Basic Horticulture
Food System	Irrigation and water management
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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)
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Influence of amino acids, bleed grape and seaweed extract on vegetative growth, yield and its quality of fig

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Abstract— *This study was conducted during the growing season of 2016 in a private orchard at AL-Abbasyia , Najaf Governorate on the local fig cv. Aswod Diala . The trees were spraying with amino acids (Amister and Gusto) at concentrations of 500 mg / L each other, Bleed of grape 100% and seaweed Ascophyllum nodosum at concentrations of 4% in single way or in combination at 15 march and 15 May,2016 . Results showed that spraying with amino acids ,bleed grape and seaweed extract treatments and their interactions caused a significant increase percentage of leaf area ,total chlorophyll, length of shoots , percentage of carbohydrate in branches , percentage of nitrogen in branches , percentage of carbohydrate / nitrogen in branches , percentage of nitrogen in leaves, percentage of phosphor in leaves, percentage of potassium in leaves ,diameter of fruit , length of fruit , weight of fruit , percentage humidity of fruit , percentage dray matter of fruit, number of days to ripening , percentage of total soluble sold , fruit firmness and total yield of trees compared with control treatment . There were significant differences between above mentioned treatments. The Bleed of grape was more effective than amino acids(Amister and Gusto) and seaweed in that respect , but the combination of amino acids(Amister and Gusto) , Bleed of grape and seaweed gave the best results in the treatment (Am + Gu+ Bg + Bs) for the season of experiment.*

Keywords— Amino acids, Bleed of grape, Seaweed extract, fig trees.

I. INTRODUCTION

Amino acids are considered as precursors and constituents of proteins (Rai, 2002), which are important for stimulation of cell growth. They contain both acid and basic groups and act as buffers, which help to maintain favorable pH value within the plant cell (Cerdana, *et al*.,2009). Amino acids can directly or indirectly influence the physiological activities in plant growth and development such as exogenous application of amino acids have been reported to modulate the growth, yield and biochemical quality of squashes and garlic plants (Hounsome, *et al*., 2008 , Abd El- Aal *et al*., 2010; Shiraishi *et al*., 2010,). Amino acids are responsible for improving physical and chemical parameters of fruits as well as increasing the productivity of trees (Mouco, et al,2009) .The spraying with amino acids led to increased leaf containing from growth regulators cytokinines (Cks) , Gibberellins (GA^3), Auxins , chemical eliminates and reducing Abscisic acid (ABA) (Talaat, et al,2013). Shehata , et al,(2011) found that spraying strawberry with amino acids and seaweed extract led to increased the length of fruit, diameter of fruit, weight of fruit, fruit firmness, percentage humidity of fruits , total yield .Datir, et al(2012) found that , spraying with amino acids has the positive effect in fruits growth and it is quality . Algae extract as a new bio fertilizer containing N, P, K, Ca, Mg, and S as well as Zn, Fe, Mn, Cu, Mo, and Co, some growth regulators, polyamines and vitamins applied to improve nutritional status, vegetative growth in different orchard such as vineyards (Elham, *et al*., 2010). Subba Rao (2008) noticed the impact of algae extract application to orchard trees was caused a significant increased total chlorophyll in leaves , total yield of tree , total soluble solids ,vitamin C , and fruit firmness . Al – Hamdawi (2016) found that ,spraying trees of fig cv. Aswod Diala with Hletab and Kelpak led to increased leaf aria and the content of leaves from total chlorophyll , shoot length ,number of shoots , total carbohydrates percentage in shoots , total yield of tree and its quality compared with control treatment . Bondok et al(2013) found that spraying grape trees with extract of alga's (Acadian , Goemar and BM86) at conc. of (0.5 , 1 and 2 %) caused increase in the vegetative growth and fruits quality with increase of concentration of extract of alga's. Foliar application of mixture of amino acids and seaweed extract at different growth stages had a positive effect on vegetative growth, reproductive growth, and berry quality of grapevines. Multiple application of 0.5 mL L-1 mixture of amino acids and seaweed extract at flowering + fruit setting + one month after fruit setting stages is quite effective to improve growth and berry physicochemical quality characteristics of 'Perlette' grapes (EKhan, *et al*., 2012) .The purpose is to study the effect of spraying with amino acids(Amister and Gusto) , Blee d of grape and seaweed Ascophyllum nodosum treatments and their interactions on vegetative growth , yield and fruit quality of local fig tree cv. Aswod Diala .

II. MATERIALS AND METHODS

This study was conducted in a private farm at AL-Abbasyia .Najaf governorate for the 2016 season on local fig tree cv. Aswod Diala , 48 trees at same size and growth were selected with 12 years of age , that planted on (5 x 5 m.) , they were spraying with Amister it was mixtures of amino acids (Arginine ,Tyrosine and Proline) and Gusto it was mixtures of amino acids (Glutathione , Aspartic acid , Glycine and Lysine) at concentrations of 500 mg / L each other , Bleed of local grape cv. Sada Batha at concentrations of 100% that containing Fe 40 mg/L , Ca 160 mg/L , P 28 mg/L , K157 mg/L total acidity 11.5 mg/L , Mg 22.7 mg/L , Succinct acid 0.130 mg/L ,Malic acid 4.50 mg/L ,Tartaric acid 2.15 mg/L ,Formic acid 0.020 mg/L ,Citric acid 5.63 mg/L ,Na 3.50 mg/L ,Zn 1.9 mg/L ,IAA26 mg/L , GA_3 40 mg /L, ,CKs 35 mg/L, olego scoris 95 mg/L, amino acid 7%, organic nitrogen 3%, organic matter 16%, Algonac acid 50%. (AL- Saidi,2000), and Brawn seaweed extract Ascophyllum nodosum that containing (Fe 46-70 mg/L , N 1400 -1800 mg/L , P 1500 -2000 mg/L , Cu 10-15 mg/L potassium 2000-2006 mg/L ,B 30-44 mg/L , Kinetin% 0.06 and organic matter 13%) at concentrations of 4% in three periods at 1April , 1May and 1 June ,2016. The experiment included 16 treatments with three replicates. It is adopted according to Randomized Complete Block Design (RCBD) , and the results were statistically analyzed according to Duncan test at the probability level of 5% (Al-Rawi and Khalf Allah , 2000) . The experiment involved the following 18 treatments :

1- Control treatment (sprayed with tap water).
2- Amister (Am)as foliar sprays at concentration of 4 % .
3- Gusto (Gu) as foliar sprays at concentration of 4 % .
4- Bleed of grape (Bg) as foliar sprays at concentration of 100% .
5- Brawn seaweed extract (Bs)as foliar sprays at concentration of 4 % .
6- Am + Gu .
7- Am + Bg .
8- Am + Bs .
9- Gu + Bg .
10- Gu + Bs .
11- Bg + Bs .
12- Gu + Bg + Bs .
13- Am + Gu %+Bg .
14- Am+ Bg +Bs .
15- Am + Gu+ Bs.
16- Am + Gu+ Bg + Bs.

Trees spraying were done early morning until wetness was full addendum. Tween 20 was added at conc. of $1cm^3/L$ as spreader material. Leaf aria cm^2 , total chlorophyll mg/1gm FW , shoot length cm, percentage total carbohydrate in branches, percentage Nitrogen in branches , percentage C/N in branches , percentage elements N,P,K, diameter of fruit cm ,length of fruit cm , length of fruit/ diameter of fruit , percentage humidity of fruit , percentage dray matter of fruit, number of days to ripening , total yield kg/tree according to (Ibrahim , 2010) .Firmness was measured on two sides of each fruit with an Effegi penetro meter (Model NI , McCormick Fruit Tech ,Yakima ,WA) Fitted with an 11.1mm tip . The percentage of total soluble solids were determined by hand refract meter.

III. RESULTS AND DISCUSSION

3.1 Leaf aria , total chlorophyll, shoot length , percentage of carbohydrate in branches , percentage of nitrogen in branches , percentage of carbohydrate / nitrogen in branches.

The data in table (1) indicate that spraying with amino acids(Amister and Gusto) , Bleed of grape and seaweed Ascophyllum nodosum in single way or in combination led to a significant increased in the leaf aria , total chlorophyll, shoot length , percentage of carbohydrate in branches , percentage of carbohydrate / nitrogen in branches compared to control treatment until reached highest rates (7.30 m^2 , 157.63 mg/1gm FW ,27.75 cm, 13.09% and 19.30 %) in the treatment (Am + Gu+ Bg + Bs) in comparison to the lowest values rates rates (6.01m^2 , 137.70 mg/1gm FW ,16.20 cm, 11.90% and 15.35 %) in control treatment , respectively . The increase in leaf area and leaf chlorophyll content , shoot length , percentage of carbohydrate in branches , percentage of nitrogen in branches , percentage of carbohydrate / nitrogen in branches that clearly obvious from the previous results could be due to the effective components of amino acids , bleed of grape and seaweed such as major and minor elements , growth regulator and vitamins which enhanced cell division, metabolism and other biological reactions, in addition to the activation effect of these components on photosynthesis and promoting

protoplasm formation including RNA and DNA that important for cell division .These idea goes in parallel with those of Attoa , *et. al.* (2002) and EL-Naggar, *et. al.* (2013) . The increase of this characterize of vegetative growth because of the treatment with concentrations of amino acids (Amister and Gusto) , Bleed of grape and seaweed Ascophyllum nodosum due to the fact that mentioned treatments led to the root system in absorption the nutrients elements in which some of them are parts of chlorophyll which led to increase its quantity in comparison control treatment . This process increases photosynthesis an activate plant growth which led to enhance hormones synthesis (Jundi , 2003) .The presence of minerals and some growth regulators in algae extract and protein, carbohydrates, vitamins increasing vegetative growth (Abed El- Hamied, 2014).

TABLE 1
EFFECT OF SPRAYING WITH AMINO ACIDS, BLEED GRAPE AND SEAWEED EXTRACT ON VEGETATIVE GROWTH OF LOCAL FIG TREES C.V ASOWD DIALA FOR SEASON 2016

Treatments	Leaf aria / tree m^2	Total chlorophyll mg/1gm FW	shoot length cm	%Total carbohydrate in branches	% Nitrogen in branches	C/N % in branches
Control	6.01 k	137.70 cd	16.20 h	11.90 c	0.775 a	15.35 g
Amister 4%	6.18 j	138.87 cd	22.40 e	12.29 bc	0.735bc	16.72 ef
Gusto 4%	6.25 ij	139.75 cd	19.00 gh	12.18 bc	0.730 bcd	16.68 ef
Bleed of grape 100%	6.59 f	144.09 abcd	22.90 cd	12.35 bc	0.706 bcdefgh	17.49 bcdef
Brawn seaweed extract 4%	6.27 ij	140.61bcd	21.70efg	12.24 bc	0.726 bcde	16.58 f
Am + Gu	6.74 e	154.55 ab	24.25 b	12.20 bc	0.718 bcdef	16.80 def
Am + Bg	6.85 cd	151.80 abc	23.19 d	12.28 bc	0.725 bcde	17.08 cdef
Am + Bs	6.78 de	149.35 abc	23.50 d	12.30 bc	0.714 bcdefg	16.93 def
Gu + Bg	6.27 ij	147.69 abc	21.64 efg	12.33 bc	0.710 bcdefgh	17.22 bcdef
Gu + Bs	6.30 hi	148.29 abc	22.80 cd	12.46 bc	0.797 efgh	17.36 bcdef
Bg +Bs	6.38 hg	140.99 abcd	23.30 cd	12.42 bc	0.697 efgh	17.87bcde
Gu + Bg + Bs	6.42 g	155.87 ab	24.71 b	12.60 ab	0.703 cdefgh	17.66 bcdef
Am + Gu +Bg	6.92 c	153.42 ab	24.97 b	12.61 ab	0.685 gh	18.39 ab
Am +Bg +Bs	6.90 c	151.24 abc	25.16 ab	12.61 ab	0.690 fgh	18.27abc
Am + Gu +Bs	7.1 b	155.30a b	25.41 ab	12.64 ab	0.700 defgh	18.05 bcd
Am + Gu +Bg + Bs	7.30 a	157.63 a	27.75 a	13.09 a	0.678 h	19.30 a

3.2 The percentage of nitrogen in leaves, percentage of phosphor in leaves, percentage of potassium in leaves, diameter of fruit, length of fruit and weight of fruit .

Data in Table (2) shows that percentage of nitrogen in leaves, percentage of phosphor in leaves, percentage of potassium in leaves ,diameter of fruit , length of fruit and weight of fruit were a significant increased when trees sprayed with amino acids(Amister and Gusto) , Bleed of grape and seaweed Ascophyllum nodosum in single way or in combination . The highest significance result were recorded in treatment (Am + Gu+ Bg + Bs) , that gave(2.86%, 0.79% ,1.40% , 4.76 cm, 3.83 cm and 38.45gm) comparison with lest rates (2.23%, 0.42 % ,1.02% , 3.63 cm, 3.40 cm and 29.15 gm) in control treatment , respectively .The higher rates of nitrogen , potassium and phosphor in the leaf contents were due to the process of spraying of the amino acids , Bleed of grape and seaweed led to increased concentration of these elements in the leaves because these material have elements in composition compared to the untreated trees . The spraying with amino acids , Bleed of grape and seaweed led to increase in the content of leaves from growth hormones and total chlorophyll , these led to increase the physical characters of fruits (Andreu,2009). The increase of this characterize of vegetative and fruit growth because of the treatment with concentrations of amino acids and seaweed extract due to the fact that mentioned treatments led to the root system in absorption the nutrients elements in which some of them are parts of chlorophyll which led to increase its quantity in comparison control treatment (Subba Rao, 2008, Abedl-Aziz et.al, 2009) .

TABLE 2

EFFECT OF SPRAYING WITH AMINO ACIDS, BLEED GRAPE AND SEAWEED EXTRACT ON VEGETATIVE GROWTH, YIELD AND ITS QUALITY OF FIG OF LOCAL FIG TREES C.V ASOWD DIALA FOR SEASON 2016

Treatments	% N in leaves	% P in leaves	% K in leaves	Diameter of fruit cm	Length of fruit cm	Weight of fruit gm
Control	2.23 F	0.42 f	1.02 i	3.63 i	3.40 j	29.15 i
Amister 4%	2.41 def	0.45ef	1.07 fgh	4.07 h	3.52 h	31.79 h
Gusto 4%	2.38 def	0.47def	0.10 fgh	4.24 fgh	3.60 ghi	33.00 g
Bleed of grape 100%	2.50 cdef	0.56 cd	1.13 efg	4.45 bcdef	3.70 cdef	34.18 f
Brawn seaweed extract 4%	2.30 ef	0.53 cd	1.07 fgh	4.14 gh	3.58 hi	32.46 gh
Am + Gu	2.54 bcdef	0.55cd	1.11 fg	4.30 efgh	3.61fghi	34.26 f
Am + Bg	2.65 abcd	0.50 cd	1.13 efg	4.34 defg	4.64 efghi	35.51 f
Am + Bs	2.68 abc	0.48 de	1.15 def	4.34 defg	3.66 defg	34.12 f
Gu + Bg	2.39 def	0.51cde	1.17 cde	4.37 cdefg	3.69cdefg	34.84f
Gu + Bs	2.71 abc	0.53 cd	1.22 cde	4.47 bcdef	3.72abcde	36.43 de
Bg +Bs	2.69 abc	0.57 cd	1.19 de	4.59 abcd	3.73abcde	36.60 cde
Gu + Bg + Bs	2.77 abc	0.61bc	1.22 cde	4.53abcde	3.73abcde	36.97 cde
Am + Gu +Bg	2.52 bcdef	0.68 ab	1.30ab	4.64 abc	3.75 abcd	36.37 bcd
Am +Bg +Bs	2.55bcde	0.62 bc	1.28bcd	4.70ab	3.80 ab	37.97 ab
Am + Gu +Bs	2.72 abc	0.70 ab	1.33 ab	4.67ab	3.78 abc	37.49 bc
Am + Gu +Bg + Bs	2.86 a	0.79 a	1.40 a	4.76 a	3.83 a	38.45a

3.3 The percentage of humidity of fruit , percentage dray matter of fruit, number of days to ripening , percentage of total soluble sold , fruit firmness and total yield of trees.

Concerning the results in Table (3), percentage of humidity of fruit, percentage dray matter of fruit, number of days to ripening, percentage of total soluble sold, fruit firmness and total yield of trees were significantly affected by all treatments.

TABLE 3

EFFECT OF SPRAYING WITH AMINO ACIDS, BLEED GRAPE AND SEAWEED EXTRACT ON YIELD AND ITS QUALITY FIG TREES C.V ASOWD DIALA FOR SEASON 2016

Treatments	% Humidity of fruit	% Dry matter of fruit	Number of days to ripening	% Total soluble solids	Fruit firmness Fruit firmness Kg/cm^2cm^2	Total yield Kg/ tree
Control	76.50 i	23.50 a	70 c	14.71 d	0.313 hij	15.50 j
Amister 4%	77.34 defgh	22.66 ab	75 bcd	15.22 cd	0.331fhj	21.60 ef
Gusto 4%	76.87ki	23.13 ab	72 de	15.39 cd	0.334efgh	17.86 hi
Bleed of grape 100%	76.99 ijk	23.01 ab	71 de	15.50 bcd	0.332 fgh	22.45 fg
Brawn seaweed extract 4%	76.95 jk	23.05 ab	72 de	15.37cd	0.322 fghi	18.21 ghi
Am + Gu	77.07 hijk	22.93 ab	72 de	16.10 cde	0.337efgh	22.90 fg
Am + Bg	77.14ghij	22.86 ab	73 cde	16.15bcd	0.339efgh	23.84 ef
Am + Bs	77.24 fgh	22.76 ab	72 de	16.06abcd	0.348efgh	23.51 efg
Gu + Bg	77.20 fghi	22.80 ab	73 cde	16.19bcd	0.351cdef	23.96 ef
Gu + Bs	77.40 cdef	22.60 ab	74 bc	16.22abcd	0.360 cde	22.93fg
Bg +Bs	77.54 bcd	22.46 ab	75 bcd	16.25abcd	0.373bcd	23.12 efg
Gu + Bg + Bs	77.46 cde	22.54 ab	77 abc	16.27 abc	0.382 ab	25.00 d
Am + Gu +Bg	77.50 bcd	22.50 ab	77 abc	16 .33 ab	0.379 bcd	24.30 de
Am +Bg +Bs	77.63abc	22.37ab	79 ab	16.79 ab	0.384 ab	25.87cd
Am + Gu +Bs	77.70 ab	22.30 ab	78 ab	16.58 ab	0.386ab	26.18 bc
Am + Gu +Bg + Bs	77.78 a	22.22 b	81 a	16.86 a	0.405 a	27.20 a

It is cleared that spraying amino acids, Bleed of grape and seaweed in single way or in combination to the fig trees increased physical and chemical characters of fruits compared with untreated trees. In addition, spraying this material in combination gave the highest parameters they were (77.78%, 81days, 16.80%, 0.405kg /$cm^2$and 27.20 kg / tree). On the other hand, untreated trees gave the lowest value they were (76.50, 70 days, 14.71%, 0.313kg /$cm^2$and 15.50 kg / tree) respectively .In

addition, the single and combination treatments led to a significant decreased in the percentage dray matter of fruit and the lowest value 22.22% in the treatment (Am + Gu+ Bg + Bs) comparison with the highest rates 23.50% in control treatment . These result are in line with (Shehata et.al, 2011, EKhan, *et al.*, 2012) on strawberry and grape they mentioned that applying of amino acids and seaweed extract to the plants improved physical , chemical of fruits and yield . The increase in diameter of fruit , length of fruit and weight of fruit is ascribed to the increased of chlorophyll contents of leaves, which increased photosynthesis and ultimately overall health of fig and this increased total yield of trees .

IV. CONCLUSION

Foliar application of mixture of amino acids, Bleed of grape and seaweed extract at 1 April, 1May and 1 June, had a positive effect on vegetative and fruiting growth, and fruit quality of fig trees cv. Aswod Diala . Multiple application of (Am + Gu+ Bg + Bs) is quite effective to improve growth and fruit physicochemical quality characteristics and total yield of trees compared with control treatment.

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Implementation of histopathological techniques and transmission electron microscopy for research of *Mycoplasma hyopneumoniae* in swine

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Abstract— *Mycoplasma hyopneumoniae is a fastidious bacterium, an important member of swine respiratory disease complex, like Porcine Enzootic Pneumonia, affecting the non-specific defense mechanism of the respiratory tract, high mucociliary system, predisposing the pigs to secondary pathogens. The objective of this study is to implement precise diagnostic techniques for identification of Mycoplasma hyopneumoniae. 19 swine lungs fragments were collected from slaughterhouses and submitted by histopathological techniques. The presence of mucocellular exudate in 78.94% of the samples was observed in the bronchi and bronchioles, absence of eyelashes in 63.15% and lymphoid tissue hyperplasia associated with the bronchus in 42.10%. In pulmonary parenchyma, thickening of alveolar wall and interstitial bronchopneumonia were observed in 68.42%, hemorrhage in 47.36%, which 36.84% had hemosiderin and 15.78% lung consolidation. The presence of mycoplasma by the negative staining technique was identified in all samples, also the labeling of epitopes by colloidal gold immunostaining, using monoclonal antibody. In immunohistochemistry techniques and in situ hybridization, the labeled epitope and genome were observed confirming the presence of Mycoplasma hyopneumoniae in the State of São Paulo. Therefore, the bronchial epithelium is the best tissue to collect the sample for an accurate diagnosis and the best method of diagnosis is the negative staining technique for screening and colloidal gold immunocytochemistry techniques to identify Mycoplasma species.*

Keywords—Mycoplasma hyopneumoniae, Porcine Enzootic Pneumonia, Immunochistochemistry and Hybridization.

I. INTRODUCTION

Mycoplasma hyopneumoniae is a small fastidious bacterium with a simple morphologically structure and absence of cell wall. (DEMINA et al., 2009; JUNQUEIRA and CARNEIRO, 2012). It is the etiologic agent of Swine Enzootic Pneumonia, causing the Porcine Respiratory Disease Complex (PRDC) (MARE and SWITZER, 1965; GOODWIN et al, 1965).

Is the most important disease that affects the respiratory system of pigs causing large economic losses for pigs (ROSS et al., 1999), due to the high morbidity and reduction of feed conversion that decreases average daily weight gain (CONCEICAO and DELLAGOSTIN, 2006).

Diagnoses for this bacterium by ELISA available on the market have low sensitivity and cross-reactions with other mycoplasmas present in the lungs of the swine (ROSS and STEMKE 1995; ERLANDSON et al., 2005).

The objective of this study is to implement precise diagnostic techniques for identification of *Mycoplasma hyopneumoniae*.

II. MATERIAL AND METHOD

2.1. Ethics Statement

This study was approved by Animal Experimentation Research Ethics Committe of the Instituto Biológico (protocol 138/14).

2.2. Experimental Design

Two lung fragments were collected from 19 male pigs weighing approximately 100 kg, at a slaughterhouse school of the University of São Paulo - Campos Pirassununga (São Paulo, Brazil).

The first fragment was submitted for analysis of electron microscopy, by negative staining techniques and electron immunocytochemistry. The second fragment was submitted to histopathological analyzes, H.E., immunohistochemistry and *in situ* hybridization.

2.3. Negative Contrasting

For this technique, 2 cm of pulmonary fragments were suspended in 0.1 M phosphate buffer - pH 7.0, placed in contact with metallic grids and previously covered in collodion and carbon films, negatively contrasted with 2% ammonium molybdate and pH 5.0 (BRENNER and HORNE, 1959; HAYAT and MILER, 1990).

2.4. Electronic Immunocytochemistry

In this paper, the immunostaining technique with negatively contrasted colloidal gold particles was used, following the protocol developed by Knutton (1995).

The copper grid was placed on 40 µl of pulmonary fragments suspension, then deposited on drops of the monoclonal antibody to *Mycoplasma hyopneumoniae* diluted 1:80. After this procedure the grids were incubated in drops of protein A conjugated with 10 nm colloidal gold (diluted 1:20 in 0.5% PBS) and contrasted with 2% ammonium molibidate, pH 5.0.

2.5. Histopathology

The lung fragments were fixed in paraformaldehyde 10% diluted in 0.1m PBS buffer, pH 7.0 for 72 hours, dehydrated in increasing series of ethyl alcohol concentrations, diaphanized in xylol and paraffin embedded in subsequent sections of 4µm and stained by the H.E. technique.

2.6. Immunohistochemistry

This technique was processed according to information from Kit LSAB + System-HRP K0690 (DAKO) and the antigen-antibody reaction was revealed by the DAB + Substrate K3468 chromogen.

2.7. In *Situ* Hybridization

The pairs of primers used for this technique were purchased by Life Technologies and performed according to the Dako K0620 - GenPoint commercial *in situ* Hybridization Kit protocol.

The sequences of the primers used for this reaction were: forward 5'-Biotin-GTC TAT CAA AAT TGC CAA TC-3 'and the reverse 5'-Biotin-TCC CAT AAC CTT GTC TTC AG-3', from nucleotides 851-870 And 1351-1370 respectively. The primers were labeled with biotin for visualization with the chromogen DAB + Substrate K3468 (KWON et al., 2002).

2.8. Observation and Images Registration

For the techniques of electron microscopy, the observation and recording of the image was performed in the transmission electron microscope Philips EM 208. In the histopathological techniques, the Carl Zeiss Axio ScopeA ® light microscope was used and the images were captured using the ZEN ® software.

III. RESULTS

3.1. Negative Contrasting

In this technique, pleomorphic particles with smooth and clear edges, gray central parts and less homogeneous, could be visualized in all 19 samples, being characteristic of M*ycoplasma* (Fig 1).

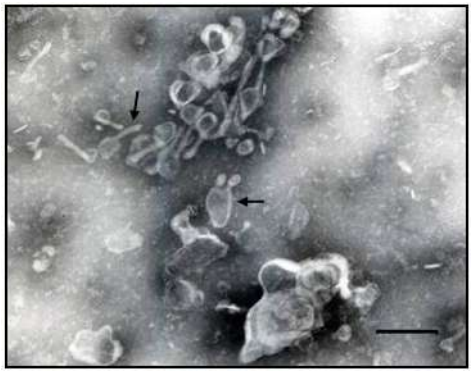

FIG 1 –PHOTOMICROGRAPHY OF MYCOPLASMA BY NEGATIVE STAINING TECHNIQUE IN ELECTRON MICROSCOPE TRANSMISSION. BAR 340 NM (SUCK ARROWS). SOURCE: ELECTRON MICROSCOPY LABORATORY STREAMING BIOLOGICAL INSTITUTE SP.

3.2. Electronic Immunocytochemistry

The specific epitope labeling for *Mycoplasma hyopneumoniae* with colloidal gold was observed in the 19 lung fragments used for this technique (Fig 2).

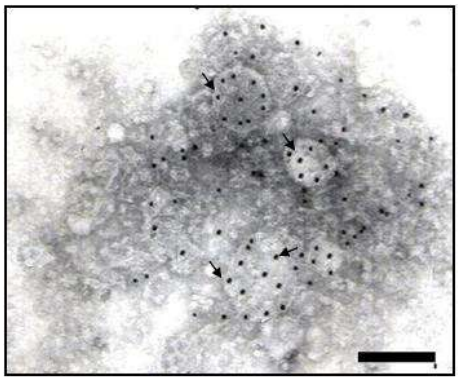

FIG 2 – PHOTOMICROGRAPHY MARKING OF MYCOPLASMA BY IMMUNOCYTOCHEMISTRY TECHNIQUE WITH COLLOIDAL GOLD IN NEGATIVE STAINING ELECTRON MICROSCOPE IN TRANSMISSION. 140 nm BAR (SUCK ARROWS). SOURCE: ELECTRON MICROSCOPY LABORATORY STREAMING BIOLOGICAL INSTITUTE SP.

3.3. Histopathology

The presence of mucocellular exudate in the bronchi and bronchioles was found in 78.94% (Fig 3), lack of eyelashes in 63.15%, bronchial associated lymphoid tissue hyperplasia (BALT) in 42.10% (Figure 4). In the pulmonary parenchyma, it was possible to visualize the thickening of the alveolar wall and interstitial bronchopneumonia in 68.42% (Fig 5), hemorrhage in 47.36%, among which 36.84% had hemosiderin and 15.78% consolidation pulmonary.

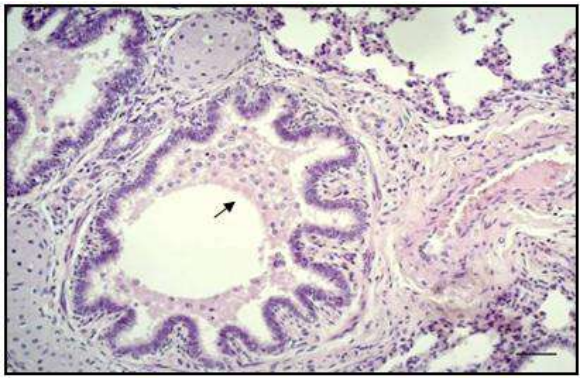

FIG 3 – PHOTOMICROGRAPH OF A HISTOLOGICAL CUT COLORED PIG LUNG BY HEMATOXYLIN AND EOSIN TECHNIQUE WITH MUCOCELLULAR PRESENCE OF EXUDATE WITHIN THE BRONCHI (BLACK ARROW) (20µm bar).

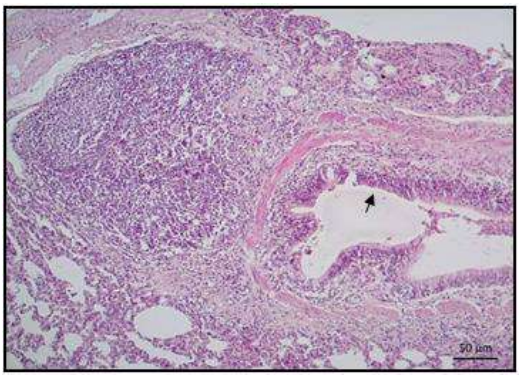

FIG 4 – PHOTOMICROGRAPH OF A HISTOLOGICAL CUT COLORED PIG LUNG BY HEMATOXYLIN AND EOSIN TECHNIQUE, WITH BALT HYPERPLASIA (ASTERISK).

3.4. Immunohistochemistry

The labeled epitope was observed by the antigen-specific antibody-*Mycoplasma hyopneumoniae* reaction in both the bronchial and bronchial epithelium and in the alveoli of the 19 histological sections of the lung fragments (Fig 6).

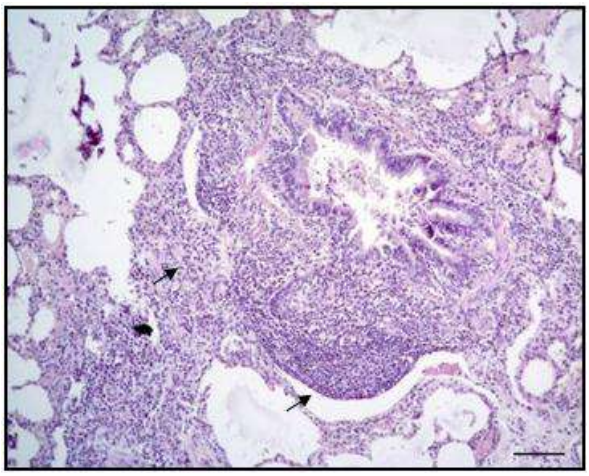

FIG 5 – PHOTOMICROGRAPH OF HISTOLOGICAL SECTIONS OF PORCINE LUNG STAINED WITH HEMATOXYLIN AND EOSIN TECHNIQUE, SHOWING AN INTERSTITIAL PNEUMONIA AND THICKENING OF THE ALVEOLAR WALLS (BLACK ARROWS) (100 µm bar).

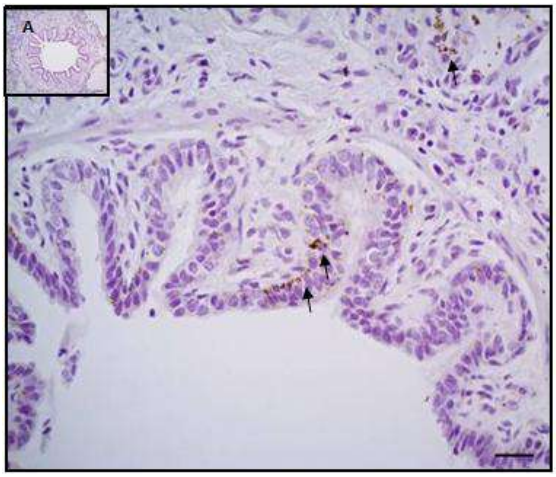

FIG 6 – PHOTOMICROGRAPH A IS SMALLER INCREASE IN PIG LUNG BRONCHIOLES (10X 10MICRON BAR). A PHOTOMICROGRAPH TO, IS THE EXPANSION OF A REGION OF THE PHOTOMICROGRAPH, DISPLAYING THE POSITIVE IMMUNOSTAINING OF EPITHELIAL CELLS OF THE BRONCHIOLES (BLACK ARROWS) (10 µm bar).

3.5. *In Situ* Hybridization

The genome of *Mycoplasma hyopneumoniae* was observed in both bronchi and bronchiole epithelium and alveoli of the 19 histological sections lungs fragments (Fig 7).

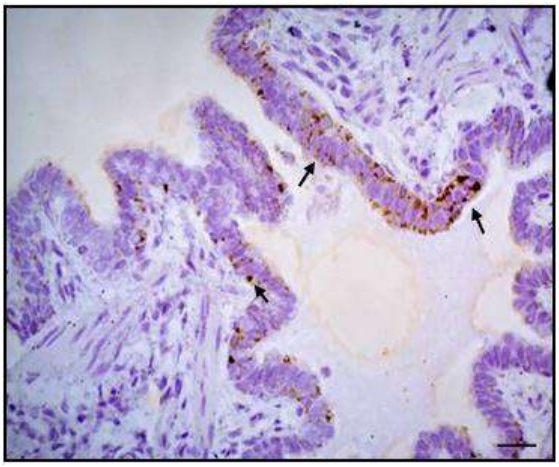

FIG 7 – PHOTOMICROGRAPH SHOWING POSITIVITY, THE MARKINGS BY MEANS OF IN SITU HYBRIDIZATION TECHNIQUE IN BRONCHIOLAR EPITHELIAL CELLS (BLACK ARROWS) (10 µm bar).

IV. DISCUSSION AND CONCLUSION

The identification of *Mycoplasma hyopneumoniae* was divided into 3 axes, according to their visualization, epitope marking and genome.

The agent was visualized through the negative staining presenting pleomorphic particles with the following characteristics: smooth and clear edges, gray in the center, less homogeneous and pleomorphic, corroborating with the characteristics described by Souza, et al. (2007). However, this technique does not allow identification at the species level, and therefore, the colloidal gold immunostaining technique was used to visualize and specify *Mycoplasma hyopneumoniae* species.

Due the small quantity of lung samples used in electron microscopy techniques, a correlation model was necessary between the identification of the agent and the histopathological lesions.

According to Sobestiansky et al., (1999) e Thacker (2006) the microscopic lesions vary with the disease progression, and the most observed lesion was bronchointerstitial pneumonia with BALT hyperplasia. In this study, it was observed that 68.42% presented interstitial bronchopneumonia and 42.10% presented BALT hyperplasia, corroborating with classic findings of the disease described above.

In addition, in 78.94% of the cases, a mucocellular exudate with neutrophils in the bronchi and bronchioles was observed, pulmonary consolidation of the parenchyma in 15.78% and thickening and hyperplasia of the alveolar walls with inflammatory infiltrate in 68.42%. Although these microscopic changes are not classic of *Mycoplasma hyopneumoniae* infection, Redondo et al. (2009) and Hillen et al. (2014) also described these findings in the discrimination of the disease. It was observed that in 63.15% there was a lack of eyelashes in the bronchi and bronchioles, an infectious characteristic also seen by Posá et al. (2013). These findings indicate that *M. hyopneumoniae* infection is extremely complex with classic and secondary characteristics that are relevant in clinical diagnosis.

Although markers are positive in histopathological lesions by immunohistochemical techniques and in situ hybridization, the fixation in 10% buffered formalin can alter the epitopes as a protein fixative as described by Souza (2007). Although the *in situ* hybridization technique is effective, Santos (2015) verified that *M. hyopneumoniae* genomics in Brazil is highly variable, identifying 39 different types of the species in 8 States, according to multiple locus variable number tandem repeat analysis (MLVA) which makes it difficult to specify probes.

Because of the possible epitope alteration and the high genotypic variability, the reliable method of histopathological findings was the positivity of both methods (immunohistochemistry and in situ hybridization) to confirm the pulmonary lesions caused by *Mycoplasma hyopneumoniae*, in the State of São Paulo, Brazil.

It was concluded that for a precise diagnosis of *M. hyopneumoniae* in São Paulo State, the best sample is the bronchial epithelium and the best diagnostic method is the electron microscopy technique (as screening) and immunocytochemistry with colloidal gold for identification level of agent species.

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Quantifying the relative impact of physical and human factors on the viticultural expression of terroir

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Abstract— *This work assesses the relative importance of the terroirs factors: climate, soil and the relation source-sink, on the vegetative development, yield, berry composition and plant sanitary status.*

The study was carried out between 2011 and 2014 in nine vineyards from six viticultural regions over the coast of Río de la Plata (Uruguay). The cultivar studied was Tannat, vertically trellised and north-south oriented. The year effect refers to climate, which was characterized using solar irradiation and three indices. The soil was characterized using pits and physico-chemical analyses, to determine three textural categories and to define soil depth and water availability. The source-sink relationship referred to four categories of relations between leaf surface and yield per vine. Statistical analyses included a Mixed Model with random effects to determine the relative importance of each factor to the total variability within the dataset.

Total yield per vine was explained by the source-sink relationship, the year and their interaction, both linked to the rainfall amount occurred during the maturation period. The synthesis of primary compounds in the berries was more dependent on the year and the interaction of soil and year with the source-sink relationship. Secondary compound concentrations in the berry depended mainly on the source-sink relationship and climate.

This study represents a significant advance to the knowledge of grapevine adaptation to the Río de la Plata terroirs, assigning a fundamental role to the vine grower actions. The growers can modulate grapevine balance as a function of the environment.

Keywords— berry composition, soil, Tannat, terroir, yield.

I. INTRODUCTION

Terroir can be defined as the interaction amongst the elements that constitute a given ecosystem: climate, soil and grapevine within a given geographical location [1], and human factors, expressed as the viticultural practices [2]. Harvest yield and quality, as well as the typicality of its wines will depend on the interaction amongst these factors along with enological practices. Knowledge about the real functioning of the vineyard and designing technical schedules stand out among the advantages of the methodological approach that implies studying terroirs,.

Due to the complexity of systematic studies, research on viticulture tends to the use of reductionists approaches and to the analysis of cause-effect relationships. In contrast, the joint study of terroir key factors, such as climate, soil and cultivar, is more complex to deal with and, hence, the amount of these studies is lower.

As examples of climate influence, several authors worked on defining climatic indices to describe the suitability of a given region for producing wine [3][4][5][6] [7][8][9]. In addition, other researchers got deep into climate effects on vine functioning [10], on wine and vintage quality [11], on yield [12][13], or on a group of variables showing vine performance [14]. Grapevine energy balance when combined with its water balance regulates the group of responses to the environment (in this case, climate variables) of a given plant population [15]. Nowadays, a great number of climate analysis related to viticulture are focused on a climate change perspective, reporting a trend to increasing temperatures in many of the most prestigious grapevine growing regions worldwide [16]. This problem led to the study of vine response to increasing temperature over the growing cycle in several climatic regions [17].

Soil factor and its influence on vine performance have been comprehensively studied [2][18][19][20][21] [22] [23][24]. Several authors proved the huge influence of water availability on vintage quality [25] [26][27][28] [29][30]. Source-sink modulation through cultural practices can be considered a key factor since it affects vine vigour, yield and berry quality. Those techniques that modify source-sink relationships have been the subject of a great number of studies [31][32][33][34][35][36][37][38][39]. Pruning, shoot thinning, defoliation, shoot trimming and cluster thinning are relevant practices for regulating source-sink relationships and grapevine balance. In this sense, the operations performed by the vine

grower represent an adaptation mechanism for rearranging vine components and directing the vineyard system in order to achieve a pre-defined goal.

Nevertheless, those studies including more than one determinant factor are scarce [40]. Recently, some authors [41] proved the significant effect of climate, soil and genetic (cultivar and rootstock) factors on a group of grapevine response variables. In particular, soil and climate had a greater incidence on the variability of the system than cultivar, likely due to their influence on grapevine water status.

From the results of partial studies it is possible to build models that allow for identifying the relative importance of each factor on the final response and, thus, generate tools that growers and technicians can use for a suitable management of the vineyard and improve its efficiency.

In order to answer these questions, the current study aimed to assess the relative importance of the climate, soil and source-sink relationship factors on grapevine vegetative growth, yield, berry composition and sanitary status. Secondly, the study aimed to establish the properties of each factor that exert more influence on vineyard performance.

II. MATERIAL AND METHOD

In the current analysis, data from four vintages on nine commercial vineyards (cv. 'Tannat') locate on the Uruguayan coast of Río de la Plata have been used. This region contains most of the vineyard surface in the country.

2.1 Study sites description and locations

Nine plots located in the 300 km of the Uruguayan coast of Río de la Plata have been selected for this study. Plots were numbered from west to east, from Colonia del Sacramento (Colonia department) to Pueblo Edén (Maldonado department) (Supplementary Figure 1).

2.2 Climate indices ("year" factor)

Weather variables were recorded in two meteorological stations managed by the Instituto Nacional de Investigación Agropecuaria: La Estanzuela (-34.3300 /- 57.6800) and Estación Las Brujas (-34.6700 /- 56.3300). Moreover, four "Vantage Pro2" (Davis Instruments, Hayward, CA, U.S.A) automated stations were installed in the plots or close to them: Mal Abrigo (-34.1167 / -56.9333), Sayago (-34.8333 / -56.2167), Empalme Olmos (-34.6667 /-55.9000), Sierra Ballena (-34.7333/-55.3000). All the stations were installed and operated according to the World Meteorological Organization (WMO). The Multicriteria Climate Classification (MCC) [42][6] was applied using the adaptations for the Uruguayan conditions [8] The following indices were estimated: Heliothermal index (HI), Dryness index (DI) and Cool Night index (CI).

Daily solar irradiation was estimated using satellite images from GOES-East satellite using the BD-JPT model [43]. Cumulative solar irradiation (RS ac) from September 1st to harvest and daily mean solar irradiation for February of each year (RS Feb) were determined.

TABLE 1
CLIMATIC INDICES FOR GRAPEVINE: HI, CI, DI AND SOLAR IRRADIATION: RS AC, RS FEB. EACH VALUE IS THE AVERAGE FOR THE YEAR ± STANDARD DEVIATION FOR EACH LOCATION

Year	HI (°C)	CI (°C)	DI (mm)	RS ac ($MJ*m^{-2}$)	RS Feb ($MJ*m^{-2}$)
2011	2331.7 ± 72.2	18.5±1.2	-24.3 ± 0.2	4347.0 ± 3.2	23.8 ± 0.1
2012	2327.7 ± 39.4	18.5±0.5	39.3 ± 24.5	4292.6 ± 44.6	21.2 ± 1.1
2013	2188.8 ± 63.9	17.3±0.4	-3.2 ± 11.1	3778.4 ± 74.5	22.6 ± 0.7
2014	2289.3 ±160.9	17.4±0.8	61.2 ± 22.9	3851.3 ±205.4	17.2 ± 0.4

2.3 Description of the study plots and plant material

The study was conducted from 2011 to 2014 in nine commercial non-irrigated vineyards. On each location, 30 vines (*Vitis vinifera* L.) cv. 'Tannat' were randomly chosen; they were distributed on three rows with 10 vines each. Grapevines were vertically trellised on a Guyot system. Rows were north-south oriented in all vineyards. Further information on the studied plots is shown in Table 2.

TABLE 2
MAIN CHARACTERISTICS OF THE STUDIED VINEYARDS

Plot	Coordinates	Location (name)	Rootstock	Plantation year	Spacing
1	34° 23' 47,40" S; 57° 52' 49,67"W	Real de Vera	3309C	2000	2.50m x 1.20m
2	34° 23' 17,40" S; 57°51' 07,37" W	Piedra de los Indios	3309C	1999	2.50m x 1.15m
3	34°07' 10,46" S; 56°56' 50,98" W	Mal Abrigo	3309C	2000	2.00m x 0.90m
4	34°36' 44,77" S; 56°14' 42,02" W	Juanicó	SO4	1998	2.50m x 1.10m
5	34°53' 04,55" S; 56°19' 24,33" W	Punta de Yeguas	3309C	2005	2.50m x 1.00m
6	34° 39' 30,43" S; 55°47' 56,11" W	Atlántida A	3309C	2006	2.50m x 0.90m
7	34°39' 36,70" S; 55°47' 55,22" W	Atlántida B	3309C	2006	2.50m x 1.00m
8	34°42' 31,74"; 55°03'31,56" W	Sierra Ballena	Gravesac	2004	2.50m x 1.00m
9	34°44'36,22" S; 55°01'16,42" W	Pueblo Edén	101-14Mg	2005	2.50m x 1.20m

2.4 Root system characterization

Roots were studied digging pits in the row in front of a selected vine. Amount, diameter and distribution of roots were determined at different distances from the vine. Roots were stained, photographed, measured and mapped over a vertical grid with a cell size of 100 cm2. Roots were classified according to their diameter: < 3 mm, 3 to 5 mm and > 5 mm. The depth to which 90% of active roots (<3 mm diameter) appeared was recorded. This depth was used as a reference for estimating Dryness Index (DI), available water capacity (AWC) and soil textural class (TCra).

2.5 Soil characterization (soil factor)

Soil from each plot was described according to FAO (2006) and classified using USDA Soil Taxonomy [44].Two samples per horizon were collected from the pits, and they were used for determining soil physical and chemical properties. These observations were complemented with, at least, five samples per horizon, collected using a manual drill on different spots in each studied plots. These samples were used for assessing soil structure, texture, color, depth, presence of active roots, amongst others. Soil texture was determined using the method described by [45]. From clay, silt and sand fractions of each horizon, their proportion was estimated for the volume of soil explored by active roots. Textural classification from these proportions was defined as TCra. Dryness index [46] [8] and available water capacity (AWC) [47] was determined. This measurement was considered as the initial volume of water in the soil (Wo) for estimating soil water balance.

TABLE 3
CHARACTERISTICS OF THE SOILS FROM THE STUDIED PLOTS

Plot	USDA Soil classification	Bedrock type	Depth* (cm)	AWC* (mm)	Textural class * (TCra)
1	Typic Argiudoll	Quaternary sediments	50	96	Silty clay loam
2	Typic Argiudoll	Quaternary sediments	56	97	Silty clay loam
3	Typic Hapludoll	Metamorphic rock (low degree)	36	76	Clay loam
4	Vertic Argiudoll	Quaternary sediments	70	123	Silty clay
5	Typic Argiudoll	Quaternary sediments	60	110	Silty clay
6	Typic Hapludert	Metamorphic rock	43	67	Clay loam
7	Abruptic Argiudoll	Metamorphic rock	36	57	Clay loam
8	Lithic Hapludoll	Metamorphic rock	36	68	Clay loam
9	Abruptic Argiudoll	Metamorphic rock/ Quaternary sediments	54	85	Clay loam

Soil depth with 90% of visible roots lesser than 3 mm in diameter.

2.6 Vegetative growth determination

Potential exposed leaf surface (SFEp) was estimated at veraison [48]. At harvest, a shoot bearing a cluster was collected from the middle of the branch in ten vines. In each of these shoots, length (LP) was measured and fresh and dry weight for each organ was recorded. Samples were dried in an oven at 50 ºC till constant weight. Dry weight was expressed by organ, total per shoot (PST) and per linear meter of the trellising system (PSesp). In order to estimate this last variable, PST was multiplied by the number of shoots per linear meter obtained from counting the shoots of all the studied plants. In addition, dry weight per cm of wood in the shoot (Psmad) was also estimated.

2.7 Yield components and rot incidence

At harvest, yield (Y) and cluster number per vine were recorded. From these data, cluster average weight was obtained. Dry weight per cluster (PSRac) was obtained from the process described in the former subsection. Those clusters that showed, at least, 5% of the berries affected by diseases (mainly *Botrytis sp*) were counted and separately weighed (Yenf). Berry weight (Pb) was obtained from 3 samples of 250 berries each, randomly collected on the studied vines at harvest.

2.8 Determination of the leaf/fruit ratio ("source/sink" factor)

The leaf/fruit ratio (FF) was established as a factor in order to analyze its influence on vine performance. Four classes were defined within this factor that, eventually, could be determined by the vine grower by performing a series of cultural practices to reduce leaf surface and/or cluster number or some parts of the clusters.

This indicator was obtained by dividing the potential exposed leaf surface (SFEp) and the yielded per vine (Y). The categories were defined accounting for the frequency distribution for six classes. Due to the low frequency of the two highest categories, they were grouped with the former one for obtaining four categories with a balanced distribution. The defined categories were the following: < 0,40; 0.40-0.60; >0.60-0,80; >0,80 (m2*kg grape-1)

2.9 Determination of berry composition

Harvest was carried out at "technological maturity" for each plot, considering pH values, the ratio between sugar content and titratable acidity of the grapes and berry weight. These parameters were determined periodically using the OIV (2007) procedures. For doing this, from veraison, weekly samples of 250 berries were collected in each plot. Berry composition was determined after manually separating the berries from the raquis and obtaining the juice by crushing the flesh with an electrical grinder (HR2290, Phillips, The Netherlands). Soluble solids contents (SS) were determined using a refractometer (Atago N1, Atago, Tokyo, Japan), pH was determined with a pH-meter (HI8521, Hanna Instruments, Italy) and titratable acidity (AT) was measured by titration and was expressed as g of sulfuric acid /L juice.

In the berry samples, we also determined total anthocyanins (ApH1), extractable anthocyanins (ApH 3.2), phenolic richness (A280) and the cell maturity index (EA) [49]. All these measurements were carried out in duplicate with a Shimadzu UV-1240 Mini (Shimadzu, Japan) spectrophotometer, using crystal (for anthocyanins) and quartz (for absorbance at 280 nm) cells with 1 cm path length. The indices were calculated considering the respective dilution of the grape extracts [50].

2.10 Statistical analysis

In order to analyze the relative importance of the different factors (and their interactions) on the total variability of vine performance, the following classes were defined:

Class	Levels	Values
Year (Year effect)	4	2011; 2012; 2013; 2014
Soil (Textural class or TCra)	3	Clay loam; Silty clay; Silty clay loam
Source-sink (SFEp*Y-1)	4	<0,40; 0.40-0.60; >0.60-0,80; >0,80

A Mixed Model with random effects was considered:

y = Soil, Source-sink, Year effect, interactions and residuals, except the intercept.

The model was run for each dependent variable and the variance was estimated by the Restricted Estimation by Maximum Likelihood (REML). The relative percentage of each one over the total sum was determined. In addition, ANOVA was used for assessing the effect of each individual factor (year, soil and source-sink) on vine performance (vigour, yield, berry composition and sanitary status). Fisher LSD test was used for mean separation ($p<0.10$). Furthermore, Pearson's correlation coefficients were calculated for the selected variables in order to further interpreter the effect of the source-sink factor. ANOVA was performed using the InfoStat software and the Mixed Models were estimated using R (R Development Core Team www.r-project.org).

III. RESULTS

Table 4 shows the relative importance of each factor ("year", "soil and "source-sink") and their interactions on the variance of the studied dataset.

Source-sink ratio was used as a factor because the determination of its magnitude has not a lineal dependence relation with the two variables that it related (SFEp/Y), as explained later.

Most of the factors, either individually or their partial interactions, did not explain the variability in the obtained results. In many cases, the percentages were equal to 0 or their values were not significant. In general, the percentages accumulated as "residual" surpassed to the studied factors and interactions. The greatest variability assigned to "residual" corresponded to the group of variables associated with vegetative vigour.

Source-sink relation explains 82% of the yield variability in the dataset; the interaction "year*soil" explained 14% of the rot incidence and 36% of the pH value in the juice. The interaction "year*FF" reflected significant effects on the variability of yield (13%), rot incidence (43%) and titratable acidity (27%).

TABLE 4
PERCENTAGE OF THE VARIABILITY WITHIN THE DATASET EXPLAINED BY THE YEAR, SOIL AND SOURCE-SINK FACTORS, AS WELL AS THEIR INTERACTIONS

Variable	Year		Soil		Source-sink		Year* Soil		Year* Source-sink		Soil* Source-sink		Residual
	%	p	%	p	%	p	%	p	%	p	%	p	%
SFEp (m^2 *$vine^{-1}$)	14	ns	11	ns	0		0		0		0		75
LP (cm)	0		12	ns	0		0		0		0		88
PSmad (mg*cm^{-1})	0		29	ns	0		0		0		0		71
PST (g)	16	ns	0		6	ns	0		0		0		78
PSesp (kg*m^{-1})	16	ns	2	ns	0		0		0		0		83
Pb (g)	42	ns	0		0		0		0		0		58
Y (kg)	0		0		82	*	1	ns	13	**	0		5
Yenf (%)	11	ns	22	ns	0		14	*	43	**	8	ns	1
PSRac (g)	27	ns	0		21	ns	0		0		0		52
SS (g*L^{-1})	22	ns	0		8	ns	0		23	ns	0		47
AT (g*L^{-1})	44	ns	2	ns	0		8	ns	27	*	0		19
PH	6	ns	1	ns	0		36	*	19	ns	21	ns	18
ApH1	16	ns	0		42	ns	0		0		0		43
ApH3,2	0		0		34	ns	0		7	ns	0		59
EA%	47	ns	0		0		0		17	ns	0		36
A280	3	ns	23	ns	0		0		33	ns	0		42

, ** indicates significant at p < 0.05 and 0.01 respectively; ns indicates not significant.

Variables associated to vegetative development tended to be influenced by soil and year factors, primarily, and for source-sink to a lesser extent. In contrast, yield variables were influenced mostly by year, source-sink and their interaction. Berry composition variables were affected by year, source-sink, the interactions year*source-sink and year*soil.

The results from the ANOVA for studying the influence of the soil factor on the response variables showed significant differences in several vegetative development variables: SFEp, LP and PSmad. The influence of soil on yield variables was relevant for Y and Yenf; finally, for berry composition, soil influenced ApH1, ApH3.2 and A280 (Table 5).

TABLE 5
VINE RESPONSE AS A FUNCTION OF SOIL TEXTURAL CLASSES (MEANS ± STANDARD DEVIATION)

Type	Variable	Soil factor: TCra		
		Silty clay N=7	Clay loam N=14	Silty clay loam N=6
Vegetative development	SFEp (m^2*$vine^{-1}$)	1.81 ab ± 0.30	1.61 b ± 0.31	1.89 a ± 0.24
	Shoot length: LP (cm)	128.95 a ± 39.35	127.10 a ± 28.28	100.23 b ± 8.97
	Shoot dry weight: PST (g)	188.21 a ± 43.93	164.39 a ± 51.00	189.09 a ± 59.06
	Dry weight per linear meter: PSesp (kg*m^{-1})	2.29 a ± 0.48	1.99 a ± 0.67	2.22 a ± 0.54
	Dry weight per cm of wood: PSmad (mg*cm^{-1})	202.93 b ± 33.17	268.69 a ± 52.88	247.71ab ± 45.53
Yield components	Berry weight Pb (g)	1.68 a ± 0.12	1.66 a ± 0.16	1.70 a ± 0.17
	Yield: Y (g*$vine^{-1}$)	5416.59 a ± 2096.38	1917.73 b ± 609.70	4774.59 a ± 2028.27
	Affected clusters: Yenf (%*$vine^{-1}$)	53.03a ±41.00	15.83b ± 19.88	7.22b ± 9.60
	Cluster dry weight: PSRac (g)	128.22 a ± 38.24	102.54 a ± 38.72	138.40 a ± 60.95
Berry composition	SS (g*L^{-1})	203.46 a±36.45	223.78 a ±24.65	216.18 a ±12.67
	TA (g H_2SO_4 *L^{-1})	5.21 a ±1.82	5.13 a ±1.29	4.21 a ±0.64
	pH	3.37 a ±0.25	3.54 a ±0.11	3.56 a ±0.09
	ApH1 (mg EMG*L^{-1})	1582.71 b ±822.30	2095.66 a ±516.72	1708.20ab ±230.28
	ApH3.2 (mg EMG*L^{-1})	776.76 b ±385.82	981.65 a ±213.06	859.67 ab ±149.83
	EA (%)	49.20 a ± 10.52	51.39 a ± 10.53	49.52 a ± 7.22
	A280	57.52 b ±17.02	73.64 a ±13.64	53.88 b ±11.92

EMG = equivalent to malvidin-3-glucoside. Different letters in the row indicate significant differences according to Fisher LSD test (p <= 0.10).

Pearson's correlation coefficients found for the proportion of sand in the soil (coarse fraction) and other variables were negative for AWC (r= -0.60, p<0.001), SFEp (r= -0.56, p<0.001), Y (r= -0.67, p<0.001) and positive with SS (r=0.36, p<0.05), ApH1 (r=0.50, p<0.01) and A280 (r= 0.52, p<0.01)

ANOVA using year as factor showed a relevant importance on vine response, influencing in 60% of vegetative development variables, in 75% of yield variables and 86% of berry composition variables. Year 2011 was the warmest, driest and with more solar irradiation over the growing cycle and during maturation. Year 2013 had the lowest register for thermal accumulation and solar irradiation, with a water balance in the soil close to 0 and high values of solar irradiation during maturation. The years 2012 and 2014 were more humid. Particularly, year 2014 had the highest rainfall values over maturation and very low solar irradiation over this period.

The year factor affected vegetative development variables (SFEp, PSesp and Psmad). Weather conditions in 2012 caused a greater development of leaf surface and a higher wood dry weight; whereas in 2014 a reduction of total dry mass per linear meter of the trellis system was observed. In the case of yield components, significant differences were detected for Pb, Tenf and PSRac. In addition, the year factor affected all the berry composition variables except for ApH3.2 (Table 6).

TABLE 6
VINE PERFORMACE AS A FUNCTION OF THE YEAR (MEANS ± STANDARD DEVIATION)

Type	Variable	"Year" factor			
		2011 N=3	2012 N=8	2013 N=9	2014 N=7
Vegetative development	SFEp (m^2*$vine^{-1}$)	1.46b ± 0,16	1.91a ±0.34	1.64b ±0.29	1.73ab ±0.25
	Shoot length: LP (cm)	107.23a ±23.31	128.67a ±37.20	117.13a ±33.61	125.45a ±20.49
	Shoot dry weight: PST (g)	247.76a ±63.63	233.68a ±63,43	261.47a ±48.76	243.22a ±49.70
	Dry weight per linear meter: PSesp (kg*m^{-1})	208.10a ±65.45	178.36ab ±40.47	191.32a ±60,84	140.06b ±21.84
	Dry weight per cm of wood: PSmad (mg*cm^{-1})	2.26ab ±0.87	2.36a ±0.56	2.21ab ±0.56	1.67b ±0.40
Yield components	Berry weight Pb (g)	1.45b ±0.13	1.75a ±0.13	1.65a ±0.12	1.71a ±0.14
	Yield: Y (g*$vine^{-1}$)	2989.48a ±1680,03	3993.62a ±1890.82	3263.12a ±2829.39	3303.76a ±2002.54
	Affected clusters: Yenf (%*$vine^{-1}$)	3.87b ±3.95	24.94ab ±32.33	10.88b ± 20.60	46.71a ±34.37
	Cluster dry weight: PSRac (g)	151.97a ±48.18	118.36a ±27.34	132.73a ±56.94	80.87b ±20.53
Berry composition	SS (g*L^{-1})	225.17a ±12.85	213.73ab ±8.80	232.92a ±22.71	196.09b ± 36.46
	TA (g H_2SO_4 *L^{-1})	4.56bc ±0.43	3.81c ±0.59	5.07b ±0.74	6.27a ±1.70
	pH	3.36b ±0.07	3.55a ±0.12	3.59a ±0.15	3.40b ±0.18
	ApH1 (mg EMG*L^{-1})	1666.07ab ±102.11	2147.84a ±402.86	2005.34a ±679.61	1491.19b ±642.58
	ApH3.2 (mg EMG*L^{-1})	782.54a ±43.20	980.47a ±174.98	898.56a ±327.59	865.73a ±323.24
	EA (%)	52.68a ±5.01	53.85a ±6.37	55.35a ±5.83	39.15b ±9.78
	A280	67.32ab ±8.39	55.08b ±14.44	70.65a ±17.50	68.35ab ±17.46

EMG = equivalent to malvidin-3-glucoside. Different letters in the row indicate significant differences according to Fisher LSD test (p <= 0.10).

The analysis of variance accounting for the source-sink factor (Table 7) determined significant differences for Psesp in the group of vegetative development variables; for Pb, Y and PSRac among yield components.

Yield was positively correlated with berry weight (Pb) and cluster dry weight (PSRac), which is the determinant of the differences in Psesp or total dry weight produced per linear meter of the trellis system.

Moreover, the source-sink factor influenced all berry composition variables, except for EA. The class ">0.80" had the highest SS values, whereas the lowest ones were observed for the "<0.40" class. The AT, pH and A280 were also higher for the ">0.80" class. Anthocyanins were greater in the intermediate categories: ApH1 was higher in the "0.61-0.80" class and ApH3.2 in "0.40-0.60" and ">0.60-0.80". Overall, the ">0.60-0.8" was associated to a response of higher quality, whereas that of "<0.40" to higher yields.

TABLE 7
VINE RESPONSE ACCORDING TO THE SOURCE-SINK FACTOR (MEANS ± STANDARD DEVIATION)

Type	Variable	"Source-sink" factor: SFEp/kg grape (m^2*kg^{-1})			
		<0.40 N=6	0.40-0.60 N=8	>0.60-0.80 N=5	>0.80 N= 8
Vegetative development	SFEp (m^2*vine^{-1})	1.86a ±0.31	1.79a ±0.26	1.63a ±0.27	1.61a ±0.37
	Shoot length: LP (cm)	109.95a ±16.95	121.04a ±38.84	130.34a ±17.41	125.47a ±36.06
	Shoot dry weight: PST (g)	229.43a ±47.09	231.43a ±55.02	255.24a ±66.46	270.54a ±46.29
	Dry weight per linear meter: PSesp ($kg*m^{-1}$)	204.86a ±63.44	182.08ab ±35.89	160.06ab ±37.03	158.42b ±57.49
	Dry weight per cm of wood: PSmad ($mg*cm^{-1}$)	2.36a ±0.35	2.24a ±0.58	1.87a ±0.63	1.97a ±0.73
Yield components	Berry weight Pb (g)	1.66ab ±0.14	1.69ab ±0.14	1.77a ±0.06	1.60b ±0.18
	Yield: Y ($g*vine^{-1}$)	6857.74a ±1380.00	3528.48b ±902.32	2317.40c ±327.92	1556.32c ±501.64
	Affected clusters: Yenf ($\%*vine^{-1}$)	20.80a ±33.25	37.26a ±40.33	21.27a ± 24.84	13.35a ± 18.59
	Cluster dry weight: PSRac (g)	153.66a ±58.80	120.91ab ±32.66	100.79b ±21.84	96.28b ±44.40
Berry composition	SS ($g*L^{-1}$)	198.38b ±35.39	218.57ab ±15.57	220.40ab ±10.09	226.68a ±32.35
	TA ($g\ H_2SO_4*L^{-1}$)	4.93ab ±2.09	4.63b ±0.67	4.17b ±0.97	5.76a ±1.19
	pH	3.35b ±0.14	3.53a ±0.21	3.60a ±0.10	3.53a ±0.10
	ApH1 ($mg\ EMG*L^{-1}$)	1276.71c ±480.83	1912.88b ±511.23	2407.25a ±224.11	1958.48ab ±588.22
	ApH3.2 ($mg\ EMG*L^{-1}$)	641.32b ±245.82	939.08a ±246.05	1116.33a ±87.61	924.53a ±237.79
	EA (%)	48.56a ±11.03	50.42a ±6.74	53.18a ±6.35	50.05a ±13.35
	A280	51.88c ±12.61	60.30bc 15.04	71.75ab ±14.24	75.57a ±14.76

EMG = equivalent to malvidin-3-glucoside. Different letters in the row indicate significant differences according to Fisher LSD test (p <= 0.10).

In order to better understand the causes that explain the source-sink factor, a series of partial correlations were analyzed (Figure 1). Soil water availability (AWC) was positively correlated with yield (r= 0.53; p= 0.004) and with SFEp (r= 0.54; p= 0.003), yield being the main explaining factor of the ratio source-sink (r= -0.57; p=0.002). In contrast, leaf surface (SFEp) did not present a correlation with this ratio (r=0.21; p> 0.1), although it was significantly correlated with yield (r= 0.36; p= 0.06). Therefore, an increasing in vegetative development explains a greater yield, although it has not a significant effect on the leaf/grape ratio. Complementarily, we observed that shoot number per linear meter of the trellis system had an incidence on vegetative development (r= 0.40; p= 0.04) and that the increase in yield was negatively correlated with the exposure of the clusters to solar radiation.

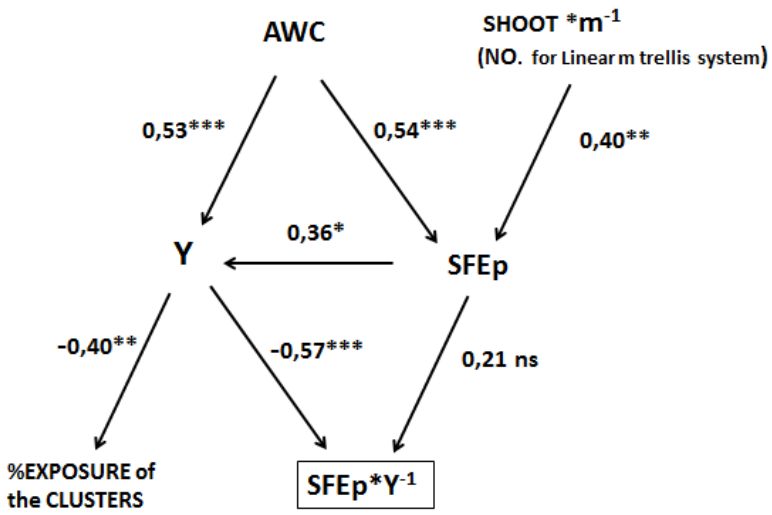

FIGURE 1: SCHEMATIC REPRESENTATION OF THE PEARSON'S CORRELATION COEFFICIENTS AMONGST THE DIFFERENT VARIABLES CONSIDERED IN THE CURRENT STUDY. THE SYMBOLS: *, **, * AND NS, INDICATE SIGNIFICANCES AT P< 0.1; 0.05; 0.001; AND NOT SIGNIFICANT, RESPECTIVELY.**

IV. DISCUSSION

The analysis of the relative importance of factors (and their interactions) to the variance allows interpreting a complex effect on determining plant performance, and that cannot be attributed only to the selected elements or to their partial interactions. The high percentages accumulated in "residual" might reveal the effect of other factors and interactions that have not been considered; for instance, soil fertility, in-row weed management, vine reserve accumulation or the occurrence of extreme climate events. In addition, the use of simple indices, such as DI or HI, for characterizing a productive cycle might not be sufficient for explaining the physiological dynamics of the plants, at least to a refined level, and the adaption processes to different constraints. In order to obtain a zonification with a more homogeneous response, it will be necessary to carry out studies that integrate climate variability at different scales, including extreme values, with key physiological processes and wine quality [10].

Apart from these limitation, the significant effects of the studied factors on the variance of our dataset, and even those that can only be taken as showing trends in the mixed model, are in accordance with the ANOVA results when the effect of each factor is analyzed independently. This accordance justifies the pertinence of the applied analysis, since it allows for a global vision of viticulture production at the territory level by jerarquizing the weight of the factors.

4.1 Effect of the studied factors on the vegetative development

Vegetative development variables were not influenced by the studied factors and their interactions. However, the "year" and "soil" factors exerted a significant influence on some vegetative development variables [41].

Water availability is the soil component with the greatest influence on grapevine physiology, and it is dependent on soil texture, depth [19] and soil organic matter content. Silty clay and Silty clay loam soils showed a greater volume explored by roots and a greater proportion of sitl and clay; whereas Clay loam soils were associated to greater water availability for the vines. A strong correlation between soil available water capacity and canopy development, yield, berry size and must quality has been detected, as observed by other authors [27] [28][29][21][22][30][23]. Weather conditions in 2012 and, especially, in 2014, with high rainfall amounts in summer, promoted a greater biomass development; this situation was more marked in those vineyards located on soils with high AWC. The different measures of plant water status (leaf water potential, carbon isotope discrimination, water balance models) were strongly correlated with soil water availability [51]. Therefore, it is possible to infer plant water status from indices such as DI and RSFeb [52].

4.2 Effect of the studied factors on yield components

The influence of the three studied factors and their interaction on the determination of yield components was evident. Yield per plant was correlated positively with rainfall amount over the maturation period. According to our results, the greatest weight in the determination of yield components corresponded to the "Source-sink" and "Year" factors, but also to their

interaction. Our results proved that the driver of the "Source-sink" factor was yield and not leaf surface, since differences on SFEp have not been detected.

In this case, the different categories of the "Source-sink" ratio were the result of the influence of weather conditions in a given year and soil type; although, usually, vine balance is also managed by the vine grower. Even though AWC affects to the vegetative and reproductive development, it induces a differential variability between both dimensions that is more beneficial to yield. Increasing crop load in the vines reduces vegetative growth and reserve accumulation in the shoots due to the competition established among the different organs, particularly in the period from fruit-set to pea-size stage, when photo-assimilate translocation is multidirectional [53]. The opposite effect was observed, for instance, in plants with severe pruning or excessive cluster thinning that promote a greater vegetative development. Clusters have greater sink strength for photo-assimilates than other organs, but also a scarce capacity for acting as a source [54]. Soil showed a lower influence on vine yield than the other factors considered in this study. Nevertheless, the analysis without accounting for the other factors reflected soil effects on this variable. Soil affected yield mainly by differences in water availability, due to soil water retention and volume explored by roots. Fine-textured soils (Clay loam and Silty clay loam), developed over quaternary sediments and with greater depths, had highest AWC values. Partial correlation analysis allowed to prove the effect of AWC on vegetative growth and, mostly, on yield.

The analysis of the relative weight of the studied factors on yield components showed that vine sanitary status was the parameter most affected by soil. In the current study, soils with favourable conditions for higher vegetative development and yield were associated with a greater incidence of diseases. Nevertheless, this response is not linear. It is important to bear in mind that, when analyzing disease incidence due to soil type, a given site is considered. Soils classified as Silty clay loam, corresponding to plots 1 and 2, located in the western part of the region, showed a lower proportion of yield losses caused by rot incidence (data not shown), than those located in the other studied areas independently of the year (this fact is known by vine growers in the area). Even though bunch rot is usually associated to *Botrytis cinerea*, it is possible that in the Colonia del Sacramento region, other low-damaging species are present. In this sense, several authors worked on the morphological characterization and on the molecular identification of *Botrytis sp*.; they found three different species that affect to a higher or lesser extent to the different grapevine organs [55] [56] [57].

Sanitary status was also affected by the "Year" factor and the interactions "Year*Source-sink" and "Source-sink*Soil". Under the conditions of the current study, the greater categories of source-sink ratio (potentially regulated by the grower) could be associated to a lower cluster volume and to a canopy microclimate with less risk of disease incidence. However, this ratio itself is not enough for reducing yield losses in years characterized by high rainfall amounts, high DI values and low solar irradiation during maturation (RS Feb). Under these conditions, high bunch disease incidence is the main cause of yield reduction.

4.3 Effect of the studied factors on berry composition

Berry composition variables were affected to different extents by the three studied factors: "Year", "Soil" and "Source-sink".

The seasonal variation in berry primary components depended less on the source-sink ratio than on vine water status [58], which is mainly associated with the "Year" factor and its interaction with "Soil", but also with "Source-sink". In the conditions of the current study, the best global quality was obtained in 2013.

The influence of the "source-sink" factor on the synthesis of primary components in the berries showed a relative linearity, when studied separately from other factors. The synthesis of SS increases with higher "source-sink" values, while AT decreases. However, when source-sink ratio is ">0.80", the trend was inverted and the AT concentration reached its maximum. This fact could be explained by the combined effect of shading inside the canopy and less exposure of clusters to solar radiation that would promote a reduction in the respiration rate of malic acid and the dilution of organic acids due to a lower dehydration of the berries [59].

The main factor determining pH values was the interaction "Year*Soil". The year acted through its influence on organic acid synthesis (malic and tartaric acids) at pre-veraison and on their degradation rate during maturation. This process is highly dependent on solar radiation, temperature and vine water status. Soil had and indirect influence through modulating water availability, thus conditioning the energy balance and vine response [15]. Water stress limits the concentration of cations in the berries, particularly K+, affecting AT and pH [58]

Regarding the synthesis of secondary components, such as anthocyanins and tannins, the separate ANOVA for the "Soil" factor allowed to observe the different responses according to TCra. Clay Loam soils, with lower water storage ability than Silty clay and Silty clay loam soils, generated moderate water stress conditions during maturation, favoring phenolic compounds synthesis. In addition, water availability differences for a given soil class, caused by the particular physical conditions and organic matter of each specific site, were evident on the level of vine water stress. For instance, plots 6 and 7, with water availabilities of 67 and 57 mm, respectively, during 2011, caused differences of 15% in the measures of pre-dawn leaf water potential over the entire growing season (data not shown), even though both plots corresponded to the same soil textural class (Clay Loam). On the other hand, the effect of soil water availability was greater than that of cultivar when accounting for the determination of yield potential in a vineyard [60], influencing in hormonal signaling from roots to shoots and on stomata control.

"Source-sink" factor was determinant for phenolic concentrations, especially those of anthocyanins, but it did not show a linear relationship with the synthesis of these compounds, which increased till the ">0.60-0.80" class and then decreased. The reduction in concentration for "Source-sink" ">0.80" could be explained by an excessive increase on leaf surface in relation to fruit load, determining a low percentage of clusters exposed to solar irradiation, limiting anthocyanins synthesis and other secondary metabolites [31]. Apart from the environment, the relative availability of carbon established by the leaf/fruit ratio affects the synthesis of both primary and secondary metabolites. In carbon-limited situations, the grape can manage the metabolic pathway of carbon and, thus, sugar accumulation remains while secondary metabolites synthesis is reduced [35]. Our results showed that, taking leaf/fruit ratio of "0.60-0.80" as a reference for the maximum accumulation of SS and ApH1, leaf/fruit ratio of "0.40-0.60" reduced SS synthesis in 0.8% and ApH1 in 20.5%. When carbon availability is lower, as in the case of leaf/fruit ratio "<0.40", SS concentration was reduced by 10%, whereas ApH1 was reduced in 47%. These results are in accordance with the report by other authors [38], who indicated that reducing canopy size would decrease the SS/AT ratio and retards maturation.

Leaf/fruit ratio values that promoted a better berry composition were associated to lower yields and similar SFEp. This result agrees with [61], who proved that berry composition was correlated to yield and berry size more strongly [30] than to canopy size.

When leaf/fruit ratio depends on viticultural practices, it is necessary to understand how this value is reached. According to [33], different pruning intensities may lead to similar leaf/fruit ratios but different preferences for the resultant wines. Similarly, leaf removal practices that lead to a given leaf/fruit ratio might cause different effects on the vines depending on the time of application and the position of the removed leaves. For instance, post-veraison removing the leaves located over the clusters might cause retarding of the maturation process [34]. When leaf removal is performed at pre-flowering, it can promote yield and cluster compactness reductions, as well as accelerating the maturation process [62]; in some cases, it can lead to an excessive decrease of berry acidity if clusters were over-exposed to solar radiation [36].

According to these considerations, canopy and leaf/fruit ratio management should be adapted to the particular environment and cultivar conditions, in order to solve site-specific productive problems. Under the current study conditions, the best SFEp/Y ratio was >0.60-0.80 m2*kg-1, with a SFEp of 1.63m2*vine-1. This balanced ratio guaranteed berry maturation and also other processes such as assimilable nitrogen accumulation and recovery of N in the reserves [39].

V. CONCLUSION

The combined analysis of "year", "soil", "leaf/fruit ratio" and their partial interactions on vine performance proved to be useful for understanding viticulture terroir functioning.

In the studied terroirs, vegetative development variables were dependent on climate and soil, but also on other factors and interactions not included in the current study, leading to the need for research including new explaining factors and their interactions.

Yield per vine was explained mainly by the "source-sink" ratio, the "year" effect and their interaction; both were linked to rainfall amount during maturation. Crop load carried by vines was determinant of this "source-sink" ratio, surpassing the leaf surface influence. In this sense, higher water availability would displace vine balance to fruit (sink).

Berry primary components synthesis depended on year and the interactions of year with soil and source-sink ratio. Concentrations of secondary metabolites in the berry were dependent on "source-sink" ratio and weather. Different features linked to "source-sink" ratio, such as vine balance, amount of available carbon during maturation and canopy microclimate,

with weather conditions that influence grapevine water status, are key processes in the synthesis of phenolic substances.

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Land use and water quality in two sub-basins

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***Abstract*—** *The study evaluated the effects of land use and occupation in water quality in two sub-basins located in the State of Paraná, Brazil. The first sub-basin has 69.8% of native vegetation (natural) and the other has 54.1% of the land cultivated (anthropic). Samples were collected from April to December 2015, analyzing the following parameters: dissolved oxygen, temperature, electrical conductivity, pH, total dissolved solids, turbidity, color, biochemical oxygen demand, total nitrogen, total phosphorus and fecal coliforms. The natural sub-basin presented, significantly, better water quality. Total phosphorus, biochemical oxygen demand and fecal coliforms exceeded the legal limits on the anthropic sub-basin. At this sub-basin correlation was found between cumulative rainfall of five days with turbidity and fecal coliforms, two days cumulative rainfall and total nitrogen, as well as between air and water temperature, affecting the dissolved oxygen, pH, electrical conductivity and fecal coliforms. In the natural sub-basin correlation was found between cumulative rainfall of two days and turbidity, total dissolved solids and electrical conductivity.*

Keywords — agriculture, native vegetation, watershed.

I. INTRODUCTION

Water is a fundamental substance and its peculiarities are important indicators of environmental quality because, during its course, it acquires characteristics derived from the environment.

The characteristics of a watershed have an important influence on water quality, such as landscape and spatial configuration (Bateni *et al*, 2013) as well as land use (Kamjunke *et al*, 2013; Wang *et al*, 2014; Meneses *et al*, 2015; Valle Junior *et al*, 2015; Durło *et al*, 2016). The watershed is the territorial unit chosen for the study and management of water resources, where the systemic view is fundamental for understanding the relative phenomena.

The removal of natural vegetation to give place to human activities affects the quantity and quality of water, since the vegetation influences the hydrological cycle, the water availability and the biogeochemical cycles. Importance has been directed to riparian vegetation that acts as a "filter", retaining substances and sediments that can be carried to water bodies (Sweeney and Newbold, 2014).

Despite the economic benefits provided by changes in land use, there is, simultaneously, a decrease in the ability of the environment to sustain human activities (Foley *et al*, 2005). In this context, public supply sources must receive special attention for their social and economic importance.

The objective of this study was to analyze the water quality in two sub-basins, with different land uses, which are part of the São João watershed - PR, used as the supply source for the city of Carambeí – PR, in southern Brazil.

II. MATERIAL AND METHOD

The São João watershed has 145 km^2 and the length of the main course is 39 km, located in the transition of Paraná first and second plateau (Fig. 1). The river is classified as class II (Paraná, 1991) and is tributary of the right bank of the Pitangui river, which belongs to the Tibagi watershed. According to the Köppen classification, the region belongs to the Cfb climate type.

The region is located in the Atlantic Rain Forest Biome in the Mixed Ombrophilous Forest (MOF), comprising the sub-formations of Montane MOF and Alluvial MOF in the general matrix of subtropical grasslands, and occasionally, cerrado (savannah type) fragments.

Twenty-one sub-basis were studied and they were delimited based on topographic map, sheet MI 2824-4 / IBGE (2001). The land use map was elaborated based on RapidEye satellite image visual interpretation, classifying: native forest (MOF), reforestation (exotic), dry and wet grasslands, agriculture, bracatinga forest (*Mimosa scabrela*), buildings for poultry and swine husbandry and other human use. To represent the native vegetation, forest, grasslands and bracatinga units were defined and mapped. The GIS component were performed with QGis® 2.6.1 software (2014).

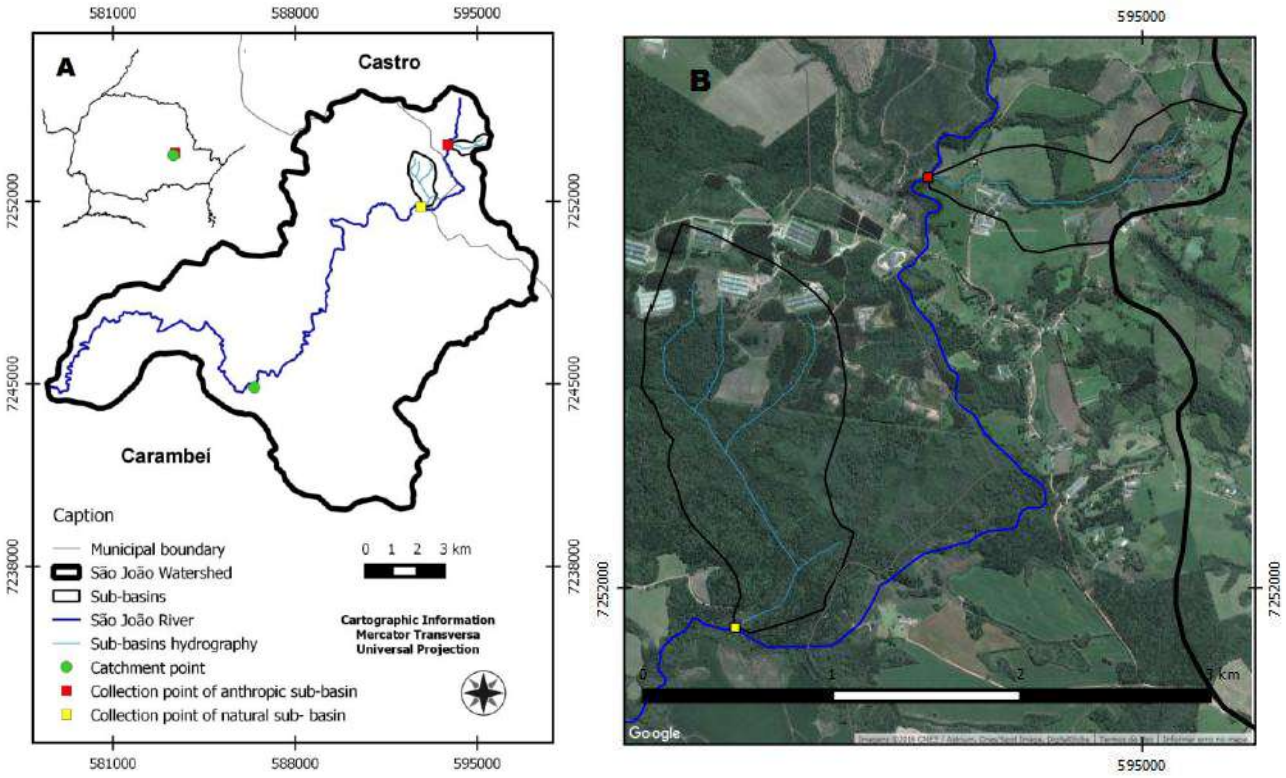

FIGURE 1. LOCATION OF THE SÃO JOÃO WATERSHED (A) AND SUB-BASINS STUDIED (B)

The collection points are located at the UTM coordinates: 592.860,28 m E and 7.251.793,18 m S for the natural sub-basin, and 593.909,4 m E and 7.254.172,43 m S for the anthropic sub-basin (22J, SAD 69). The water samples were collected monthly, from April to December 2015. With YSI 556 MPS® multiparameter probe it was *in situ*, analyzed, dissolved oxygen (DO), temperature, electrical conductivity (EC), pH and total dissolved solids (TDS). The parameters turbidity, color, biochemical oxygen demand (BOD), total nitrogen (TN), total phosphorus (TP) and fecal coliforms (FC) were analyzed at the Water Resources Laboratory (LRH) of the State University of Ponta Grossa (UEPG). Hach® turbidimeter was used for turbidity evaluation and DM-Color Digimed® colorimeter for color analysis. For the bacteriological examination Colilert® substrate and Quanti Tray/2000® Idexx card were used. For BOD, VelpScientifica® equipment was used. For the TN and TP DRB-200 reactor and DR-5000 Hach®, spectrophotometer were used for digestion and reading the analysis.

The meteorological data were obtained from the Maracanã Agrometeorological Station in Castro - Brazil (UTM coordinates 609554,0844 x and 7246980,6670 y, WGS84 - 22J) (ABC Foundation, Agricultural Research and Development).

In the evaluation, descriptive analysis, analysis of variance and Pearson correlation were performed. The variables were tested for homogeneity of variance and normality. When these values were not observed, the values were transformed: Ln for TDS and EC, square root for FC and 1/x for TP, color and turbidity. For such software Sisvar 5.6 was used (Ferreira, 2011). The design was completely randomized blocks. The two sub-basins were considered as treatments and the date of collection as blocks (nine dates). The replicates ranged from three to twenty per variable.

The results were compared against the values established by the CONAMA Resolution nº 357 for class II freshwater rivers (Brasil, 2005).

The values of precipitation, mean, maximum and minimum temperature were used for correlation analysis with water quality parameters. Cumulative precipitation values of two and five days prior to sampling were used, and for the temperature values of the mean of the collection day and the mean of two previous days, for the mean, maximum and minimum temperatures.

III. RESULTS AND DISCUSSION

From the sub-basins studied, one was selected to represent natural land use, with a 69.8% area covered by native vegetation (forest 51.5% + bracatinga 18.3%) and another representing anthropic use, with 54.1% of area covered by agriculture (Fig.1).

Both sub-basins are shaped by second-order rivers and the hydrographic network pattern is dendritic (Strahler, 1952). The main course length of the natural sub-basin is 2.3 km and the anthropic is 1.6 km. The soil types that occur in the São João watershed are Histosol, Cambisol and Nitosol.

The hypsometric and topographic gradient maps were elaborated from ASTER GDEM/NASA (2011) image, demonstrating that the natural sub-basin presents altitudes ranging from 1.018 to 1.162 m and the anthropic sub-basin between 1.066 m and 1.163 M. The relief of the natural sub-basin ranges from undulating (3-8%) to rolling (8-20%) and in the northern part is steep (20-45%) to very steep (> 45%). The anthropic sub-basin presents relief ranging from flat (0-3%) to rolling (8-20%).

Land use and cover are presented in Table 1. At the head of the natural sub-basin there are buildings for livestock production located very close to the springs, within Permanent Preservation Areas (PPA). In the midst of native vegetation there are forest plantations based on exotic species (*Eucalyptus spp and Pinus spp*) and native (*Mimosa scabrela*), and this river is intersected by a vicinal road. The sample collection point was located few meters before the mouth into São João river. In this place the water body presents 15 cm of depth, 2 m of width and a sandy bed.

TABLE 1
USE AND RELATIVE LAND USE IN THE NATURAL AND ANTHROPOGENIC SUB-BASINS

Classes of land use and occupation	Sub-basins			
	Natural		Anthropic	
	%	Area (ha)	%	Area (ha)
Agriculture	0.0	0.0	54.1	37.3
Bracantiga (*Mimosa spp*) forest	18.3	29.6	0.0	0.0
Grassland	0.0	0.0	1.4	0.9
Livestock buildings	5.3	8.6	6.2	4.3
Native forest	51.5	83.6	26.5	18.3
Reforestation	29.4	40.5	0.0	0.0
Other farm buildings and houses	0.0	0.0	11.8	8.2
Total	100	162.3	100	69.0

At the head of the anthropic sub-basin there are buildings for human use near the springs and inside the PPA. The riparian vegetation accompanies most of the hydrography, except in the lower part where it was clear-cut. On the left bank of the main river there are livestock buildings near the water body, also inside the PPA. A vicinal road cuts the watershed lower portion. At the sample collection point the river bed is rocky, the water depth is 15 cm and the width of the channel is 1.5 m.

The minimum DO value for the anthropic sub-basin was 4.4 mg L^{-1} and for the natural sub-basin 6.6 mg L^{-1} (Table 2). The anthropic sub-basin presented lower values, most of these measurements presented high DO concentrations due to a small waterfall located some meters before the collection point and to the rocky substratum, resulting in turbulence and replenishing water with oxygen (Von Sperling, 1996).

In the natural sub-basin there was variation in temperature from 10.5 °C to 17.4 °C and in the anthropic sub-basin from 14.2 °C to 20.1 °C (Table 2). These values are low due to the climate of the region (Cfb) and the altitude (± 1000 m).

The anthropic sub-basin presented greater variability for most of the parameters (Table 2), which is directly related to the predominant type of land use (Table 1). The BOD and FC showed great variation in the two sub-basins (Table 2).

TABLE 2

MAXIMUM, MINIMUM, MEDIAN AND COEFFICIENT OF VARIATION OF THE DEPENDENT VARIABLES FOR SUB-BASINS WITH NATURAL AND ANTHROPIC USE

Parameters*	Natural sub-basin				Anthropic sub-basin			
	Maximum	Minimum	Median	Coefficient of variation (%)	Maximum	Minimum	Median	Coefficient of variation (%)
DO (mg L^{-1})	9.1	6.6	7.2	11.92	7.9	4.4	6.3	16.14
Temperature (ºC)	17.4	10.5	15.4	14.97	20.1	14.2	18.8	12.33
pH	8.0	6.4	7.3	9.68	7.4	6.5	7.0	5.95
Color (Pt-Co)	3.4	1.4	1.9	31.85	210.0	5.5	38.1	118.07
Turbidity (NTU)	4.5	1.9	2.7	32.82	81.1	3.4	9.6	107.84
TDS (mg L^{-1})	26.9	20	25.6	11.74	77.5	40.1	58.1	24.02
EC (µS cm^{-1})	33.9	25.6	31.1	11.45	108.0	52.1	76.0	25.4
TP (mg L^{-1})	0.6	0.3	0.4	28.27	2.5	0.6	1.9	38.96
TN (mg L^{-1})	2.7	0.6	1.3	90.23	5.5	0.4	1.6	92.29
BOD (mg L^{-1})	24.8	0.0	5.2	105.79	29.3	5.2	16.5	80.6
FC (MPN)	866.4	54.6	193.5	96.04	2419.6	235.9	1553.1	60.16

Dissolved Oxygen (DO), Total Dissolved Solids (TDS), Electric Conductivity (EC), Total Phosphorus (TP), Total Nitrogen (TN), Biochemical Oxygen Demand (BOD), Fecal Coliforms (FC)

According to analysis of variance all parameters presented significant differences between sub-basins (Table 3). In both sub-basins, the DO was above the minimum established by the Brazilian legislation. The natural sub-basin presented higher average than that of the anthropic sub-basin, with values of 7.5 mg L^{-1} and 6.2 mg L^{-1}, respectively. The relationship between the amount of native vegetation and DO concentration tends to be positive (Sweeney and Newbold, 2014).

TABLE 3

ANALYSIS OF VARIANCE AND COEFFICIENT OF VARIATION OF THE VARIABLES FOR SUB-BASINS WITH NATURAL AND ANTHROPIC USE, AND WATER QUALITY STANDARDS ESTABLISHED BY CONAMA RESOLUTION Nº 357 (BRASIL, 2005)

Parameters*	Natural sub-basin mean	Anthropic sub-basin mean	Coefficient of variation (%)	p-value	CONAMA (Brasil, 2005)
DO (mg L^{-1})	7.5	6.2	5.7	<0,00001	> 5
Temperature (ºC)	15.0	17.8	3.2	<0,00001	-
pH	7.3	6.9	6.0	<0,00001	6,0 - 9,0
Color (Pt-Co)	2.1	68.1	19.3	<0,00001	-
Turbidity (NTU)	3.0	26.5	23.7	<0,00001	< 100
TDS (mg L^{-1})	24.1	56.6	5.1	<0,00001	< 500
EC (µS cm^{-1})	29.9	76.1	4.4	<0,00001	-
TP (mg L^{-1})	0.4	1.7	28.2	<0,00001	< 0,1
TN (mg L^{-1})	1.1	2.1	38.9	<0,0011	-
BOD (mg L^{-1})	8.0	18.2	55.4	<0,0001	< 5
FC (MPN)	308.6	1451.9	39.4	<0,00001	< 1.000

Dissolved Oxygen (DO), Total Dissolved Solids (TDS), Electric Conductivity (EC), Total Phosphorus (TP), Total Nitrogen (TN), Biochemical Oxygen Demand (BOD), Fecal Coliforms (FC)

The anthropic sub-basin has 27.9% of native vegetation cover and in many places does not present riparian vegetation. In water bodies where the riparian forest is non-existent the water temperature tends to be higher (mean water temperature of 17.8 °C in the anthropic sub-basin and 15.0 °C in the natural sub-basin), showing an inverse relation with DO and decreasing its concentration in water (Table 3).

The pH in both sub-basins was close to neutrality in accordance with the legislation (Table 3). The color and turbidity presented higher averages for the anthropic sub-basin, 68.1 Pt-Co and 26.5 NTU respectively, in relation to the natural sub-basin, 2.1 Pt-Co and 3.0 NTU respectively, where the CONAMA Resolution nº 357 establishes a maximum value of 100 NTU for turbidity (Table 3). The results are directly related to the amount of vegetation in the sub-basins, where less vegetation allows the carrying of a greater amount of sediments (Sweeney and Newbold, 2014).

The mean for TDS in the anthropic sub-basin (56.6 mg L^{-1}) was higher than the natural sub-basin (24.1 mg L^{-1}), lower than that established by legislation (Table 3). The EC mean was higher for the anthropic sub-basin. This difference must be related to the vegetation cover, since the sediment transport containing soluble ions to the water increases the value of these parameters (Lima *et al*, 2015).

The TP presented averages of 0.4 mg L^{-1} and 1.7 mg L^{-1} for the anthropic and natural sub-basins, respectively, values higher than those define by the legislation (Table 3). Mean TN of 1.1 mg L^{-1} for the natural sub-basin and 2.1 mg L^{-1} for the anthropic sub-basin, are considered high. Even with greater presence of vegetation in the natural sub-basin, there are animal confinements that generate a large amount of manure and the relief at the headwaters ranges from steep to very steep. In the anthropic sub-basin these values were expected. It is largely occupied by intensive farming and high use of chemical fertilizers, pesticides and animal manure.

The BOD means for the natural and anthropic sub-basins presented values of 8.0 mg L^{-1} and 18.2 mg L^{-1}, respectively, higher than those stipulated by the legislation (Table 3). In the stabilization of organic matter the DO is consumed in the microorganisms metabolic processes, reducing its availability (Von Sperling, 1996). The high BOD of the anthropic sub-basin indicates the presence of a greater amount of organic matter, compromising the DO in this sub-basin.

The FC had mean of 308.6 MPN in the natural sub-basin and 1.451.9 MPN in the anthropic sub-basin, a parameter that should not exceed 1.000. The highest mean for the anthropic sub-basin is due to the lack of regular riparian vegetation and in the natural sub-basin may be related to the presence of livestock farming.

The turbidity presented a negative correlation with the accumulated precipitation of five days in the two sub-basins (Table 4). The water transparency decreased after rainfall, with higher turbidity in the early stages of precipitation. In the natural sub-basin the correlation is associated with a higher percentage of native vegetation.

TABLE 4
PEARSON CORRELATION ANALYSIS BETWEEN DEPENDENT VARIABLES AND ACCUMULATED PRECIPITATION FOR TWO AND FIVE DAYS PRIOR TO COLLECTION

Parameters*	Two days precipitation		Five days precipitation	
	Natural	Anthropic	Natural	Anthropic
DO (mg L^{-1})	-0.35	-0.35	-0.44	-0.27
Temperat. (ºC)	0.2	0.38	0.33	0.45
pH	-0.26	-0.32	-0.28	-0.29
Color (Pt-Co)	-0.42	-0.33	-0.49	-0.52
Turbidity (NTU)	-0.41	-0.37	-0.7	-0.71
TDS (mg L^{-1})	-0.57	0.47	-0.88	0.49
EC (µS cm^{-1})	-0.48	0.51	-0.74	0.52
TP (mg L^{-1})	0.28	-0.33	0.33	-0.6
TN (mg L^{-1})	0.25	0.66	-0.02	0.63
BOD (mg L^{-1})	-0.31	-0.15	-0.41	-0.23
FC (MPN)	-0.15	0.38	-0.34	0.67

Dissolved Oxygen (DO), Total Dissolved Solids (TDS), Electric Conductivity (EC), Total Phosphorus (TP), Total Nitrogen (TN), Biochemical Oxygen Demand (BOD), Fecal Coliforms (FC)

Although the positive correlation between TN and accumulated precipitation of two days in the anthropic sub-basin is not strong (Table 4), it was observed that after rain events there was foam on the water surface, strong odor of manure and sludge in the river bed. It can be concluded that the runoff from the agricultural area and the lack of riparian vegetation contributes to the transportation of nutrients to the water.

The TDS and EC in the natural sub-basin showed a negative correlation with the accumulated rainfall of five days (Table 4), reflecting the importance of vegetation in the river bank stability and sediment retention (Sweeney and Newbold, 2014).

In the anthropic sub-basin there was a positive correlation between accumulated rainfall of five days and FC (Table 4), being related to the farms, the use of manure fertilization and lack of riparian vegetation. Positive correlations between these indicators and precipitation of one and three days prior to collection have already been reported (Liang *et al*, 2013).

Observing the correlations between air temperature and DO (Table 5), the inverse relationship between these variables is evident, showing strong and negative correlation for the anthropic sub-basin. The lack of riparian vegetation exposes the

river to higher solar radiation, increasing the water temperature and decreasing the solubility of the DO (Tables 2 and 3). In the natural sub-basin, correlations (Table 5) reinforce that the presence of vegetation promotes water shading, decreasing temperature and increasing DO concentration (Tables 2 and 3).

The correlations between water and atmospheric temperature (Table 5) shows seasonal influence (Silva *et al*, 2015). The higher temperatures in the anthropic sub-basin (Tables 2 and 3) tend to catalyze chemical and biological reactions, increasing the activity of the organisms that release CO_2, forming carbonic acid and hydrogen ions that lower the water pH (Esteves, 1988), justifying the observed correlations (Table 5).

TABLE 5

PEARSON CORRELATION BETWEEN THE DEPENDENT VARIABLES AND THE MAXIMUM, MINIMUM AND MEAN TEMPERATURE ON THE DAY OF COLLECTION AND TWO DAYS PRIOR TO COLLECTION

Parameters*	Maximum temperature				Minimum temperature				Mean temperature			
	of the day		Two days		Of the day		Two days		Of the day		Two days	
	Ant.	Nat.	Ant.	Nat.	Ant.	Nat.	Ant.	Nat.	Ant.	Nat.	Ant.	Nat.
DO (mg L^{-1})	0.67	0.81	0.54	0.8	0.84	0.68	0.75	0.52	0.83	0.92	0.8	0.9
Temperature (ºC)	00.63	00.69	00.5	00.54	000.89	00.88	00.7	00.76	00.8	00.85	00.76	00.78
pH	-0.36	-0.66	-0.36	-0.67	-0.45	-0.48	-0.48	-0.45	-0.52	-0.72	-0.52	-0.78
Color (Pt-Co)	-0.27	-0.56	-0.18	-0.39	-0.69	-0.65	-0.87	-0.72	-0.55	-0.63	-0.5	-0.64
Turbidity (NTU)	00.29	-0.39	00.51	-0.17	-0.43	-0.41	-0.67	-0.53	00.01	-0.37	00.2	-0.37
TDS (mg L^{-1})	00.03	00.3	00.36	00.17	-0.18	00.32	-0.55	00.59	00.04	00.39	00.11	00.34
EC (µS cm^{-1})	00.33	00.41	00.6	00.27	00.2	00.53	-0.25	00.74	00.4	00.56	00.46	00.48
TP (mg L^{-1})	-0.23	-0.46	-0.38	-0.31	-0.48	-0.43	-0.07	-0.51	-0.46	-0.46	-0.44	-0.48
TN (mg L^{-1})	00.45	00.38	00.53	00.28	00.27	00.22	00.14	00.45	00.52	00.39	00.47	00.34
BOD (mg L^{-1})	-0.33	-0.21	-0.09	-0.06	-0.08	-0.05	-0.47	-0.33	-0.16	-0.11	-0.21	-0.16
FC (MPN)	-0.5	00.35	-0.38	00.12	00.18	00.53	00.03	00.74	-0.17	00.42	-0.31	00.39

Anthropic (Ant.), Natural (Nat.), Dissolved Oxygen (DO), Total Dissolved Solids (TDS), Electric Conductivity (EC), Total Phosphorus (TP), Total Nitrogen (TN), Biochemical Oxygen Demand (BOD), Fecal Coliforms (FC)

Turbidity interferes with the penetration of light into water (Von Sperling, 1996) as well as color, demonstrating consistency in the correlations found (Table 5).

The EC of water is dependent on its temperature, increasing 2% at each ºC (Esteves, 1988). In this sense, the positive correlation in the anthropic sub-basin is justified (Table 5). The higher water temperatures found for the anthropic sub-basin (Tables 2 and 3) tend to promote bacteria proliferation, with higher concentrations of FC (Table 2), explaining the correlation found (Table 5) (Liang *et al*, 2013).

Concerning the water quality, the land use has shown to be relevant, observed in the difference of the parameters between the sub-basins, which agrees with Mori *et al* (2015), and the greater compliance with legislation for the natural sub-basin.

IV. CONCLUSION

The natural sub-basin presented better water quality than the anthropic sub-basin. Upstream farms and the lack of Permanent Preservation Area in springs contribute to fecal contamination and high values of total phosphorus, total nitrogen and biochemical oxygen demand in the natural sub-basin. The predominant use of agriculture, the absence of riparian vegetation and the use of synthetic and natural fertilizers degrade water quality in the anthropic sub-basin. Precipitation and atmospheric temperature tended to influence the analyzed parameters.

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Mycotoxin production by entomopathogenic fungus *Conidiobolus coronatus*

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Abstract— *Qualitative and quantitative analysis of selected mycotoxins has been performed in extracts of Conidiobolus coronatus pathogenic fungus cultivated under optimal and stress conditions. Furthermore, the analyses of these compounds in post-incubation filtrates were done. For identification purposes the analytical method allows identification and quantitation of selected mycotoxins including beauvericin , fumonisin B1, enniatin A and B and destruxin A based on high performance liquid chromatography coupled with tandem mass spectrometry was developed. Only beauvericin was detected in very low amounts in C. coronatus mycelium extract cultivated under optimal condition. In the extract of C. coronatus mycelium grown on LB 12.3 ± 0.1 µg/g of beauvericin was determined, while in the extract of C. coronatus mycelium grown on MM medium beauvericin content was lower and amounted 4.6 ± 0.1 µg/g. Also the presence of beauvericin was confirmed in postincubaction filtrate extract (MM). The content of this compound was 2.2 ± 0.1 µg/g. In other extracts beauvericin was not detected. In addition, in the tested extracts other compounds were not detected.*

Keywords— Mycotoxin Production, Conidiobolus, quantitative analysis.

I. INTRODUCTION

The use of chemical insecticides can cause a variety negative effect on the environment. They exhibit high toxicity, but also a low biodegradability and thus accumulate in the environment. As a result of drift by the wind or flushing them torrential rains, these compounds get into reservoirs and waterways. Therefore, it is necessary to search for alternative methods of pest control, which will not have a negative impact on the environment, including humans and animals. The solution to this problem may be use of entomopathogenic fungi. Entomopathogenic fungi are ubiquitous in the environment and plays an important role due to its ability to spontaneous infection reduce the amount of many plant pests [1]. Currently, there are about 3,000 known species of fungi that can cause diseases of living arthropods. Only 30 of them are used as biological agents to limit the number of plant pests [2]. The most of these products is based on the fungi species such as *Metarhizium anisopliae, Beauveria bassiana, Beauveria brongniartii, Paecilomyces fumosoroseus, Lecanicillium longisporum and Lecanicillium muscarium* [3]. These entomopathogenic fungi can be use as bioinsecticides, due to their ability to mass propagation on artificial media [1]. Entomopathogenic fungi produce a number of secondary metabolites which have a different effect on insects [4-7]. *Beauveria bassiana* produces bassianolides - depsipeptide which proved to be important factors in the insect infection [4]. Destruxins produced by the *Metarhizium anisopliae* causes paralysis and death of the infected host [5]. There are also metabolites do not cause the death of organisms, but does not exclude the importance of these compounds in the infection process, e.g. beauverolides not show the insecticidal activity and the immune response [7]. Fumonisins are produced by fungi of the genus *Fusarium*, for example: *F. moniliforme* and *F. proliferatum* occurring primarily in corn grain and its processing products intended for food and feed. The most important analogues found in naturally contaminated corn are fumonisin B1, fumonisin B2 and fumonisin B3. Several strains of fungi *Fusarium* spp produce secondary metabolites belonging to the enniatin group. They are six-membered cyclic depsipeptides having ionophoric, phytotoxic, antiparasitic and antibiotic properties. Beauvericin (BEA) is a toxic metabolite produced by entomopathogenic fungi. This mycotoxin was isolated from an entomopathogenic fungus *Beauveria bassiana* and several other species belonging to the family *Cordycipitaceae* in the *Hypocreales* (*Ascomycota*) [8-11]. *Fusarium* species infecting maize, rice, and wheat are also known as beauvericin producers [9]. There is only one report of BEA occurrence and co-occurrence with fumonisin B1, fumonisin B2 and ochratoxin A. BEA is cyclohexadepsipeptide fungal metabolite with a wide range of biological activities, such as insecticides, anthermintic, antibacterial, antifungal, antiplasmodial, antimycobacterial and anticancer activities. It is the most potent specific inhibitor of cholesterol acyltransferase and possesses ionophoric properties. BEA increases ion permeability in biological membranes by forming a complex with some cations (Ca^{2+}, Na^{+}, K^{+}), which may affect the ionic homeostasis [8]. The insecticidal activity of BEA was first discovered by Hamill et al.[12].

BEA was confirmed as the active compound against *Artimia salina*, which was considered as a model organism to insecticidal activity study. Subsequently, the insecticidal effect of BEA on a microgram level was investigated on *Calliphora erythrocephala, Aedes aegypti, Lygusspp., Spodoptera frugiperda* and *Schizaphis graminum* [13-16]. BEA exhibits toxicity to bacteria: *Bacillus subtiilis, Escherichia coli, Mycobacterium phlei, Sarcinea lutea, Staphylcoccus aureus* and *Streptococcus faecalis* [73]. Furthermore, it is an effective integrase inhibitor of HIV-1 [71]. A very important feature of bEa is the antitumor effect [71]. It interferes with the motility of tumor cells which reduces the speed of many processes in the development of the disease, including the formation of new blood vessels in the tumor cells, and metastasis [53, 77]. It also inhibits the acetyltransferase and cholesterol results in programmed cell death, similar to apoptosis, as well as cytolysis [72, 73, 74].

Conidiobolus coronatus is an opportunistic pathogen with a fairly wide range of infected hosts. For the first time this species was described in 1897 by Costantin'a in France. In contrast, *C. coronatus was* isolated in 1961 by Chester Emmons and Charles Bridges [13, 14]. It occurs commonly in soil and decaying plant material [14]. This entomopathogenic fungus causes the disease process in many arthropods, eg. greater wax moth *(Galleria mellonella)*, pine lappet moth *(Dendrolimus pini)*, springtails and other [13-16]. After penetrating into the body cavity of the fungus kills the insects within 1-2 days, resulting in tissue damage, which is caused by the depletion of nutrients and the production of mycotoxins, which are considered to be the main factor causing the death of an insect. Because of their insecticidal potential *C. coronatus* can be used as a source of new generation bioinsecticides. However, to date, a mycotoxin produced by *C. coronatus* not have been identified and described.

Therefore, the main goal of this study was to assess selected mycotoxins content in extracts of pathogenic fungus *C. coronatus*. As first, different solvents and solvent mixtures were applied to extraction of target analytes. Then, the analytical method based on high performance liquid chromatography coupled with tandem mass spectrometry was developed for qualitative and quantitative determination of these compounds in obtained extracts. The productions of selected mycotoxins by *C. coronatus* grown in rich and poor media were determined. Moreover, the impact of changes in culture conditions, including temperature and pH on mycotoxins production were also assessed. The results of this study provide useful information to assess the potential use of *C. coronatus* as a source of new generation bioinsecticides.

II. EXPERIMENTAL PART

2.1 Chemicals

Beauvericin, fumonisin, enniatin A and B, destruxin were obtained from Sigma Aldrich. HPLC-grade acetonitrile and methanol, dichloromethane, hexane, ammonium acetate, acetic acid was purchased from POCh. Stock solutions containing all tested mycotoxins were prepared at concentration 50 µg/mL, working solution were prepared by appropriate dilution of stock solution.

2.2 Conidiobolus coronatus

The subject of the studies was the entomopathogenic fungus *C. coronatus* belonging to the class of *Zygomycota* and the order of *Entomophthorales*. Mycelium was obtained from cultures of the insecticidal fungus *C. coronatus* grown in 30 L of liquid minimal medium (MM pH 7.0, consisting of 0.1 % of $(NH_4)_2SO_4$, 0.45 % of KH_2PO_4, 1.05 % of K_2HPO_4, 0.05% of sodium citrate dehydrate, 0.2 % of glucose and 0.025 % of MgSO4 all as mass %) and liquid nutrient-rich medium (LB consisting of 1 % of tryptone, 0.5 % of yeast extract, 0.5 % of NaCl and 0.1 % of 1 M NaOH)) in optimal and stress condition. After 3 weeks, the mycelium was filtered on filter papers, collected and stored at -20^{o}C.

2.3 Optimization of extraction conditions

To 1 g of finely ground sample 50 µl of a mixture solution containing target analytes in MEOH at concentration 5 µg/ml were spread over the surface. The flask was shaken manually to distribute the added standards as evenly as possible. The sample was left open at room temperature for 2 h. Three extraction solvent mixtures such as: methanol-water (70:30, v/v), ACN: MeOH: H_2O (16: 3: 1, v/v); and ethyl acetate were used. After 12 h, the extract was filtered through the filter paper. The extraction procedure was repeated once again. Then 25 ml of hexane was added and the mixture was shaken for 10 min. The hexane layer was discarded, and the remainder layer was concentrated to dryness on a rotary evaporator. The residue was dissolved in a mixture of MeOH: H_2O (1: 1, v / v) at a volume of 15 ml and extracted twice with 7.5 ml of dichloromethane. The layers of dichloromethane were combined, then evaporated to dryness using a rotary rotator. The

residue was then dissolved in 1 ml of ACN and transferred to vials. As described above, the native sample of the *C. coronatus* mycelium was also prepared. All extracts were analyzed by LC-MS/MS.

2.4 Extraction of *C. coronatus* mycelium grown in optimal and stress conditions

A samples of *C. coronatus* mycelium grown in optimal and stress conditions were lyophilized. To the resulting lyophilisate 20 ml of a mixture ACN: MeOH: H_2O (16: 3: 1, v / v) was added and left for 12 h. After that, the extract was filtered and extracted once again using 20 ml of solvent mixture mentioned above (12 h). Then 25 ml of hexane was added and mixture was shaken for 10 min. The hexane layer was discarded, and the remainder layer was concentrated to dryness on a rotating rotor. The residue was dissolved in 15 ml of MeOH: H_2O (1:1, v/v) and extracted twice with 7.5 ml dichloromethane. The layers of dichloromethane were combined, evaporated to dryness. The residue was then dissolved in 1 ml of ACN and analyzed by LC-MS/MS. Table 1 summarizes the culture conditions and the weight of the extracts of *C. coronatus* mycelium nad post-incubation filtrates.

TABLE 1
***C. CORONATUS* MYCELIUM MASSES BEFORE AND AFTER EXTRACTION BY ACN: MeOH: H_2O (16:3:1, v/v/v)**

The culture conditions	Masses of *C. coronatus* mycelium [g]	Masses of extracts [mg]
MM medium, optimal condition	6.91	50.9
LB medium, optimal condition	4.16	25.7
MM medium, pH 5, temperature 30 °C	1.49	12.6
MM medium, pH optimal, temperature 30 °C	1.87	18.9
MM medium, pH 5, temperature 20 °C	1.29	22.9
MM medium, pH optimal, temperature 20 °C	1.09	17.9
LB medium, pH 5, temperature 30 °C	1.51	11.9
LB medium, pH optimal, temperature 30 °C	0.99	10.8
LB medium, pH 5, temperature 20 °C	1.04	15.7
LB medium, pH optimal, temperature 20 °C	2.9	14.1
Post-incubation filtrate (MM)	100 ml	89.7
Post-incubation filtrate (LB)	100 ml	124.7

2.5 Extraction of post-incubation filtrates

Post-incubation filtrates obtained after *C. coronatus* culture on MM and LB medium were lyophilized and weighted. Then 75 ml of a mixture ACN: MeOH: H_2O (16: 3: 1, v / v) was added and samples left for 12 h. At this time extracts were filtered and extraction was repeated with a mixture of solvents for 12 h . After this time extracts were filtered, combined, and shaken with 25 mL of hexane for 10 min. The hexane layers were discarded, whereas the layer of ACN: MeOH: H_2O was concentrated to dryness using a rotary rotator. The resulting residue was dissolved in a mixture of MeOH:H_2O (15 ml, 1:1, v/v) and extracted twice with 7.5 ml dichloromethane. The dichloromethane layers were combined, and then evaporated to dryness using a rotary rotator. Thus prepared extracts were dissolved in 1 ml of ACN and analyzed by LC-MS/MS.

2.6 LC-MS analysis

Chromatographic analysis was performed using liquid chromatography Series 1200 (Agilent Technologies) with Ultra HCT mass spectrometer (Brucker Daltonics) equipped with a column Hypersil Gold a Q C18 (150×4,6 mm, 5 µm). As the mobile phases: ACN (phase A), 1 mM aqueous solution CH_3COONH_4 (phase B): ACN (90:10, v / v) at pH 3.5 were used. The separation was carried out under gradient elution conditions from 10% B to 90% phase B (20 min.) and then 90% of phase B by 5 minutes. The following mass spectrometer parameters were applied: drying gas – nitrogen, drying the gas pressure - 10 psi, drying gas flow rate - 7 l/min., drying gas temperature - 300 ° C, capillary voltage - 4 kV.

III. RESULTS AND DISCUSSION

Quantification of mycotoxin is usually carried out by liquid chromatography coupled with mass spectrometry (LC-MS(/MS)) or gas spectrometry coupled with mass spectrometry often within a multi-analytes approach without any clean-up procedure. For accurate quantification, liquid chromatography equipped with UV-Vis detector can be also applied because of using these techniques allows to avoid of matrix effects. None of the applied methods has been formally evaluated in inter-laboratory validation studies and there are no certified reference materials available for beauvericin determination in natural samples. In our work the LC-MS method for determination of beauvericin in mycelium and post-incubation filtrates was developed. The optimum parameters of the mass spectrometer and a liquid chromatography allowing the isolation and detection of all analytes, ie.: beauvericin, destruxin A, fumonisin, enniatin A and B were selected. Optimization of the mass spectrometer was done in full scan range of *m/z* 50-1000 Da. Analysis of mass spectra obtained in positive ion mode for beauvericin shows the presence of the ion $[M + Na]^+$ *m/z* 806.7. In addition, ions $[M+NH_4]^+$ at *m/z* 801.7 and $[M+K]^+$ at *m/z* of 822.7 were also present. In the mass spectrum recorded in negative ion mode, the signal with the highest intensity corresponds to the ion $[M-H]^-$ at *m/z* 782.6. The mass spectrum recorded in the positive ion mode for destruxin A presence of a signal of *m/z* 600.6 which corresponds to $[M + Na]^+$ ion was detected. Furthermore, in this mass spectrum the ions: $[M + H]^+$ at *m/z* 578.6 and $[M + K]^+$ at *m/z* 617.6 were also denoted. As a result of negative ionization of the analyte a signal for *m/z* corresponding to the ion $[M-H]^-$ was not present. For compounds from the group enniatin (enniatin A and B) both positive and negative ionization mode gave signals of pseudomolecular ions. in the negative ion mode signals at *m/z* 680.6 and 638.6 were present whilst in the positive ionization mode the presence of ions $[M + Na]^+$, $[M + NH4]^+$ and $[M + H]^+$ was observed. Analysis of mass spectra obtained in positive ion mode for fumonisin B1 shows the presence of the $[M + H]^+$ ion at *m/z* 722.7. Moreover, the ions: $[M + Na]^+$ at *m/z* and 744.7 $[M + K]^+$ at *m/z* 760.6 were detected. In the spectrum registered in negative ion mode, a signal corresponding to $[M-H]^-$ at *m/z* 720.6 was observed. In the next stage of the research the conditions of chromatographic separation of analyzed compounds was studied. In the application of available software, complete separation of the chromatographic signals is not necessary however; their separation can significantly improve the sensitivity and selectivity of developed method. Furthermore, the retention time is an additional parameter to permit identification of analytes in real samples. The use of gradient elution from 10% to 90% phase B, then 90% of phase B for 5 minutes allowing obtaining optimum conditions in terms of the retention time, separation of the compounds and symmetry of signals. Pseudo-molecular and fragmentation ions selected for qualitative analysis were summarized in Table 2. Validation parameters of the LC-MS method were summarized in Table 3.

TABLE 2
SELECTED PSEUDO-MOLECULAR AND FRAGMENTATION IONS FOR MYCOTOXINS DETERMINATION

Mycotoxin	Retention time [min.]	Pseudomolecular ion		Fragmentation ions
Fumonisin B1	16.8	$[M+H]^+$	*m/z* 723	352.4 528.6 704.7
Destruxin A	19.8	$[M+H]^+$	*m/z* 579	178.1 437.4 465.5 550.5
Enniatin B	30.8	$[M+NH_4]^+$	*m/z* 658	196.3 640.7
Beauvericin	32.4	$[M+NH_4]^+$	*m/z* 802	244.2 262.3 542.5 784.7
Enniatin A	33.9	$[M+NH_4]^+$	*m/z* 700	210.3 455.4 682.7

TABLE 3
METHOD VALIDATION PARAMETERS OF THE FINAL MYCOTOXINS DETERMINATION BY LC-MS/MS

Mycotoxin	Coefficient of determination R^2	Accuracy [%]	Precision [%]	IQL [µg/ml]	IDL [µg/ml]
Beauvericin	0.9998	103.7 – 109.1	1.47 – 5.32	0.06	0.02
Enniatin A	0.9998	101.26 –113.21	1.83 –2.97	0.06	0.02
Enniatin B	0,9992	97.92 –103.75	1.53 –4.03	0.06	0.02
Fumonisin B1	0,9982	94.33 –101.43	2.38 –5.73	0.06	0.02
Destruxin A	0.9964	92.32 –106.64	0.88 –2.85	0.06	0.02

3.1 Identification and quantitation of selected mycotoxin in extracts of *C. coronatus* mycelium and post-incubation filtrates

Fungi in *Entomophthorales*, including *C. coronatus*, are subject to interest of researchers due to the possibility of their use in biological pest control. This is related to relatively short infection cycle, high index of reproduction and the ability to induce epizootic in ecosystems. Most of them are specialized in infecting a specific group of hosts, so that does not pose a risk to non-target organisms. Mycotoxins produced by entomopathogenic fungi have insecticidal properties, and preparations containing the fungal spores capable of produce them are now increasingly being used in biological pest control. In addition, this kind of insecticides is an alternative to chemical compounds, the use of which in excess is hazardous to the environment and living organisms. This is the first report of the detection of beauvericin in extracts of *C. coronatus*. Several extracts of *C. coronatus* were analysed and the impact of the grown conditions on beauvericin production were assessed. The isolation of mycotoxins from *C. coronatus* mycelium and post-incubation filtrates was done by solvent extraction with different methods. As first ethyl acetate was used due to the fact, that it is one from four most commonly used solvent for the extraction of mycotoxins from biological samples. Extraction by mixture of solvents ACN: MeOH: H_2O (16: 3: 1, v / v) and MeOH: H_2O (7:3, v/v) was also applied to mycotoxin isolation from *C. coronatus*. As was shown in Table 4, the extraction efficiency of target analytes were the highest using ACN: MeOH: H_2O (16: 3: 1, v / v). The recoveries of selected mycotoxin were in the range from 63.5% to 75.5 %. Therefore this mixture was applied as a extraction solvent to isolation mycotoxins produced by *C. coronatus*.

TABLE 4
PERCENTAGE OF RECOVERY FOR SELECTED MYCOTOXINS IN SPIKED MYCELIUM OF *C. CORONATUS* BY USING DIFFERENT EXTRACTION SOLVENTS

Mycotoxin	Recovery [%]		
	Ethyl acetate	ACN: MeOH: H_2O (16:3:1, v/v/v)	MeOH: H_2O (7:3, v/v)
Fumonisin B1	46.4	63.5	73.4
Destruxin A	43.7	67.8	62.3
Enniatin B	55.3	72.4	58.4
Beauvericin	53.8	75.3	67.4
Enniatin A	58.5	69.4	54.8

The results of LC-MS analysis indicated the presence of beauvericin in the extract mycelium of *C. coronatus* and the post-incubation filtrates. In the mass spectrum obtained in the LC-MS analysis of the extract of the *C. coronatus* mycelium, the molecular peak at m/z 802 and a signals at m/z 244.2; 262.3; 541.5; 542.6; 784.6; 811.4 confirmed the trimeric structure of beauvericin and clearly indicates the presence of this compound in analyzed extracts. Results of beauvericin production by *C. coronatus* isolates are summarized in Table 5. In the case of extract of C. coronatus mycelium grown on LB medium was 11.8 ± 0.1 µg/g, while grown on MM medium - 5.2 ± 0.3 µg/g. Also the presence of beauvericin was confirmed in post-incubaction filtrate extract (MM). The content of this compound was 2.2 ± 0.1µg/g. In extracts of *C. coronatus* mycelium

grown under conditions other than the optimal beauvericin content was detected in extracts of *C. coronatus* mycelium grown on both LB and MM medium, at temperature 20 °C and optimal pH condition. In the extract of *C. coronatus* mycelium (MM medium 20, pH optimum) the presence of 1.5 ± 0.1 µg/g beauvericin was confirmed. While in mycelium of *C. coronatus* grown on LB medium (20, optimal pH) beauvericin content was 4.9 ± 0.1 µg/g in the extract. The highest BEA yield was detected in extract of *C. coronatus* mycelium cultures grown in LB medium in optimal condition. Three times lower contents of BEA was determined in the extract of *C. coronatus* mycelium grown on MM medium under optimal pH and temperature conditions. The lower content of BEA mainly attributed to the type of medium which contains only those components that are essential to sustain vital functions. The effect of changes in the pH of the culture medium on BEA content in *C. coronatus* mycelium was also observed. Temperature changes also affect the BEA content in the extracts, it was observed that in the case of temperature reduction the contents of the BEA is lower than in optimal conditions, while with an increase of temperature BEA was not detected. Beauvericin was originally identified in entomopathogenic fungi, such as *Beauveria bassiana* and *Isaria fumosorosea* (formerly *Paecilomyces fumosoroseus*)[9]. As the main beauvericin producers and the species responsible for its accumulation *F. subglutinans* and *F. proliferatum* were identified. Previous studies reported the natural occurrence of beauvericin in maize infected by entomopathogenic fungi [17-21].

TABLE 5
MYCOTOXIN CONCENTRATION FOUND IN HOMOGENATES AND POST-INCUBATION FILTRATES OF *C. CORONATUS* CULTIVATED AT DIFFERENT CONDITIONS

The culture conditions	Fumonisin [µg/g]	Beauvericin [µg/g]	Destruxin [µg/g]	Enniatin A and B [µg/g]
MM medium, optimal condition	nd	5.2 ± 0.3	nd	nd
LB medium, optimal condition	nd	11.8 ± 0.1	nd	nd
MM medium, pH 5, temperature 30 °C	nd	nd	nd	nd
MM medium, pH optimal, temperature 30 °C	nd	nd	nd	nd
MM medium, pH 5, temperature 20 °C	nd	nd	nd	nd
MM medium, pH optimal, temperature 20 °C	nd	1.5 ± 0.1	nd	nd
LB medium, pH 5, temperature 30 °C	nd	nd	nd	nd
LB medium, pH optimal, temperature 30 °C	nd	nd	nd	nd
LB medium, pH 5, temperature 20 °C	nd	nd	nd	nd
LB medium, pH optimal, temperature 20 °C	nd	4.9 ± 0.1	nd	nd
Post-incubation filtrate (MM)	nd	2.2 ± 0.1	nd	nd
Post-incubation filtrate (LB)	nd	nd	nd	nd

The results of qualitative and quantitative analysis of the BEA in five mycelium samples of different strains of *Fusarium subglutinans* and in corn samples infected by this species are described in the literature [16]. *F. subglutinans* strains produced BEA at a level of 140 mg/kg to 700 mg/kg, while in the maize samples revealed the presence of the BEA content from 7.6 to 238.8 µg/kg. BEA production capacity by various *Fusarium* strains were also described by A. Logrieco et al.[9]. Among the 94 strains belonging to 25 different species of the *Fusarium*, the ability to beauvericin production revealed: *F. sambucinum, F. longipes, F. subglutinans, F. anthophilum, F. oxysporum, F. poae, F. avenaceum, F. beomiforme, F. dlamini, F. nygamai*. The highest beauvericin producer was one of the two tested strains of *F. longipes* Wollenw. et Reinking (ITEM-3202) (200 µg/g). Other beauvericin-producing species were *Fusarium acuminatum* Ell. et Ev. var. acuminatum (one of four isolates), *Fusarium acuminatum* var. armeniacum Forbes et al. (one of three isolates), and F. equiseti (Corda) Sacc. (two of three isolates). In the *Sporotrichiella* section, all four tested strains of *F. poae* (Peck) Wollenw. Produced the toxin, ranging from traces to 63 µg/g.

In the literature, there is little information about the effects of external factors on the mycotoxins production by entomopathogenic fungi. According to Hodgson (2000) optimal temperature for the production of mycotoxins by *F. langsethiae and F. sporotrichioides* is lower than a temperature that is optimal for their growth [22]. In 2006 the effects of temperature on the effectiveness of fungal pathogens in reducing the number of different developmental stages of *Frankliniella occidentalis* were carried out. The aim of study was to determine the effect of temperature: 20 °C, 25 °C and 30 °C on the effectiveness of *Beauveria bassiana, Metarhizium anisopliae, Lecanicillium lecanii* and *Paecilomyces lilacinus*. After the experiment, it was found that the temperature has an effect to reduce the number of the western flower thrips by tested entomopathogenic fungi. The obtained results shown that *P. lilacinus* is effective pathogen for western flower thrips reduction in all the temperatures and their effectiveness was in the range of 68% to 76%. *M. anisopliae* was the most

effective at 25 ° C, and its efficiency was 82%. An increase or decrease in temperature caused a decrease in efficiency of this fungus. Similarly was in case of *B. bassiana*. The fungus *L. lecanii* was more effective in reducing of western flower thrips population with increasing temperatures, for example, at 30 °C caused 84% mortality of larval stages, but in relation to the adult insects its effectiveness at 30 °C does not exceed 50%. The highest mortality of western flower thrips, i.e. 92% was noted by the pathogens *B. bassiana* and *M. anisopliae* at 25 °C [23].

The results of LC-MS analysis revealed no ions indicating the presence of other compounds, including enniatin A and B, destruxin A, fumonisin B1. Therefore, it can be concluded that in extracts of mycelium and post-incubation filtrates of *C. coronatus* the above-mentioned compounds have not been present.

IV. CONCLUSIONS

Economic losses caused by harmful insects are a serious economic problem. Reducing of the pests are still made mainly using non-selective chemical insecticides, the accumulation of which in the environment is a serious threat to biodiversity and human health. Accompanying this process the growing resistance of harmful insects to classical insecticides, tends to reduce the use of chemical insecticides and the search for new, selective and less environmentally harmful methods of pest control. In this work the isolation and qualitative and quantitative analysis of mycotoxins in the mycelium and post-incubation filtrates of the *C. coronatus* using chromatographic methods were done. In the extracts C. coronatus mycelium grown in LB and MM medium the presence of BEA was confirmed (12.3 ± 0.1 µg/g and 4.6 ± 0.1 µg/g, respectively). In turn, the content of BEA in the post-incubation filtrate (MM) was 2.2 ± 0.1 µg/g. On the other hand, in the test samples of mycelium and post-incubation filtrates the presence of compounds from the group of enniatin, fumonisins and destruxin were not determined.

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Effects of heavy metals' toxicity on plants and enhancement of plant defense mechanisms of Si-mediation "Review"

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Abstract— *Today's [e.g., "heavy metals (HMs)"] caused by anthropogenic activities have negative impacts on our environment and food productions. HMs can be classified as either essential or nonessential. A trace of essential HMs, such as Cu, Mo, and Zn, can be necessary for plant metabolism, but excess of them can harm the plant growth and development. Nonessential HMs, however, are toxic for plant metabolism and have damaging effects on enzyme activity, photosynthetic properties, cell membrane, permeability and eventually plant growth. Plants with avoidance and tolerance against stress could manage extreme HM stress in soils so that with special mechanisms, such as specific translation and metal accumulation, can elevate abiotic and biotic stress in plants. Moreover, in cells with mechanisms such as [e.g., "Metallothionein (MTs)"] (metal binding proteins) or [e.g., "Phytochelatin (PCs)"] storage and crystallization could reduce the HM stress in the cell wall, plasma membrane, cytosol, tonoplast and vacuoles. Meanwhile, the role of Si-mediation in detoxification of HMs is so bold. Si-mediation with increasing antioxidant, reducing lipid peroxidation, and increasing efficiency of photosynthetic properties elevates the HMs and other biotic and abiotic stresses in plants.*

Keywords— HMs, stress, cells defense mechanism, Silicon.

Abbreviation:*HMs(heavymetals);MTs(Metallothionein);PCs(Phytochelatin);WHO(WorldHealthOrganization);ROS(ReactiveOxygenSpecies);BBYs(enrichedthylakoidsmembranes);PsII(PhotosystemII);SOD(Superoxidedismutase);CAT(catalase);APX(ascorbateperoxidase);DHAR(dehydroascorbatereductase);GR(glutathionereductase);WUE(wateruseefficiency);ELP(Euph orbia characias latex peroxidase);WCE (whole chain electron transport activity); LHC(light harvesting complex).*

I. INTRODUCTION

HMs are known as biotic stress and hazardous chemical that could affect human health by influencing the food chain and aquifers. They are, also, known as one of the reasons to inhibition of plant growth [1]. There are two types of metals in the soil: essential and non-essential. Essential HMs plays an important role in many enzyme activities as cofactor and in other protein structures which plants need them for growing and development [2,3]. However, HMs concentration is an important factor in the growth of plants so that the excess of HMs can lead to a reduction in plant growth. Heavy metals with binding to sulfhydryl group could lead to ions' substitution on protein structure [4]. In the other hand, enhancement of HMs can initiate the oxidative stress by generating ROS form oxidative stress, which in turn may disrupt the balance between pro-oxidant and antioxidant homeostasis. Additionally, observation obtained by oxidative attacking to DNA in cultured cells, and animals indicated that metal has this ability to interact with nuclear proteins and DNA [5]. Plants use a number of defense mechanisms for detoxification of toxic when encounter with abiotic stress caused by high concentration of HMs. This can help to recover and ameliorating in cells. As the first step, plants start with some avoidance and hemostasis mechanisms to prevent the onset of stress in extracellular, including binding them to micronize, cell wall, and extracellular exudates or with control efflux pumping of metals in plasma. This includes membrane and mechanisms of storage and detoxification in the vacuole and protoplasm. Among these mechanisms, the most important ones are transferring and sequestration to the vacuole, chelation mechanisms and reducing the damage heat shock proteins with renovating their [4,6]. It has been found in recent decades that Si could play an important role as one useful element in plant resistance which copes with abiotic and biotic stress and improves the plant growth [7]. In plants, silicon amplifies water-use efficiency [8], enhances cell wall rigidity [9], increases antioxidant enzyme activities, and reduces lipid peroxidation [10]. The aim of authoring this paper is to first recognize the impact of HMs on plants, and then investigate the toxicity of some non-essential HM on plants and study the plant defense mechanisms. Eventually, it is aimed to assess the Silicon as a reduction and amelioration of biotic and abiotic stresses.

II. HEAVY METAL TOXICITY IN PLANTS

HMs in plants could be due to the increase in free radicals and consequent occurrence of oxidative stress that lead to oxidation of membrane protein and lipids, or directly due to disturbance of plant activities by interacting with DNA [11]. Some of the HMs, such as Ni(nickel), Cu(copper), Zn(zinc), Fe(iron), Mn(manganese), and Mo(Molybdenum) depend on their concentration, could act as nutrient that are essential for some enzyme activities as cofactor and very beneficial for growing organisms in the plant. However, there are some other HMs that their efficiencies of the plants are not well understood; in many studies, they are known as metalloids and considered non-essential for plant growth [12,13,14]. Identification of non-essential HMs can give us a better understanding of how they operate in a different plant culture (recognition of their performance in different plants) [15] In this section, we consider three nonessential HMs including Pb(lead), Cd(Cadmium) and Hg(mercury), which have been named as the most toxic HMs in an environment[16].

2.1. Lead(Pb)

Lead as a non-redox active metal [17], by positioning in group 14 (IVA) of the periodic table and having a low melting point is one of the important metals in a variety of industrial products, including paints, weights, ammunitions, and leaded glass [18]. However, because of the protective role against acid and radiation, the most important application of lead is in recyclable car batteries [16]. In other hand, lead is known to be one of the most hazardous materials in the soil and the air so that a trace of lead can cause problems for the environment and human life [19,20] . In the human body, excess of Pb can cause problems in body skeleton, nervous system, circulatory, enzymatic, endocrine, and immune systems [21]. It can also seriously affect children's brain activities [22]. Having accumulation properties for hundreds of years, untapped soils can act as a reservoir of lead [23]. With the development of technology and appearing new generations of surplus-lead in smelting industry, mining [20], agricultural activities, Urbanism [24], and paints [25] lead has been turned into a serious problem for this century [20]. Lead is considered as an immobilized property in the soil so that plants can easily access it; however, it should be noticed how lead enters the plant body. Because the roots do not have any sites for Pb uptake, and lead would be absorbed through the root surface by carboxylic structures of mucilage uranic acids [24]. One of the consequences of increasing lead is the production of ROS in plant cells, which can cause the replacement of essential ions in the cell and impair other processes such as cell adhesion and cell signaling [25]. In the cell, nuclear by binding with DNA, lead can reduce the role of repairs in DNA and lead to a disturbance in mitotic stage and prolongs interface and consequently, increase the period of the cell cycle [26]. Pb (lead toxicity) in plants can decrease the growth of roots and increase the roots' suberized [27]. Pb (lead), with impact on the Reaction Centre and Antennae, decreases the efficiency of photosystem II [28], which can negatively affect plant metabolism.

2.2. Cadmium(Cd)

Cadmium, as a non-essential element [29], is one of the aggravating factors in soil salinity, which plays a major role in inhibition of plant growth by accumulation in plant [30]. Resources of cadmium in nature are volcanic emissions and weathering of rocks [31]. Cadmium naturally exists at trace amounts in soil (0.8-3.5 mg kg^{-1} soil); but, because of human manipulation of environment, such as mining, polluting water, and using fossil fuels, it can be observed in large quantities (up to 4-50 mg kg^{-1} soil) [32]. In fact, it turned into one of the main pollution in the soil caused by phosphate fertilizers and sewage sludge [33,8]. Since Cadmium is a mobile heavy metal that could be transferred easily between plants, investigating the effects of Cadmium in the environment is considerably important [33,34]. Because of powerful toxicity, even at low concentrations, mobility property, and simple entrance into the human food chain, Cadmium (Cd) can be named as one the most dangerous heavy metals [35,36]. Normal amount of Cd in agricultural lands is 1 mg kg^{-1} that could be increased due to human activities such as pesticides, irrigation and industrial activity [36]. Cadmium is absorbed by minerals, gets into the plants, and accumulates at different levels in plants; it, then, influences the human food chain and causes human carcinogen [37]. [e.g., "World Health Organization (WHO)"] has announced that the permitted level of cadmium in a normal human body is 70 μg [38]. Itai-Itai disease is one of the well-known diseases caused by accumulation of Cd in plant [39]. Cadmium was more toxic than chromium [40]. Cadmium in the plant could intervene in plant chemical synthesis processes such as ammonification, nitrification, DE nitrification, and microbiological process that affect the quantity and quantity of the crop products [41]. It also leads to the generation of[e.g., "Reactive Oxygen Species (ROS)"] and oxidative stress so that it can impact on the performance of protein and lipids [8]. Cadmium in leaf leads to leaf chlorosis [36], photosynthesis inhibition with the decline of pigment content, chlorophyll a, and phycobiliproteins [42,43] and then reduce the plant biomass [42]. In an experiment, effect of cadmium on *P. Flagellifera* showed that the excess amount of Cd could decrease the plant growth and photosynthesis pigment and damage thylakoid membranes and then disturb the cell wall activity [43], chlorophyll

content, and stomata size of Schinus molle trees [44]. In another experiment, uptaking Cu on two Cypress Varieties indicated that with increasing 100 mg kg^{-1} of Cd, the plant growth approximately decreased 37.6 % in P. *Orientalis* and 40.6 % in J. *Chinensis* [45].

2.3. Mercury (Hg)

Elemental mercury and its industrial derivatives, as a non-essential HM with high toxicity [46,47], are one of the detrimental factors in human health and plant growth [48,49]. Because of high volatility and water solubility, mercury [Hg0 (g)] is really hard to be removed [50]. Studies have shown that approximately 2320 t of Hg releases in atmosphere per year [51]. Anthropogenic activities, including smelting, mining and other industrial activities are the major sources of Hg in the environment [52,53]. However, one of the most important sources of mercury made by human activities is coal combustion and coal-fired power plants [54,55,56]. The average amount of mercury in Chinese coal is 0.22 mg/kg and in US is 0.09-0.126 mg/kg [54]. Although mercury is essential in many industrial applications, such as producing ultraviolet radiation in fluorescent lamps [57,58], but it is harmful for human health and could easily leave a negative effect on the nervous system; moreover, its development can hardly affect the renal system, immune system, reproductive system, and kidneys, especially for infants, children and pregnant women [59,60]. There are three toxic forms of mercury in the environment, including elemental mercury (Hg^0), miraculous ion (Hg_2^{2+}) and mercuric ion (Hg^{2+}) [46]. Mercury may exist in environment as gas, liquid, or solid so that it can be exposed to plant [61]. The main source of mercury in agriculture is anthropogenic activities, including pesticides, manure, lime, fertilizers and low quality urban compost [62]. Mercury in soil can be accumulated in the plant roots and then transferred to shoot, or it can be absorbed by the stomata in the leaves during the process of transpiration stream as gas [61]. In plants, mercury leads to a reduction in plant growth [62], especially in the root because of accumulation [63], disturbance in membrane structures, mineral nutrient uptake, photosynthesis, and transpiration and generation of reactive oxygen species (ROS) and oxidative stresses [62].

III. THE EFFECTS OF HEAVY METALS ON PLANT GROWTH

Plants, in the life cycle, need some essential micronutrients for growth and development; but, this issue depends on the dose and concentration of micronutrients. Trace concentration of some essential HMs would stimulate the plant growth [64] and act as a regulator and cofactor in enzyme activates [65]. A previous study on the effects of different doses of Cd (II), Cr(Chromium) (VI), Cu (II), Ni (II), and Zn (II) on the growth of the alfalfa plants (*Medicago sativa*) indicated that the seed germination significantly increased in Cd and Cr to 10 ppm, and in Cu and Ni to 20 ppm; it also showed that the shoot size increased to 14.0%, 60.0%, 36.0%, and 7.7% in Cr (VI), Cu (II), Ni (II), and Zn (II), respectively [64]. In other hand, increasing of heavy metals due to fracture makes both old and young roots to be thick and brown and decreases the length and elongation of roots; due to the important role of roots in water absorption, it consequently decreases the water absorption in plant [11]. In another experiment, effect of five HMs on seed germination and plant growth in *alfalafa* were studied [66]. The results indicated that all five heavy metals, except Zn with 40 ppm concentration, decreased the seed germination, root and shoot elongation in *alfaalfa* [66]. Shivhare and Sharma (2012) [67] investigated the effects of HMs on Georgina Wild (Dahlia). Their results indicated that increasing of HMs concentration decreases the root and shoot elongation and consequently, leads to inhibition plant growth and development [67].

IV. THE EFFECTS OF HEAVY METALS ON PLANT PHOTOSYNTHESIS

Many different studies indicated that HMs inhibit the net photosynthetic rate (Pn) and intracellular CO2 concentration [68,69]. HMs, directly (with accumulation in leaves) and indirectly, disturb stomata structure and decrease net photosynthesis and transpiration [70]. They induce alteration in chlorophyll a (chl a) and chlorophyll b (chl b) ratio, and is especially decreasing the chlorophyll content and biosynthesis [70]. Disturbing the electron transport system activities, heavy metals decrease the Photosystem II (Ps II) activities. High concentrations of heavy metals decrease the energy dissipation in reaction centers with affecting the reaction center or light harvesting complex (LHC), alternating state 1-9 state 2 transition in the LHC, and disturbance in the antenna complex [71,72]. Babu et al, (2010) investigated the effects of two heavy metals (Cr and Ag) on Cyanobacterium, Spirulina platensis. Their results indicated that heavy metals at Ps II decrease the [e.g., "whole chain electron transport activity (WCE)"] as 17% with inhibition of absorption light and energy in the reaction center and [e.g., "light harvesting complex (LHC)"] [71]. Moreover, in other studies, similar results obtained on chloroplasts, [e.g., "enriched thylakoids membranes BBYs"],thylakoid membranes and [e.g., "PhotosystemII PsII"] complexes [73,74].

V. THE EFFECTS OF HEAVY METALS ON ENZYME ACTIVITIES

According to previous studies in contaminated areas, diminishing the amount of HMs could influence the micro-organisms and enzyme activities and lead to an increase in enzymatic and microbial activities of soils [75,76]; in contrast, excess of HMs could lead to a decrease in the affluence of soil microbial community [77] and enzyme activities in soil [78,79,80]. In soil, HMs can disturb the structure, alteration, diversity, population, size and overall activity of microbial and bacterial community [81], cause an inhibition on synthesizes enzymes [81,82], decrease the bacterial species richness and consequently, change the chain and cycle of nutrients in soil. In other hand, HMs with effect on enzymes in plant restrict water and nutrient absorption by the roots, disturb the photosynthesis process, cause a morphological alteration in plants and consequently, decrease the plant growth [81]. In one study on seven different tree species at five enzymes involved in carbon, phosphorus and nitrogen cycle, including phosphates, â-glucosidase, cellobiohydrolase, chitinase, and xylosidase, HMs strongly decreased all activities of these five enzymes in soil, and also, according to tree species, in fine roots [83]. Khan et al (2007) investigated the effects of Pb and Cd on some individual enzymes such as catalase, alkaline phosphatase, dehydrogenase and found that they could significantly reduce enzyme activities. In other hand, the changes of microbial community structure can reduce the range of microorganisms and enzyme activities; this process is conducted with binging HMs to amino acids [84]. Evaluating Ni in some enzyme activities, researchers indicated that Ni decreased the enzyme activity, according to enzymes sensitively so that urease > dehydrogenases > alkaline phosphatase > acid phosphatase > catalase > arylsulphatase > β-glucosidase. This showed different reactions of enzymes to excess HMs [85]. Moreover, antioxidant enzyme activities may increase with the excess of HMs. Kumchai et al (2013) investigated the excess of Mo on cabbage seedling. Their results showed a positive effect on antioxidant enzyme activities such a [e.g., "Superoxide dismutase (SOD)"] , [e.g., "catalase (CAT)"] and [e.g., "ascorbate peroxidase (APX)"] that can be counted as plant nature reaction to excesses of HMs; this means that plants produce antioxidant enzyme activities under stress condition to overcome the cell damages [86]. As a conclusion, it could be said that HMs is the most harmful element on enzymatic activities that can be summarized to affluence of soil microbial and bacterial communities and lead to inhibition on synthesizes enzymes in soil and restrict the water and nutrient absorption by roots as well as the photosynthesis process in plants.

VI. THE EFFECTS OF HEAVY METALS ON MEMBRANE PERMEABILITY

One of the outcomes of extra HMs is the effects of HMs on the efficiency of cell membranes in plants. HMs have the ability to penetrate the cell membranes and bound with cell membranes constitutive such as proteins and phospholipids groups; They also distribute the functions of cell membranes, disrupt the transporting activities and substitution of calcium ions at essential sites, and reduce the level of plasma membrane H^+-ATPase MRNA [87] so that the availability of the substrate of the ATPase is reduced by binding with ATP [88]. One experiment showed that the permeability in root cells with K^+ efflux increased when they are exposed to excess of Cd^{2+} [89]. Evaluating the role of floc size in membrane permeability, Amiri et al (2010) concluded that excess of HMs on the pores of cake layer in the membrane and the toxic affects the sludge properties and decreases the permeability in cells so that the excess of HMs prevents the formation of larger floc and decreases the membrane permeability [90].

VII. PLANTS' RESISTANCE MECHANISMS AGAINST HEAVY METAL STRESS

Avoidance of stress and tolerance to stress are general mechanisms of plant reactions encountering abiotic stress, such as extreme HMs in the plant. Therefore, plants induce avoidance and tolerance mechanisms against heavy metal stress with some strategies such as alternation in permeability regulation and plasma members. Additionally, plants can be detoxified with mechanisms such as metallothionein (MTs) (metal binding proteins) and phytochelatin (PC) [91]; Phytochelatin (PC) mechanism in Cytosol is one of the best detoxification mechanisms under stress conditions [92]. In the cell, plants accumulate the heavy metals with special mechanisms. However, in high concentrations, heavy metals are transferred to the cytoplasm and removed rapidly, so that cations are broken into complex compounds by Thiol-containing molecules. Tonoplast decreases the heavy metal efflux to cells with some permeability mechanisms. Then, in the vacuole, remaining heavy metals will be stored and converted to crystal. Finally, the heavy metal toxicity is decreased [93] (Fig 1). This mechanism (accumulation and transport of HMs to cytoplasm), which is expressed as an indicator in face with extra heavy metals, keeps the gate of cells open [93]. The total tolerance ability of HMs of plants depends on plant species tolerance**.** Some species can prevent penetrating of excess heavy metal to aerial part; some species also accumulate the excess of HMs in their above ground tissue that could be toxic to most plants [94]. Meanwhile, the role of some mediation in raising the defense mechanism of plants against stress is important that we address it below.

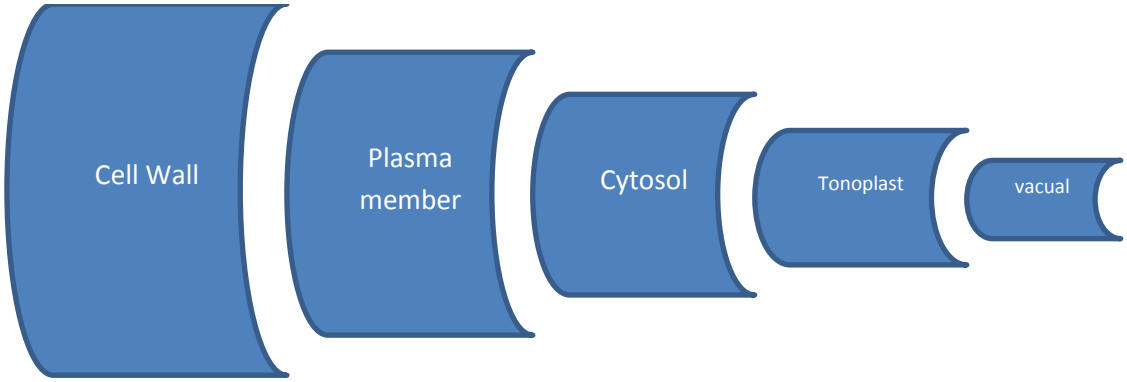

FIG 1: PLANT CELL MECHANISMS TO HMS STRESS: CELL WALLS ARE THE FRONT LINE OF HMS STRESS SO THAT WITH MECHANISMS SUCH AS SEDIMENTATION, BIND TO PECTIN CAN ELEVATE HMS STRESS. IN PLASMA MEMBER, PLANTS, WITH REGULATION OF METAL TRANSPORTATION, REDUCE THE INFLUENCE OF HMS ON THE CELL, AND THEN IN CYTOSOL, REMOVE THE COMPLEXION AND CAUSE A RAPID COMPLETION AND RAPID REMOVAL FROM CYTOSOL. TONOPLAST DECREASES THE HEAVY METAL EFFLUX TO CELLS WITH SOME PERMEABILITY MECHANISMS, AND FINALLY, IN VACUOLE, REMAINING HEAVY METALS WILL BE STORED AND CONVERTED TO CRYSTAL.

7.1. Si-mediated ameliorate plant tolerance against to stress

Silicon is one of the most important elements in the soil [95,96] taking 28% of the total earth surface [97.98]. It is considered as an important fertilizer component to ameliorating effect on plant growth in abiotic and biotic stress [95,96,97,99,100]. In plants, roots can take Si with silicic acid form $Si(OH)_4$ [101,102,103,104] with doses of 0.1 to 0.6 mmol L^{-1} in the soil [102] that is translocate to shoot by transpiration flow in the xylem [9], and depending on different cultivars, plants have an ability to accumulate Si between 0.1% to 10.0% Si (dry weight) [101]. There are many mechanisms to reduce HMs stress by Silicon, including HMs armature binding to the wall of Sully [95], stimulation of enzyme and non-enzyme, antioxidant that consequently decrease the lipid and H_2O_2 [95,102] peroxidation, and the positive variety of sub-cellular distribution of HMs [95]. Si-mediation improves the quantity and quality of crops. In one study, Si promotes the photosynthetic rate and chlorophyll content with positive alternation in leaf anatomy in banana [105]. Gottardi et al,(2012) investigated the effect of silicon on corn salad (*Valerianella locusta* (L.) Laterr). They found that it can increase edible yield, quality of crops and shelf life [98] Si-mediated alleviation of abiotic and biotic stresses including HMs, salinity, drought, disease, chilling and freezing stresses [106,103] that summarized below.

7.1.1. Si-mediated against heavy metal stress

Si with the rising of pH solution and inhabitation of metal Phyto-availability [97] influences in bioavailability metals and regulates them [99]. Stimulating antioxidant enzyme, Si reduces the necrotic spots caused by superoxide anions and free radicals in the leaves of both Zn and Mn plants [107]. On the other hand, Si improves the growth and development of cotton crop exposed to Zn stress by limiting Zn bioavailability and oxidative damage [97]. Moreover, the role of Si in the alleviation of iron soybean and cucumber plant growth is revealed by reducing the iron choruses and impact of iron distribution [99]. Si in the cell wall with the effect of cation binding capacity decreased concentration of Mn in apoplastic in cowpea [106]. Reduction of Mn toxicity in cucumber is happened by Si because reduction of lipid peroxidation is caused by stimulation enzymatic (e.g. SOD, APX, DHAR and GR) and non-enzymatic antioxidants (e.g. Ascorbate and glutathione) [106]. But , effect of Si on HMs changes according to cultivars and tissues [102] that can attribute to different Si uptake by the roots [101]. Evaluating the role of Si on uptake and translocation of arsenic and entry into the fruit indicates that different cultivar of tomato can show the opposite reaction to a combination of silicon [108].

7.1.2. Si-mediated against salt stress

Salt stress is one of the agriculture soil problems that have an adverse effect on plant growth by inducing oxidative stress [109]. Si-mediation can elevate the salt stress in plant by improving the antioxidant enzymes, decreasing lipid peroxidation, reducing permeability of the plasma membrane of leaf cells, ameliorating the ultra-structure of chloroplasts [110], improving shoot plants, and increasing gas exchange rate such as stomata conductance, net photosynthetic rate, and transpiration [111]. Additionally, Si can elevate the salt stress with apoplastic sodium absorption to adjust the stomata and

spaces [112]. A previous study revealed that application of Si is beneficial in improving the salt tolerance of tomato, grass, and *Spartina densiflora* plants with balance in mineral nutrient, [e.g.,"water-use efficiency (WUE)"] and increasing photosystem properties [96,113]. The result of another study indicated that application of Si reduces the effect of salt stress on potato with conservation plant water content due to increasing water-use efficiency [114]. The results of a study on cucumber showed that Si-mediated amelioration salt stress with increasing antioxidant defense enzyme and reducing [e.g., "Euphorbia characias latex peroxidase (ELP)"] and H2O2 [115]. Later, in another study, it is shown that Si can improve the salt tolerance in barley (*Hordeum vulgare* L.) by increasing the antioxidant enzymes and consequently, decreasing the lipid peroxidation [110].

7.1.3. Si-mediated against disease stress

Si plays an important role in alleviates plant disease and their control [7] and prevents the entry of fungi and disease to plants [112]. There are two mechanisms for Si against disease: the first is that Si forms a cuticle-Si double layer and prevents the influence of fungi on plants; the second is that Si acts as one adjuster host resistance to pathogens [101]. It is shown in a study that Si decreased the spread of root-rot pathogen *Pythium aphanidermatum* in bitter gourd [116]. Si can rise the ability of plants in preventing the leaf and neck blast, sheath blight, brown spot, leaf scald, and stem rot in rice [101]. Si decreases the brown rust incidence in sugarcane with increasing the leaf Si concentrations [117].

7.1.4. Si-mediated against drought stress

The role of silicon in plant is known as one osmoregulation to regulate the water deficit, which is related to the efficiency of photosynthesis and antioxidant enzymes in plants [118]. Silicon in the leaf surface reduces the loss of water by transpiration, and thus, reduces the drought stress [116]. Moreover, Si with a cooling mechanism by mid-infrared thermal emission of Si can adjust the leaf temperature [116]. Drought stress usually reduces the of crops yielding with interfacing in photosynthetic pigments, proteins, lipids, and some enzyme activities and leads to an oxidative damage in plants. The results of another study on wheat (*Triticum aestivum* L.) showed that Si can elevate drought stress by increasing the antioxidant defense mechanism and consequently, increase the photosynthesis properties [119]. Si concentration has a positive impact on the improvement of water resources in rice plant [120] and seed germination in tomato under water deficit stress by enhancing the antioxidant defense [121]. Generally, Si is not categorized as one essential heavy metals ; however, that is a beneficial element for improving the plant growth and development. It can also be expressed that Si-mediated is one proper application to raise the plant defense mechanism in confronting with biotic and abiotic streets [101].

VIII. CONCLUSION

HMs are one of the most important abiotic stresses that inhibit the growth and development in living organisms, lead to an early senescence of them [122], and menace safe food product for human over the world [123]. In plants, the excess amount of HMs leads to some symptoms such as necrosis, chlorosis, alterations of plants' phenotype, and genotype, causes an oxidative stress, and subsequently reactivates oxygen species (ROS) so that they stimulate the plant defense mechanism such as increasing antioxidant enzymes and non-enzyme activities [124]. Hence, that is counted as the most important defense mechanism of plants against stress for cell protects [125].

Plants, in response to HMs, follow three different strategies: 1- metal excluders: it covers a majority of mechanisms, including a large group of plants in which plants prevent the HMs stress by limiting the translocation of HMs into plant aerial parts; 2- metal indicator: plant is one indicator of soil HM that accumulates in biomass and other parts of plant; 3- metal accumulation: plants are counted as an accumulator in soil so HMs transudate and accumulate in plant tissue [126].

In cells, using strategies such as metal binding to cell wall and chelation, transforming HMs to low levels, and eventually accumulation and crystallization of them, plants could detoxify the HMs stress [127].

Si-mediation can help plant biomass production and plant growth [128]. One of the avoidance mechanisms of Si-mediation in the root is to reduce the uptake of HMs (Cd) with increasing the root oxalate exudation by enhancing the number of root tips [129].But, the major role of Si, when encounters with abiotic stress, is the elevation of the plant resistant by increasing the antioxidant enzyme activity [130].Si-mediation in cells decreases the toxic concentrations caused by HMs, symplast, apoplast, and incensement Si-absorbed in cell walls, and limits the root to shoot HMs translocations [131].

Generally, excess of HMs in agricultural soil caused by anthropogenic activities has made serious problems on the way to boost the agricultural products and improve their quality. In recent centuries, numerous studies carried out by researchers to alleviate and ameliorate the HMs toxicity in plants, which have revealed new ways for research communities to understand

and solve this problem. Si-mediation is one of the beneficial elements in stress conditions that helps to increase the efficiency of antioxidant enzyme activity. In this study, we tried to identify some non-essential elements and express some plant mechanisms caused by Si-mediation in coping with abiotic and biotic stress.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper.

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The Influence of Soil Organic Matter on the Uptake of Silver Nanoparticles in a Terrestrial System

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Abstract— *The uptake of silver from silver nanoparticles in soil was investigated in the presence of increasing concentrations of soil organic matter. Especially, the effect of Humus component of soil organic matter on the uptake of Ag from silver nanoparticles was studied. Two insect species, Acheta domesticus and Tenebrio molitor, and two plant species, Helianthus annuus and Sorghum vulgare, were exposed to silver nanoparticles(25 ppm in the presence of increasing concentrations of Humus (0, 1, 5, 10, 15, and 20% Humus) in soil (by weight). The techniques of transmission electron microscopy, dynamic light scattering, and powder X-ray diffraction were used to characterize the silver nanoparticles used in the study. An inductively coupled plasma-optical emission spectrometer was used to measure the levels of silver in test samples. Increasing concentrations of Humus in soil has resulted in an increase in the sulfur content and cation exchange capacity of the soil. A general decrease in the concentrations of silver was observed in Acheta domesticus and both the plant species, as a function of increasing concentrations of Humus in soil. In the case of plant species, the accumulation of silver nanoparticles was predominantly observed in the root tissue. Additionally, the translocation of silver from the roots to other plant tissues was observed in the case of Helianthus annuus. Results from this study suggest that the presence of Humus in soil could possibly decrease the uptake of silver from silver nanoparticles by insect and plant species.*

Keywords— Silver nanoparticles, Acheta domesticus, Tenebrio molitor, Helianthus annus, Sorghum vulgare, inductively coupled plasma-optical emission spectrometer, Soil organic matter, Humus.

I. INTRODUCTION

Silver nanoparticles (Ag NPs) are among the most commonly used metal nanoparticles today with an estimated global market projected to be worth USD 2.45 billion by 2022 (https://www.grandviewresearch.com/press-release/global-silver-nanoparticles-market) [1]. The range of applications of Ag NPs includes consumer products such as detergents, textiles, home appliances, nutritional supplements, etc (www.nanotechproject.org) [2]. Such widespread use of Ag NPs may inadvertently facilitate their entry into various ecosystems. Predominantly, Ag NPs often find their way in to terrestrial ecosystems through the application of sewage sludge to land [3-5].

Metals in soil are found to be present in eight different fractions: 1) free metal cations; 2) inorganic complexes; 3) organo-metal complexes; 4) organo-complex chelates; 5) in association with high molecular weight organic materials; 6) bound as diverse colloids; 7) adsorbed to colloids and 8) within the soil particles [6]. Spurgeon and Hopkin 1996 [7] observed that metals in the first four fractions remain in soil solution and subsequent uptake [7]. The fate and toxicity of metal nanoparticles in soil is governed by the physicochemical properties of both the metals and soil. The size and shape of nanoparticles, the aqueous solubility and acid-base character of nanoparticles, and the presence of any surface coatings on nanoparticles are some of the factors that influence their fate in a terrestrial system [8]. On the other hand, the many different properties of soil such as pH, texture of the soil, cation exchange capacity and the soil organic matter (SOM) govern the mobility, bioavailability and toxicity of metals in a terrestrial system [9,10].

Decomposing plant material predominantly serves as a source of organic matter in soil [11]. SOM is composed of humic substances (often referred to as humus) and non-humic substances. Humus is comprised of humic and fulvic acid (Foth 1978, cited in [12]). SOM plays a key role in determining the mobility, fate and toxicity of metals in terrestrial systems [11,12]. Complexation and adsorption are the phenomenon involved in the retention of metals by SOM [13]. Additionally, SOM

influences many difference functional properties of soil that include: 1) water holding capacity; 2) aggregate stability; 3) compaction characteristics and friability; 4) soil erodibility; 5) nutrient cycling; 6) buffering capacity to acidification and 7) cation exchange capacity[14] . SOM and clay content of soil play a major role in decreasing the availability and subsequent uptake of metals because of the negative charges and these negative charges are observed to be temporary in the case of SOM (Wild 1993 cited in [7]). The negatively charged functional groups in SOM include phenols, carboxyl(-ate), and amino groups. It was also observed that the negative functional groups increase in number during the process of humification of organic matter (Foth 1978, cited in [12]). However, SOM is also known to chelate metals [15], a phenomenon that may enhance their availability and uptake in soil.

This study attempted to measure the effects of Humus on the uptake of Ag from Ag NPs in soil by insect and plant species in a terrestrial system. The chelating ability of SOM may increase the bioavailability to plants; conversely, the increased S content and CEC could decrease the overall bioavailability.

The interaction between nanoparticles and SOM was thoroughly reviewed by Grillo et al. 2015 [16]. The present study investigated the uptake of Ag from Ag NPs in a terrestrial system using two insect and two plant species. The insect species used in the study include *Acheta domesticus* and *Tenebrio molitor*. The plant species employed in the study include *Helianthus annuus* (a dicot plant) and *Sorghum vulgare* (a monocot plant). All insect and plant species employed in the study are native to the region where the soil was collected. Insects serve a crucial role in the food chain of insectivorous birds, especially during the breeding season [17,18]. Similarly, plants serve as an important food source for grainivorous birds [19]. Results from this study would enable the understanding of the uptake of Ag from Ag NPs in soil by the insect and plant species under consideration and their subsequent role in bioaccumulation and bioconcentration of Ag NPs.

II. MATERIAL AND METHOD

2.1 Soil collection and preparation

All soil used during the insect and a plant exposure experiment was collected from the Colorado City, Texas at the following coordinates: Universal Transverse Mercator (UTM) 14 S 0319752 mE 3557792 mN. The soil was collected from the top 10 cm in to clean plastic containers and and transported back to The Institute of Environmental and Human Health (TIEHH) at Texas Tech University (TTU) in Lubbock, TX. The soil was then processed for homogeneity. Large rocks, roots, living organisms and other organic matter was removed, and large clumps of soil was crushed. This was followed by sifting the soil through a 2 mm wire screen into another clean plastic storage container. All processed soil was covered and stored indoors until further analysis.

2.2 Analysis of soil properties

The analysis of soil properties was performed at the Midwest Laboratories Inc. (Omaha, NE). The soil was characterized by evaluating the soil texture, percent humic matter, percent organic matter, exchangeable cations (K^+, Mg^{2+}, Ca^{2+}), available phosphorus (P), soil pH, percent base saturation of cations (K^+, Mg^{2+}, Ca^{2+}, H^+), cation exchange capacity (CEC), and S content. The effect of increasing concentrations of humic acid on some important properties of soil was also investigated.

2.3 Characterization of Ag NPs

Ag NPs (30-50 nm) containing $\geq$ 99.99% Ag were purchased from the US Research Nanomaterials, Inc. (Houston, TX). Additionally, the Ag NPs were characterized by studying their size, shape and composition.

The size and shape of Ag NPs was confirmed using transmission electron microscopy (TEM) was used. Sample preparation included dispersing the Ag NP powder in ethanol (EtOH). The mixture was sonicated for 10 minutes before being drop cast onto a carbon coated copper grid. Samples were air dried and analyzed on a Hitachi H-8100 TEM operated at 200 kV using a tungsten filament side-mounted camera.

The size of Ag NPs was also confirmed using the technique of Dynamic light scattering (DLS). 10 ml of reagent grade acetone (Fisher Chemical) was added to 10 mg of Ag NP powder and the mixture was sonicated until Ag NPs remained suspended in solution. Samples were analyzed using a Nanotrac NPA252 Combination (Microtrac Inc. Montgomery, PA) and Mictrotrac Flex Software (Version: 10.3.14).

The composition of Ag NPs was confirmed using the technique of Powder x-ray diffraction (PXRD). A Rigaku Ultima III X-Ray Diffractometer was used and samples were analyzed using a Cu Kα radiation as x-ray source. Parallel-beam geometry

with a step width of 0.03° and a count time of one second was used. The divergence, scattering, and receiving slits were set at one. The diffraction patterns were compared and matched to the phases in the International Center for Diffraction Data (ICDD) powder diffraction file (PDF) database.

2.4 Exposure of insects to increasing concentrations of Humus in soil

37.8 L terrariums were used to conduct the exposure studies of *T. molitor* and *A. domesticus* to increasing concentrations of Humus in soil. The treatment groups include concentrations of 0 (control), 1, 5, 10, 15 and 20% of Hapi-Gro Peat Humus (by weight) in 2.5 kg of soil. The soil was then spiked with 62.5 mg of Ag NPs to obtain a final concentration of 25 ppm Ag NPs in soil. Either 300 crickets or 400 large mealworms were added to each treated soil groups to conduct the exposure studies. The exposure study was carried out over a period of 28 days and all insects were provided with fresh food and water for the entire duration of study. After 28 days, insects were removed from individual terratiums in to glass jars. The jars were placed in a -80°C freezer to ensure all insects are deceased. The insects were then freeze dried (FreeZone 2.5 Liter Freeze Dry System, Labconco, and Corp. Kansas City, MO) for at least 48 hours to ensure the removal of all moisture. Freeze dried insects were then crushed into a fine powder and stored in a freezer until further analysis.

2.5 Exposure of plants to increasing concentrations of Humus in soil

The plant exposure studies were performed in commercially available plastic nursery containers filled with approximately two inches of pond pebbles to aid in adequate drainage. The preparation of soil with increasing concentrations of Humus and the subsequent spiking of soil was performed in a similar fashion to that of the insect exposure studies. Seeds for two plant species, *H. annuus* and *S. vulgare* were planted into the prepared nursery containers and were transported to the TTU greenhouse. The plants remained in the greenhouse until they reached maturity, approximately three months for *H. annuus* and six months for *S. vulgare*. While in the greenhouse, plants received shaded sunlight and were maintained at 60°F or above. Once plants reached maturity, the entire plant was harvested. The roots were separated from the remainder of the plant and rinsed using tap water for a full minute to remove all attached soil. The shoot system of the plant was separated into leaves, stems, and seeds. The plant samples were stored in a freezer until further analysis.

2.6 Sample Digestions

A modified version of EPA Method 3050B was used to perform all sample digestions. No hydrochloric acid was used during the process of sample digestions in order to prevent the formation of silver chloride.

2.7 Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) Analysis

All samples were analyzed using a Teledyne Instruments (Hudson, New Hampshire) Prodigy High Dispersion ICP-OES. The samples were analyzed for silver at three different wavelengths: 224.643, 328.068, and 338.289 nm. Ultimately, the data from 338.289 was chosen for statistical analysis.

2.8 Statistical Analysis

All statistical analyses was performed using MINITAB 17 software [20]. All samples were analyzed in relicates (n=2). A two-way ANOVA was performed to analyze the data for effect of insect species and percent humus content on the uptake of AgNPs. A multi- way ANOVA was performed to examine the effect of plant type, percent humus, and plant tissues on the uptake of Ag NPs. The post analysis comparison was performed with Tukey test. Any statistical significance was established at an alpha level of 5% (p=0.05).

III. RESULTS AND DISCUSSION

3.1 Soil Characterization

The control soil was found to be sandy loam in nature, with 54% sand, 36% silt, and 10% clay. Additional analyses revealed that the soil contains 0.01% humic matter, 1.7% organic matters, and 9 ppm S. The pH of the control soil was slightly basic (8.1 pH units) and the CEC of the soil was calculated to be 18.0 meq/100g.

The effect of increasing concentrations of humus in soil on the various properties of soil is summarized in TABLE 1. As is evident from TABLE 1, increasing concentrations of humus in soil has resulted in an increase in the humic matter (%), sulfur content (ppm), CEC (meq/100g), and the organic matter (%) of soil.

TABLE 1
EFFECT OF INCREASING CONCENTRATIONS OF HUMUS ON PROPERTIES OF SOIL.

Treatment Group	Humic Matter (%)	Sulfur Content (ppm)	CEC (meq/100 g)	Organic Matter (%)
0% Humic Content	0.01	9	19.8	1.7
1% Humic Content	0.01	17	18.2	1.6
5% Humic Content	0.03	36	19.0	1.9
10% Humic Content	0.07	82	21.4	2.5
15% Humic Content	0.07	101	20.7	2.6
20% Humic Content	0.11	127	23.1	2.9

3.2 Transmission electron microscopy analysis

The 30-50 nm uncoated silver nanoparticles were found to be heavily aggregated after being dispersed in EtOH. However, the TEM was able to confirm the spherical shape of the nanoparticles (Figure 1). The average particle size of Ag NPs was found to be 30-50 nm, with outliers on either side of the range.

3.3 Dynamic light scattering analysis

The size of Ag NPs used in the study is confirmed by DLS. It was observed that approximately 95% of the 30-50 nm Ag NPs had a size between 30.70 to 52.90 nm (Figure 2). The average size of Ag NPs used in the present study was found to be 41.80 nm.

3.4 Powder X-Ray Diffraction analysis

The composition of Ag NPs used in the present study is confirmed using the technique of Powder X-ray diffraction (Figure 3). The diffraction patterns of Ag NPs obtained matched both those in the ICDD and those provided by the manufacturer.

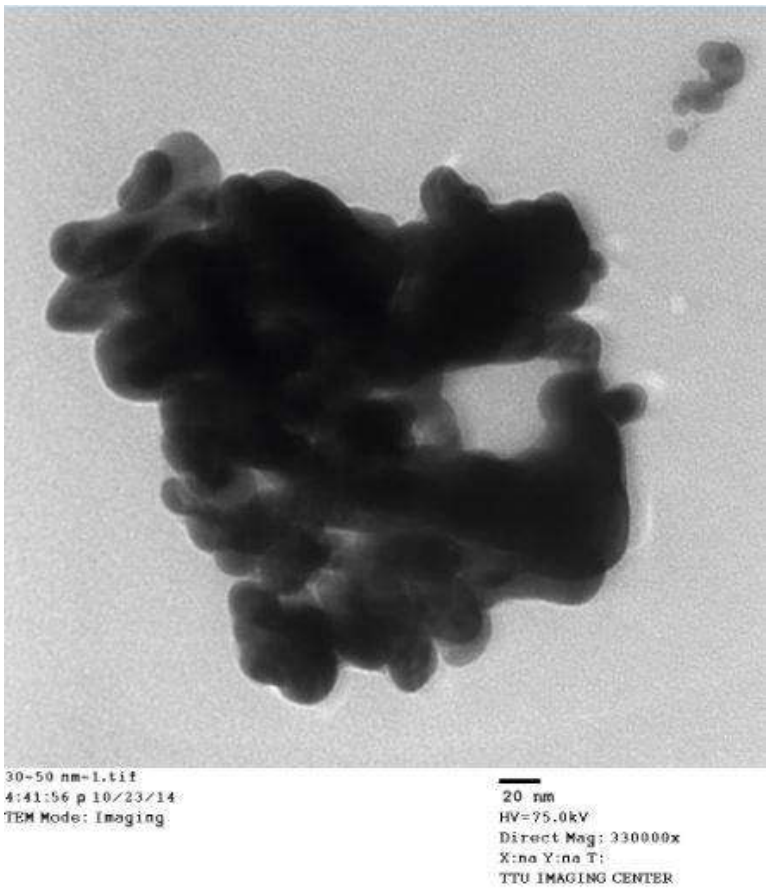

FIGURE 1: REPRESENTATIVE TRANSMISSION ELECTRON MICROSCOPY IMAGE OF 30-50 nm Ag NPs USED IN THE STUDY.

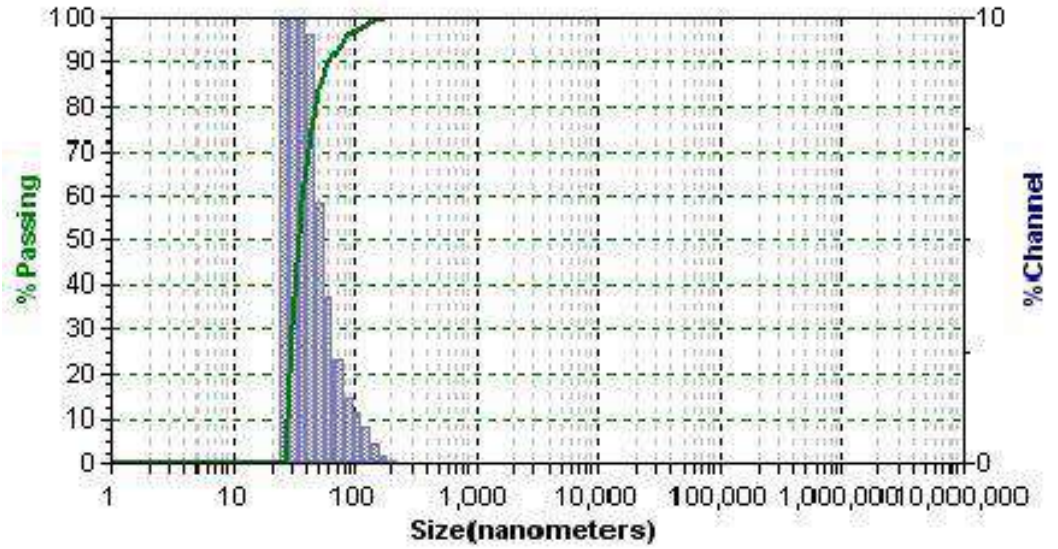

FIGURE 2: REPRESENTATIVE SIZE DISTRIBUTION OF 30-50 nm Ag NPs USED IN THE PRESENT STUDY OBTAINED USING DYNAMIC LIGHT SCATTERING.

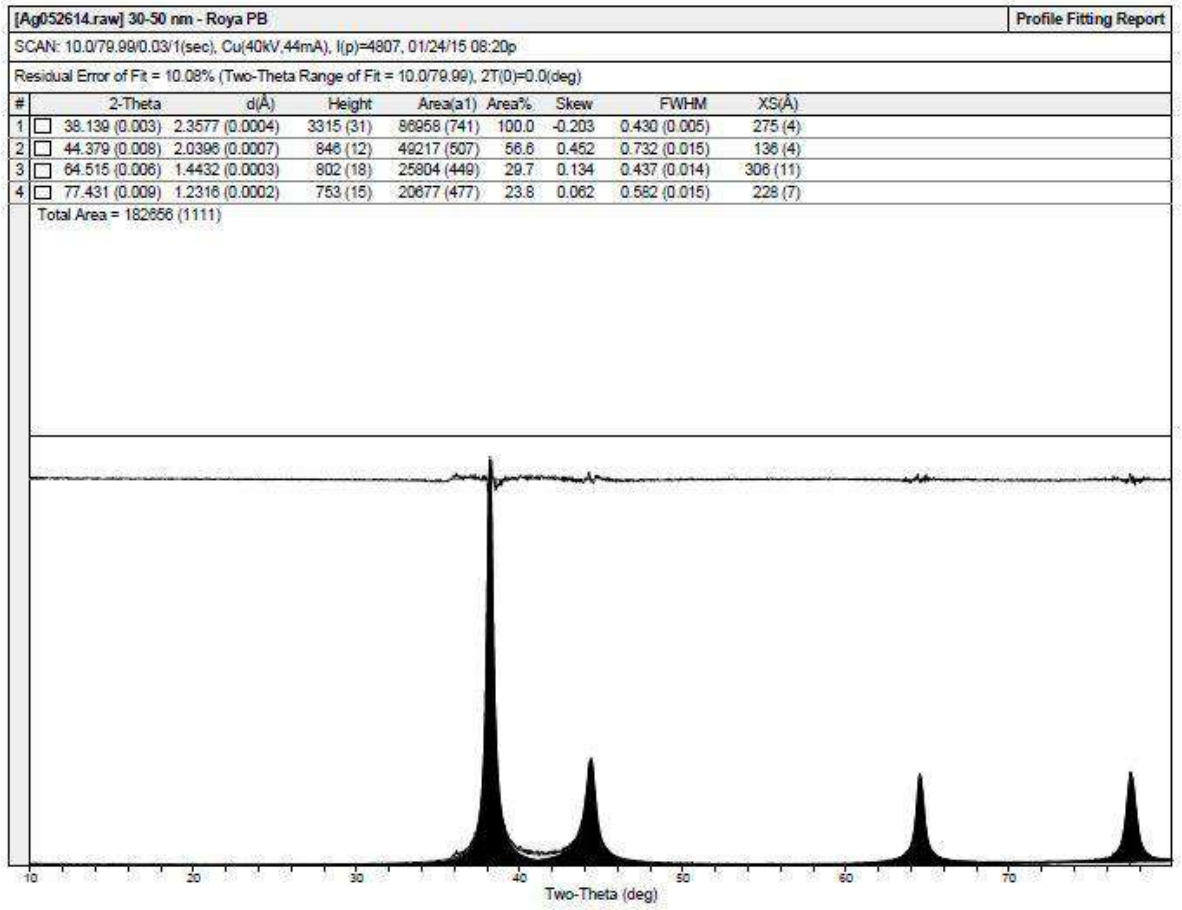

FIGURE 3: REPRESENTATIVE DIFFRACTION PATTERN OF 30-50 nm Ag NPs USED IN THE PRESENT STUDY AS DETERMINED BY POWDER X-RAY DIFFRACTION.

3.5 Effect of increasing concentrations of humus on the uptake of Ag from Ag NPs in soil by insect and plant species

Figure 4 summarizes the effect of increasing concentrations of humus in soil on the uptake of Ag from Ag NPs in soil by *A. domesticus* and *T. molitor*.

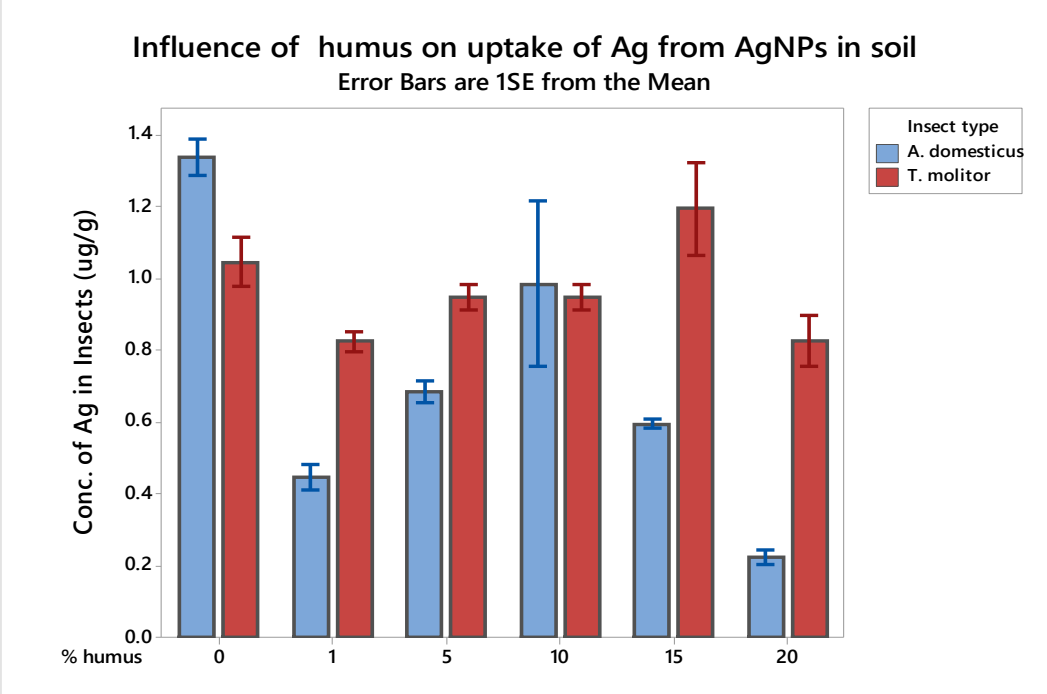

FIGURE 4: INFLUENCE OF INCREASING CONCENTRATIONS OF HUMUS ON THE UPTAKE OF Ag FROM Ag NPs IN SOIL BY INSECT SPECIES (n=2).

The control samples from the insect species were found to contain no detectable concentration of silver. No apparent trend was observed to decipher the effect of increasing concentrations of humus in soil on the uptake of Ag from Ag NPs in soil by the insect species. However, a decrease in the uptake of Ag from Ag NPs in soil was observed in the case of *A. domesticus*, especially at high concentrations of humus in soil. The uptake of Ag from Ag NPs in soil by *A. domesticus* was observed to be significantly lower when the soil had 20% humus compared to the remaining soil treatment groups. However, the overall uptake of Ag from Ag NPs in soil was found to be higher in the case of *T. molitor* when compared to *A. domesticus*.

Figures 5 and 6 summarize the effect of increasing concentrations of humus in soil on the uptake of Ag from Ag NPs in soil by H. annuus and S. vulgare, respectively.

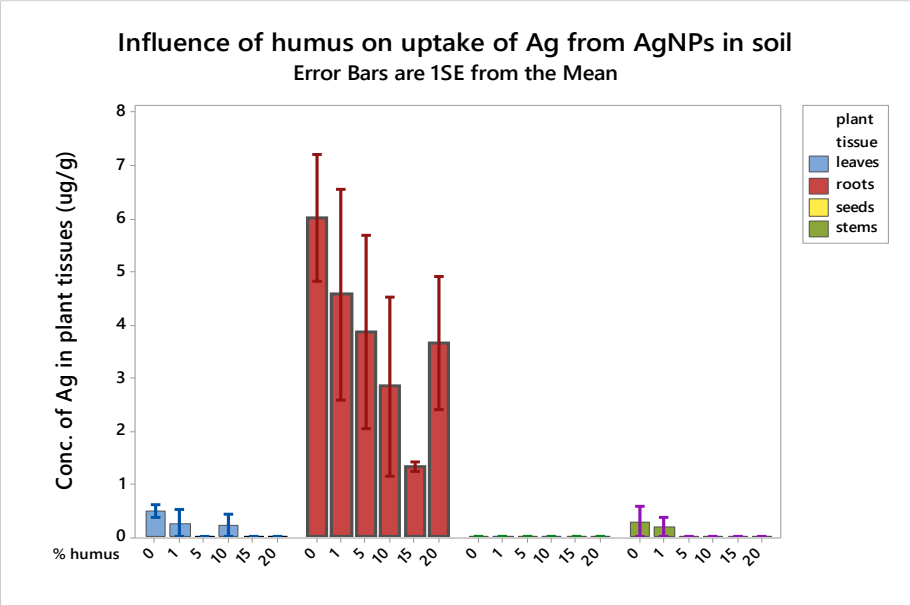

FIGURE 5: INFLUENCE OF INCREASING CONCENTRATIONS OF HUMUS ON THE UPTAKE OF Ag FROM Ag NPs IN SOIL BY *H. annuus* (n=2).

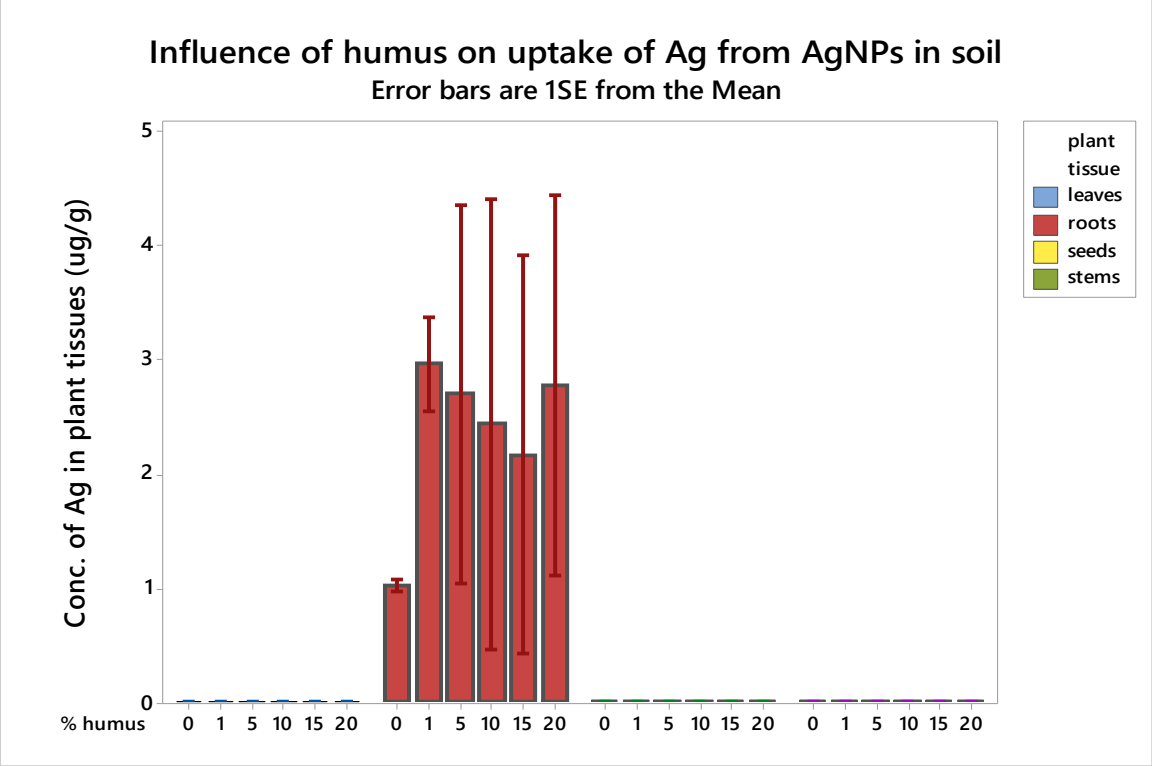

FIGURE 6: INFLUENCE OF INCREASING CONCENTRATIONS OF HUMUS ON THE UPTAKE OF Ag FROM Ag NPs IN SOIL BY *S. vulgare* (n=2).

The blank samples from both the plant species used in the study were found to have no detectable levels of silver. In the case of both the plants, the concentrations of Ag in the roots at all treatment levels was found to be significantly higher than the concentrations of Ag in the remaining plant tissues ($p<0.05$). The translocation of Ag from the roots to other plant tissues like leaves was observed in the case of *H. annuus*. Nevertheless, a general decrease in the levels of Ag in roots of both plants was observed as a function of increasing concentrations of Humus in soil.

Unintentional contamination of glassware during the process of sample digestions has compromised the use of all three replicates for statistical analyses. Hence, only two replicates per sample were used in the present study. This explains the lack of any definitive trend in the concentration of Ag in insects and plants as a function of increasing concentrations of Humus in soil. Regardless, a general decrease in the concentrations of Ag in both the plants and *A. domesticus* was observed as a function of increasing concentrations of Humus in soil. The decrease in the uptake of Ag in the presence of increasing concentrations of Humus in soil could be explained using TABLE 1. As is evident from TABLE 1, increasing the concentrations of Humus in soil has resulted in an increase in the CEC and Sulfur content of soil. CEC of soil is defined as the quantity of positively charged ions that could be help by the soil [21].It provides electrostatic binding sites for cations like silver (Ag+) thus inhibiting their availability for uptake in a terrestrial ecosystem [22]. Hence, an increase in the CEC of soil due to increasing concentrations of Humus (TABLE 1) in soil does result in a decrease in the availability of Ag for uptake by plants and insect species in a terrestrial system.

Additionally, it is also evident from TABLE 1 that increasing concentrations of Humus in soil has resulted in an increase in Sulfur content of soil. The increase in Sulfur content could be of significance especially in the case of Ag NPs. Silver has a very high affinity for S and will form Ag_2S which is highly insoluble ($K_{sp}= 8.0 \times 10^{-51}$) . To be available to uptake by plants and insect species in a terrestrial system, Ag ions ought to be in soil solution. However, Ag_2S being highly soluble in water renders Ag unavailable for uptake [23-29]. Finally, the presence of negatively charged functional groups in SOM (Foth 1978, cited in [12]) may also decrease the availability of Ag ions in a terrestrial system

IV. CONCLUSIONS

The effect of increasing concentrations of soil organic matter (Humus) on the uptake of Ag from Ag NPs in soil by two insect and plant species was investigated. Ag NPs were thoroughly characterized using the techniques of transmission electron microscopy, dynamic light scattering and powder X-ray diffraction analyses. In general, it was observed that the

presence of Humus decreases the uptake of Ag from Ag NPs in soil by insect and plant species. Hence, it can be inferred that the presence of increasing concentrations of Humus in soil decreases the bioavailability of Ag from Ag NPs in a terrestrial system.

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SERS of insecticides and fungicides assisted by Au and Ag nanostructures produced by laser techniques

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***Abstract*—** *This study deals with the use of laser techniques for preparation of advanced Au and Ag nanostructures on SiO_2 (001) substrates to be applied to high-resolution analyses, namely, surface enhanced Raman spectroscopy (SERS) analyses. The optical and morphological properties of the nanostructures are compared with those of the PLD thin films. The activity is tested of the structures fabricated as substrates for SERS covered by small quantities (usually applied in agricultural medicine) of the Aktara 25 BG (thiamethoxam) insecticide and the Dithane DG (mancozeb) fungicide. To the best of our knowledge, Raman spectra of Aktara 25 BG are presented for the first time. The study has a direct bearing on the human health and food quality by way of assisting the detection of small amounts or residue of harmful pollutants.*

***Keywords*— laser deposition and annealing, Ag and Au nanostructures, SERS, insecticide Aktara 25 BG, fungicide Dithane DG.**

I. INTRODUCTION

The properties of noble metallic nanostructures (NSs) have been the subject of considerable fundamental and technological interest. Metal nanoparticles (NPs) play an important role in scientific investigation and nanotechnology. As a result of the progress in nanotechnologies during the last two decades, nanosystems find nowadays application in many areas, such as chemistry, optics, biology, agriculture, medicine, microelectronics, etc. [1].

The excitation spectrum of noble metallic sub-wavelength structures is determined by its surface plasmon resonance. The energy of the plasmon resonance depends strongly on the shape and composition of the nanostructures. The tunability of the plasmon resonances of noble metallic NPs can be exploited to position the optical resonances at specific wavelength regions of interest and has led to a wide range of applications. The strong local electro-magnetic field enhancement accompanying the surface plasmon resonances has also been used to manipulate light-matter interactions, so that noble metallic sub-wavelength structures have been widely applied in surface enhanced Raman spectroscopy (SERS) [2,3]. The enhancement of the Raman signal may reach a factor of 10^8-10^{12} – the method is thus capable of detecting even a single molecule [2]. The increase of the Raman signal is a result of a local electromagnetic field enhancement in the vicinity of a structured surface due to the excitation of local and surface plasmons. In addition to their fundamental importance, plasmonic nanostructures are receiving a great deal of attention for their potential applications in areas such as sub-wavelength waveguides, optical nanoantennas, photovoltaic technology for efficient light coupling into solar cells, metamaterials, chemical and biological sensing, and biomedical applications [4-7].

Among the physical techniques, laser-based syntheses of nanomaterials have constituted a continuously growing field of research. In particular, pulsed laser ablation of solid targets in different environments, e.g. vacuum, background gas or liquid, has become an attractive method for the generation of NPs and deposition of NPs-assembled materials [8]. Among the successfully applied techniques one can mention the ns-laser deposition of thin metal films and the post-deposition structuring [9-13]. The nanostructuring of thin metal films by excimer laser pulses has been introduced as a technique for production of nanoparticles on different substrates [12,14]. The fragmentation of the metal surface into nanosized droplets during the melting is due to the poor wetting between the substrate and the liquid phase [15]. Moreover, femtosecond laser nanostructuring of silicon-based SERS substrates has also been reported [14,16].

Au and Ag NSs produced by laser methods have been used successfully in SERS analyses. Thus, an enhancement has been observed of the R6G Raman spectrum on Au nanocolumns formed by off-axis pulsed laser deposition (PLD). Concentrations of R6G as low as 1 nM [3] have been measured and a maximum enhancement higher than 10^5 has been achieved.

SERS has been used for trace analyses and detection of residue of different organophosphorous pesticides and insecticides [17-22] by employing various nanostructures, such as a solution of 100 nm Ag nanocubes [18] and a Klarite Au-coated SERS-active substrate [19-21]. The SERS analyses have been compared with the traditional analyses, as chromatography, fluorescence polarization immunoassay, multi-enzyme inhibition assay, and biosensors. Although the traditional methods can be used to detect trace amounts of pesticides, they are time-consuming, labor-intensive and expensive, which makes them less attractive and limiting to a certain extent the laboratory, real-time, and field detection [19].

Among the large variety of pesticides, dimethoate is a widely-studied substance. The lowest concentration registered has been 5÷10 μg mL^{-1} using confocal Raman micro-spectrometry with Klarite substrates [19]. Generally, a low concentration at about 10^{-6} can be detected.

The Dithane DG fungicide (mancozeb as an active substance) and the Aktara 25 BG insecticide (thiamethoxam as an active substance) are among the chemicals used extensively in agricultural medicine as protective substances for vegetables, fruits, crops etc. The fungicides from the dithiocarbamate group, as thiram, ferbam, ziram, metiram and zineb, have been widely studied in different SERS configurations using Au or Ag NSs [23-27]. Moreover, chemically produced gold nanorods have also been used as active SERS substrates for detection of ultra-low levels of fungicides from the dithiocarbamate group, i.e. thiram, ferbam and ziram [28]. Limits of detection as low as several nM have been achieved depending on the type of material tested. Furthermore, recording of Fourier transform Raman and surface-enhanced Raman spectra of a silver colloid of the mancozeb dithiocarbamate fungicide has been reported [29]; a slight decomposition has been observed of this material because of a metal exchange with the silver on the surface. As for the insecticide Aktara 25 BG (thiamethoxam), to the best of our knowledge no SERS study has been reported.

The aim of this paper is to describe a study on and the results of the development of laser technologies for formation of advanced Ag and Au nanostructures (NSs) by annealing PLD films on quartz. The as-produced NSs were used as active substrates for high-resolution analyses (SERS) to detect the Dithane DG fungicide and the Aktara 25 BG insecticide. Two types of concentrations of the two materials were analyzed and compared – low, as in routine agricultural use, and high, as offered on the market. The SERS spectra of Au and Ag NPs-covered areas and films were compared with the Raman spectra taken from high concentrations deposited directly on glass.

II. EXPERIMENTAL

2.1 Ag and Au active substrates synthesizing

The Ag or Au nanostructures were fabricated by means of a laser-based technique. As a first step, approximately 100-nm thick films were deposited by a standard PLD on 10×10 mm quartz (001) substrates at room temperature in vacuum (ambient pressure of ~10^{-3} Pa) using a THG Nd:YAG laser (Lotis TII) operating at λ = 532 nm at a repetition rate of 10 Hz with a pulse duration of 12 ns. The targets used were Au or Ag (both of 99.99 % purity, Alfa Aesar). The laser radiation (fluence of 1.5 J/cm^{-2}) was focused on the surface of the targets by a lens with a focal distance of 20 cm. The second step, i.e. producing the NPs, consisted of annealing the as-prepared films by a single pulse of the same laser wavelength with a fluence of 0.3 J/cm^2 incident on an area on the film with a diameter of ~4 mm, again in vacuum at room temperature. The as-prepared active samples were subjected to μ-Raman analyses.

2.2 Materials and Instrumentation

The Aktara 25 BG insecticide (Syngenta, Switzerland) and the Dithane DG fungicide (Indofil Industries Limited, India) were purchased from a specialized agricultural drugstore. The in-stock standard concentrations of the active chemicals are as follows: thiamethoxam $C_8H_{10}ClN_5O_3S$ in Aktara 25 BG is 250 g/kg (i.e., ¼ of the entire mass); and mancozeb $(C_4H_6MnN_2S_4)_x(Zn)_y$ in Dithane DG, 750 g/kg (i.e. ¾). In fact, mancozeb is a combination of two dithiocarbamates – maneb and zineb. Maneb is a polymeric complex of manganese with ethylene bis (dithiocarbamate) anionic ligand and zineb is the same, but with the manganese substituted by zinc. Figs. 1 a and b present the chemical structures of thiamethoxam and mancozeb, respectively.

Two drops of aqueous solution of each of the analytes, at the concentrations summarized in Table 1, were deposited on the Au and Ag active samples (the films and NPs sections). The samples were then dried at 50 ^oC. For comparison with the properties of the active substrates prepared, a drop of each of the analyte solutions at their highest concentrations (as obtained from stock) was deposited on a glass substrate and dried under the same conditions for μ-Raman analyses. In all cases, the areas on the substrates covered by the dried analytes had a diameter of ~4 mm, i.e. ~12 mm^2.

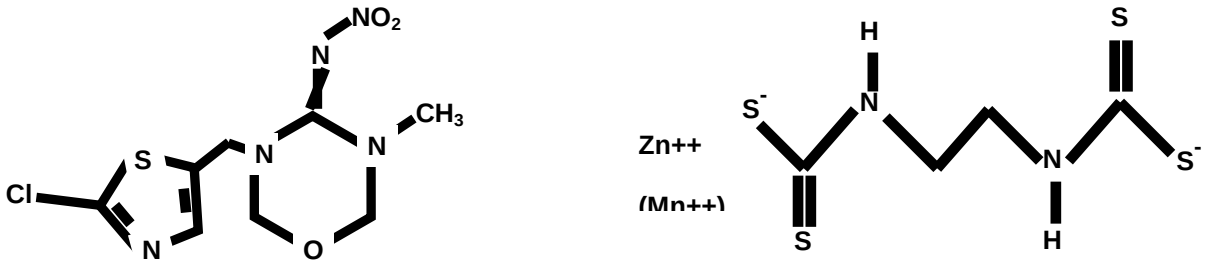


FIG. 1 CHEMICAL STRUCTURE OF: A) thiamethoxam; AND B) mancozeb (maneb + zineb).

TABLE 1
SUMMARIZED DESCRIPTION OF THE TESTED CHEMICALS AND THEIR CONCENTRATION

Name of the product studied	Active Chemical	Analyte, deposited on film or NPs areas	Analyte, deposited on glass	On sale
Insecticide Aktara 25	thiamethoxam	75 $\mu g \cdot mL^{-1}$ active substance; 0.26 mM	12.5 $g \cdot L^{-1}$ active substance; 43 mM	Powder; active substance 250 g/kg
Fungicide Dithane DG	mancozeb	0.75 $mg \cdot mL^{-1}$ active substance; 2.8 mM	37.5 $g \cdot L^{-1}$ active substance; 0.14 M	Powder; active substance 750 g/kg

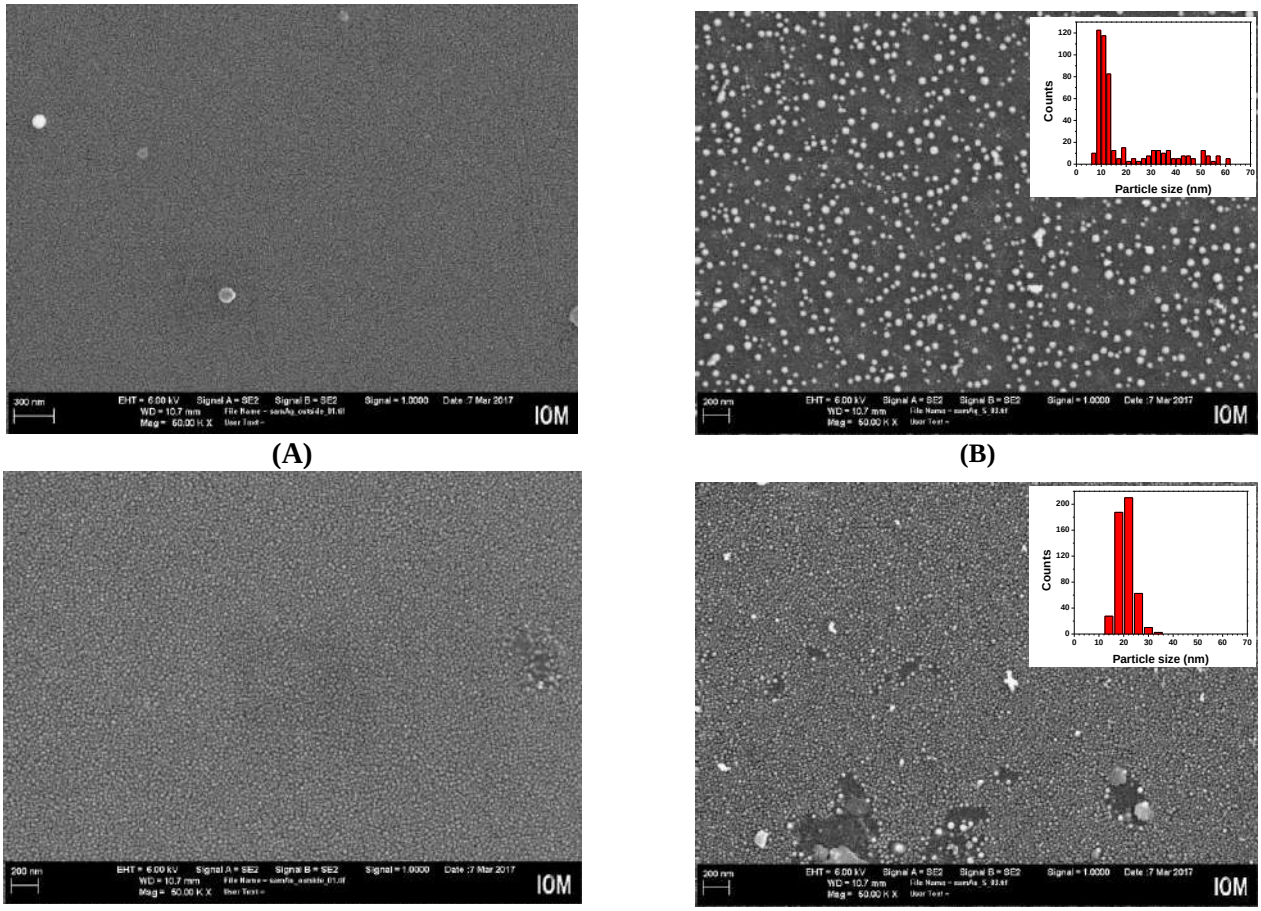
The morphology of the samples was observed by scanning electron microscopy using a SU 8000 FE-SEM (Hitachi, Japan) and a FE-SEM (Zeiss Ultra55, Gemini) microscopes. The optical properties of the thin films and the NPs areas were measured by an optical spectrophotometer (Jasco V-670, Japan). The Raman spectra were obtained by an RMS-310 μ-Raman spectrometer (Photon Design, Japan). It uses an excitation power of 0.5 mW (image dimension of ~1 μm^2) at a wavelength of λ = 532 nm and has a resolution of 0.2 cm^{-1}.

III. RESULTS AND DISCUSSIONS

3.1 Morphological and optical properties of the Ag and Au substrates

The morphology of the samples was observed and analyzed by FE-SEM. Figs. 2 a-d present SEM images of the Ag and Au films and NPs arrays, respectively. As is evident, the Ag film was much flatter than the Au one. The insets in Figs. 2 a and d display histograms of the Ag and Au NPs size distributions evaluated by counting 500 particles. One can see that the Au NPs size distribution was quite narrow with a maximum at ~22 nm. Additionally, areas where the Au NPs had been completely evaporated were present, because of "hot spots" in the laser-beam energy distribution (Fig. 2 d). The Ag NPs size distribution (see inset in Fig. 2 b) had two maximums – one at ~10 nm corresponding to the smaller NPs, and second one at 30 ÷ 55 nm, corresponding to the larger NPs.

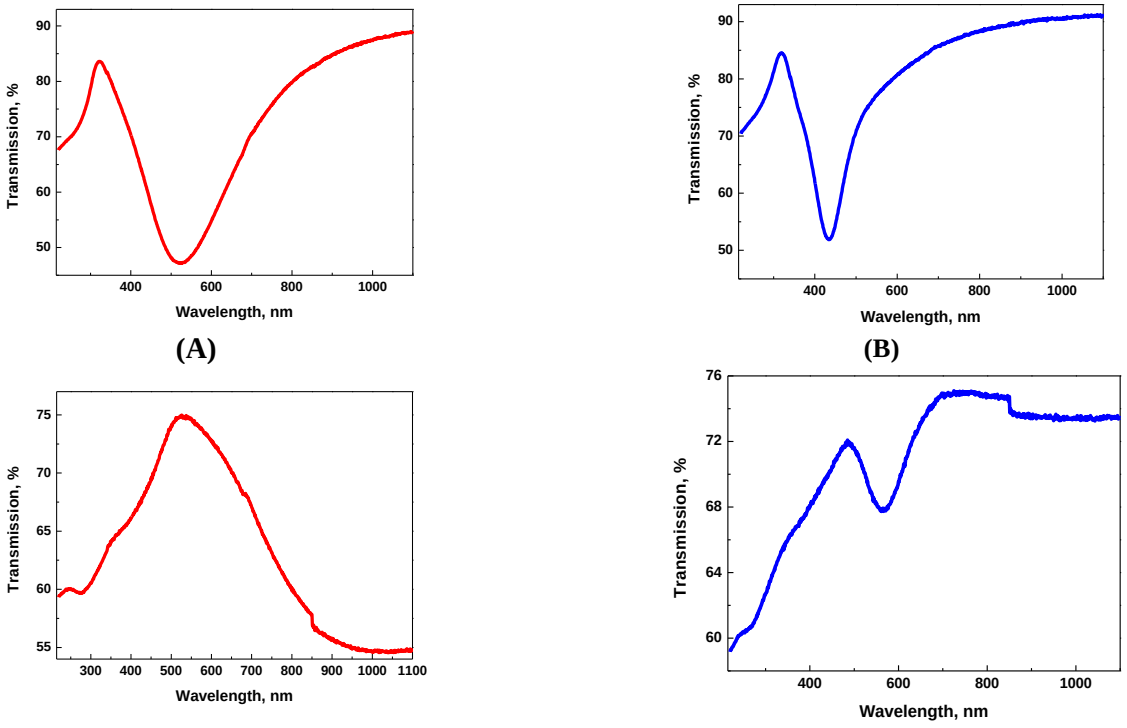
The optical properties of the Ag and Au films, as well as of the nanoparticles areas, were also studied. Figs. 3 a-d show the transmission spectra of the samples presented in Fig. 2. As is seen in Fig. 3, the plasmon resonance was very well pronounced in both cases, i.e. the Ag and Au NPs areas. However, it was much stronger and narrower in the case of Ag NPs (Fig. 3 b). It is worth noting that a plasmon resonance was also achieved in the case of the Ag film (Fig. 3 a); it had the same intensity as that in the Ag NPs area (Fig. 3 b), but was much wider. In what regards the Au film, the plasmon resonance was very shallow. Further, the plasmon resonance appeared at 525 nm for the Ag films and was shifted towards the shorter wavelengths, namely, 438 nm, for the Ag NPs. In the cases of the Au NPs and films, the respective values were 566 nm and ~681 nm. We assume that the features described above could be attributed to the characteristics of the structures explored.



(A) (B)

(C) (D)

FIG. 2 Fe-SEM IMAGES OF: A) AG film; B) Ag NPs area; C) Au film; D) Au NPs AREA.



(A) (B)

(C) (D)

FIG. 3 TRANSMISSION SPECTRA OF: A) AG film; B) Ag NPs area; C) Au film; D) Au NPs.

3.2 SERS of Aktara and Dithane

The analytes in the active Ag and Au substrates (films and NPs areas) and the samples on glass were examined by μ-Raman spectrometry. Figs. 4 a-c present μ-Raman spectra of Aktara (thiamethoxam). As is seen, several strong peaks were detected from the Ag NPs area, while the Ag film gave rise to some shallow peaks. Additionally, several very week and broad peaks were observed when analyte with a higher concentration (more than two orders of magnitude) was deposited on the glass substrate. The parameters of the SERS peaks observed originating from the Ag NPs and the Ag film and the μ-Raman peaks recorded from the glass substrates are summarized in Table 2. To the best of our knowledge, a SERS study of the Aktara insecticide (thiamethoxam) is reported for the first time. The strongest peaks in the SERS spectra arising from the Ag NPs sample were located between 840 cm^{-1} and 1238 cm^{-1}. Additionally, peaks at 545 cm^{-1}, 583 cm^{-1} and 590 cm^{-1} of intermediate intensity could be seen. According to Nicholas et al. [30], who studied other structurally related halogenated (Cl) pesticides, several peaks below 600 cm^{-1} can be ascribed to the C-Cl linkage. Moreover, the activity between 600 cm^{-1} and 1600 cm^{-1} is characteristic for hydrocarbons. The peak at 1619 cm^{-1} can be related to a distortion of the benzene-type ring.

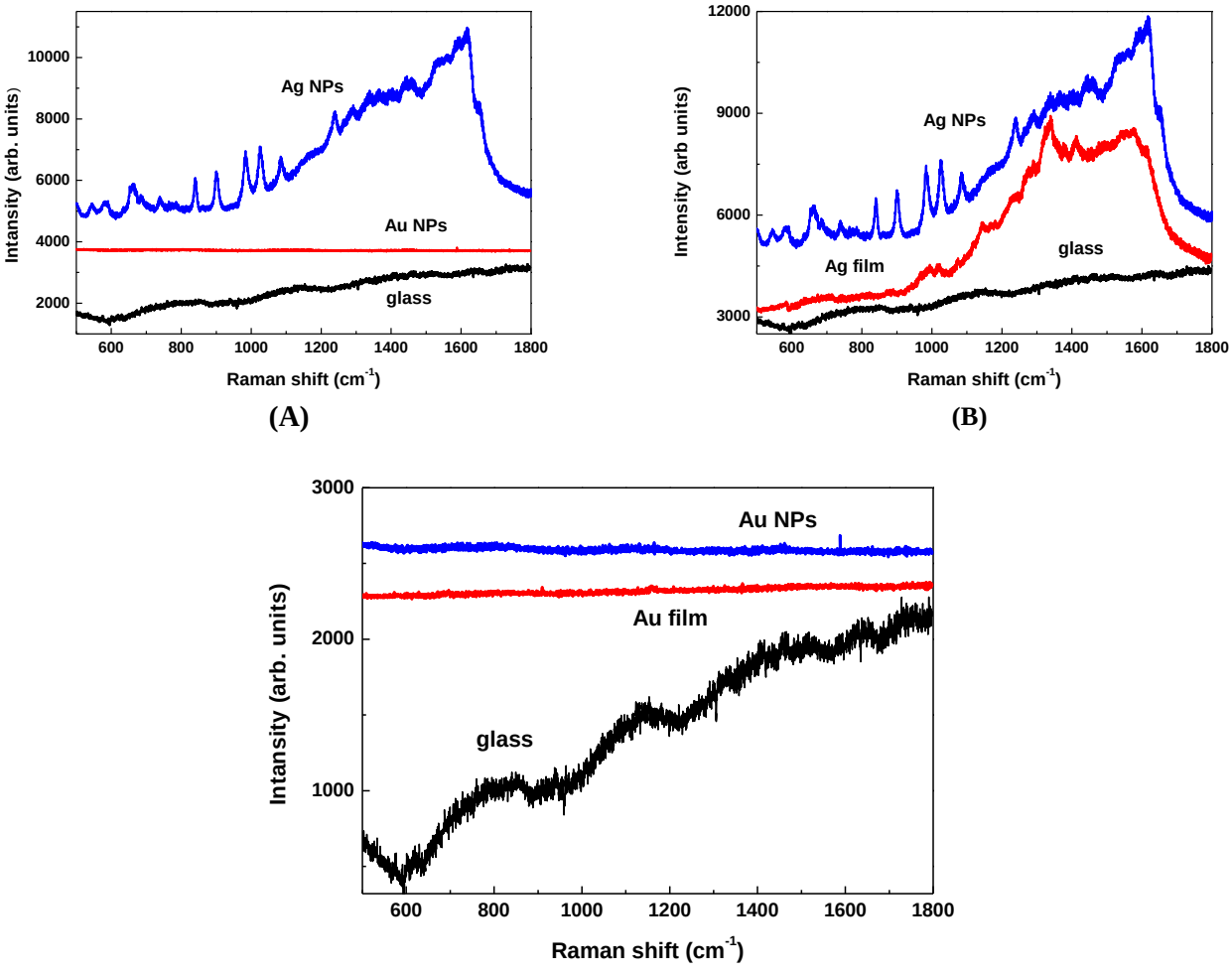


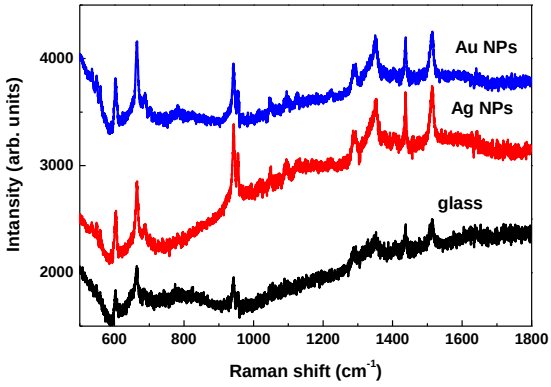
FIG. 4 μ-RAMAN SPECTRA OF AKTARA 25 (thiamethoxam) DEPOSITED ON: A) Ag and Au NPs and glass; B) Ag NPs and Ag film and glass; C) Au NPs and Au film and glass. Note that the concentration of the analyte deposited on glass is much higher compared to that deposited on Ag, Au films or NPs areas – see Table 1.

TABLE 2
SUMMARY OF THE VIBRATION MODES OBSERVED (cm^{-1}) of Aktara 25 (thiamethoxam). Relative intensity: vs – very strong; s – strong; m – middle; w – weak; vw – very weak; vwb – very week and broad.

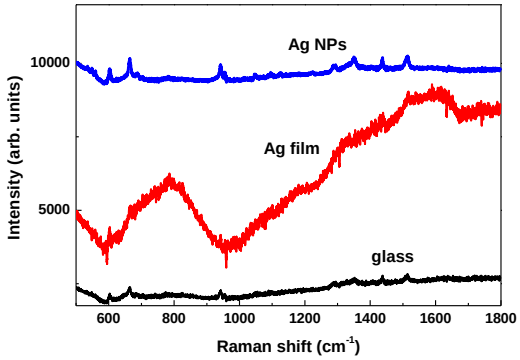
SERS Ag NPs, cm^{-1}	SERS Ag film, cm^{-1}	μ-Raman spectrum, Glass, cm^{-1}
545, m	-	-
583, m	583, w	576, vwb
590, m	-	-
634, vw	634, vw	629, vwb
654, 663, m	663, vw	-
684, w	684, vw	-
738, w	-	-
840, vs	-	-
901, vs	-	-
983, vs	992, vw	-
1026, vs	1026, vw	-
-	1074, w	-
1084, s	-	-
-	1143, w	1144, vwb
1238, s	1238, vw	-
1290, w	1290, w	-
1339, w	1339, s	-
1366, vw	-	-
1413, vw	1413, m	1413, vwb
1443, w	1443, vw	-
1458, w	1458, vw	1458, vwb
1593, vw	-	-
1619, m	1619, w	-
1651, w	-	-

Regarding the results obtained, we did not reach the lower limit of detection. However, it is evaluated to be <6 ng of Aktara (thiamethoxam), as a result of the enhancement caused by the plasmon resonance in Ag NPs.

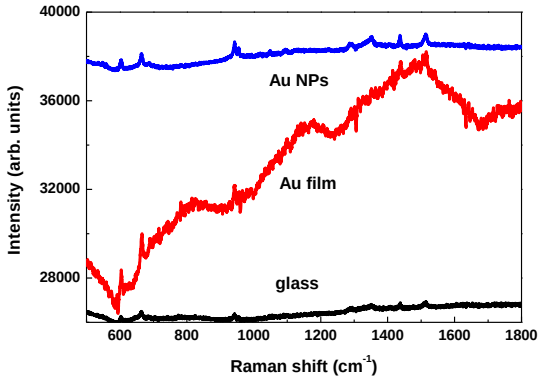
Au and Ag NPs, thin films and glass samples were also used for μ-Raman spectrometry analyses of the Dithane DG fungicide. Figs. 5 a-c show μ-Raman spectra of Dithane DG (mancozeb). As is seen, the enhancement in the case of Ag NPs was slightly higher than that of the Au NPs, while the Ag and Au films' SERS spectra had the same bands with a reduced intensity. It is worth noting that some shallow peaks appeared in the Raman spectrum when Dithan DG was deposited on glass. However, the concentration of the analyte there was much higher – more than three orders of magnitude (see Table 1). The characteristics of the SERS spectra originating from the Ag and Au NPs, the Ag and Au films, and the μ-Raman spectra of the glass substrate samples, are summarized in Table 3. The salient parameters of the Fourier transform Raman and SERS spectra (denoted by an asterisk) of mancozeb on a Ag colloid from Ref. [29] are also given for comparison. As is seen (Fig. 5 a and Table 3), the strongest six lines in the Ag NPs and Au NPs SERS spectra were: 603 cm^{-1}, 665 cm^{-1}, 942 cm^{-1}, 1350 cm^{-1}, 1438 cm^{-1} and 1514 cm^{-1}; the line at 1286 cm^{-1} was of intermediate intensity. They completely coincided with those reported in Ref. [29]. However, they were present in the Fourier transform Raman spectrum and missing in the SERS. The authors connect this effect with the degradation of the mancozeb on the Ag surface into the colloid used. In contrast, in our case the analyte was in a solid form on the surface of the active substrates, so that it probably did not degrade sufficiently. This was why the bands observed by us were visible in the SERS spectra with a high intensity. It is also worth noting that all seven bands, but with a lower intensity, were present in the μ-Raman spectrum of the analyte deposited on glass, or in the Ag and Au films' SERS spectra. The enhancement in the case of the Au film was very weak, as illustrated by the shallow plasmon resonance recorded (Fig. 3 c).



(A)



(B)



(C)

FIG. 5 μ-Raman spectra of Dithane DG (mancozeb) deposited on: A) Ag and Au NPs and glass; B) Ag NPs and Ag film and glass; C) Au NPs and Au film and glass. Note that the concentration of the analyte deposited on glass is much higher – see Table 1.

TABLE 3

SUMMARY OF THE VIBRATION MODES OBSERVED (cm^{-1}) of Dithane DG (mancozeb). Relative intensity: vs – very strong; s – strong; m – middle; w – weak; vw – very weak. All bands given in the last column are recorded by Fourier transform Raman spectroscopy; those marked by * are SERS [29].

SERS Ag NPs, cm^{-1}	SERS Ag film, cm^{-1}	SERS Au NPs, cm^{-1}	SERS Au film, cm^{-1}	μ-Raman spectrum, Glass, cm^{-1}	Ref. [29]
536, w	536, vw	536, vw	-	536, vw	-
546, w	-	546, vw	-	546, vw	-
560, w	560, vw	560, vw	-	560, vw	-
603, s	603, m	603, s	603, w	604, m	603, s
665, vs	665, w	665, vs	666, w	665, m	665, vs
689, w	689, vw	689, w	-	689, vw	-
783, vw	783, vw	783, vw	783, vw	783, vw	-
942, vs	942, m	942, vs	943, vw	942, m	945, vs
953, m	953, w	953, w	-	953, w	954, m*
1048, w	1048, w	1048, w	-	1048, vw	1051, m
1095, w	1095, vw	1095, w	-	1095, vw	-
1286, 1293, m	1286, vw	1286, 1293, m	-	1286, 1293, w	1292, s; 1282, m*
1350, vs	-	1350, s	-	1350, w	1351, s
1438, vs	1438, w	1438, vs	1438, w	1438, m	1438, s
1514, vs	1514, m	1514, s	1515, w	1514, m	1515, s

Based on the above results, several vibrations could be identified in the SERS spectra. The strong bands in the 600-700 cm^{-1} region, i.e. 603 cm^{-1} and 665 cm^{-1}, can be attributed to the different interactions of Zn and Mn with the CSS group [29]. The most intensive bands at 1514 cm^{-1} and 1438 cm^{-1} can be attributed to the (C=N) stretching coupled with deformation (NH) and (CH_2) vibrations [24,29]. The band monitored at 1286 cm^{-1} having intermediate intensity is probably connected to the NH motion [29]. Moreover, several peaks between 900 cm^{-1} and 1050 cm^{-1}, including the strongest bands at 942 cm^{-1} and 1048 cm^{-1}, correspond possibly to the stretching (C=S) motion. It could also be affected by the simultaneous presence of the bidentate and monodentate complex on the Ag or Au surfaces of the active substrates [29].

Finally, the limit of mancozeb detection was evaluated to be <60 ng for the Ag and Au NPs. However, the μ-Raman and SERS spectra indicated that this was not the lowest possible detection limit.

IV. CONCLUSION

The results obtained can be summarized as follow:

- Laser annealing of Au and Ag films on quartz substrates resulted in areas containing nanoparticles. The size of the Au NPs exhibited a quite narrow distribution with a maximum at ~22 nm; that of the Ag NPs was much broader with two maximums at ~10 nm and 30÷55 nm. A plasmon resonance was very well pronounced in both cases, but was much stronger in the case of Ag NPs. Additionally, a plasmon resonance with the same intensity as for the Ag NPs, although much wider, was also seen in the case of Ag films;

- To the best of our knowledge, for the first time a strong enhancement of the μ-Raman spectra was detected in the case of Aktara 25 BG deposited on the Ag NPs area caused by plasmon resonance in Ag NPs; thus, the minimum detectable Aktara (thiamethoxam) amount was estimated to be <6 ng.

- A strong enhancement of the μ-Raman spectra was registered in the case of Dithane DG deposited on Au NPs and Ag NPs areas, as compared with the case of Dithane DG deposited on glass, its concentration being much higher in the latter case notwithstanding. The limit of detection of mancozeb achieved based on μ-Raman SERS was evaluated to be <60 ng for Ag NPs or Au NPs, although there were indications that this is not the lowest limit possible.

Further improvement of the active substrates is needed in order to increase the sensitivity of the SERS analyses.

The experimental results reported are very promising and demonstrate a great potential for application as an additional and competitive method for analyses compatible with the existing chemical methods. Moreover, suitable and enhanced Ag or Au nanostructures of improved properties must be produced to improve the interaction with such molecules. This will be the subject of further investigations aimed at obtaining the higher sensitivity required for SERS analytical applications.

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Effects of Bradyrhizobia and Phosphate-solubilizing bacteria on soybean (*Glycine max* L. Merrill) cultivated on Ferrasols of Cujut district, DakNong province, Vietnam

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Abstract— *A field experiment was conducted in Summer-Spring cropping season 2016 at Cujut district, DakNong province, Vietnam to study the effects of rhizobia and phosphate-solubilizing bacteria (PSB) on soybean (cv. Cujut) cultivated on ferralsols. The experiment consisted of six treatments as follows: control (no fertilizer, no inoculant), 240 kg/ha NPK 15-15-15, rhizobial inoculant [with liquid cover seeds] + 20 kg N/ha applied at 10 days after sowing [DAS], PSB inoculant [with liquid cover seeds] + 20 kg N/ha at 10 DAS, rhizobial and PSB inoculant [with liquid cover seeds] + 400 kg fertilizer/ha + 20 kg N/ha at 10 DAS and endophytic bacteria inoculant [with liquid cover seeds] + 400 kg fertilizer/ha + 20 kg N/ha at 10 DAS from June to August, 2016. The results showed that application of rhizobial inoculant and/or PSB inoculant produced significantly higher yield component, grain yield than control and did not differ from 240 kg/ha NPK 15-15-15. Consequently, application of rhizobia and PSB improved soil fertility after harversting however using mixture of rhizobia and PSB inoculation plus 400 kg biofertilizer/ha +20 kg N/ha for soybean cultivation supported yield component, grain yield and oil, protein in seed than control and equivalent with treatment of chemical fertilizer (240 kg/ha NPK 15-15-15). This technique not only increased grain yield, incomes for farmers but also improved soil fertility.*

Keywords - Endophytes, Ferralsols, Phosphate-solubilizing bacteria, Rhizobia, Soybean.

I. INTRODUCTION

Symbiotic nitrogen fixation, a key component in biological nitrogen fixation, has not been as successful in substituting for chemical fertilizer as initially expected. Rhizobial inoculants seem to be an attractive and cost effective source of N for soybean cultivation in the Mekong Delta, Vietnam [1]. Phosphorus plays an important role in the plant's energy transfer system since phosphorus deficiency retards growth and tillering [2]. In soil, phosphorus is quite abundant but it reacts readily with iron, aluminum and calcium to form insolubly compounds. These reactions results in very low phosphorus avability and low efficiency phosphorus fertilizer used by plants [3].

Soybean (*Glycine max*) is one of the most important oil seed crop in the world. It contains 18 to 22% oil, highly desirable in diet and have 40 to 42% of good quality protein [2]; Soybean protein is rich in valuable amino acid lycine (5%) in which most of the cereals are deficient [4]. Soybean, like other legumes, fixes atmospheric nitrogen in association with gram-negative soil bacteria of the genera *Bradyrhizobium* and *Sinorhizobium* [5][6]. Many rhizobial inoculant products have been applied for soybean cultivation for along time [7]. However there were many researches showed that many PGPR as PSB supported good nodulation and rhizobia-legume symbiosis [8][9], the results led to high grain yield and protein content in seeds [10]. The biofertilizer (consisted of rhizobia and PSB) was not only as well as soybean grain yield applying with 100 kg N and 60 kg P_2O_5/ha but also quality soybean seed [protein and lipid content in seed] was higher than soybean seed using of chemical fertilizers at Dong Thap province, Mekong Delta, Vietnam [11].

DakNong province is situated in the highland of Vietnam, it locates from 107°42'03"to 107°44'44" E and from 11°59'01" to 12°40'56" N and CuJut is a district of DakNong province, it locates the north of province (Figure 1) [DakNong province locates in Central High Land of Vietnam]. The soils are mainly red latosols (from origin of volcanic mountain) or ferralsols (FAO classification) with a pH range of 4.61 - 4.91. They are considered a good nutrient, with an average organic matter of 2.75 – 4.06%, a total nitrogen range of 0.11 – 0.13%, but it has concentrations of low available phosphorus, cation exchange

capacity, exchangeable K [12](WASI, 2014). Many kinds of crop such as rubber, coffee, pepper, upland-rice, corn and soybean have been cultivated on ferralsols permanently.

In this study, selected bradyrhizobia strain CJ02 [13] and PSB strain S31 [14] were evaluated on yield component, grain yield and soil characteristics [after harvesting] and biofertilizer technology has taken a part to minimize production costs with granule fertilizer which suitable for soybean cultivation mechanise.

II. MATERIAL AND METHOD

A field experiment was conducted at Nam Dong village, CuJut district, DakNong province in Summer-Autumn cropping-season 2016 (June to August).

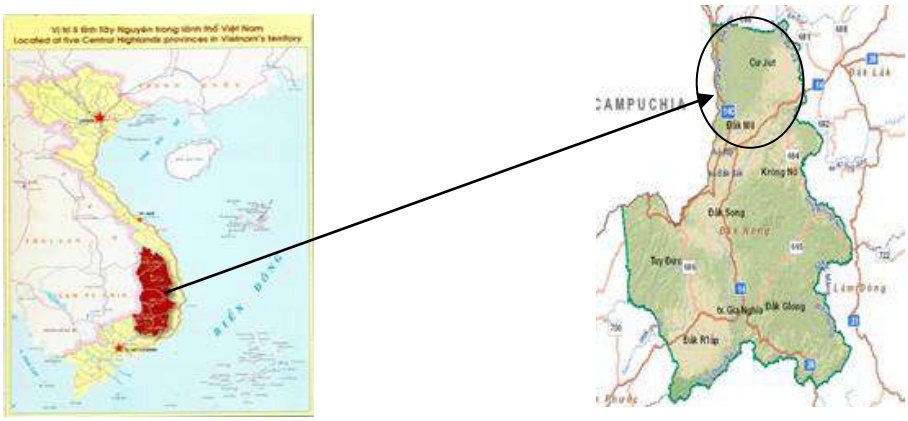


FIGURE 1. THE LOCATION WAS EXAMINED IN THIS STUDY - CUJUT DISTRICT (DAKNONG PROVINCE) [WITH DARK BLUE] AND FERRALSOLS WERE PRESENTED SOILS WITH REDDISH BROWN LATOSOLS AND RED & BROWN LATOSOLS

2.1 Soil characteristics

The soil was ferralsols (or red latosol) in pH of 4.64, low in organic matter (3.575%), nitrogen total (0.135%) and available P_2O_5 (8.177 mg/kg) in the first experiment and pH=5.42, organic matter (3.822%), N total (0.137%) and available P_2O_5 (6.322 mg/kg) in the second experiment (Origin: Soil analysis Lab., Institute of Western Agriculture-Forestry Science [WASI]).

2.2 Rhizobial and PSB inoculant, biofertilizer

Rhizobia and PSB strains: *Bradyrhizobium japonicum* strain CJ02 [13] was produced in YEM broth in 4 days, reached to $>10^9$ cell/ml and *Burkholderia* sp. S31 strain [15] was produced in NBRIP broth in 2 days reached to $>10^9$ cell/ml.

Biofertilizer consisted of organic matter (35%), thermophosphate (15% P_2O_5) (5%) + Dolomite (0.5% P_2O_5, 50% $CaCO_3$, 10% $MgCl_2$)[dolomite is by-product of cement factory](45%), ground black rice-hull ash (15%) and PSB liquid at moisture 25% and the mixture was made to granule with size 5-7 mm diameter (Figure 2).

2.3 Experimental design

The experiment was arranged with completely block randomized design with four replications; each plot was a treatment with 20 square meter (4x5 m) (Figure 3), total was 24 plots. Weed control two times (20 and 40 DAS), pest control according to the guide of Department of Plant Protection, DakNong province, the experiment was watered by rainy. The experiment had six treatments as follows control (no fertilizer, no inoculant) [Treatment 1], 240 kg/ha NPK 15-15-15 without inoculation [Treatment 2], rhizobial inoculant [with liquid cover seeds] + 20 kg N/ha applied at 10 days after sowing [DAS] [Treatment 3], PSB inoculant [with liquid cover seeds] + 20 kg N/ha at 10 DAS [Treatment 4], rhizobial and PSB inoculant [with liquid cover seeds] + 20 kg N/ha at 10 DAS + 400 kg biofertilizer [Treatment 5], Endophytic bacteria [15][with liquid cover seeds] + 400 kg biofertilizer + 20 kg N/ha at 10 DAS [Treatment 6].

FIGURE 2. BIOFERTILIZER (GRANULE)

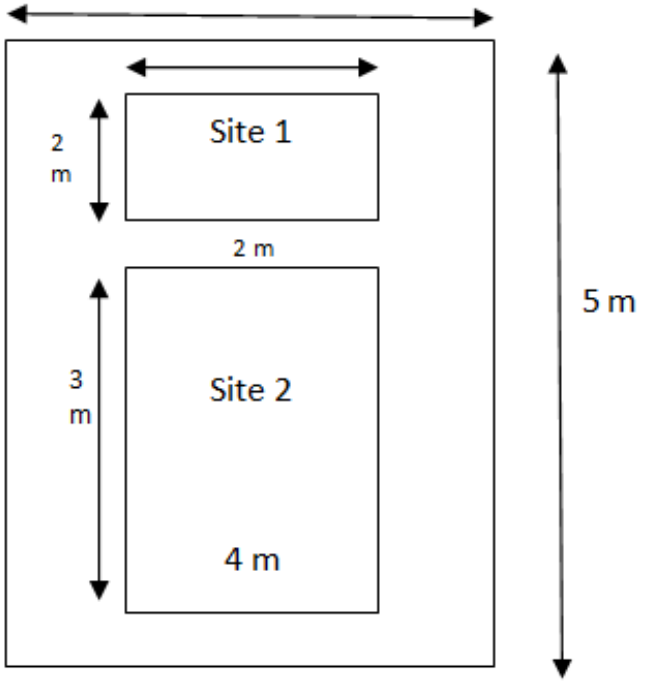


FIGURE 3. LAYOUT OF 1 PLOT WITH SITE 1 AND SITE 2

2.4 Yield component and Grain yield

Plant height and yield component were recorded at the time of maturity. Five randomly plants were taken at site 1 (Figure 3) to measure plant height, number of pods/plant, hundred seed weight. All plants in site 2 (Figure 3) were harvested to calculate grain yield after soybean seeds were oven dried at 70^{o}C for constant weight. Soybean seed was also chosen to ground for determination of total nitrogen and oil contents.

2.5 Estimation of protein and oil

Total nitrogen content of soybean seed was determined by Micro-Kjeldahl method as recommended by AOAC, 1975)[16]. Nitrogen contents were multiplied by dry matter-based factor 5.71 to determined total protein content [17] and Oil content of soybean seeds was estimated by adopting Soxhlet Ether Extraction method [17].

2.6 Soil analysis

After harvesting, soil samples of each treament were collected to analyse as soil pH with pH meter, N total by Micro-Kjeldahl method, Available P_2O_5 by Colorimetric method [18], Organic matter with Walkley-Black method.

2.7 Statistical Analysis

All the data pertaining to the present investigation were statiscally analyzed as per the method described by Gomez and Gomez [19]. The stastistically significance of various effects was tested at 5 per cent level of probability.

III. RESULTS AND DISCUSSION

3.1 Effect of Rhizobia and PSB on Nodule number and Shoot Dry Weight (DW)

Inoculated rhizobia on soybean seeds enhanced nodule number/plant however nodule number/plant of control and PSB together with endophytic bacteria treatments also appeared (Table 1), this showed that high native rhizobia population in soil and they infected into young soybean roots and formed the first nodules at main root in Treat 1 and Treat 2. (Figure 4).

TABLE 1
EFFECTS OF RHIZOBIA, PSB, ENDOPHYTIC BACTERIA AND MINERAL FERTILIZERS ON NODULATION AND DRW WEIGHT (DW) OF SHOOT OF SOYBEAN (CV. CUJUT) CULTIVATED ON FERRALSOLS OF CUJUT DISTRICT, DAKNONG PROVINCE IN SUMMER-SPRING 2016

Treatment	Nodule number / plant *	DW of nodule /plant (mg) *	DW of shoot/plant (gr) *
Treat. 1	11.35 d	44.35 d	3.429 b
Treat. 2	13.43 c	44.54 d	3.430 b
Treat. 3	15.30 b	52.35 c	**4.240 a**
Treat. 4	18.33 a	**91.81 a**	**4.007 a**
Treat. 5	18.25 a	64.42 b	**4.230 a**
Treat. 6	17.20 a	68.63 b	3.260 b
F calculated	**	**	**
C.V (%)	**8.04**	**9.28**	**6.19**

Treat.1: *control (no fertilizer and without inoculation),* ***Treat. 2:*** *Application 240 kg NPK (15-15-15)/ha,* ***Treat 3:*** *Rhizobial Inoculation + 20 kg N/ha,* ***Treat. 4:*** *PSB Inoculation + 20 kg N/ha,* ***Treat. 5****: Rhizobial + PSB Inoculation + 400 kg biofertilizer (granule)/ha + 20 kg N/ha;* ***Treat. 6:*** *Endophytic bacteria inoculation + 400 kg biofertilizer (granule)/ha + 20 kg N/ha.*

**The numbers followed by the same letter do not differ at 1% level significantly*

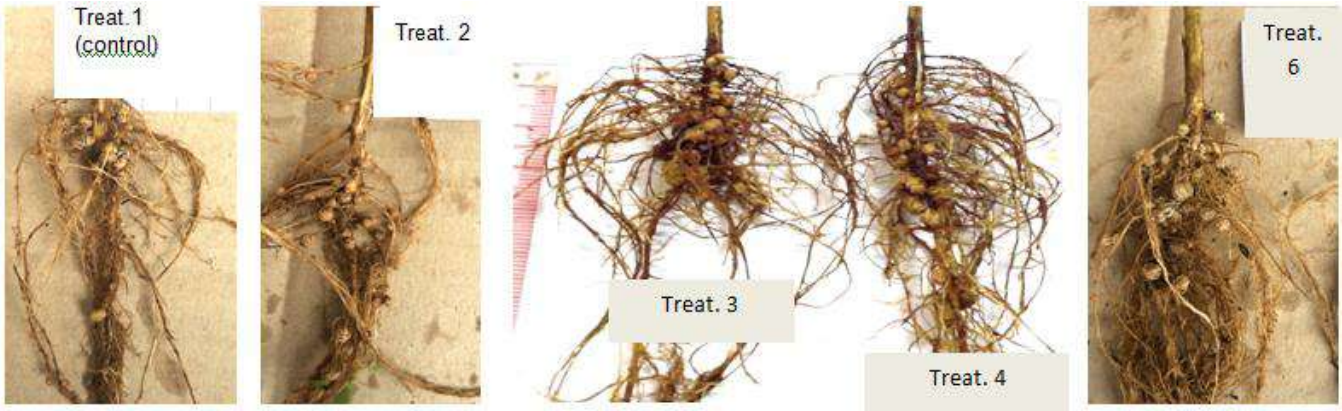


FIGURE 4. EFFECTS OF RHIZOBIAL, PSB AND ENDOPHYTIC BACTERIA ON SOYBEAN NODULATION OF SOYBEAN CULTIVATED ON FERRALSOLS

PSB inoculated on soybean seeds before sowing had the highest DW of nodule/plant in comparison to rhizobial and mixture of rhizobia and PSB treatments, this result showed that phosphorus requirement in nodulation and development of nodule was important in this stage however no difference about the growth of soybean plant (DW of shoot) between these three treatments while endophytic bacteria did not affect on DW of shoot of soybean (Table 1)

Interestingly, DW of nodule root had correlation with DW of shoot (Figure 5) at 5% level, this showed that the development of nodule or the effectiveness of rhizobia affected to the growth of soybean plant.

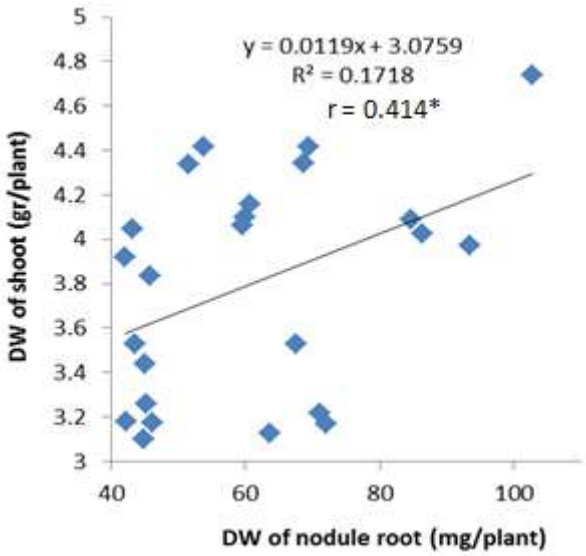


FIGURE 5. THE CORRELATION BETWEEN DW OF NODULE ROOT (mg/plant) AND DW OF SHOOT (gr/plant)

The result from Table 2 showed that inoculation of rhizobia, PSB, Endophytic bacteria and chemical fertilizers did not affect to plant height, effective branche number/plant and 100-seed weigh of soybean however these factors influenced to effective pod number/plant and total of pod/plant especially in the treatments of chemical fertilizers (Treat. 2) and rhizobial inoculant (Treat. 3).

TABLE 2

EFFECTS OF RHIZOBIA, PSB, ENDOPHYTIC BACTERIA AND MINERAL FERTILIZERS ON YIELD COMPONENT OF SOYBEAN (CV. CUJUT) CULTIVATED ON FERRALSOLS OF CUJUT DISTRICT, DAKNONG PROVINCE IN SUMMER-SPRING 2016.

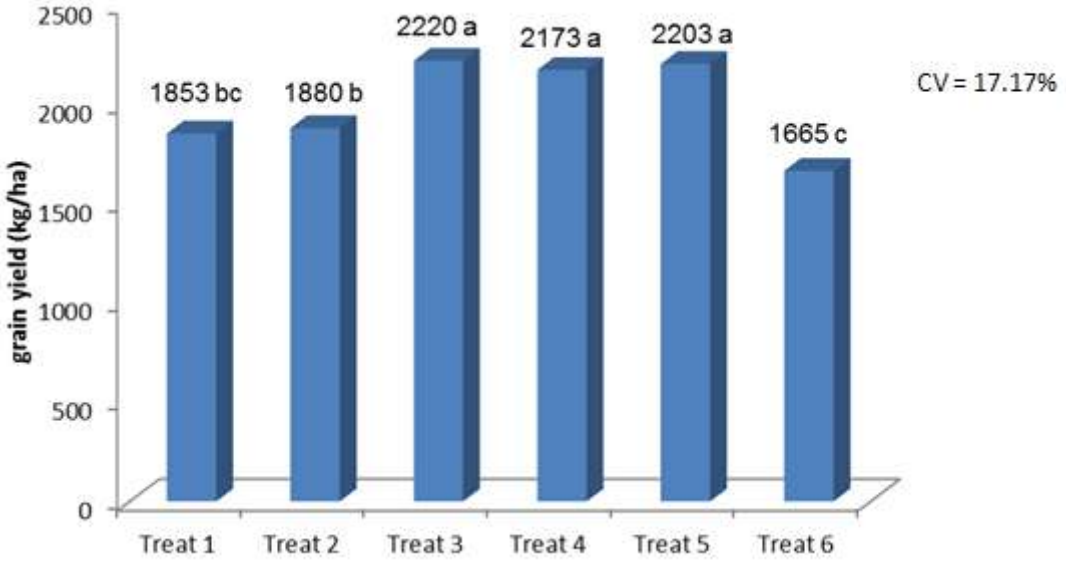
Treatment	Plant height (cm)	Effective branche number/plant	Effective Pod number/plant	Total of pod / plant	100-seed weigh (gr)
Treat. 1	75.75	1.60	8.10 a	20.35 b	16.58
Treat. 2	77.15	1.85	7.75 ab	22.68 ab	17.96
Treat. 3	77.75	2.10	7.95 a	24.55 a	17.02
Treat. 4	76.45	1.80	8.05 a	21.93 b	16.34
Treat. 5	74.70	1.50	7.45 b	21.95 b	16.86
Treat. 6	68.90	1.50	7.05 bc	17.87 c	15.89
F calculated	**n.s**	**n.s**	*****	******	**n.s**
C.V (%)	**8.76**	**26.43**	**5.51**	**7.22**	**7.07**

n.s : not significant

***Treat.1:** control (no fertilizer and without inoculation), **Treat. 2:** Application 240 kg NPK (15-15-15)/ha, **Treat 3:** Rhizobial Inoculation + 20 kg N/ha, **Treat. 4:** PSB Inoculation + 20 kg N/ha, **Treat. 5**: Rhizobial + PSB Inoculation + 400 kg biofertilizer (granule)/ha + 20 kg N/ha; **Treat. 6:** Endophytic bacteria inoculation + 400 kg biofertilizer (granule)/ha + 20 kg N/ha.*

**The numbers followed by the same letter do not differ at 1% level significantly*

These yield components affected to grain yield (Figure 6) and these treatments as rhizobial inoculation (Treat. 3), PSB inoculation (Treat. 4) and mixture of rhizobial and PSB inoculation (Treat. 5) had the highest grain yield and they differed from other treatments significantly (Figure 6).



Means within a column follwed the same letter/s are not significantly diferrent at p<0.05

Treat.1: *control (no fertilizer and without inoculation),* ***Treat. 2:*** *Application 240 kg NPK (15-15-15)/ha,* ***Treat 3:*** *Rhizobial Inoculation + 20 kg N/ha,* ***Treat. 4:*** *PSB Inoculation + 20 kg N/ha,* ***Treat. 5****: Rhizobial + PSB Inoculation + 400 kg biofertilizer (granule)/ha + 20 kg N/ha;* ***Treat. 6:*** *Endophytic bacteria inoculation + 400 kg biofertilizer (granule)/ha + 20 kg N/ha.*

FIGURE 6. EFFECTS OF RHIZOBIA, PSB, ENDOPHYTIC BACTERIA AND CHEMICAL FERTILIZERS ON GRAIN YIELD (KG/HA) OF SOYBEAN (CV. CUJUT) CULTIVATED ON FERRASOLS OF CUJUT DISTRICT, DAKNONG PROVINCE, VIETNAM IN SUMMER-SPRING CROPPING-SEASON 2016

Grain yield of chemical fertilizer treatment (Treat. 2) did not differ significantly with grain yield of control treatment (Treat. 1) therefore application chemical fertilizers for soybean cultivation in this ferrasols (Cujut district) had no efficiency while application of endophytic bacteria had the lowest grain yield. DW of soybean shoot had close correlation very significantly with pod number/plant (Figure 7) and grain yield (Figure 8) while DW of nodule root had correlation with DW of shoot at different <0.05, therefore DW of nodule root had correlation with pod number/plant and grain yield indirectly.

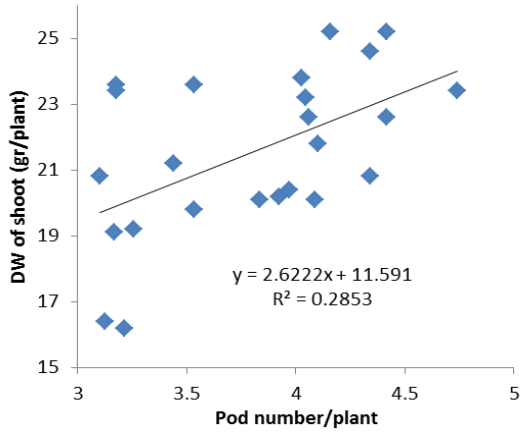


FIGURE 7. CORRELATION BETWEEN DW OF SHOOT (gr/plant) WITH POD NUMBER/PLANT

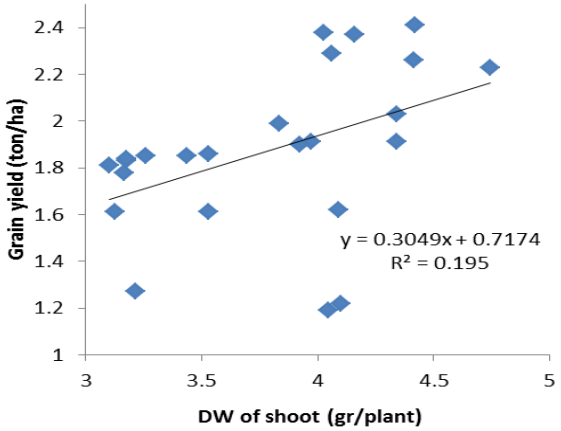


FIGURE 8. CORRELATION BETWEEN DW OF SHOOT (gr/plant) WITH GRAIN YIELD (ton/ha)

Application of chemical fertilizer (Treat. 2) and rhizobial and PSB inoculation (Treat. 5) for soybean cultivation increased protein content in seed but total of protein in seed/ha of treat. 2 was lower than total of protein of treat. 5 because grain yield of this treatment was low, this lead to low total of protein of treat. 2 (Table 3); Protein content in seed of treatments of rhizobial inoculation (Treat. 3) and PSB inoculation (Treat. 4) were lower than protein content in seed of treat. 2 and treat. 5 and total of protein/ha of two treatments were also lower than treat. 5 however lipid content in seed of these two treatments were higher than and this led to total of lipid in seed/ha of two treatments were the highest while endophytic bacteria

inoculation for soybean seed before sowing did not enhance yield component, grain yield, protein centent in seed but increasing lipid content in seed (Table 3).

TABLE 3

EFFECTS OF RHIZOBIA, PSB, ENDOPHYTIC BACTERIA AND MINERAL FERTILIZERS ON PROTEIN CONTENT, LIPID CONTENT IN SOYBEAN SEED AND TOTAL OF PROTEIN AND TOTAL OF LIPID IN SOYBEAN SEED/HA CULTIVATED FERRALSOLS OF CUJUT DISTRICT, DAKNONG PROVINCE IN SUMMER-SPRING CROPPING-SEASON 2016

Treatment	Protein content in seed (%)	Total of protein in seed /ha (kg/ha) *	Lipid content in seed (%)	Total of lipid in seed/ ha (kg/ha)
Control (no fertilizer, without inoculation	29.16 c	53.995 d	20.841 c	38.607 b
240 kg NPK (15-15-15)/ha without inoculation	32.34 a	60.841 c	20.512 c	38.554 b
Rhizobial inoculation, 20 kg N/ha	30.88 b	68.424 b	21.600 b	47.917 a
PSB inoculation, 20 kg N/ha	30.12 b	65.469 b	21.408 b	46.495 a
Rhizobial + PSB inoculation + 400 kg biofertilizer + 20 kg N/ha	33.13 a	72.964 a	20.686 c	45.560 a
Endophytic bacteria inoculation + 400 kg biofertilizer + 20 kg N/ha	28.32 d	47.205 e	22.144 a	36.888 b
F calculated	**	**	**	**
C.V (%)	**3.29**	**7.03**	**2.25**	**6.60**

Total protein in seed/ha = protein content in content (%) x grain yield

****Total Lipid in seed/ha = lipid content in content (%) x grain yield***

+The numbers followed by the same letter do not differ at 1% level significantly

Soybean cultivation on ferrasols improves soil pH and organic matter in soil but decreased N total in soil perhaps requirement of soybean plant use a big amount of nitrogen and available phosphorus for the development of shoot, root and pod especially soybean seed (contains more than 34% protein) (Table 4). Application of chemical fertilizer in soybean cultivation supported soybean plants and enhanced soil pH, chemical parameters as N total, P available and organic matter in soil however rhizobial inoculation (Treat. 3), PSB inoculation (Treat. 4) and mixture of rhizobia and PSB inoculation (Treat. 5) also had good effectives as chemical fertilizer treatment (Treat. 2) while endophytic bacteria inoculation (Treat. 6) only improper soil pH and N total.

TABLE 4

EFFECTS OF RHIZOBIA, PSB, ENDOPHYTIC BACTERIA AND MINERAL FERTILIZERS ON PH AND CHARACTERISTICS OF FERRALSOLS BEFORE AND AFTER CULTIVATED SOYBEAN.

Treatment	pH	N total (%)	P available (mg P_2O_5/soil kg)	Organic Matter (%)
Initial	4.91 c	0.141 bc	30.98 cd	2.36 c
Control (no fertilizer, without inoculation	5.87 ab	0.135 c	28.33 d	2.53 b
240 kg NPK (15-15-15)/ha without inoculation	5.96 a	0.160 ab	38.67 ab	2.71 ab
Rhizobial inoculation, 20 kg N/ha	5.89 ab	0.178 a	37.45 ab	2.69 ab
PSB inoculation, 20 kg N/ha	5.87 ab	0.155 b	36.40 b	2.83 a
Rhizobial + PSB inoculation + 400 kg biofertilizer + 20 kg N/ha	5.93 a	0.160 ab	40.19 a	2.62 b
Endophytic bacteria inoculation + 400 kg biofertilizer + 20 kg N/ha	5.89 ab	0.165 ab	32.79 c	2.39 c
F calculated	**	**	**	**
C.V (%)	**1.38**	**0.079**	**13.62**	**5.24**

The numbers followed by the same letter do not differ at 1% level significantly

3.2 Economical efficiency

Based on grain yield, application of NPK (240 kg 15-15-15) in soybean cultivation did not differ from control (no fertilizer, without inoculation) while rhizobia inoculation plus 20 kg N/ha or PSB inoculation plus 20 kg N/ha and mixture of rhizobia and PSB inoculation plus 400 kg biofertilizer and 20 kg N/ha enhanced grain yield from 367 kg, 320 kg and 350 kg soybean seed/ha, respectively in comparison to control (Table 5) and farmers earned income 12,845,000; 11,200,000 and 12,250,000 VND, respectively while they must pay to cost of fertilizer as 367,000; 347,000 and 762,000 VND, respectively.

TABLE 5
ECONOMICAL EFFICIENCY IN SOYBEAN CULTIVATION WITH CHEMICAL FERTILIZERS, RHIZOBIA, PSB, ENDOPHYTIC BACTERIA INOCULATION

Treatment	Grain yield (kg/ha)	Enhanced seed weigh* (kg/ha)	Soybean seed price (35,000 VND/kg)	Cost of fertilizer** (for 1 ha)	Benefit income (VND/ha)
Control (no fertilizer, without inoculation	1853	0	0	0	
240 kg NPK (15-15-15)/ha without inoculation	1880	27	945,000	1,680,000	- 735,000
Rhizobial inoculation[1], 20 kg N/ha	2220	367	12,845,000	367,390	12,478,000
PSB inoculation[1], 20 kg N/ha	2173	320	11,200,000	347,390	10,852,610
Rhizobial[2] + PSB inoculation[2] + 400 kg biofertilizer + 20 kg N/ha	2203	350	12,250,000	762,390	11,487,610
Endophytic bacteria inoculation[3] + 400 kg biofertilizer + 20 kg N/ha	1665	-188	0	767,390	0

**enhanced seed weigh = grain yield (treat) – grain yield (control)*
*** 1 kg NPK = 7,000 VND/kg, 1 litre rhizobia liquid, endophytic bacteria = 50,000 VND, 1 litre PSB liquid = 25,000 VND*
20 N = 43,3 kg urea x 7,300 VND/kg
15 litres of rhizobial inoculant/ha, 22.5 litres rhizobial liquid and 2.5 PSB liquid, 35 litres of endophytic bacteria inoculant/ha,
1 kg biofertilizer = 1,000 VND

Low soil pH and N total are characteristics of ferralsols [20]; while concentration of available P_2O_5 and organic matter in ferralsols depended on cultural practices. Besides N requirement for soybean growth through nitrogen biological fixation, soybean plants require many other nutrients as phosphorus and P had quite prominent effects on nodulation, growth and yield parameters [21]. Many soils throughout the world are P-deficient because the free phosphorus concentration (the form available to plants) even the fertile soil is generally not higher than 10 µM even at pH 6.5 where it is most soluble [22]. To circumvent the problem of P deficiency, chemical fertilizers are added to the soils but cost of chemical phosphatic fertilizers is high [23] and low efficiency (<0.1%) [24]. Phosphorus biofertilizers in the form of microorganisms, especially phosphate-solubilizing bacteria in rhizosphere, can help in increasing the availability of accumulated phosphates for plant growth by solubilization [25][26].

Application of phosphorus along with PSB improved phosphorus uptake by plants and yields indicating that the PSB are able to solubilize phosphates and to mobilize phosphorus in crop plants [27]. PSB enhanced the phosphorus availability to plants by mineralizing organic P in soil and by solubilizing precipitated phosphate [28][29][30]. Dubey et al. [31] have also reported significant increased in grain yield of soybean due to co-inoculation of phosphorus solubilizers. Our results showed that application biofertilizer (mixture of thermophosphate and PSB) for soybean cultivation had high grain yield, protein yield and oil yield as applying NPK or inorganic fertilizer without inoculation [14]. PSB also are capable of transforming soil phosphorus to the forms available to plant and oil and protein yield were also maximum with PSB inoculant or biofertilizer. It is reported that soybean inoculated by *Bradyrhizobium* bacteria and phosphate solubilizing bacteria increased the seed yield [32][33]. Rana et al. [4] calculated that the highest B:C ratio (1:39) were obtained from the crop sown with 45 kg P_2O_5/ha, *Rhizobium* and phosphorus solubilizing bacteria followed by the crop sown with 60 kg P_2O_5/ha, *Rhizobium* and phosphorus solubilizing bacteria and therefore saving of 15 kg P_2O_5/ha; our results also recognized mixture rhizobia and PSB inoculants + 400 kg biofertilizer and 20 kg N/ha had grain yield, oil, protein in seed was equivalent with treament of 100 kg/ha thermophosphate (15% P_2O_5) + 25 kg/ha NPK 16-16-ha for soybean cultivation on ferralsols of DakLak province [14].

In the Mekong Delta, Son et al. [34] reported that application of *Bradyrhizobium japonicum* and PSB *Pseudomonas* spp. can enhance the number of nodules, dry weight of nodules, yield components, grain yield, soil nutrient availability and uptake of soybean crop. This result showed that application of rhizobia or/and PSB inoculation in soybean cultivation on ferralsols of Cujut district, DakNong province not also enhanced yield component, grain yield, incomes but only improved soil fertility.

IV. CONCLUSION

Rhizobial inoculation or/and Phosphate-solubilizing bacteria inoculation are good, cheap, effective techniques in soybean cultivation on ferralsols of Cujut district, DakNong province. These techniques not only increased grain yield, incomes for farmers but also improved soil fertility.

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Renewable energy resource of Sri Lanka! A review

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Abstract— *Energy and related sectors play a key role in the developmental profile of Sri Lanka. Energy demand is going up and up with time due to population growth and industrial revolutions. In order to meet such growing needs probably we are relying on fossil fuel in a large fraction, which is leading to many negative environmental impacts. Hence the government decided to take off many long term as well as short term planes to promote renewable energy sector in Sri Lanka as it gives minimum impact to environment and its quality than the fossil fuel. This paper gives basic information about renewable energy resources of Sri Lanka and their development &future perspectives.*

Keywords— Renewable Energy, Fossil fuel, Sri Lanka, Environment.

I. INTRODUCTION

Renewable energy is generally defined as energy that comes from resources which are naturally replenished on a human timescale. Types of renewables include solar energy, wind energy, tidal energy and geothermal energy. Energy can be called renewable if it can neither run out nor be easily replaced (like slow-growing trees).Renewable energy is any form of energy, which can be used by the present generation without affecting the future generation's ability and right to use the energy resource. The basic forms of renewable energy are based on solar energy. All these renewable energy forms depend on the solar radiation, except geothermal energy, which again is a renewable energy resource, independent of solar energy. (11)

Renewables are great for our energy portfolio because they are inexhaustible and won't pollute the planet. However, nearly every town has access to sunlight, wind, and the geothermal power from the Earth. therefore harnessing these sources of renewable energy could be a great democratizing force, offering affordable and plentiful power to every corner of the globe. It is therefore important to propose possible renewable energy resources to meet the energy demand without polluting the environment and quality.

II. GROWTH AND DEVELOPMENT OF RENEWABLE ENERGY SECTORS IN SRI LANKA

Sri Lanka has a long history of using renewable energy for its power generation from early 20th century by most of the tea plantation companies. with the installation of small hydro power plants.(10)

It is now recognized that for developing countries like Sri Lanka, meeting the energy needs of an economy aspiring to achieve rapid growth, while ensuring environmental sustainability, is a key challenge. 'Energy Challenges in the Knowledge Economy' was an important theme. With steady economic growth, the demand for energy is of paramount importance especially for a developing country like Sri Lanka. The national planners should give priority to energy security and formulate a national energy policy by identifying a sustainable energy mix keeping in mind the various growth sectors specially industry and services.(5)

Sri Lanka is on the path towards becoming an internationally competitive middle-income country. This power and energy sector development plan is aligned to the country's development drive, and has been prepared to provide affordable, high quality and reliable energy for all citizens, rich or poor, equally by conserving country's precious natural environment, giving priority to the indigenous energy sources, and minimizing regional disparities in energy service delivery.(9)

The power and energy sector vision is to capture the full potential of all renewable and other indigenous resources in order for Sri Lanka to become a nation self-sufficient in energy. The total energy requirement of the country was around 11,125 ktoe in 2013, and the primary energy supply mainly consisted of 4,814 ktoe of biomass, 4,582 ktoe of fossil fuels, and 1,442 ktoe of hydro. Accordingly, 56% of total energy consumption is from indigenous (biomass + hydro).(9)

2.1 Biomass Energy

Sri Lanka has a vast potential of producing biomass from a unit area as a result of high plant growth rate due to high incidence of solar energy and rainfall. It is estimated that approximately 40 Billion kg of biomass can be generated by converting marginal land to fuel wood plantations, and improving productivity of other crop land and home gardens [Energy Conservation Fund. 2005). Total potential of this resource is estimated to be about 16 Million ton oil equivalents (Mtoe) per

annum. But around 70% of biomass energy production is informal.(8) Most widely-used renewable energy in Sri Lanka is biomass. (8)

TABLE 1
PLANT FACTORS OF BIOMASS POWER PLANTS

Badalgama BMP	42.93%
Tokyo BMP	26.03%
Kottamurichchana BMP	15.11%

2.2 Hydro power

As of the geographical configuration with a rainfed central hills, Sri Lanka enjoys a good hydropower potential. Hydro-power potential is defined by two aspects of the water resource which is either moving or flowing body of water, i.e., the amount of water that passes through a point during a given period and the vertical drop through which the body of water passes through. Hence, large volumes of water and sudden drops are termed good hydropower resources. The country has used this resource for conveyance of irrigation water for many millennia, and for electricity generation during the last two centuries. Early days of grid electricity generation saw hydro as the major component in electricity generation, accounting for more than 65% of the total. Recently, this component has been reduced to 35% mainly due to the exponential load growth, which cannot be met by this limited resource. (8)

Graph 1 indicates the contributions of different energy sources in electricity production in Sri Lanka

TABLE 2
PLANT FACTORS OF SMALL HYDROPOWER PLANTS

Rathganga MHP	67.56%
Hapugastenna - 2 MHP	66.70%
Somerset MHP	61.90%
Kotanakanda MHP	61.85%
Batatota MHP	58.47%
Wee Oya MHP	57.30%
Palmerston MHP	54.82%

GRAPH 1: CONTRIBUTIONS OF DIFFERENT ENERGY SOURCES IN ELECTRICITY PRODUCTION IN SRI LANKA

2.3 Solar Energy

Energy from sunlight is captured in solar panels and converted into electricity. Sri Lanka is blessed with an impressive solar energy resource (Located near the equator) . From the earliest times, this resource had been utilized for drying purposes such

as crops, clothes, etc., and has remained largely a non-commercial energy resource. Two thirds of the country's lowland area receives a radiation of 4-5.5 kWh/m2 per day, whilst the remaining area in the central hills receives a lower radiation of 2-3.5 kWh/m2 per day, due to persistent cloud cover in those areas(1). It is interesting to compare these energy yields with daily electricity consumption of a typical household as both are in the same range of 4-5kWh per day (7). However, it is dangerous to assume that all houses can be provided with a solar panel of 1m2 , as conversion losses and energy storage requirement of such a system is well beyond the reach of a typical family.

TABLE 3
PLANT FACTORS OF SOLAR POWER PLANTS

Solar PV SPP	69.56%
Gonnoruwa I SPP	13.81%
Gonnoruwa II SPP	10.68%

2.4 Wind Energy

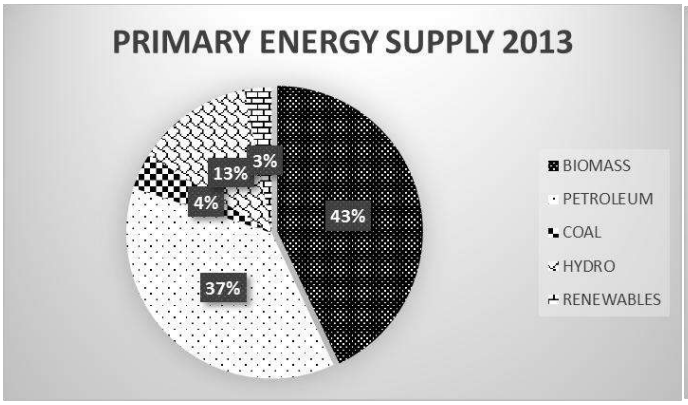
Sri Lanka is located in the Indian Ocean facing a vast Swath of uninterrupted ocean, providing solid wind energy potential. The country experiences two main wind climates, namely the South-Westem monsoon (May-August) and the North-Eastern monsoon (October-December). Archaeologists have proven that iron smelting using South-Westem monsoon without the use of a bellow to pump air to smelting furnaces had been widelyused technology during the period of 300-200 B.C. in the Balangoda area, making Sri Lanka the earliest country to utilise wind energy for productive work(Gill Juleff 2003) As in the case of hydropower, wind energy is derived from a moving body of wind, by transferring the kinetic energy of the moving body of wind to a turbine rotor, Due to the very low density of wind.(8)

TABLE 4
PLANT FACTORS OF WIND POWER PLANTS

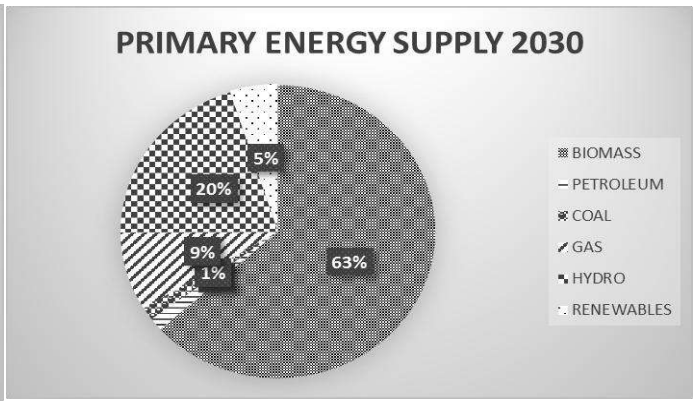
Nirmalapura WPP	49.36%
Vidatamunai WPP	35.93%
Seguwantivu WPP	31.69%
Mampuri WPP	31.12%
Willpita WPP	13.92%
Hambantota WPP	10.13%

2.5 Other Renewable Energy Sources

In addition to the four resources mentioned above, there are other forms of renewable energy such as wave energy, ocean current energy, geothermal energy and (Ocean Thermal Energy Conversion (OTEC) energy which could be useful for Sri Lanka in the distant future. Presently, these resources are evaluated and technologies being developed for energy conversion, and early breakthroughs are eagerly awaited by the research communities worldwide. Hence, no attempt will be made to describe these forms of energy. (8)



GRAPH 2 INDICATES PERCENTAGE CONTRIBUTION OF EACH SOURCES FOR ENERGY SUPPLY IN 2013



GRAPH 3 INDICATES PERCENTAGE CONTRIBUTION OF EACH SOURCES FOR ENERGY SUPPLY IN 2030

III. BARRIERS TO RENEWABLE ENERGY

- Market -Highly controlled energy sector, Lack of information and awareness,Restricted access to technology, High investment requirements.(2)
- Economic and financial-Economically not viable, High initial capital cost Lack of access to capital, Lack of financial institutions to support RETs, lack of instruments (2)
- Technical-Lack of standard and codes and certification, Lack of skilled personnel/training facilities, System constraints. (2)
- Social, Cultural and Behavioural - Lack of consumer acceptance of the product, Lack of social acceptance for some RETs, Environmental externalities (2)

IV. RENEWABLE ENERGY TOWARDS FUTURE NEEDS

Sri Lanka's new government expects a big increase in renewable energy investment by the public and private sectors as it unveiled an ambitious plan to gain energy self-sufficiency in the next 15 years. By 2030 the island aims to phase out fossil fuel imports which account for 25-30 percent of the total import bill and burns up 40 percent of export earnings, according to the Sri Lanka energy sector development plan for 2015-2025.

"If current trends in fossil fuel imports continue, soon export earnings will not be enough to meet the fuel import bill," Power and Energy Minister Patali Champika Ranawaka said. About half the island's primary energy supply is imported today, making it vulnerable to international supply shocks, he said. The government intends to replace these imports by developing indigenous energy sources and reducing energy usage and waste, Ranawaka told a forum held to launch the new energy plan. (4)

About 20 percent of imports would be replaced by developing to the fullest potential non-conventional renewable energy such as wind and solar by 2030, he said. "We will also reduce the technical and commercial losses of the electricity transmission and distribution network from 11 percent to eight percent, which is the international norm, by 2020."The government also aims to reduce annual energy demand growth by two percent through "through very rigorous conservation of energy and efficient use," he said. (4)

The plan aims to increase the share of electricity generation from renewable energy sources from about half today to 60 percent by 2020 and finally to meet the total demand from renewable and other indigenous energy resources by 2030.(3)

Current total installed power generation capacity is about 4,050 MW, consisting of 900 MW of coal power, 1,335 MW of oil burning thermal power, 1,375 MW of hydro power and 442 MW of non-conventional renewable energy sources such as wind, mini hydro, biomass and solar power plants. (9)

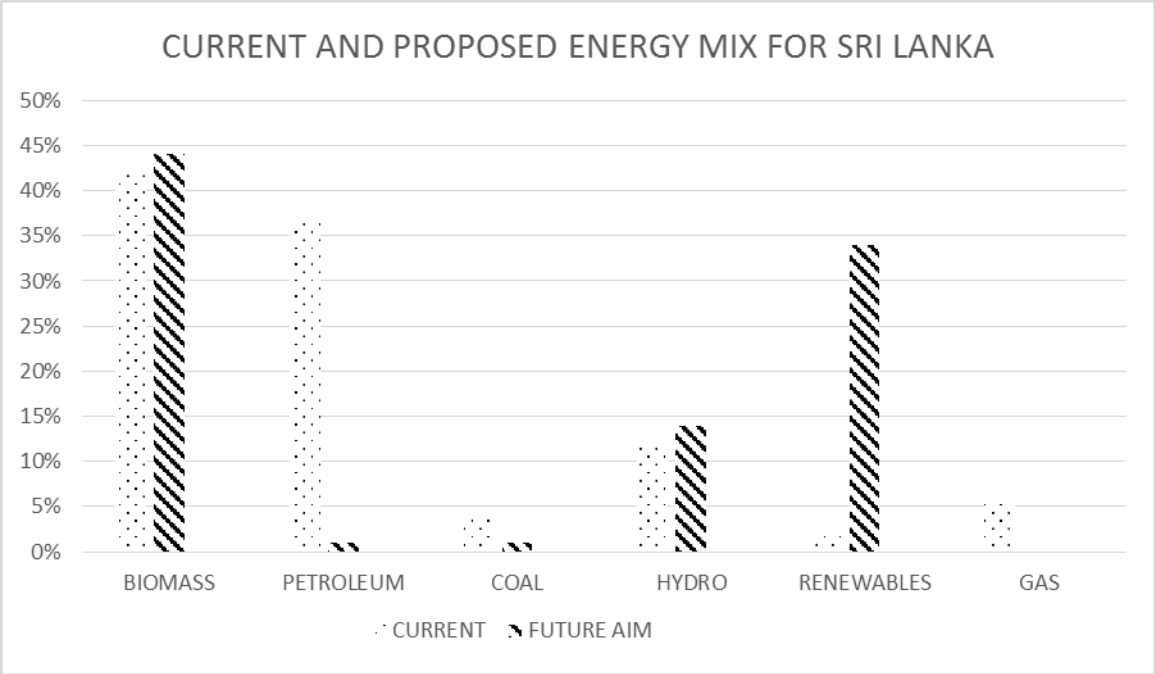
V. CAN SRI LANKA BECOME AN ENERGY INDEPENDENT COUNTRY?

Sri Lanka annually imports 2 MMT of crude oil, 4 MMT of refined petroleum products and 2.25 MMT of coal. This costs approximately 5 billion USD and covers 44% of the energy requirements. It also accounts to 25% of the import expenditure and almost 50% of the total export income. As a result, Sri Lanka’s energy demand puts immense pressure on the national budget and on exchange rates. In addition, oil burning emanates various gases, intensifying air pollution. (8)

Therefore, it is essential that the country’s energy policy puts more emphasis on renewable energy production to ensure the country does not depend on imported oil and oil products or contribute to air pollution. However, whether there is sufficient motivation to do this and whether it is a possibility are interesting questions to be debated. The Ministry of Power and Energy has developed an energy sector development plan that looks into increasing the use of renewables in energy production while reducing the dependence on imported oil and oil products. Analysis of the current composition of energy production and the aims of the new plan would provide a better idea on what areas Sri Lanka can expand on with respect to renewable energy and how soon that can be done.

Graph 4 indicates that the new energy sector development plan aims to reduce the petroleum contribution from 37% to 1%. The dependence on coal is to be reduced from 4% to 1%. Contributions from bio-mass and hydro are to remain more or less the same. In correspondence with the reduction on the dependence on imported oil, the new plan aims to increase the contribution from 3% to 34% with more emphasis on wind and solar power generation. Additionally, Sri Lanka hopes to

provide a 6% contribution from the recently discovered gas resources. While this is an ideal vision for Sri Lanka to answer its issues on establishing energy independence, there are several key concerns over the proposed future development.



GRAPH 4: CURRENT AND PROPOSED ENERGY MIX FOR SRI LANKA

VI. CONCLUSION

Requirement of energy in Sri Lanka also increasing in an increasing rate like other countries, So Energy needs to be conserved to protect our environment from drastic changes, to save the depleting resources for our future generations. The rate at which the energy is being produced and consumed can damage our world in many ways. In other words, it helps us to save the environment. We can reduce those impacts by consuming less energy. The cost of energy is rising every year. It is important for us to realize how energy is useful to us and how can we avoid it getting wasted.

Other prominent strategy is utilization of renewable energy. The vast renewable energy resource base of Sri Lanka will be developed to increase the dominance of indigenous energy in both electricity and thermal energy supplies. This initiative will cover the whole value chain of the electricity sector from electrification of remote locations through off-grid solutions to large scale infrastructure development to absorb wind, solar, remaining hydro and other renewable energy resources based power generation to the national grid.

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The Factors of Land use Conversion from Settlement Area to Commercial Area at IR. Soekarno/ Merr Street, Rungkut Street, and Medokan Ayu Street, Surabaya

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Abstract— *Land use that does not conform to its city plan is a common phenomenon that happens on fast growing cities. Land use conversion usually caused by discrepancy between the reasoning that underlie a city plan and market's judgement. At the area around Ir. Soekarno Street / MERR that designed as a part of Surabaya City Ring Road, the land use is gradually changing from settlement area into commercial area. In urban planning, it is important to understand the cause of land use change in order to be able to handle the implication well. Therefore, the research objective was to identify the factors that affecting land use conversion from settlement area into commercial area at Ir. Soekarno Street/MERR, Rungkut Madya Street, and Medokan Ayu Street. The research's methods are descriptive. The descriptive analysis was used to describe the land use change pattern at Ir. Soekarno Street/MERR, Rungkut Madya Street, and Medokan Ayu Street. Multiple regression analysis was used to analyses the factors that affecting land use conversion from settlement area into commercial area. The result showed that the factors that affecting land use conversion are land's value, average income of the land owner, and land use in the surrounding area.*

Keywords— conversion factor, land use, settlement, commercial area, regression analysis.

I. INTRODUCTION

Land use planning is the main guide for determining the direction and the management of activities and land use in the city. Land use planning usually took a form as city structural planning that was established by considering all the factors affecting it. Even so, since there are so many factors that could affect land use formation, oftentimes the land use formed not the way it was planned to be.

Land use that does not conform to its city plan is a common phenomenon that happens on fast growing cities. Land use conversion usually caused by discrepancy between the reasoning that underlie a city plan and market's judgement. In one hand, land use should be considering public needs and all the technical rules. In other hand, market's demand has the power that couldn't be controlled easily.

Land limitation and high land prices are common on places that had been set as commercial area in the middle of the city. It caused the invasion of commercial activities into settlement area. Accesibility toward settlement area makes it easier to convert settlement area into commercial area, as it happened at Ir. Soekarno Street/MERR. The street is part of Surabaya City road network system that formed a ring road of Surabaya (Surabaya City Spatial Plan 2013-2032)[1]. The street is passing settlement areas on the east side of the city and goes through to the north (Kenjeran). On the area surrounding the street, the land use is gradually changing from settlement area into commercial area.

The change was indicated by the increasing land prices on settlement areas surrounding Ir.\ Soekarno/MERR Street, Rungkut Madya Street, and Medokan Ayu Street. Based on Maulana (2013)[2], land prices at Kedung Asem on 2012 was about Rp5.000.000,00 – Rp6.000.000,00 / m2. And, land prices at Deles, Semolowaru, Medokan Semampir areas on 2012 are about Rp5.000.000, 00 – Rp7.000.000, 00 / m2. Before the developing of Ir. Soekarno/ MERR Street, the land prices on those areas are about Rp750.000, 00 – Rp5.000.000,00 / m2. The increasing land prices were indicating the uplift of land values caused by land use conversion from settlement area into commercial area. Whereas this change would bring up some implications such as decreasing green area ratio (GAR), increasing vehicle volumes and traffic jam, also parking problems that often happen at commercial area because of the use of roadside as parking site. Those phenomenons could affect the city's dynamics as a whole.

In urban planning, it is important to understand the cause of land use change in order to be able to handle the problems effectively. Therefore, in order to establish Surabaya City land use planning that is more accommodative to dynamical

changes, the research objective was to identify the factors that affecting land use conversion from settlement area into commercial area at Ir. Soekarno Street/MERR, Rungkut Madya Street, and Medokan Ayu Street. The research's methods are descriptive.

II. METHODS

2.1 Research Variables

The research variables were obtained from theoretical review and policies review. The research variables and sub variables are mentioned at Table 1.

TABLE 1
RESEARCH VARIABLES

Indicator [3][4] [5][6][7][8][9][10][11]	Variable	Sub Variable	Operational Definition
The type of land use change	The type of land use change	-	Categories of the type of commercial activities at research area
Land use change rate	Land use change rate	-	The speed of land use change in the periods of 5 years (2012-2016)
Location distribution	Location distribution	-	The orientation of commercial area distribution based on the occurrence of land use change
physics	Land area per lot	-	Land area of the lots in research area.
Economic	Land value	-	The land value of the lots in research area.
Social	Land owner characteristic	Land owner earning	The average earnings of land owner of the lots in research area.
Policy	Land use guide plans	-	Policies that governing the land use in research area.
Accesibility	Transportation facilities	Public transportation	Public transportation which route goes through research area.
	Access to the city center	The distance to main road	The distance of research area to main road.
	Access to the central activities	The distance to central activities on the area	The distance of research area to central activities on the area, that is the university (educational facility).
	Road function	-	Road function classification in research area.
Spatial	Land use in surrounding area	Settlements	Settlement area around the research area.
		Comercials	Commercial area around the research area.
		offices	office area around the research area.
	Lot image	Type of activities	People's judgment about the commercial area on research area based on the type of activities.

2.2 Population and Samples

Population is the whole analysis unit that is the target of the research. The populations of the research are all the land owners of areas on the street corridor of Ir. Soekarno Street/MERR, Rungkut Madya Street, and Medokan Ayu Street, that changed their land use from settlement to commercial building.

Sample is part of population that taken by some methods and posses certain characteristics, clear, and thorough so that it can be assumed to represents the whole population (Nazir, 2003)[12]. To determine the sample used for this research, the method used were purposive sampling and simple random sampling. Purposive sampling has the benefit as the ability to obtain information-rich cases. Purposive sampling aims to take on subject not based on level, random, or spatial, but based on certain purpose. The use of this technique was for determining land use change actors at research area. The numbers of samples were calculated using equation (2.1).

$$n = \frac{N}{1+n(e)^2} \quad (2.1)$$

Explanation:

n = the number of samples
N = the number of population
e = Margin error (10%)

Based on, the equations, the number of samples used to appraise the causative factors of land use conversion are 78 land owners.

2.3 Research Methods

The research's methods were descriptive. There were two analysis technic used in the research, i.e. observing land use pattern changes by descriptive analysis and identifying factors of land use change by regression analysis. The land use change was observed and produced a pattern. The analysis was used to identify a spatial pattern within a certain time which builds upon time factor (t). The land use change pattern was observed based on:

1. The speed rate of commercial land use development that was to observe the land use development from land's building area based on the formation periods of the building.
2. The commercial area location distribution that was to observe the land use development based on the location of commercial buildings.
3. The type of land use change that was to observe the land use change from previous land use to current land use.

The observing of land use change on the research area was divided into 2 parts, namely:

- The north zone
- The south zone

The analysis was presented on the form of maps that describing the land use change at research area.

On determining the factors affecting land use conversion from settlement area into commercial area, regression analysis was used by utilizing SPSS software for windows. The analysis tool was used to determine causative relation in between variables. On table 1 are the variables used in the regression analysis.

TABLE 2
REGRESSION ANALYSIS'S VARIABLES

***Dependent Variable* (Y)**	***Independent Variable* (X)**
Land use change (Y) • Land use change rate (Y_1) • The type of land use change (Y_2)	Land area per lot (X_1)
	Land value (X_2)
	Land owner earnings (X_3)
	Land use guide plans (X_4)
	Transportation (X_5)
	Access to the city center (X_6)
	Access to the central activities (X_7)
	Road function (X_8)
	Land use in surrounding area (X_9)
	Lot image (X_{10})

III. FINDING AND ARGUMENTS

3.1 Research Area Characteristic

3.1.1 Area Orientation

The research area is street corridor of Ir. Soekarno Street/MERR, Rungkut Madya Street, and Medokan Ayu Street. The targeted area is the lot that had undergoes land use change from settlement area into commercial area. The boundary of research area, namely:

- North : Surabaya River
- South : Rungkut Manggala and Puri, Mas Residential Areas
- East : Royal Town Regency, Residential Area
- West : Rungkut Asri Barat, Residential Area

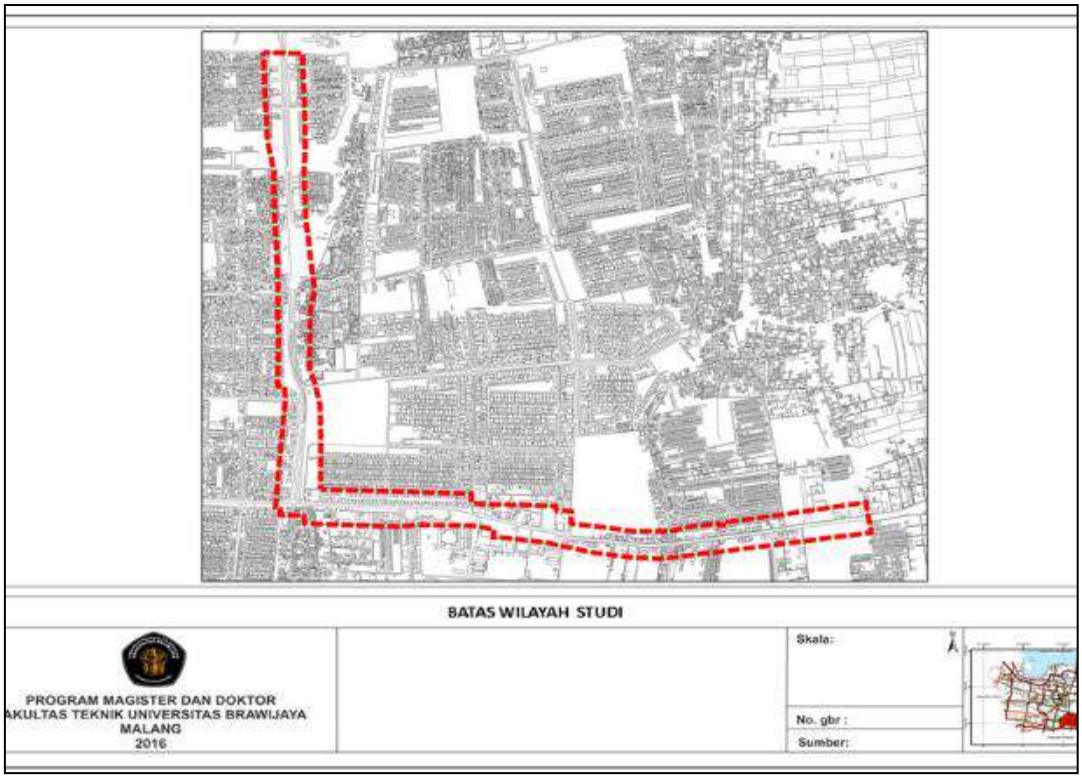

FIGURE 1. RESEARCH AREA ORIENTATION

3.1.2 Land Use Pattern

The observing of existing land use pattern of commercial area, the research area was divided into 2 zones, i.e:

a. North Zone

The north zone spans from Guna Wangsa Apartment until Rungkut Madya Street intersection. The land use on north zone was dominated by commercial activities (stores, minimarkets, restaurants, etc), also services industries, such as banks, workshops, carwashes. The existence of commercial activities would encourage land use change on the back layer area to be secondary commercial area.

b. South zone

The south zone spans from Rungkut Madya Street intersection to Medokan Ayu Street intersection. The land use on north zone was dominated by commercial activities (stores, minimarkets, restaurants, etc), services industries, such as banks, workshops, hair salons, and carwashes, also office complex. On this area, there was agglomeration of commercial building and transportation routes, resulting on high rate of accessibility.

3.2 Land Use Pattern Change Analysis

3.2.1 Land Use Change Process Analysis

Generally, the land use change at research area was started by "penetration", that is a breakthrough of new functions in a homogeneous function. The people changed their home (residential function) into commercial places (restaurant/café, laundy place, stores, etc). The success of the people, who originally opened the business together with the residential home, attracted more people to do the same thing. The phenomenon was stimulating the occurrence of "invasion". Invasion is greater than the penetration stage, but still not dominating. The accumulation of land use change was leading to the invasion of commercial activities on the main road, intersection and the surrounding area, until the proportion of commercial area increased gradually.

In the end, the new function (commercial) would be dominating the previous function (residential). On the last step, succession, the old function were already wiped out and be replaced by the new function. The change, would be responded by the government with the legalization of land use planning of the area in regards to the land use change occured.

3.2.2 Land Use Change Distribution Analysis

The distribution of commercial activities at the research area was not clustered on one location at the same time frame. The identification was observed on 5 years periods, i.e 2012-2016. The observation was divided into 2 zones, the north zone and the south zone.

The distribution of commercial areas on 2012, namely:

a. On the north zone, the distribution of commercial activities were gathered on one spot, which is the road intersection. It happened because the road intersection has higher accessibility level than the other places without a crossing. The intersections that close to city center has higher accessibility level than the further places. This condition was resulting to the road intersections that undergo the land use change.

b. On the south zone, the distribution of commercial activities were gathered on along the way of Rungkut Madya Street and Medokan Ayu Street in a linear form (ribbon shaped development). In this matter, it clearly showed the role of transportation system that affecting the development of commercial area so that the area spread was longer than it widen. The area along the main road is the area that got the most severe pressure of land use change. The high price of land along the road corridor encouraged the land owners to change the land use from residential into commercial.

The development of commercial area on the south zone had begun earlier because Rungkut Madya Street was established before Ir. Soekarno Street/ MERR was built.

The distribution of commercial activities on 2016 became more intense,with explanation:

a. On the north zone, the location distributions of commercial area were gathered along the way of Ir. Soekarno Street/ MERR corridor in a linear form (ribbon shaped development).

b. On the south zone, the distribution of commercial area that has been identified, was divided into 2 patterns:

- At the corridor of Rungkut Madya Street and Medokan Ayu Street, the distribution of commercial activities were gathered along the way of Rungkut Madya Street and Medokan Ayu Street corridor in a linear form (ribbon shaped development).

On the enclave of the south zone, the pattern distribution of commercial activities was spread, generally they spread among the center of activities, near the educational center Pembangunan Nasional University (UPN). The pattern was formed as the result of domino effect from the land use change on Rungkut Madya Street and Medokan Ayu Street corridor

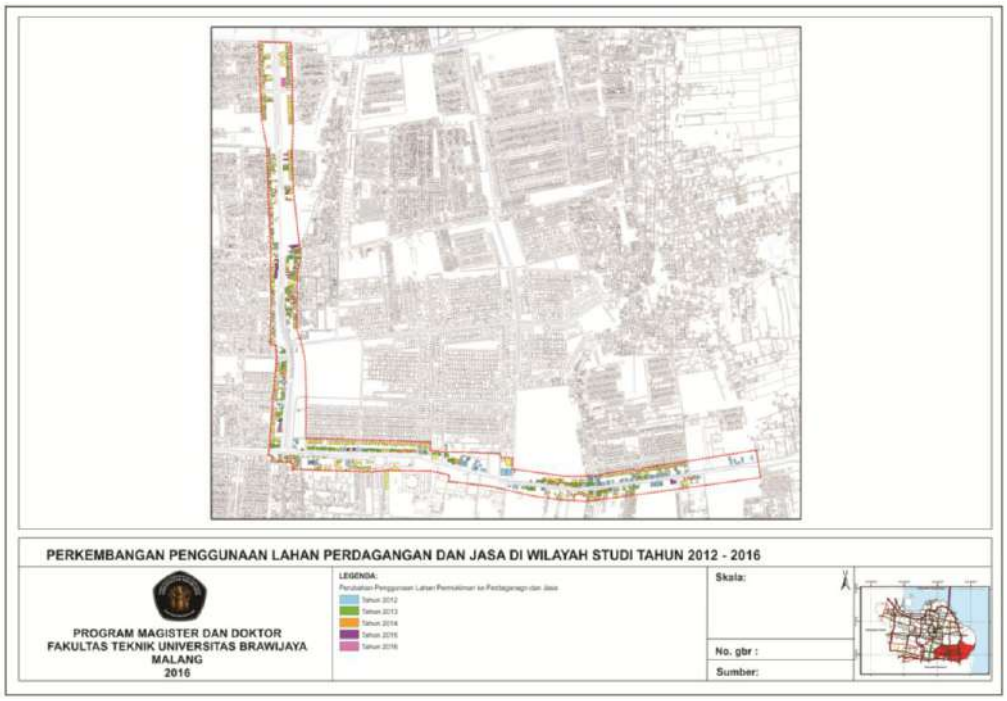

FIGURE 2. ANALYSIS OF THE DEVELOPMENT OF COMMERCIAL ACTIVITIES AT RESEARCH AREA 2012-2016

3.3 Analysis to Determine Factors Affecting Land Use Change from Settlement Area to Commercial Area

The analysis to determine factors affecting land use change from settlement area to commercial area was using multi linear regression analysis. There were 2 dependent variables studied, land use change rate (Y_1) and The type of land use change (Y_2).

3.3.1 The Land Use Change Rate

Based on the result of multi linear regression analysis showed on table 3, formed the regression model of the factors affecting the land use change rate.

TABLE 3
COEFFICIENT VARIABLES OF LAND USE CHANGE RATE

Coefficients(a)

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	Collinearity Statistics	
		B	Std. Error	Beta			Tolerance	VIF
1	(Constant)	4.177	1.372		3.045	.003		
	luas lahan per kapling	-.255	.202	-.177	-1.264	.210	.571	1.752
	nilai lahan	.384	.163	-.295	-2.362	.021	.719	1.390
	penghasilan	.293	.121	.283	2.416	.018	.817	1.224
	arahan guna lahan	.280	.203	.167	1.375	.174	.760	1.316
	sarana transportasi	-.094	.163	-.098	-.578	.565	.390	2.563
	akses menuju pusat kota	.269	.167	.233	1.614	.111	.542	1.844
	fungsi jalan	-.222	.153	-.189	-1.450	.152	.665	1.503
	akses menuju pusat kegiatan	-.154	.171	-.108	-.901	.371	.781	1.281
	guna lahan sekitar	.259	.207	.178	1.253	.215	.559	1.790
	image kapling	.118	.176	.077	.671	.504	.853	1.173

a. Dependent Variable: perubahan pemanfaatan lahan

The coefficients table was used to identify the influence of independent variables to dependent variables. If the significance value was smaller than 0, 05, and then the variable was affecting the dependent variable, which is the years, spent on land use change. Based on table 3, the variable with value less than 0, 05 were land value and earnings. The regression model is:

$$Y_1 = 4{,}177 + 0{,}384\ X_2 + 0{,}293\ X_3 \quad (3.1)$$

Explanation:

Y_1 = land use change rate

X_2 = Land Value

X_3 = Earnings

Based on equation (3.1), it was known that all of variables as predictor have positive influence towards the years spent on land use change. It means, the higher land value and land owner's earnings, the faster land use change rate happened.

3.3.2 The Type of Land Use Change

Based on the result of multi linear regression analysis showed on table 4, formed the regression model of the factors affecting the type of land use change.

TABLE 4
COEFFICIENT VARIABLES OF THE TYPE OF LAND USE CHANGE

Coefficients(a)

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	Collinearity Statistics	
		B	Std. Error	Beta			Tolerance	VIF
1	(Constant)	2.722	1.001		2.719	.008		
	luas lahan per kapling	.031	.147	.030	.213	.832	.571	1.752
	nilai lahan	.070	.119	.074	.594	.554	.719	1.390
	penghasilan	.229	.089	.304	2.584	.012	.817	1.224
	arahan guna lahan	.099	.149	.081	.664	.509	.760	1.316
	sarana transportasi	.069	.119	.099	.584	.561	.390	2.563
	akses menuju pusat kota	-.106	.122	-.125	-.870	.388	.542	1.844
	fungsi jalan	-.054	.111	-.063	-.482	.631	.665	1.503
	akses menuju pusat kegiatan	-.080	.125	-.077	-.643	.523	.781	1.281
	guna lahan sekitar	.311	.151	.293	2.064	.043	.559	1.790
	image kawasan	-.103	.128	-.092	-.804	.424	.853	1.173

a. Dependent Variable: jenis perubahan pemanfaatan lahan

The coefficients table was used to identify the influence of independent variables to dependent variables. If the significance value was smaller than 0,05, then the variable was affecting the dependent variable, which is the type of land use change. Based on table 4, the variable with value less than 0, 05 were earnings and land use in surrounding area. The regression model is:

$$2{,}722 + 0{,}229\ X_3 + 0{,}311\ X_9 \qquad (3.2)$$

Explanation:

Y_2 = type of land use change

X_3 = earnings

X_9 = land use in surrounding area

Based on equation (3.2), it was known that all of variables as predictor have positive influence towards the type of land use change. It means, the more relevant land use in surrounding area (with commercial activities) and the higher land owner's earnings, the more diverse the type of land use change.

As in each regression analysis, the value of R square (R2) of the model resulted were 22,2%. It means that on determining the factors that affecting land use conversion from settlement area to commercial area at Ir. Soekarno Street/MERR, Rungkut Madya Street, and Medokan Ayu Street could be explained by the predictor variables (land value, earnings, land use on surrounding area), but the value was relatively small. The rest of it, 77,8% of the factors were influenced by other factors that did not included in this research.

IV. CONCLUSION

4.1 Summary

The reseach's conclusion were described as below:

a. Land use change pattern from settlement area to commercial area at Ir. Soekarno Street/MERR, Rungkut Madya Street, and Medokan Ayu Street was divided into 2 types, i.e. linear pattern (ribbon development) along the way on street corridor and spread pattern and clustered around center of activities on enclave part.

b. The factors that affecting the land use conversion from settlement area into commercial area are land value, earnings, and land use in surrounding area.

4.2 Recommendation

Based on the research results, recommendation are given, i.e:

a. The existing commercial activities are to be maintained and encouraged to provide a parking area on the lot.

b. On the enclave, the one which still dominated by residential area, it need to be controlled, so that commercial activities, especially the big ones, won't caused a negative impact.

c. Participation of the stakeholders, which are bureaucrats, governments, scholars,and the people, are needed in order to implement regulation and land use planning.

d. The advanced research related to the impact of land use change is needed.

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Added Value on Sade Village and Bau Nyale Festival in Autoimmune Diseases Immunization Travel: Supported by Aptamers Technology

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Abstract*—Prevalence of Autoimmune diseases (AD) are high in developed countries and low in developing countries. Wall of worm were associated with lower level of allergy and support the hygiene hypothesis (HyHy). Long term use of Interferon as drug for AD and synthetic vaccine made by epitope mapping are global economic burden. Adolescence from developed countries traveling to Ghana forwarding AD-HyHy. Fecal microbiota transplantation (FMT) for fighting AD have been developed in hygiene countries such as UK, Japan and Korea, meanwhile the aims of this study are reveal an added value to Sade village 'FMT culture' and Bau Nyale/Mandalika Princess festival for Nature Vaccination on AD. These local nature vaccination needs to be known by policy maker and political doer and could be a translational-medicine education topic to lessen AD economic global burden. Method: Systematic-review and Bayesian network analysis. EBSCO host search engine were used. Result: Description of 9 Publications of meta-analysis are chosen. Conclusions: The increasing of immunity in developing countries and decreasing in clean habit educated family is based on hygiene hypothesis, could be answered by local life-style and festival. These added value for local tour package has been supported by affimers and aptamers contemporary technology based on bacteria and worm epitope mapping.*

***Keywords*—hygiene hypothesis-autoimmune diseases, bacteria and worm cell wall, aptamers, epitope mapping, Fecal Microbiota Transplantation (FMT).**

I. INTRODUCTION

Wall of worm cell have many epitope as well dormant bacteria (Fecal Microbiota Transplantation or FMT),[1,-5] is now locally neglected and globally abandoned. Usage of natural antigen for vaccination is hygiene hypothesis (HyHy) based on prevention such as in allergy epidemic in US.[6-8] Decreasing autoimmune disease (AD) by natural AD vaccination as added value of Sade and Bau Nyale festival, could lessen AD burden. Meta-analysis on Rheumatoid Arthritis (RA) molecule has ethnicity relation[9] parallel with new loci RA in Japan[10,11] and Korean and European eight new RA risk loci.[11] Meta-analysis on FMT in IBD[12] and hygiene lifestyle are associated with allergic, atopic sensitization,[7] and asthma. Hand vs. machine dishwashing have significantly different AD prevalence.[13] A number of references show that AD and climate[14,15] and socioeconomic status[14] are related to HyHy.[8,16] Do these factors contribute to the occurrence of increasing prevalence of AD in developed countries? Meanwhile, protocols in synthetic vaccine industry for AD or treatment strategies for people with AD, with interferon longterm usage are still unclear. No publication before that summarizes and describes the association of HyHy, AD, FMT, Genome Wide RA,[17] RA in developed countries,[18-20] Epitope-RA-SLE,[21] Helminth-Hygiene-AD,[22,23] vaccine-epitope/antigen.[22,24-26] Just by implementing local wisdom such as Sade village and Bau Nyale–Mandalika Princes festival can lower risk of AD. Effective preventive activities and treatments could be initiated for high risk person's AD caused by clean habit of living.[6,13,27-31]This prevention and therapy model is the first effective natural vaccination idea published.

II. MATERIAL AND METHOD

Systematic review and Bayesian network analysis using EBSCOhost search engine. The search was performed up to Sept 2016 in 3 steps. First, searching with Google search engine for the definition of known key words, then with CINAHL plus with full text for developing the knowledge for the aims of study. Using the following search terms: Autoimmune Diseases, Hygiene Hypothesis, Epidemiology and AD, Atopic Sensitization, Asthma, T1D, IBS, MS, Rheumatoid Arthritis, Ulcerative

Colitis, Allergic Diseases. AD AND Meta-Analysis (372), high prevalence in UK (Epidemiology and AD and UK (237), Japan (247), Korea (51). Low in Sierra Leone (0, SL ancestor (1), Egypt (0), Ghana (0). Where FMT are growing in high prevalence 3 countries, FMT (554), FMT AND UK (14), FMT AND Japan (12), FMT AND Korea (9). In molecular level using terms of: exposure of unhygienic bacteria, worm, epitope with some topics: high in tropical rainforest area (wet and warm) and savana dry and warm climate; combination of worm antigen-prevent T1D[32]; Epitope E. coli-vaccine[33]; wet and warm countries: Ghana[14]; making of synthetic vaccination HBV aptamers: next generation sequencing[34]; synthetic long peptide vaccine[24]: long peptide synthetic vaccines with just one protective epitope[22]; FMT for extra-intestinal disorders AD[35] (FMT industrialization)[35]. recent therapy and education on HyHy: Exposure 1: sade village cultural: bacteria and parasitic cell wall as natural ligand[3]; Exposure 2: bau nyale-putri mandalika cultural event (annelids) : Effect of helminth-induced immunity on infections,[36] : helminth can protect IBDS, an Meta-analysis (MA)[36]; Schitosoma induce Treg[37]; Ghana: Longitudinal studies increase in immunological disorders as it grew cleaner[37]; helminth therapy for allergy[39]; probiotic-helminth[1]; the ability of countries to respons of infectious and chronic diseases[40]; lower prevalence on asthma and allergies in childhood exposed to infections, as postulated in the HyHy to fight AD[41]; School children in the beginning and the end of rainy season: wake up of dormant cell (April-October is the dry season) in Flores could be the Next Prevention on Autoimmune and TB vaccine[42]: Immunizations by Traveling.

In the second Step, searching for meta-analysis of 2 key words related to the researcher Okada and Orenstein and one key words of helminth AND Autoimmune; EBSCO:CINA HL plus (Medline/PubMed) for FMT-MA (12), Helminth AND AD (137), Worm AND AD (33), Worm AND HyHy (17); Epitope AND Helminth AND Vaccine (80); and in the molecular level RA (Epidemiology AND MA AND RA (28.). All are Screening abstract or title: excluded the not relevant and the duplicates by Bayesian analysis. Since we were interested in explanatory factors of bacteria and worm epitope sequencing are filtered.

In the third step, screening full text publications, excluded since not relevant and publications has been filtrated for table 1. The selection was mainly performed by one reviewer and confirmed by the co-authors.

III. RESULTS AND DISCUSSIONS

3.1 Discussion

From clinical to molecular clue of inflammation markers in savanna (warm and dry climate) with dormant bacteria and worm in dry season, and also in tundra (cold and dry) where no worm no bacteria in all season are searched by us. Meanwhile AD have been found in low prevalence in wet and warm or hot and humid countries, whereas epidemic in clean countries. Dormant bacteria in dry season are specific physics-chemistry antigens and broad spectrum of variable microbiota implies rich of epitope of protein from known FMT. Fig. 1 and Table 1. showed the association of HyHy-allergy and AD.[3,6-8,13-15,22,24,32,35,36,39,42,43] Whereas deepest understanding of inflammation reaction increase correlation with the generation of antibody and affimers based product based product induced by antigen based on epitope mappng[51-56] Immune Modulation and Prevention of Autoimmune Disease by Repeated Sequences from Parasites Linked to Self Antigens has reported.[56]

Studies that have developed FMT and helminth probiotic for prevention and therapy of these diseases[1], and has been supported by recent studies,[32,43,56] The association of Epitope mapping of similarity structure and the size length to the making of immunological properties has been reported[57-60] Reduced Asthma produced from early childhood exposure is relied in developing country.[61] Clean habit has support these AD and any potential association between bacteria and helminth infection.[35] In Sade village area which peoples expose to dormant bacteria and Bau Nyale cultural event in which sea-worm were catch to eat, prompt us to propose a specific HyHy to explain how dormant bacteria and helminth wall cell play a role as good antigen similar to high technology for making vaccine and antibiotics. Basically, using small or length peptide sequence from parasites has been an industrial economic income.[22,24,35,46] People resistant from AD and the broad range of bacteria and worm might have contributed to the uprising national economic income for the most common infectious diseases burden countries like Ghana and Indonesia.[38,40,42,55] With the recognition that FMT as well treatment for ulcerative colitis due to supported by epitope mapping-immunology technologies, and parallel with urban rich-urban poor epidemiology,[62-64] Traveling to Sade village and participate in Bau Nyale–Mandalika princess festival will be good for AD patients, family, tribe and country with high prevalence of atopic sensitization, asthma, IBD, SLE, MS, psoriasis, Rheumatoid Arthritis.[9,13,15,16,29,40,49,55,61,65]

TABLE 1
DESCRIPTION OF 9 IDENTIFIED LITERATURES ON META-ANALYSIS IN AD-FMT-GENOME WIDE VERY SIMILAR-EPITOPE-ONE SHOOT VACCINE-PRIMARY THERAPEUTIC AGENT

Study	Variables of Interest	Adjustment for other variables	Comparative risk measures for HyHy	Influence of co-morbidities	Influence of infectious and worm parasite
Okada 2016[9]	Ethnic Rheumatoid Arthritis Molecule	Ethnology, genetics, metabolism	Genome-Wide Association	Hygiene Hypothesis of Autoimmune Diseases	In Human meta-analysis
Sakai Bizmark 2016[64]	Autoimmune in Developing countries	Asthma, allergic diseases World wide	Industrialized countries in Childhood	More prevalent in affluent countries	Epidemiology reduce in developing countries
Shi 2016[65]	FMT	Efficacy Safety	Ulcerative colitis	Clinical remission	Clinical response
Lopez-Isac 2016[44]	Genome wide RA Systemic Sclerosis	same direction, opposite-direction	Allelic effect Rheumatoid Arthritis	Incl. several genomic regions identified T1 Interferon and IL-12	Usefulness of a cross-disease GWAS meta-analysis strategy in the identification of common risk loci
Burr 2010[63]	Rheumatoid Arthritis UK vs. Asian	RA in an independent group of RA cases and control	PADI4_94 Anti-CCP ab PTPN22	-	R PADI4-RA In Asian pop but no R in European ancestry
Dieguez-Gonzalez 2008[21]	Epitope Meta-analysis RA and SLE	ACPAs anti-citrullinated protein antibodies –vs.+	Rs2004640 IRFS Shared epitope OR 0.88 vs. controls	Very similar in SLE	OR haplotype 1.8 vs. controls, protective haplotype OR 0.76 vs. controls
Gorman 2004[29]	Epitope Meta-analysis RA	SE shared epitope	Erosive disease in many ethnic groups OR 2.0; Greek)R0.8, South Eu Caucasians 6.2, Asians OR 5.4 with 2 SE alleles	Genetic and environmental differences in the clinical expression of RA	DRB1*0401 frequency among different ethnic group
Colman 2014[12]	FMT Meta-analysis	Primary therapeutic agent for IBD	Donor selection and microbiome analysis	Ulcerative Colitis, Crohn's diseases	Clinical remission and or mucosal healing
Orenstein 2014[49]	Vaccine IBD	Additional diseases	Supply and delivery	Improving global health one shot at a time	Particularly in developing countries

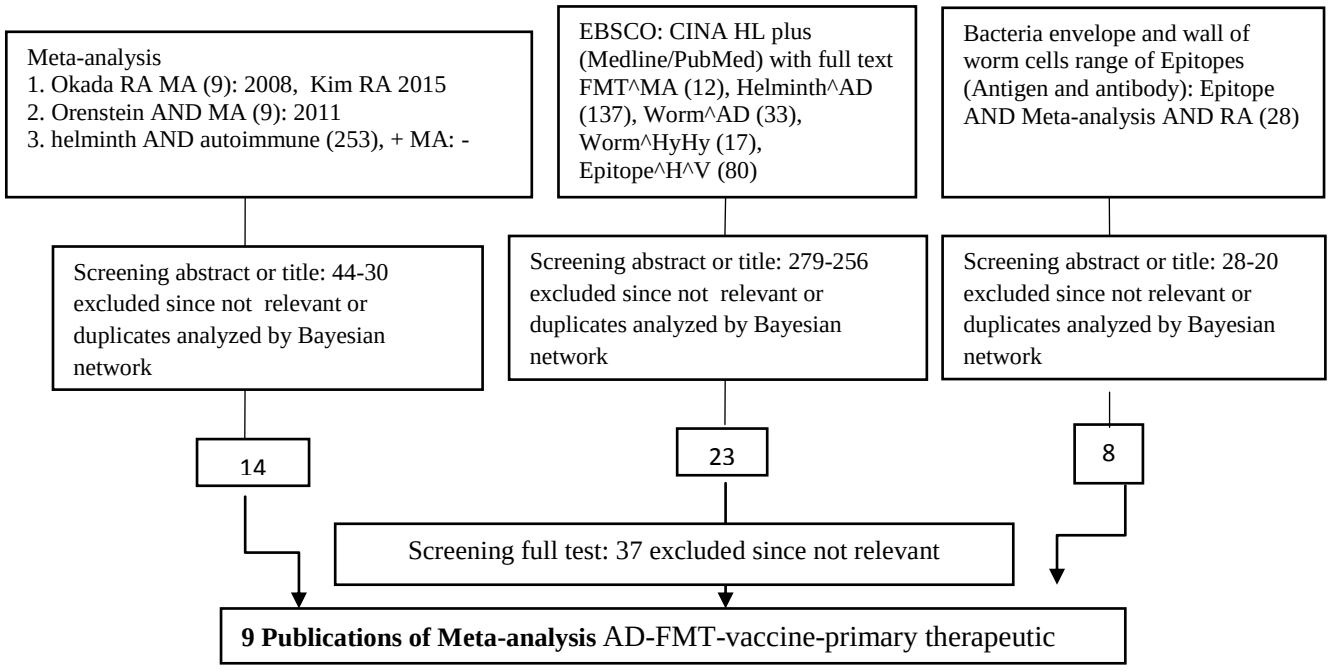

FIGURE 1. FLOWCHART OF THE IDENTIFIED LITERATURE
RA: Rheumatoid Arthritis; MA: Meta-analysis; ^: AND`

The historic antibody level in developing countries is closely associated with worm enteric infection and in the other hand the emerging allergic epidemic is associated with low exposure of epitopes. In 2015, AD affects up to 50 million Americans, according to the American Autoimmune Related Diseases Association has 80 types, 70% women, and in the last few decades increase in the global prevalence.[39] A better comprehension of the association between helminthes and allergies is pressing as research orientation progress closer to harnessing the preventive potential of worms and derivational relations in also allergic disorders therapy.[39]

The body produces Antibody against some of own tissue is the definition of AD. Epidemiology of T1D[16] and AD to geography[8] vs. Hyhy[32], helminth infection has associated negatively with AD[5,14,23,39,42,43] has proven that geography and HyHy associate with socioeconomic status. FMT associate with AD was in HyHy topic,[16] where as helminth associate with AD was in Epitope mapping topic.[9,57,58,60] The increasing of inflammation cytokine associated with AD is also reveal in Anti TNF antibody; anti TNF-a treatment response in RA has been reported[66] and supported by others.[43,56,67]

The same trends of HyHy-AD association in developing countries is proven in schoolchildren,[38] whereas animal studies demonstrated that worm antigens increase the protective effect and represent a new potential management therapy fighting AD.[33] The use of pills of frozen microbiota are already used in clinical trials, will be effective as the leading for abundant use and large industrialization of FMT.[35] These pills of frozen microbiota are similar with dormant bacteria in dry and hot area like Sade village, which is forbidden done in early rainy season where the dormant bacteria become active living bacteria (*pageblug*/ epidemic disease). Synthesis vaccine of HBV are produce by Aptamer AND miRNA (43 publications). Aptamer AND vaccine (23); aptamer AND antibiotic (54); vaccine AND epitope (6941); antibiotic AND epitope (135) are found with EBSCOhost search engine. Treatment of several AD with worm and worm eggs mitigate the clinical symptoms and inhibited production of proinflammation cytokines and increase anti-inflammatory cytokines.[43] The hopes of creating vaccine based on new protective antigens not only for AD, but also in TB vaccine.[47] Vaccination for Mycobacterium tuberculosis[47] and antimicrobial peptides for vaccine nanolipoprotein have been reported [24,25] but for AD were already answered by meta-analyses[36]; and supported by others in table 1. Since 2010, de-Graft[40] convinced the double burden of infectious and chronic diseases which is a risk by FMT diversity microbiota but could be safe in Sade village and Bau Nyale festival. It is eastern to Wallace's Line, between tropical rainforest area (Bali island, humid and hot) and Savanna area (Sumbawa Island, dry and warm). One shot contemporary vaccine challenges could be improving global health vaccine, at a time,[49] cross diseases[44] and same IRF5 polymorphisms could influence RA similar to SLE patients.[21]

3.2 Bacterial and Parasite wall

Prevention of AD with immune modulation using repeated sequences from parasites associated to anti TNF or decrease TNFa associate with T cell epitope in RA Therapy known as affimer technology,[66] and Jhonson 2012[51] made affimer which linked with impedance CRP immunoassay and TNFa.[43,51,56,67] An intestinal worm infections longitudinal studies and allergy in rural vs. semi urban areas of Flores, Indonesia have been reported,[42] which could help develop evidence-based policy making.[42]

FMT beyond intestinal disorders,[22] is supported by allergy reduction due to increase microbial exposure.[13] Caucasian RA were lower than Japanese, which is statistically significant in the Japanese population[15] by HyHy approach could now be simply understood. Another autoimmune reaction associate preeclamsia-EBV epitope[54] and the global multimorbidity pattern in multi country has been made.[55] Epitope mapping of tuberculosis antigen,[57] and other epitope mapping,[58] could now be help by Mass Sepctrometric epitope mapping[59] and short non-epitop vs. epitope prediction antibody binding,[60] but FMT is an effective therapy for recurrence CDI reduce the cost effectiveness therapy for CDI,[50] Qualitative and quantitative antigen induce immune responses,[2] and a linear surface epitope in a proline rich reg of HeV could be a model.[4] Invariant NKT cells on Parasitic HyHy and cell wall of Bacterial and Parasit is a cellular structure in variety highly enriched of glycolipids and lipoprotein, is a cross talk of Th1, Th2, Th17, Treg, inate lymphoid cells.[3]

IV. LIMITATION

These Systematic review has several limitation 1) Non published literature on Sade village and bau nyale/princess Mandalika may limit the validity of our findings, but these cultural indigenous study are a real phenomenon which is already attracted peoples from developed and clean countries with AD burden. However, we used meta-analysis with partly overlapping data bases for our literature search on bacteria and worm cell wall antigens. And we found a large number of papers fulfilling the criteria of our search in these HyHy-AD prevention, diagnosis and therapeutic. 2) Another potential problem in this systematic review is that studies mainly used in the searching steps are depended on the vocabulary synonyms (not find in

general, but many in specific such as autoimmune diseases could be Rheumatoid arthritis, recurrent CDI, IBS, T1D, atopic sensitization, asthma etc.). Different chances changes choices used give different results may be misleading. We intended to manage this problem in an additional work, by dividing the using chosen specific to generic into 2 broad categories: measurements and epidemiological findings.

V. CONCLUSION

Epitope of FMT, dormant bacteria, protein from the wall of worm are all rich of ranges epitopes and could be present in cultural event like Sade village and Bau Nyale 'Mandalika princess' festival. These indigenous study from local, present to global is similarly paralleled with epitope mapping and contemporary industrial affimers and aptamers based on epitope biotechnology for diagnostic, vaccination and therapeutic fighting against autoimmune diseases. Further education to policy maker and global politic doer from local to global will support this added value idea.

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DISCLOSURE

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Red Algae (Rhodophyta) in Biomonitoring of Coastal Ecosystems

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Abstract— *The formation, species composition and functional role of red algae were studied in the Black Sea's coastal zone. In sublittoral plant communities, red algae are dominant both by their number of species in the phytocenosis and by their active thallus - the one that ensures their high metabolism. Algae with a large specific surface area of thallus can accumulate heavy metals in higher concentrations. In the paper, we discuss possibilities of using red algae as biomarkers of marine pollution, and as bio filters in the processes of water natural.*

Keywords— biological monitoring, biomarkers, heavy metals, red algae (Rhodophyta), sublittoral zone.

I. INTRODUCTION

Biomonitoring as a system of long-term observations and measures of control over marine environment goes down to two key components: diagnosis and prognosis. When carrying out diagnostic monitoring, we study the "here and now" condition of the ecosystem polluted by the most widespread and hazardous marine contaminants. At that, such monitoring is focused both on environmental factors and on various biological responses that testify to derating of a biotic community's parameters. Methodology that underlies prognostic monitoring is active experimenting in situ that allows us to cross-relate - within a well-planned study - the results of forecasting environmental implications of existing impacts upon the ecosystem [8].

Recently, the use of biomarkers has taken on special significance for assessing the implications of pollutant effects upon water ecosystems. In seashore ecosystems, algae has a leading role in autotrophic synthesis of organics, as it is precisely algae that determine the biological efficiency level and take part in determining the quality of natural sea water. In this connection, we see that algae's role is more than logical as monitoring indicators for seashore ecosystems pollution by the most hazardous substances - heavy metals, i.e. pollutants that can get into sea water by many a way. Algae response to heavy metals presence in water is very often the key informant in assessing the marine community condition, if compared to other types of hydrobionts. [4].

It was algae that were there first indicator organisms in qualitative assessments of marine environmental conditions: presence or absence of certain species is a testimony to the environmental status of a water basin. Algae can also be used as biological markers of long-term toxic effects, while their response is what allows us to both forecast changes in the composition of a biotic community and provide well-founded guidance for an active intervention by way of bioremediation and recovery of the affected biocenotic communities before irreversible processes begin in the ecosystem that suffers from anthropogenic impacts [6].

With this in mind, we have carried out studies aimed at providing rationale to the idea of using red algae (with different lifecycles) as biomarkers, when doing biological monitoring of the coastal zone.

The key goal of this study was as follows: to investigate the formation of sublittoral phytocenoses and to determine the specific surface area of red algae's thallus. Special care was taken for heavy metals distribution in the abiotic component of the ecosystem and for accumulation of the metals in algae of the coastal zone's biocenosis. Our findings paved the way for shaping the species composition and subsequent reconstruction of a benthic community on an artificial reef.

II. MATERIAL AND METHOD

The study was done in the littoral area of the Black Sea's north-western district, using lightweight diving equipment. Algae samples were taken in the zone spanning from the water edge down to 30 m depths, using area frames 0.1 and 0.25 sq/m, and differentiated by species and size; later the species abundance and dry/wet weight of each species' biomass were determined [7 ; 5].

When determining specific thallus area of red algae, we chose 10 specimen, dissected them singling out the basal part, medpart and apical part of each plant. Then clipping of thallus was done, their parameters measured, and subsequently squared by known formulae [9] and using computer-aided systems of image analysis: «Image analysis system», «Video

TesT-Morphologia» and «MaxSoft 3.0». The weight of thallus clippings was measured on electronic scales to within 0.001 g. The specific surface area of a plant was expressed as a thallus square per weight unit (S/W)[10].

When analyzing the epiphytic complex of diatoms, algae's thallus suspension was concentrated by filtering through membranes, and the sediments fixed by 2.5 per cent glutaric aldehyde. The diatoms' species composition was determined in vivo and on permanent preparations prepared in the following way. The algae were washed free of the fixative and placed on a cover-glass with a tiny splash of H_2O_2, and scorched in open flames through an asbestos blanket. The cover-glass with plants was placed onto the object plate with Canada balsam [1].

Water samples taken by a plastic sampler were then filtered through a membrane and fixed by HNO_3 up to 0.1 per cent by volume. Extraction of metals from the water was done using a 0.01 M methylisobutyl ketone solution of in an acetyl-acetate hexamethyl dithiocarbamate hexa methylammonium buffer at pH 6.0. Sediment samples were taken by a Teflon tube stratameter, dried up and brought to constant weight at 105^{o}C. Metals from bottom sediments containing fractions of oxidized, reduced and helate compounds were recovered by the method of sequential extraction [3].

Metal accumulation in algae thallus was determined using an atomic absorption spectrophotometer. Samples were prepared by incineration using the «Digester system». A weighed quantity was treated by the mixture of HNO_3+H_2SO_4+$HClO_4$ according to the pattern as follows: 20 minutes at 30^{0}C, 5 hours at 75^{0}C and 30 minutes at 150^{0}C. Then the samples were filtered out and diluted to volume of 10 ml. Subsequently the solution was cooled off, pH brought to 3.0, and analyzed using «Hitachi» and «Perkin-Elmer» equipment, relying on calibration standards CRM «Sea lettuce» [2].

Artificial reefs up to 20 sq/m were erected at depths down to 10 m, chessboard, on mud-sand bottom, no other algae around; the structures were made of shell stone covered with a mesh.

The findings were finally processed in software packages «Microsoft Exel» and «STATISTICA 5.0 for Windows».

III. RESULTS AND DISCUSSION

In the north-western area of the sea, red algae either form independent communities or belong to phytocenoses whose edificator species may be brown or green algae. Plant formations where edificators are *Cystoseira crinita (Desf.) Bory*, *Phyllophora nervosa (DC) Grev.* or *Ulva rigida Ag.* do belong to the most widespread communities in the sublittoral sea zone.

When studying multilayer plant formations made up by brown algae *C.crinita* having projective cover of 80 per cent, we identified 20 species of brown and red algae. The basic stratum of them was formed by *Cystoseira crinita*, in the second one were found *Ph. nervosa* and *Laurencia obtusa*, in the third layer - *Corallina mediterranea* and *Gelidium latifolium*. Among the aerial plants on the thallus of large algae having the biggest biomass, *Ceramium rubrum*, *Polysiphonia subulifera* and *Laurencia coronopus* (Table 1) were found.

TABLE 1
ALGAE BIOMASS AND THALLUS SURFACE AREA IN PLANT FORMATION *CYSTOSEIRA CRINITA*

Depth, m	*Cystoseira crinita*		Lower layer algae		Epiphytic algae	
	biomass, g/m²	S of thallus, m²	biomass, g/m²	S of thallus, m²	biomass, g/m²	S of thallus, m²
1 m	1209-6920 4148±48	11.5-65.7 39.4	754-1753 1364±21	7.5-45.5 25.4	0-311 169±10	0-31.8 15.9
3 m	3950-8163 6696±71	37.5-77.5 63.5	609-3102 1433±18	35.2-136.9 57.4	205-2032 1194±21	16.0-30.6 40.0
5 m	1830-8609 5373±42	17.4-81.7 51.0	330-1085 809±15	12.8-54.1 24.5	165-3109 1709±24	13.1-77.3 47.9
7 m	1519-2845 2322±35	14.4-27.0 22.0	165-1400 799±17	7.9-10.3 9.1	129-4211 2214±30	11.7-81.5 49.2
9 m	712-1250 989±21	6.8-11.8 9.3	500-1480 835±17	8.3-14.3 9.9	115-1552 867±17	11.7-64.6 38.8

In another multi-layer phytocenosis growing on a stony substrate at the depth of 10 m, red algae were also dominant. The upper level with projective cover of < 20 per cent was formed by *C.crinita*; at the same time the basis of the plant formation was made up by perennial photophilous red sea-grass: *Ph.nervosa, G.latifolium, Laurencia pinnatifida*. In the composition of the phytocenosis, we found certain forms of sciaphilic red algae *Apoglossum ruscifolium* and *Polysiphonia elongata*, along with filiform and lamellate epiphytic red algae growing on *Cystoseira* and *Phyllophora*, including *Rhodochorton purpureum*.

In a two-layer phytocenosis (3 m deep) with green algae *Ulva rigida* as edificator, red algae also occupied over 70 per cent of the formation, many of them (*Callithamnion corymbosum, C.rubrum, P.subulifera, Ph.nervosa*) having 100 per cent occurrence.

The specific thallus area (S/W) of algae is a very important indicator that determines the level and rate of metabolism in aquatic organisms. With very few exceptions, perennial species-edificators had relatively small specific thallus area: *Cystoseira crinita* – 95 cm^2/g, *Phyllophora nervosa* – 136 cm^2/g, *Ulva rigida* – 355 cm^2/g. At the same time, seasonal and annual algae usually had a slightly higher thallus S/W. Among perennial algae, certain sciaphilic forms and organisms living in the lower sublittoral (*A. ruscifolium, Gracilaria verrucosa, Gelidium crinale*) also had a larger thallus area (Table 2).

TABLE 2
SPECIFIC THALLUS AREA OF DOMINANT RED ALGAE SPECIES IN THE BLACK SEA (cm^2/g)

Algae species	Specific surface area sm^2/g
Apoglossum ruscifolium (Turn.) J. Ag.	630±39
Callithamnion corymbosum (J.E.Smith) Lyngb.	1815±123
Ceramium ciliatum (Ell.) Ducl.	321±24
Ceramium rubrum (Huds.) Ag.	262±10
Ceramium strictum Grev. et Harv.	418±33
Chondria tenuissima (Good et Wood) Ag.	259±16
Corallina mediterranea Aresch.	667±50
Corallina officinalis L.	429±43
Gelidium crinale (Turn.) Lamour.	712±58
Gelidium latifolium (Grev.) Born. et Thur.	171±39
Gracilaria verrucosa (Huds.) Papenf.	606±64
Laurencia coronopus J. Ag.	68±7
Laurencia obtusa (Huds.) Lamour.	66±10
Phyllophora nervosa (DC.) Grev.	136±14
Polysiphonia denudata (Dillw.) Kütz.	598±47
Polysiphonia elongata (Huds.) Harv.	405±27
Polysiphonia subulifera Harv.	273±12

It was established that the surface area of algae populations in phytocenoses undergoes significant change in different seasons subject to the vegetation depth. Maximum values of thallus surface in sublittoral plant formations are characteristic of late summer when the weight of summer and annual algae reaches its top.

Thus, in plant formations of the investigated sea area, by range of species - and in deeper waters also by biomass - prevailing are red algae with divided and filiform thallus that are structural and functional dominants in these plant communities.

When determining heavy metal contents in water and in bottom sediments in the area, we found that heavy metal concentrations in the abiotic substance are by and large typical for slightly polluted sea zones. The content of dissolved metal forms in water was slightly above the average concentrations in coastal waters. In bottom sediments, metal concentrations were by a huge ratio higher than in water per se. At that, about half the metals found in the sediments were bound in strong complexes that can be extracted only by an EDTA solution and, apparently, were not available to many an osmotrophic aquatic organism. At the same time, the oxidized and reduced metal forms we revealed can travel to the environment thus enriching bottom waters. It was found out that metal concentrations in water vs. sediments are slightly different, including such pairs of elements close to each other in their chemistry as Zn: Cu and Zn : Cd, which is a sign that some biological way may exist of removing them from the solution, since high metal concentrations in bottom sediments can be hardly accounted for solely by absorption processes (Table 3).

TABLE 3
HEAVY METAL CONTENT IN WATER AND BOTTOM SEDIMENTS

Metal	Water, µg /l	Bottom sediments, µg /g of dry weight	Metal content in bottom sediment fractions, %		
			I	II	III
Cu	4.9±0.8	35.5±7.2	46.0	14.6	39.4
Zn	15.4±3.0	33.8±4.6	45.2	-*	54.8
Cd	0.7±0.2	3.2±0.5	41.2	-*	58.8
Ni	3.4±0.4	24.8±5.6	42.9	13.4	43.7
Pb	1.9±0.3	9.4±2.3	40.1	12.4	47.5

Note: -*- not found F I – extraction: 25% NH^3OHCI + 35% CH_3COOH
F II- extraction 30% H_2O_2, F III – extraction 0.1 M EDTA at 70^0 C

Analysis of algae belonging to different taxonomic ranks in a plant community revealed differences in heavy metal content: red algae generally accumulate more Zn, Cu, Ni, Cd and Pb than brown and green algae. At that, metal content differences were revealed not only between different algae groups, but also orders, families and geni. Thus, the content of some metals in algae tissue cells of genus *Laurencia (L. coronopus, L.obtusa)* sampled for analysis in one of the bays differed considerably. A similar way of accumulating certain heavy metals was also observed in algae having a thallus surface area close to the specific one.

Analysis of the dynamic pattern of some heavy metals content in water and algae showed that high concentrations of copper, cadmium and lead in water do not always go hand in hand with maximum accumulation of these in algae's thallus. It seems to be obvious that in the absence of local pollution, algae preferentially accumulate the metals that are necessary for their metabolism. The top content of copper in algae was observed in April and September, whereas that in water occurred in July and November. The content of cadmium in algae thallus went up during summer time and considerably dropped in autumn. Maximum content of lead in algae fell on June-July when the man-induced impact of transportation in coastal ecosystems goes up radically.

Of agar-producing algae (*Ph.nervosa, G.verrucosa , G.latifolium*) sampled for analysis in different areas, it is characteristic to have a rise in metal content from January till September [2]. A similar pattern in metal accumulation is characteristic of red algae of the genus *Polysiphonia* (*P.sanguinea* и *P.subulifera*): during the period from April till September the rate of metals in thallus tissues perceptibly increased. To theses algae is an also peculiar species difference in metal accumulation, which is first and foremost associated with their formation: the specific thallus surface area of *P.sanguinea* is considerably larger than that of *P.subulifera*.

On the basis of measurements concerning metal content in algae, we could identify species with similar patterns of metal bioaccumulation - of the metals belonging to a high-priority list of potentially hazardous chemical substances. Relying on cluster analysis, we can count the following species as similar in metal accumulation: *C.corymbosum, C.strictum, P.sanguinea* and *Gelidium crinale*. At that, to the same cluster belong the algae having highly divided thallus, i.e. the ones with large specific surface area of thallus and S/W ratio. At the same time, mineral composition of the algae varied in quite a wide range: ash content of the dry weight made up from 17.9% in *G.latifolium* and 24.6% in *C.strictum* to 80.7% in *C. mediterranea* (Table 4).

TABLE 4
HEAVY METALS IN THE THALLUS OF DOMINANT RED ALGAE SPECIES

Algae	Zn	Cu	Ni	Cd	Pb
Ceramium corymbosum	83.4±12.0	27.0±3.3	19.3±2.6	3.4±0.7	4.9±1.3
Ceramium rubrum	66.5±11.6	19.4±2.0	8.3±1.5	2.2±0.3	3.8±0.5
Ceramium strictum	77.8±7.0	18.8±2.9	13.6±1.2	2.6±0.5	5.8±1.0
Gelidium crinale	73.3±10.2	23.5±1.7	17.1±1.4	2.9±0.4	4.0±0.6
Gelidium latifolium	58.1±9.6	15.0±1.8	10.1±1.0	2.0±0.5	6.1±1.9
Gracilaria verrucosa	83.4±8.2	11.5±2.1	7.7±1.8	1.9±0.3	2.3±0.6
Laurencia coronopus	27.6±2.0	7.8±1.3	6.9±1.2	1.8±0.4	2.6±0.3
Laurencia obtusa	16.6±1.9	5.9±1.2	5.6±1.0	1.3±0.3	2.0±0.4
Polysiphonia elongata	60.8±8.1	20.1±3.0	18.8±3.1	3.1±0.6	3.0±0.4
Polysiphonia sanguinea	75.8±3.9	25.8±2.3	26.3±3.0	4.7±0.3	3.5±0.8
Polysiphonia subulifera	53.7±5.0	18.7±2.0	8.3±1.6	0.9±0.2	2.2±0.4
Phyllophora nervosa	63.2±8.2	21.4±7.0	19.2±3.9	1.9±0.3	3.0±1.0
Cystoseira crinita	50.0±6.3	11.9±1.5	3.3±0.5	1.1±0.2	1.7±0.3
Ulva rigida	61.8±6.9	12.6±1.9	4.1±0.8	1.2±0.2	1.0±0.2

IV. CONCLUSION

The established facts about metal accumulation in sea grass - linked to thallus formation, along with species- and environmentally specific peculiarities of the latter - turn out to be determinative for choosing monitor plants in different taxons and ecological groups in the context of sea ecosystems pollution. This is particularly important for coastal ecosystems that are most susceptible to human impact.

In the sublittoral zone of the sea, red algae belonging to various bottom plant formations play the role of functional dominating species of the biocenosis. High biodiversity of red algae occupying different biotopes in the coastal area is what allows us to use them as indicator/monitor/biomarker-plants when assessing the ecological state of the community when water is contaminated by most dangerous substances. Red algae with a large specific surface area of thallus are capable of accumulating high concentrations of hazardous pollutants including heavy metals. It is exactly this peculiarity that allows to successfully use the algae - along with other types of sea organisms - in bio-filters involved in the processes of self-purification of natural water, relying at that both on the group-specific and selective pattern of heavy metal accumulation [6].

The above statement is based on the following established facts: First, algae provide us with a comprehensive picture of the ecosystem state, which is particularly important when the latter's pollution demonstrates a discreet pattern. Second, algae have an explicit capability of accumulating heavy metals, which both contributes to the accuracy of chemical examinations and indicates that bio-available forms of the former are present in the environment. Third, using algae as monitor plants opens up a promising perspective for developing practical guidance on how to remove heavy metals from polluted water basins via the use of bio-filters implanted in artificial reefs.

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Factors Affecting the Acceptance of Integration Tend Rice Fields

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Abstract— *Land fragmentation is one of the country's traditional agricultural structural elements which nowadays as one of the main obstacles to agricultural development. Therefore, the aim of this study was to investigate the willingness to accept integration of rice fields by the lifters who strive in Behshahr interest and identify factors affecting it. In this respect, the required data through the completion of a questionnaire among lifters who strive in Behshahr interest collected and analyzed using Logit analysis. The results of the study showed that the amount of variables, age, education, experience, income, capital, area under cultivation and the distance of the Earth from each other parts have a significant difference. At the end of the research based on the results, decide to present.*

Keywords— consolidate, the tendency toward admission, rice, Behshahr.

I. INTRODUCTION

Agriculture sector of our country today, responsive in many new internal and external needs. The transformation of the Iranian society to society that its people consume more of their manufacturer plays the role of their manufacturer is not only causing the exit of the unfortunate consequences of the Exchange will be reduced, but other sources, such as increasing the national capital to the following process. Domestic consumption per capita energy and renewable natural resources in Iran is very high. Comparison of statistics related to production and consumption within and outside of the country, indicating the lack of coordination of the process of with domestic production, consumption as compared to other countries. In other words, the efficiency of biological resources in Iran due to the lack of true management system of production is very low. Perhaps it could be said, of natural resources available in the country, but to reach more than correct and leads to the production of agricultural products, the growth and development and are looking for a positive result, without arzavari and that, which they had expected, will process the header (technology network, 2009; Kohansal and Akbari, 2013).

Consolidate a process that aims to help land to communities for the optimum use of resources, and also the Organization of spatial parts, in accordance with the agreement of the public again that eventually led to the reconstruction of society in all economic, social and political dimensions may be fitted, is (Kopya et al, 2002; Mousavi et al, 2011).

Consolidate the land provides the means for rural development, because it can operate with the efficiency and healthy competition to create better integration and commonality of agriculture we have. Also, the process of economic conditions of the villagers by providing greater production of non-agricultural activities, access to markets and credit, etc. And the social conditions of employment with the creation of opportunity, participation, access to education, health services, etc. Improve (FAO, 2003).

Land for agriculture purposes survival consolidate necessary infrastructure could be fitted and the standard of living of the villagers and upgraded to provide sustainable development (Akbari et al, 2017; Mullen et al, 2005; Kohansal et al, 2013). Consolidate the possibility to upgrade the level of management of the farm land in the total consumption of water and the fathe farm operation and the use of new technology to provide an appropriate, especially irrigation system under pressure due to the drought and the recent drought of loaders are provided (the Office furnishing and renovation of land, 2001; Mousavi and Akbari, 2014).

Rice as a main food, play an important role in Iranian calaiy household basket. Mechanized cultivation of this crop in the country with many problems and obstacles encountered, which is one of the most important factors of retail property. From the perspective of mechanization, the various aspects of retail property on cropping of rice mechanized company from which its effects are direct and indirect. Establishment of limitations on substrate suitable for mechanized rice cultivation such as to the design and implementation of the grading feature and consolidate paddy land commensurate with the demands and conditions of the machine and production system and supply the product to the market, the lack of possibility of comprehensive utilization of machinery system (in order to increase the capacity of the farm and optimum

use of energy) and the restriction on the use of appropriate equipment and machinery to increase the conversion of rice production and product quality. Including retail ownership, problems are in effect happen.

In total retail property can be used as a way of developing mechanization rice outlined, because the country is not only providing the platform and implementation mechanization faced with limitations, but even makes it is possible because of the lack of the possibility of the use of machinery and technology, product quality, the production loss in the family.

To prevent the existing ownership level of more small compilation of comprehensive and stable laws, data on utilization system shifts to a group of solo instead of machine operation, utilizing a farm with machines and higher capacity in the form of production and services cooperatives of mechanization, the reduction of production figures at the level of regional variability, changes in how to deliver and supply the product to the market and also the changes in how to deliver and supply the product to be the factories In the direction of the implementation of the agricultural mechanization with retail are ownership should seriously study and discuss.

In Iran due to the fragmentation of ownership and being small it, consolidate and equipping land renovation and drorti ankarnapzir for the thdidehay the way of fleeing from the rice production. So with regard to the importance of the above mentioned items in this research to examine the most important factors affecting farmers desire to integrate agriculture lands fitted paid Behshahr.

Zarifiyan damavandi (2012), to examine factors affecting land in the villages of consolidate proposal Kabudarahang engaged in Hamedan Province, the results of the study showed 18 variables: consult with experts of agricultural extension, history, membership organization, the number of pieces of land, the amount of income and land, of the effective factors in the acceptance of the plan by the consolidate farmers.

Ashkar Ahangar Kalaei et.al (2006), during the study of the history of the Mazandaran in paddy soils: laws, agriculture, intellectual property, know-how, timely delivery of integrated land, making the right of way between the right of canal construction, farms, water consumption and reduction in the cost of production factors in the acceptance of and adherence to cultural factors, attitudes and traditional beliefs, level of literacy and the ensuing lack of technical knowledge and knowledge of effective factor in the failure of the plan might consolidate.

Gonzalez Garcia, (2014), in Spain showed that the program is an important step forward in the building of an integrated to improve workforce productivity and not the work of agricultural land may be fitted and the farmers awareness about integrated economic and social results of useful information, the transfer of land to the farms, and the program of state support factors in accepting this application may be fitted to the relevant integration records. In the case of other varieties in other areas has been working but in the fields related to Behshahr record view is not so cultivated product in preference to the terms discussed topic Behshahr farms.

II. MATERIAL AND METHOD

This research is exploratory and descriptive-type collection method is used, in terms of the criteria being applied, in terms of functionality, cross-sectional and time criteria, level, phananagar. The scroll method is a tool to get the response of the sample answers and Guyana has four feature fitted below;

1. Scroll is a form the samples examined population, especially through the potential sample (randomly).
2. Navigating directly to Guyana response evaluated fitted.
3. Method of navigation is to collect sample data because of the crowd support will be fitted.
4. Scroll with a natural head set and work (Hosseini, 2000).

Therefore, in this study, according to the statistics of the community and navigation benefits span the scroll method was used.

Statistical research community will be fitted Behshahr, and number of samples based on the statistical mean and variance of the society before completing the questionnaire and by using the formula of Cochran. In this case that was initially based on simple random sampling from the land between they had not yet integrated the number 5 village then based on simple random sampling size of sample size were selected. In fact, in this study, sampling of clusters of two-stage.

To complete the theoretical basics, fitted library (books, articles, and various research study) was used and the tools used to collect the data in this study, a questionnaire, to determine validity, multiple copies of the questionnaire to the agricultural

extension and training teachers, and some experts of agricultural Jihad organization of Behshahr and supervisors, and other masters of research methodology.

Also, to analyze the data collected in the research of the method of assessment was that the Logit economy using Spss software, coding and data entry program from the forklift to the average types, frequency, percentage, SD, data, descriptive and inferential statistics level and then check the software for profile measurements Variables data Shazam analysis. To examine the relationship between the variables of econometric methods used were Logit at the bottom of the model is described.

2.1 Logit regression model

Possible models of normal distribution and Logit of interest Logit and predicted probability values, between zero and one fact. What are the factors that affect the possibility of choosing one option will affect, the assumption that the average achieved desirability of a selection to select the traits that differ according to individuals, depends? If the choice of desirability of each medium as a component of random disorder in addition to the definition of the desirability of the investment, we have the following relations (Jaj and partners, 1988):

$$U_{i1} = \overline{U}_{i1} + e_{i1} = z'_{i1}\delta + w'_i\gamma_i + e_{i1} \tag{1}$$

$$\mathrm{U_{i0}} = \overline{\mathrm{U}}_{\mathrm{i0}} + \mathrm{e_{i0}} = \mathrm{z'_{i0}}\delta + \mathrm{w'_i}\gamma_{\mathrm{i}} + \mathrm{e_{i0}} \tag{2}$$

In it and in desirability of selecting moderate, and desirability of, and by the individual specifications of vector fields i download, is a vector of social economic profile of a person, and i Amin - components and random disorder. With regard to the contents, and were mentioned and i Amin the first option, if you will, or if you choose to see the proof, we have variable, the following values were as visible as are determined:

$$\left[y_i = \begin{cases} 1 & if \quad y_i^* > 0 \\ 0 & if \quad y_i^* \le 0 \end{cases} \right] \tag{3}$$

We have active review as follows:

$$y_i^* = (z_{i1} - z_{i0})'\delta + w'_i(\gamma_1 - \gamma_0) + (e_{i1} + e_{i0}) \tag{4}$$

(5)

$$y_i^* = \left[(z_{i1} - z_{i0})' w'_i\right] \begin{bmatrix} \delta \\ \gamma_1 - \gamma_0 \end{bmatrix} + e_i^* = X_i^* \beta + e_i^*$$

The idea behind the variables and unknown parameters, respectively, and random errors. The possibility that the phrase is:

$$\mathrm{P_i} = \Pr[\mathrm{y_i} = 1] = \Pr(\mathrm{y_i^*} > 0) = \Pr(\mathrm{e_i^*} > \mathrm{X_i^*}\beta) \tag{6}$$

It is clear that filling top model must be selected for a particular probability distribution of investment. Two examples of the most common distribution that is used for this purpose, the standard normal distribution function and logistic. The cumulative distribution (C.D.F) 1 is the normal standard as follow

$$F(t) = \int_{-\infty}^{\infty} (2\pi)^{-\frac{1}{2}} \exp(-\frac{X^2}{2}) dx \tag{7}$$

The cumulative distribution function of the random variable logistic and as follows:

$$F(t) = \frac{1}{1 + \exp^{(-t)}} \tag{8}$$

The cumulative distribution function, both the symmetrical and has a mean of zero. Normal standard against the variance of random variable, the variance and is equal to logistic. It is near to the normal distribution lajastic distribution and comfortable

with it. Select the logistic if the level of distribution, statistical model that will be gotten from that model logit applicable. With respect to the standard normal distribution, functions are symmetric and logistic the possibility of failing to choose one of the following options with the use of the relationship will be achieved:

$$F(-t) = 1 - F(t) \tag{9}$$

In addition, so can the relationships (the possibility of) the level below to review:

$$P_i = \Pr(e_i^* > -X_i^* \beta) = 1 - (e_i^* < -X_i^* \beta) = 1 - F(-X_i^* \beta) = F(X_i^* \beta) \tag{10}$$

With regard to the cases of the expression, in the model that i logit the possibility to consolidate individual land-use proposal a. accept, will be achieved as follows:

$$P_i = F(Z_i) = F(X_i^* \beta) = \frac{1}{1 + e^{-X_i^* \beta}} \tag{11}$$

Also with regard to the relationship between the probability that the person above I propose to consolidate Amin the land book, the author's acknowledgement shall be calculated below:

$$1 - P_i = \frac{1}{1 + e^{Z_i}} = \frac{1}{1 + e^{X_i^* \beta}} \tag{12}$$

One of the important objectives of the Logit model in the prediction of effects, the estimated change in the probability of acceptance of the proposal by the description of variables on the individual i. For evaluation of the effects of a change in any of the independent variables on the probability of acceptance of the proposal, should the relationship (11) partial derivative to be taken as follows (Madal, 1991):

$$\frac{\partial P_i}{\partial X_{ik}} = \frac{e^{Z_i}}{(1 + e^{Z_i})^2} \beta_k \tag{13}$$

The parameter K is my independent variable. Having partial derivatives of the relationship (3-13) I K, more variables that are specific are read as follows.

$$\varepsilon_i = \left[\frac{e^{Z_i}}{(1 + e^{Z_i})^2} \beta_k \right] . \frac{X_{ik}}{P_i} \tag{14}$$

The relationship works (3-14) shows more ability are not fixed, and since the values depends on the model used in the description of variables.

2.2 Estimation of model Logit:

If a sample is available with the T, the function will be defined as the following:

$$L = \prod_{i=1}^{T} f(y_i) = \prod_{i=1}^{T} P_i^{y_i} (1 - P_1)^{(1 - y_i)} \tag{15}$$

Function (3-15) also can be used as indicated below:

$$L = \prod_{i=1}^{T} F(X_i' \beta)^{y_i} \left[1 - F(X_i' \beta) \right]^{(1 - y_i)} \tag{16}$$

In the event that the first option is selected and otherwise. The following is a classical book function:

$$LnL = \sum_{i=1}^{T} y_i LnF(X_i^{\prime}\beta) + \sum_{i=1}^{T}(1-y_i)Ln\left[(1-F(X'_i\beta))\right] \tag{17}$$

According to the studies of Broan et al (1994), the following expression, a view:

$$(1-d_1)(1-d_2)\int_{-\infty}^{b_2} dF(x) + d_1(1-d_2)\int_{b_1}^{b_2} dF(x) + (1-d_1)d_2\int_{b_2}^{b_1} dF(x) + d_1d_2\int_{b_2}^{\infty} dF(x) \tag{18}$$

Cumulative distribution F (x) that tends to consolidate. In this phrase, if the proposal is accepted and, i otherwise.

2.3 Independent variables include:

The amount of the age, level of education, the history of rice cultivation, the income, the amount of capital, the level of mechanization, the attitude toward land, consolidate the history of agriculture, the distance between the Earth and the education and extension pieces.

$$Y= f(x1, x2, x3, x4, x5, x6, x7, x8, x9, x10, D1) \tag{19}$$

The dependent variable y)) in this study, a variable is a variable that is zero or an imaginary will that takes the value of a farmers that you want to consolidate, and the value of zero to farmers that do not have a tendency to consolidate in terms of. In addition, the demo variable is a variable, education and extension, which is Passeda value of course farmers and not the value zero.

III. RESULTS

3.1 A linear analysis

In a multivariate analysis of each variable separately, and no evaluation of its relationship with the other variables studied. This method is only necessary for a general understanding of the variables that are obtained from the statistical community is used.

3.1.1 Age

The combination of age and physical forces, with their experience. Adults have been made due to experience of hue, compared with young people with coherent and this is based on the necessity of continuous efforts and reasonable justification to change is based on the necessity of continuous efforts and reasonable justification to change their inclination and towards emphasizing specific subject. On the other hand, increasing age is associated with decreased physical. This property, in particular for farmers that their income due to the severe environmental conditions of dependency and economic policies the Government constantly fluctuate, causing the ratio to a permanent hazard and risk coupled with life, are chronically vulnerable (Rahjoi, 2010). Sincethe statistical community case study includes patients in different parts of the city in Behshahr. Therefore, statistical calculations related to farmers.

TABLE 1
FREQUENCY DISTRIBUTION OF AGE PATIENTS BEHSHAHR COUNTY

SD	Mean	Frequency	%	Age
14.3	52.5	5.25	3	<35
		21.6	21	35-40
		31.7	36	41-50
		25.8	31	51-60
		10.3	9	61-70
		5.25	4	>70
		100	97	Total

3.1.2 Education

Different ways of education on the tendency and effect decisions of the people. With the increasing education of a person in order to obtain information and enjoying it, his understanding of the economic and social environment on extra his broader views and make improvement that will be rendered in the selection frarvish. Peoples mind for creativity education tract may

increase productivity with repatriated Cubans and their potential to achieve higher levels of life, and thus the possibility to upgrade to create new opportunities for them are possible.

TABLE 2
DISTRIBUTION OF FREQUENCY OF BEHSHAHR PATIENT'S LEVEL OF EDUCATION

Frequency	%	Education
41.3	41	Non
46.5	45	Diploma
12.2	11	University
100	97	Total

3.2 Estimating the relationship between related factors affecting land to consolidate tendency

In this study, dependent variable, the tendency has been consolidate the rice land. Thus, if the lifters who strive to consolidate the interest had a value you want and if you want a zero value you did not. For this purpose, the relevant regression based on 100 patients Behshahr questionnaires that had been collecting, estimating, and the result were presented in table 3.

TABLE 3
FACTORS AFFECTING LAND TO CONSOLIDATE TENDENCY BEHSHAHR PATIENTS USING LIGT METHOD

Variable	Coefficient	t	e	ME
C	-4.36	-1.51	-	-
Age (year)	-1.43*	-1.95	-1.13	-1.108
Education (year)	1.89*	2.52	2.101	1.95
Area of agricultural land	-1.94*	-2.67	-1.73	-0.15
Thinking about agricultural land	0.97	1.22	1.15	1.103
Income	-1.74*	-1.91	-2.106	-1.905
Capital	-1.809*	-2.201	-1.904	-1.59
Meccanization	0.97	1.22	1.15	1.103
Experience (year)	-1.86*	-2.24	-1.91	-1.67
Distance between the Earth parts	-1.96	-2.45	-1.83	-1.41
Extension	0.55	1.18	0.64	0.48
LIKELIHOOD RATIO TEST= 57.5364 **MADDALA R-SQUARE=** 0.75346 **MCFADDEN R-SQUARE=** 0.81157				

IV. DISCUSSION

- Negative effects of age and rate-variable were significant on the tendency to consolidate land use by patients Behshahr.
- Variable rate-the history of rice cultivation is also a negative effect on the tendency to consolidate land use by patients, but have not been significant Behshahr.
- Variable-level of education was a significant and positive effect of beneficiary on the tendency to consolidate land use by patients Behshahr.
  - Negative effect also proportional acreage on a significant tendency to consolidate land use by patients have side Behshahr.
  - Variable attitude towards land consolidate the positive effect on the tendency to consolidate land use by patients, but have not been significant Behshahr.
  - The highest income variable impact on the willingness to consolidate the land with significant negative effect shows that higher income farmers interest that have less tendency to consolidate the land.
- Variable level of mechanization with the plus sign, but no statistically significant on the land by tendency to consolidate Behshahr patients.

- Interest rate variable significant capital and negative effect of the lifters who striveon the tendency to consolidate land use by patients, and indicating the topic of Behshahr is farmers that have a higher investment than other patients have less tendency to consolidate land.

- Variable interest agricultural history and significant negative effect on lifters who strive on the tendency to consolidate land use by patients Behshahr.
- Variable rate distance between the Earth and the effects of negative pieces on a significant tendency to consolidate land use by patients Behshahr.
- Positive education and extension with variable symptom but no statistically significant on the land by the tendency to consolidate Behshahr patients.

V. CONCLUSION

As we know in the Probit and Tobit models of lajite, that the dependent variable for virtual variables may wind up and are allowed to do in the interpretation of coefficients and coefficients of the mark can only study to characterize the interpretation. So according to this more important to provide more precise analysis of the triglyceride and the final effects of a change in the amount of variables such as age, level of education, the history of rice cultivation, the income, the amount of capital, the level of mechanization, the attitude toward land, consolidate the history of agriculture and the distance between the Earth and the education and extension parts on land they want to consolidate the interpretation. That results in the last column of the table (4) with the title of the drawing and the final effect. Therefore, based on the results of this table to interpret and render final effect only:

-The final effect of a variable amount of change in the age on the tendency to consolidate land use by patients against 108.1-Behshahr which footnotes in a rate increase in the age of the beneficiary being assuming the other conditions for the land by the inclination to consolidate Behshahr County 108.1 patients.

-The final effect of a variable amount of change in the history of the rice cultivation is also on the land by the tendency to consolidate Behshahr patients against 13.0-which is due to the lack of interpretation does not render it to be significant.

-The final effect of a change in the beneficiary's level of education variables tend to consolidate land use by patients against 95.1 which Behshahr footnotes per unit increase in the level of education of a beneficiary being assuming the other conditions for the land by the inclination to consolidate Behshahr County 95.1 patients.

-The final effect of a change in acreage is also variable on the land by the tendency to consolidate 15.0-Behshahr patients against which footnotes in a unit increase for acreage being assuming the other conditions for the land by the inclination to consolidate 15.0-Behshahr County patients.

-The final effect of a change in attitude to consolidate variable on the land they want to consolidate the land by patients against 103.1 which Behshahr due to lack of interpretation does not render it to be significant.

-The final effect of a change in the highest income level variable impact on the tendency to consolidate is equal to 905.1 land-and shows that per unit of an increase in interest income being assuming the other conditions for the vector inclination to consolidate land use by patients 905.1 Behshahr.

-The final effect of a change in variable units the level of mechanization on the tendency to consolidate land use by patients against 103.1 which Behshahr due to lack of interpretation does not render it to be significant.

-The final effect of a change in the interest rate on variable capital lifters who strive to consolidate the land desired by patients against 59.1-Behshahr and indicating the subject is in a capital increase in the beneficiary being assuming the other conditions for the land by the inclination to consolidate Behshahr County 59.1 patients.

-The final effect of a variable interest in agricultural history on lifters who strive to consolidate the land desired by patients against 67.1-Behshahr which footnotes per unit increase in agricultural history one beneficiary being assuming the other conditions for the land by the inclination to consolidate Behshahr County 67.1 patients

The final effect of a unit changes in the variable-in the amount of space between the pieces of land on the land they want to consolidate by patients against 41.1-Behshahr which footnotes in one-unit increase in the amount of space between the pieces of land being assuming the other conditions for the land by the inclination to consolidate Behshahr County 41.1 patients.

-The final effect of a variable changes in education and extension on the land by the tendency to consolidate Behshahr patients against 48.0 which, due to the lack of interpretation does not render it to be significant.

In the end, we are to interpret the R2. With iodine noted that in logit and Tobit models, we are faced with in the conventional R2 and R2 R-SQUARE and MCFADDEN are used.

R MC here against 81.0 that shows we have been able to read the variables, 81 percent of the changes performed on the explanation of the dependent variable.

5.1 Suggestions:

According to the results obtained can be applied specifically to provide as follows:

1. As described in the results became clear, the age and history of agriculture, the farmer on the tendency to have negative effects on the consolidate that with regard to this matter, we must first lower the ages of farmers and history are less integrated in the agricultural land to encourage and support to other farmers, he has experience with higher age and according to the main positive of this process interesting to consolidate in this matter.
2. Considering the distance pieces on the Earth would have negative effects should consolidate in the first identification of reasons for such intervals, and in this regard, the policy of some farmers ' interests to adopt.
3. Furthermore, considering that the amount of capital and income, causing a negative tendency is based on the siasti State must consolidate in order to keep a higher proportion of farmers ' income and capital income and capital with the farmers that the payinteri is adopting to increase the tendency of farmers to consolidate.
4. Finally it should be mentioned, that education was not statistically significant in this study but should of educational policies and knowledge about laws and the disseminative giving in to consolidate farmers was unaware because this is very weak though, but can be influential.

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Seroepidemiological Investigation for Chagas Disease in Two Municipalities of Goiás, Brazil

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Abstract— *This study evaluated the risk of transmission of Chagas disease following implantation of the Brazilian National Health System (Sistema Único de Saúde – SUS) in two municipalities considered endemic risk areas. A seroepidemiological and entomological survey was conducted as part of the Triatoma infestans eradication program. According to a previous survey conducted in 1980, seroprevalence of Chagas disease was around 0.82% in São Luís dos Montes Belos and around 2.35% in Novo Brasil. In the present serological survey conducted in 303 schoolchildren born after the control phase in these regions, one of the children tested positive for the disease. In the 236 homes surveyed (150 in São Luís dos Montes Belos and 86 in Novo Brasil), all of which were infested by triatomine bugs, none of the triatomine bugs captured tested positive for Trypanosoma cruzi. Although Triatoma infestans is under control, there has been a considerable increase in secondary vectors such as Triatoma sordida; therefore, continuous epidemiological surveillance is fully justified within the current context of the SUS, and should be considered for inclusion as routine practice within the Family Health Program.*

Keywords— Chagas disease, serology, epidemiology, triatomine bugs, surveillance.

I. INTRODUCTION

As part of a Chagas disease control program, the Brazilian Ministry of Health conducted a serological and triatomine survey throughout most of the country between 1975 and 1983. Data on the prevalence of the infection and on the distribution of its vectors were obtained. The areas in which the disease was endemic were identified, allowing control actions to be defined and prioritized [1, 2].

Chagas disease is an endemic disease caused by the protozoan parasite *Trypanosoma cruzi*, transmitted to humans and other animals by triatomine bugs. The condition is characterized by a chronic generalized infection. Chagas disease is found throughout the entire American continent from the southern United States to the south of Argentina [3].

Domestic transmission of the disease depends on the following factors: the vector must be present, it must be infected and it must have colonized human households. This situation results primarily from circumstances related to the environment, to man's effect on the environment and to the attributes of the vector itself, all of which will end up favoring the presence of the vectors in households [4].

American trypanosomiasis, or Chagas disease, is the most prevalent zoonosis in Goiás, a Brazilian state that is considered a region at risk of vector transmission. Natural transmission has always been associated with close contact between humans and triatomine bugs, with rural populations being those most affected [5, 6].

Triatoma infestans was the principal household vector of Chagas disease in Brazil between 1975 and 1980 and it is now believed to be eradicated from the country. In 2006, the Pan American Health Organization/World Health Organization certified that, as a result of the Southern Cone Initiative against Chagas Disease, transmission by the principal household vector, *Triatoma infestans*, was halted [7].

From an epidemiological viewpoint, the state of Goiás ranked third with respect to the prevalence of Chagas disease in a nationwide survey conducted in 1980. In the municipality of São Luís dos Montes Belos, seroprevalence was around 0.82%, while in the municipality of Novo Brasil, the rate was around 2.35%. In Novo Brasil, a serological survey was conducted in children up to 10 years of age in 1980; however, no such survey was conducted in São Luis dos Montes Belos. At that time,

the most prevalent species of triatomine bug were *Triatoma sordida*, *Rhodnius neglectus*, *Panstrongylus diasi*, *Panstrongylus megistus* and *Triatoma pseudomaculata*. [1].

Oliveira et al. reported that of 32,437 triatomine bugs examined in Goiás between 2000 and 2003, 276 (0.85%) tested positive for *Trypanosoma cruzi*. Of these 276 triatomine bugs, 234 (84.78%) were of the *T. sordida* species (50 intra- and 184 peridomestic) and 21 (7.61%) were of the *Rhodnius neglectus* species (18 intra- and 3 peridomestic) [4]. A triatomine survey conducted in April 2013 found six specimens of *T. sordida* infected by trypanosomatids in the town of Trombas (in northeastern Goiás), corresponding to a rate of infection of 0.6%. These trypanosomatids were morphologically similar to *T. cruzi*. [8].

Based on these previous serological and entomological data recorded by the regional offices of the National Health Foundation, this study aimed at evaluating the seroepidemiological and entomological patterns in towns considered areas of risk, although located within areas in which the transmission of Chagas disease has been controlled. The results of this study will be helpful when making decisions on the implementation of low-cost, effective and viable control measures, as well as the establishment of continuous entomological and epidemiological surveillance.

II. MATERIAL AND METHOD

The present methodology complied with the technical guidelines of the Ministry of Health's nationwide Chagas disease control program [3] and with the routine fieldwork conducted by the National Health Foundation. All municipal rural schools in the selected towns were included in the study. Consent for the children to participate in the survey was obtained from their parents at meetings held at the schools.

2.1 Eligibility criteria with respect to the towns and to the study sample

Based on seroepidemiological and entomological parameters established by the official government agency between 1995 and 1999, for logistical reasons (proximity, the support provided by the municipal councils, the active presence of the Family Health Program in the town), and by consensus, two out of a total of twenty towns were selected for inclusion in the present study: São Luís dos Montes Belos and Novo Brasil. (Fig. 1).

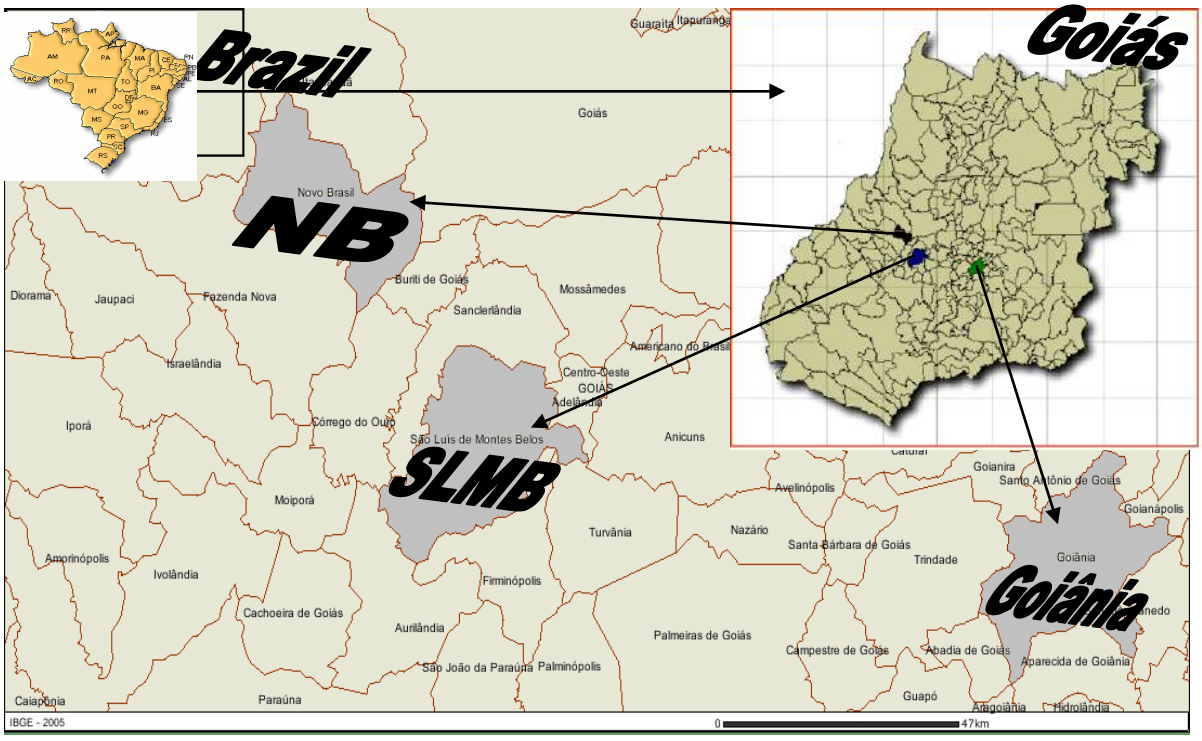

FIGURE 1 – LOCATION OF THE MUNICIPALITIES INVESTIGATED IN RELATION TO THE STATE CAPITAL, GOIÂNIA.

NB: Novo Brasil; SLMB: São Luís dos Montes Belos.

TABLE 1
LOCALITIES INVESTIGATED AND THOSE INFESTED BY TRIATOMINE BUGS: NOVO BRASIL AND SÃO LUÍS DOS MONTES BELOS, 2003-2005

	2003	2004	2005
Municipality	**Novo Brasil**		
Localities investigated	82 / 86	21/86	11/ 86
Percentage of localities investigated	95.35%	24.42%	12.79%
Localities infested	21/ 82	11/21	09/12
% of localities infested	25.61%	52.38%	75.00%
Municipality	**São Luís dos Montes Belos**		
Localities investigated	50/69	50/69	36/69
Percentage of localities investigated	72.46%	72.46%	52.17%
Localities infested	69/93	69/81	69/97
% of localities infested	74.19%	85.19%	71.13%

TABLE 2
LOCALITIES INVESTIGATED AND LOCALITIES INFESTED BY TRIATOMINE BUGS: NOVO BRASIL AND SÃO LUÍS DOS BELOS MONTES, 2003-2005.

Novo Brasil							
Year	**Domestic infestation**		**Peridomestic infestation**		**Households investigated**	**Households infested**	**Households infested (%)**
	T. sordida	*R. neglectus*	*T. sordida*	*R. neglectus*			
2003	2	0	0	0	86	02	02.33%
2004	6	13	10	5	86	11	12.79%
2005	1	10	0	3	86	9	10.46%
São Luís dos Montes Belos							
Year	**Domestic infestation**		**Peridomestic infestation**		**Households investigated**	**Households infested**	**Households infested (%)**
	T. sordida	*R. neglectus*	*T. sordida*	*R. neglectus*			
2003	41	0	346	22	91	58	63.74%
2004	45	1	677	8	91	77	84.62%
2005	32	6	594	3	91	54	59.34%

2.2 Description of the study area

The town of São Luís dos Montes Belos (latitude 16.57868°, longitude 50.31041°) is located at an altitude of 320 feet above sea level, 150 kilometers east of the state capital, Goiânia (latitude 16.7267°, longitude 49.25481°, altitude 2,780 feet above sea level). The town is within an endemic risk area. According to data supplied by the municipal council, the principal economic activities consist of agriculture, animal farming, and commercial activities.

São Luís dos Montes Belos had a population of 26,383 inhabitants in 2005, distributed over 114 localities and consisting of 2,513 household units. *T. infestans* was not detected during previous study years, with a prevalence of Chagas disease of 0.82% being found in a serological survey conducted in 1980. However, children under ten years of age were not included in that serological evaluation [1].

Novo Brasil, also considered an endemic risk area, is situated at 320 feet above sea level, 199 kilometers to the east of the capital city, Goiânia. According to the city council, its principal economic activities are related to agriculture, animal farming and commerce. In 2005, its population consisted of 4,086 inhabitants distributed over 82 localities and 1,515 households. *T. infestans* was not detected during the previous study years; however, the secondary vector rate was high, reaching 33.33% in 1998 [4]. In Novo Brasil, 30.8% of the population lives in rural areas (1,153 inhabitants or 395 families) [10].

2.3 Study sites

It was decided to focus on rural areas in the municipalities due to the higher domestic infestation by secondary vector species there. The municipal schools were strategic in the project, since schools serve as a link between the community, children, parents, educators, healthcare professionals (the National Health Foundation and the Family Health Program) and local community leaders.

Two reference laboratories conducted the study tests: a) the *Fundação Nacional Ezequiel Dias* of the Minas Gerais State Health Department (FUNED), a national reference laboratory for Chagas disease situated in Belo Horizonte, Minas Gerais screened filter paper blood samples from schoolchildren under 10 years of age; while b) the Chagas Laboratory of the Federal University of Goiás Teaching Hospital in Goiânia, Goiás tested the full blood samples collected in tubes. These full blood samples were obtained from a random sample of the same schoolchildren under 10 years of age and were used as quality control.

2.4 Study sample

The sample consisted of 303 children ≤ 10 years of age enrolled at municipal schools in rural or urban areas (Fig. 2).

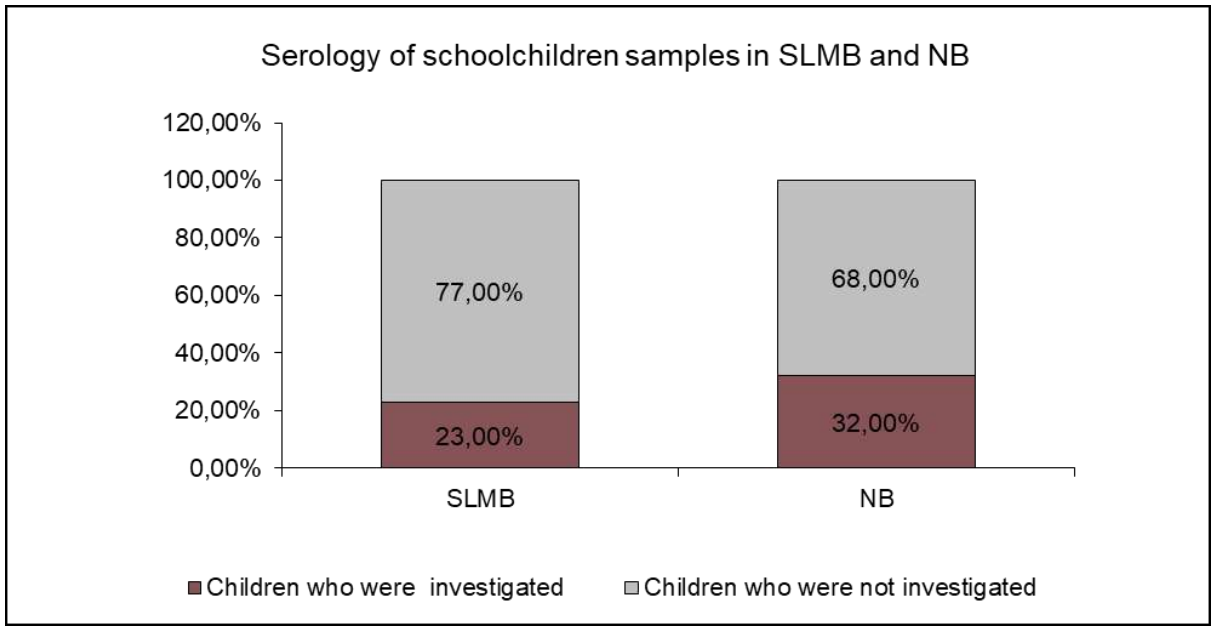

FIGURE 2: SEROLOGY PERFORMED IN THE SAMPLE OF SCHOOLCHILDREN EVALUATED IN THE MUNICIPALITIES.
SLMB: São Luís dos Montes Belos; NB: Novo Brasil.

In São Luís dos Montes Belos, 175 blood samples were collected (140 on filter paper and 35 in tubes), representing 23% of the children ≤ 10 years of age living in rural areas and attending school regularly. These children were living in 69 of the rural household units included in the study.

In Novo Brasil, 128 blood samples were collected (103 on filter paper and 25 in tubes), representing 32.41% of the children ≤ 10 years of age living in rural areas and also attending school regularly. These children were living in 82 of the rural household units evaluated.

2.5 Instruments used in the study

2.5.1 Mapping and description of the study area

The townships of São Luís dos Montes Belos and Novo Brasil are located east of the state capital, Goiânia, and are endemic risk areas. According to the respective council authorities, the principal economic activities of the region are related to agriculture, animal farming and commerce.

In São Luís dos Montes Belos, 15.9% of the population (4,109 inhabitants or 1,182 families) lives in a rural area compared to 30.8% in Novo Brasil (1,153 inhabitants or 395 families).

2.6 Techniques and Procedures

After each child's parent or legal guardian had provided authorization for his/her participation in the study by signing the informed consent form, the following procedures were carried out.

A trained clerk from the National Health Foundation captured triatomine bugs manually for entomological evaluation. A minimal surveillance unit was established and the study forms were completed. In addition, triatomine bugs detected by chance by the inhabitants were collected in a plastic bag attached to a minimal surveillance unit consisting of an illustrated detector calendar and a collection device. These were then duly identified with the investigator's code, the micro-area to which the household belonged, the family, date, and the name and address of the inhabitant, and sent to the triatomine bug data collection point. The data were recorded (triatomine bug notification record) and the material was then sent to the state entomologic center or to the National Health Foundation for detailed evaluation. All the households infested with triatomine bugs were then scheduled to be sprayed by personnel from the municipal health department. Overall, 150 minimal surveillance units were established in São Luís dos Montes Belos and 86 in Novo Brasil, making a total of 236 household units (Fig. 3).

FIGURE 3: MINIMAL SURVEILLANCE UNIT. THE PICTURE SHOWS A TRIATOMINE BUG CAPTURED USING THE INSTRUCTIONS PROVIDED IN THE MINIMAL SURVEILLANCE UNIT. GOIÁS, 2003-2006.

Samples were progressively collected from the target population (children under 10 years of age born after the massive attack phase involving insecticides and individuals with symptoms compatible with those of Chagas disease). In the first phase, conducted with samples collected on filter paper (Watman #1) for use with the techniques with greater sensitivity (IFA or IHA), 103 samples were collected in Novo Brasil and 140 in São Luís dos Montes Belos. Later, 25 and 35 samples, respectively, were collected in tubes for the techniques with greater specificity (ELISA was used in all inconclusive cases). The dilution used in indirect IFA was 1:20, while the cut-off point used in ELISA was <0.9 (Fig.4).

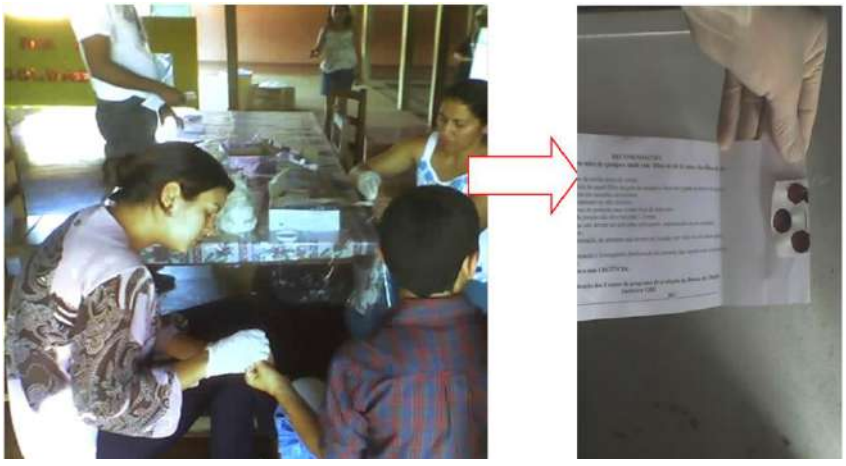

FIGURE 4: COLLECTION AND PROCESSING OF THE SAMPLES ON WHATMAN FILTER PAPER NO. 1.

2.7 Community participation

A health education program was implemented to encourage the communities to participate actively in the survey. A campaign was initiated to increase community awareness of the study and to involve the community in the process by implementing a program in the rural schools, with the collaboration of the teachers, in the form of lectures, fliers and posters. The family health units (7 healthcare units in São Luís dos Montes Belos and 2 in Novo Brasil), together with the National Health Foundation, were transformed into health multipliers. Twelve community leaders from Novo Brasil and 54 from São Luís dos Montes Belos participated in the study. Initially, they, together with the community health agents and, later on, the teachers from the municipal schools, the schoolchildren themselves and, finally, the general population, formed the components of each family health unit (Fig. 5).

FIGURE 5: A HEALTH EDUCATION PROGRAM WITH THE COMMUNITY PARTICIPATION

2.8 Referral of infected patients

Any infected patients were to be referred to one of the municipal health clinics within the National Health Service (SUS) network. If necessary, they would then be referred to a state referral center for treatment of the disease, preferably the Chagas laboratory at the Federal University of Goiás Teaching Hospital, for clinical evaluation and follow-up. The health authorities responsible for health surveillance in the municipality would be notified.

III. DISCUSSION

The results of the present survey are of considerable value, since they provide a picture of the current situation in the two municipalities evaluated, both Novo Brasil where the prevalence of the infection in this age group fell from 42.5% to 0% between 1996 and 2006 [9] and São Luís dos Montes Belos where no previous survey had been conducted with that age group. Nevertheless, investigation into this population found one single case that tested positive for the disease. Confirmation of the positivity of that single sample from a 10-year old child in the São Luís dos Montes Belos region leads us to believe that although Chagas disease is under control, it has yet to be completely eradicated.

In Goiás, the epidemiological profile of Chagas disease changed following implantation of the *T. infestans* eradication program in 1991; however, in 2003, 20 municipalities were considered to be at risk of transmission of the disease in the state, from an original total of 221 in 1980. Traditionally, the regional office of the National Health Foundation in Goiás was responsible for maintaining this program, with the Goiás State Health Department having inherited this responsibility in 1998 following decentralization of the National Health Service [9].

The choice of the municipality of São Luís dos Montes Belos for evaluation in this study was due to the fact that no serological investigation had been conducted in children under 10 years of age there; because *T. sordida* had been found there in all the years evaluated, with its prevalence reaching 17.89% in 1998; and because of the presence of 25 community

health agents who would be available to help in the study. On the other hand, in Novo Brasil, *Triatoma infestans* was not detected in any of the years covered by the study; however, the index of the secondary vector was high, reaching 33.33% in 1984 [4, 9].

Evaluating the entomological situation in the municipality of Novo Brasil, the presence of secondary vectors such as *T. sordida* and *R. neglectus* is notable. However, in São Luís dos Montes Belos, the risk is even greater, with nymphs of *T. sordida* being found in 60% of the cases of infestation. In the municipality of Posse in Goiás, the peridomestic indexes of infestation by *T. sordida* were also found to be high, particularly in henhouses; nonetheless, no nymphs were found in any of the homes [8].

According to Diotaiuti, the epidemiological importance of triatomine bugs is defined by their degree of association with humans. Among the species of secondary importance, *T. sordida* is the most important due to its high peridomestic density. In fact, this is currently the most commonly captured species throughout the entire country [11]. In the municipality of Posse in the state of Goiás, of the 1,059 specimens of triatomine bugs captured, 99% consisted of *T. sordida* [8]. In towns in the southeast of Bahia, *T. sordida* was also the most common species found (96.5%, n=8,657) and of all the specimens captured, 19 were found to be infected by *T. cruzi* [13]. Epimastigote forms of *T. cruzi* were found in *T. sordida* specimens in the district of Santo Inácio in Bahia. Inoculation of BALB/c mice showed low infectivity, with results revealing only mild inflammation, with no sign of parasitism in the tissues [14].

There was great interest in the results of this study from all the sectors involved - the population, healthcare professionals and municipal councils, all of which form the basis for the control and prevention of the disease. In the municipality of Totolga in Nicaragua, the integration of primary healthcare into the services was also the key to improving the surveillance and response system required to eliminate Chagas disease in the country [15].

IV. CONCLUSION

In conclusion, the measures required to control the transmission of Chagas disease consist of simultaneous seroepidemiological and entomological surveillance, continuous spraying of residual insecticides in endemic areas, and now the continuation and strengthening of health education by the Family Health Program.

In general, the data show how the control measures adopted up to the present time have been effective in controlling the disease; however, according to data obtained from the Epidemiological Surveillance Department of the Ministry of Health in December 2006, acute cases of the disease had been confirmed in Brazil.

Although the state of Goiás has been certified as free from *T. infestans*, the principal vector in previous years, the fact that other species of synanthropic triatomines have been captured that tested positive for *T. cruzi* (Chagas, 1909) is a warning that the natural transmission of Chagas disease continues in the state.

Further serological studies need to be conducted to increase the effectiveness of the program and its results, since in some municipalities no investigation has yet been carried out. Failure to conduct such studies may result in the non-identification of some areas in which Chagas disease continues to be transmitted, with the result that attack efforts to eradicate the disease may focus on the wrong strategic points in the state or even in the transfer of a large number of personnel to a region that is not so badly infested. It is vital to prioritize careful epidemiological surveillance, including the involvement of the population and local health authorities, and to have sufficient resources available to do so.

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Responses of wheat seedling to varying moisture conditions and relationship between morphological and molecular characterization

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Abstract— *The following study was conducted to estimate the genotypic differences among 30 wheat (Triticum aestivum L.) genotypes under different moisture regimes and relationship between morphological and molecular characterization. Eight seedling parameters root length (RL), shoot length (SL), root fresh weight (RFW), shoot fresh weight (SFW), root dry weight (RDW), shoot dry weight (SDW), chlorophyll rate (CR) and survival rate (SR) were studied at four different soil moisture conditions ($T_1$40%,$T_2$60%,$T_3$80%,$T_4$100%) using two factor factorial complete randomized design (CRD). Significant differences among genotypes were observed by analysis of variance. For heritability estimates, survival rate showed lowest heritability under all the treatments. Principal components analysis accounted 81.4% variation in T_1, 81.9% in T2, 87.7% in T3 and 84.7% in T4 conditions in first PC. Selected diverse genotypes were further fingerprinted with 10 ISSR markers. A total of 74 DNA fragments were detected and 72.7% of was polymorphic. The amplified DNA fragments were ranged from 4 (UBC-809) to 11 (UBC-808). PIC values were ranged from 0.32 to 0.81. Cluster analysis grouped the genotypes into 4 clusters on the basis of molecular and phenotypic characterization under T4 normal conditions whereas under T1 (moisture stress) conditions genotypes were grouped into 5 clusters explaining genotypic differences under different moisture conditions. The present results showed that phenotypic difference in wheat seedling expression under different water regimes is accompanied with molecular basis, which offer a prospective to enhance wheat adaptation under moisture stress conditions.*

Keywords— Principal component, Dendogram, Genotypes, Polymorphism.

I. INTRODUCTION

Different types of biotic and abiotic stresses are affecting the efforts of researchers working to evanesce the increasing demands of wheat. Drought may cause 10% to 90% yield losses depending upon the intensity of drought and the stage of plant development (Dhanda et al. 2004; Reynolds et al. 2004). The decreasing water resources demands immediate actions for the genetic improvement of crops which requires plant evaluation under stress conditions and their genetic exploration. Drought stress retards plant growth, reduces performance, and has negative impact on development (Shao et al. 2009). Moisture stress not only affects the morphology but also badly affects the metabolism of plant.

The genetic basis for drought tolerance can be predicted by evaluating genotypes under stress condition (Ceccarelli and Grando 1997). Genetic improvement involves selection of genotypes with favorable alleles. Furthermore, screening techniques should be precise to evaluate plant performance at suitable developmental stage. Seedling survivability is a simple and well documented method used to screen wheat germplasm (Singh et al. 1999; Tomar and Kumar 2014). It discriminates between drought susceptible and tolerant genotypes under artificial moisture conditions. Uniform and rapid germination and good seedling emergence are necessary components of crop establishment. Root system helps plants to maintain their growth under moisture stress conditions. Limited water conditions can reduce seedling germination and growth which leads to less plant population per unit area. Khan et al. (2004) analyzed that drought adapted plants are often characterized by deep and vigorous root systems. Therefore, genetic basis of these seedling traits should be exploited to know the inheritance of these traits.

Development of molecular markers have provided new possibilities to evaluate genetic diversity, inter and intra species genetic relationship and to locate QTLs responsible for specific trait development (Sofalian et al. 2003). Inter simple sequence repeats (ISSR) are the DNA based markers which are being used for molecular characterization of different crops. Najaphy et al. (2012) showed that ISSR markers provide adequate polymorphism and reproducible fingerprinting profile for genetic characterization of wheat.

To analyze the genetic diversity various biometric tools are being used by plant breeders. Multivariate techniques which are commonly used to explore genetic diversity include cluster analysis, principal coordinate analysis (PCoA) and principal component analysis (PCA) (Brown-Guedira et al. 2000; Melchinger, 1993; Thompson and Nelson, 1998). The following study was conducted to gain a better understanding of different seedling traits under different moisture conditions and to measure the extent of genetic diversity contributing to drought tolerance at seedling stage.

II. MATERIALS AND METHODS

2.1 Phenotypic characterization:

Thirty bread wheat (*Triticum aestivum* L.) genotypes collected from Regional Agriculture Research Institute (RARI), Bahawalpur were sown in polythene bags of (6``L x 4``W) in the glass house of the Department of Plant Breeding and Genetics, Bahauddin Zakariya University, Multan during 2012-2013. The experiment was carried out in two factor factorial complete randomized design (CRD) with three replications. After 21-days of planting, treatments with different soil moisture conditions (T_1=40%, T_2=60%, T_3=80%, and T_4=100%) were applied until 50% mortality appeared. Hoagland solution was applied to strengthen the weaker plants to obtain data regarding the survival rate. Next day the data for survival rates of different treatments were recorded by following formulae.

Survival rate (%): The dead plants per genotypes were counted and the data for their survival rate was calculated by using the following formula:

$$SR = \frac{Number\ of\ alive\ plants}{Total\ number\ of\ plants\ grown} * 100$$

In order to conduct the data for root shoot architecture the plant seedlings were up taken from the polythene bags following thorough washing with distilled water. The following seedling parameters were recorded, shoot length (cm, SL), root length (cm, RL), Shoot fresh weight (gm, SFW), Shoot dry weight (gm, SDW), Root fresh weight (gm, RFW), Root dry weight (gm, RDW), Chlorophyll rate (%, CR) with chlorophyll meter.

2.2 Genotypic characterization:

Fourteen diverse wheat genotypes were used to extract genomic DNA from young leaf tissues as described by Sofalian et al. (2009). DNA concentration was estimated using spectrophotometer. DNA concentration was calculated using following formula

Concentration of DNA µl/ml= OD at 260x 50 x DF

To characterize the 14 wheat varieties 10 ISSR primers (UBC-807, 808, 809, 810,811,812, 813, 815, 816, and 817) were used to conduct the PCR reaction. The PCR reaction was performed in 20µl volume. The PCR products were separated and scored by agarose gel electrophoresis (Ahmad et al. 2014)

2.3 Statistical Analysis

To find out significant difference among genotypes, analysis of variance was performed as described by Steel et al. (1997). Principal component analysis (PCA) was performed on the basis of correlation matrix to determine diverse genotypes (Ogunbayo et al. 2005). By eigen value as determined by Kaiser (1960) statistically significant principal components (PC_S) were selected. Genotypes were further grouped on the basis of ward's linkage cluster analysis (Sneath and Sokal, 1973).

III. RESULTS

Analysis of variance showed significant genetic differences for all the characters under all the treatments except SR which showed no significant differences under $T_2$60% and $T_3$80% soil moisture conditions (Table 1-4). SR showed lowest heritability under all the treatments (Table 1-4) whereas highest heritability estimates were observed in RL, SL and RFW (0.98) under $T_1$40%, RL and SL (0.99) under $T_2$60%, RFW (0.99) $T_3$80% and RL (0.99) under $T_4$100% soil moisture conditions. Values of genetic advance were ranged between (2.36 for SL and 0.15 for SFW) under $T_1$40%, between (3.05 for SL and 0.17 for SR) under $T_2$60%, between (2.91 for SL and 0.13 for SFW) under $T_3$80% and between (3.86 for SL and 0.13 for SR) under $T_4$100% soil moisture conditions (Table 1-4). Observed heritability was higher than 70% of all parameters except SR exhibiting heritable deviation of genotypes.

TABLE 1

MEAN VALUES AND ANALYSIS OF VARIANCE FOR 8 CHARACTERS AMONG 30 WHEAT GENOTYPES IN T_1 40% SOIL MOISTURE CONDITIONS.

Parameters	MS(Rep)	MS(V)	F. value	h^2	G.A	CV (%)
RL	0.107	238.16	71.69**	0.98	2.28	1.92
SL	0.103	235.37	79.20**	0.98	2.36	4.68
RFW	0.001	3.41	79.21**	0.98	0.27	2.38
SFW	0.001	1.02	24.52**	0.96	0.15	3.08
RDW	0.003	2.59	29.99**	0.97	0.23	8.01
SDW	0.001	1.14	30.99**	0.97	0.15	19.72
CR	0.131	222.47	54.82**	0.98	2.19	2.24
SR	0.171	8.96	1.69*	0.44	0.19	10.26

TABLE 2

MEAN VALUES AND ANALYSIS OF VARIANCE FOR 8 CHARACTERS AMONG 30 WHEAT GENOTYPES IN T_2 60% SOIL MOISTURE CONDITIONS.

Parameters	M.S(Rep)	M.S(V)	F. value	h^2	GA	CV (%)
RL	0.075	313.16	133.22**	0.99	2.64	1.23
SL	0.120	418.79	112.28**	0.99	3.05	2.58
RFW	0.002	1.71	30.21**	0.97	0.18	2.01
SFW	0.006	3.22	15.88**	0.94	0.25	4.91
RDW	0.002	1.89	25.66**	0.96	0.19	4.00
SDW	0.002	2.17	39.07**	0.97	0.22	6.01
CR	0.179	267.39	48.15**	0.98	2.41	2.00
SR	0.155	7.91	1.65^{NS}	0.41	0.17	7.69

TABLE 3

MEAN VALUES AND ANALYSIS OF VARIANCE FOR 8 CHARACTERS AMONG 30 WHEAT GENOTYPES IN T_3 80% SOIL MOISTURE CONDITIONS

Parameters	MS(Rep)	MS (V)	F. value	h^2	GA	CV (%)
RL	0.209	264.97	40.85**	0.97	2.38	1.59
SL	0.190	386.18	65.50**	0.98	2.91	2.13
RFW	0.001	3.83	179.53**	0.99	0.29	0.99
SFW	0.001	0.82	20.61**	0.95	0.13	1.65
RDW	0.002	1.43	22.08**	0.95	0.17	2.59
SDW	0.001	1.07	27.11**	0.96	0.15	3.01
CR	0.141	152.42	34.68**	0.97	1.80	1.43
SR	0.406	20.37	1.62^{NS}	0.41	0.27	11.76

TABLE 4
MEAN VALUE AND ANALYSIS OF VARIANCE FOR 8 CHARACTERS AMONG 30 WHEAT GENOTYPES IN T_4 100 % SOIL MOISTURE CONDITIONS.

Parameters	MS(Rep)	MS(V)	F. value	h^2	GA	CV (%)
RL	0.068	416.47	1.96**	0.99	3.05	0.74
SL	0.457	687.98	48.53**	0.98	3.86	2.43
RFW	0.001	3.72	87.29**	0.98	0.28	1.09
SFW	0.003	3.97	41.89**	0.97	0.29	2.10
RDW	0.002	1.67	25.41**	0.96	0.18	2.09
SDW	0.002	1.69	26.22**	0.96	0.19	2.76
CR	0.398	396.74	32.15**	0.97	2.90	1.98
SR	0.054	3.27	1.93**	0.49	0.13	3.95

3.1 Principal component analysis

The data matrix was standardized to make the variable traits unit less for computing PCA (Principal Component Analysis). Individual accession component scores were accounted by following character loading. The sum of Eigen values resulted in total number of variables. Eight PCs were accounted to analyze the available genetic variation in the wheat genotypes. Out of eight PCs, 1st PC accounted maximum variation for the studied traits. In treatment (T140%, T260%, T380%, and T4100%) contribution of 1st PC was 81.415%, 81.955%, 87.775%, and 84.731% of the variability in different genotypes estimated for root shoot architecture components (Table 5). In case of treatment T140% the first PC was more related to SFW, RDW, CR, SL, RFW, SDW, RL and SR. Under $T_2$60% soil moisture conditions the PC1 was more related to SR while rests of the attributes were not contributing to cause variability.

TABLE 5
PRINCIPAL COMPONENTS (PCS) FOR 8 CHARACTERS IN 30 WHEAT GENOTYPE IN T_1 40%SOIL MOISTURE CONDITIONS.

Traits	PC1
Eigen value	6.513
Proportion of variance	6.513
Cumulative variance	81.415
Eigen vectors	
PC1	
SFW	0.994
RDW	0.993
CR	0.993
SL	0.991
RFW	0.988
SDW	0.976
RL	0.798
SR	0.054

Among thirty wheat genotypes, fourteen diverse genotypes were selected on the basis of accession component scores. To analyze genetic differences the selected genotypes were analyzed with molecular markers. The characterization and genetic identification of fourteen wheat accession were carried out by 10 ISSR primers (Table 6). The PCR amplification results of ISSR primers indicated characteristic differences among genotypes. A total of 74 DNA fragments were amplified, whereas 66 fragments were polymorphic and 8 fragments were monomorphic. Therefore, out of 74 DNA fragments 72.7% were polymorphic. The amplified DNA fragments were ranged from 4 (UBC-809) to 11 (UBC-808). The lowest level of polymorphisms (72.7%) was represented by ISSR primer UBC-808 and markers UBC-807, UBC-809, UBC-811, UBC-816, and UBC-817 showed 100% polymorphism (Table 7). PIC values were ranged from 0.32 to 0.81.

TABLE 6
PRINCIPAL COMPONENTS (PCS) FOR 8 CHARACTERS IN 30 WHEAT GENOTYPES IN T_2 60% SOIL MOISTURE CONDITIONS.

Traits	PC1
Eigen value	6.556
Proportion of variance	6.566
Cumulative variance	81.955
Eigen vectors	
	PC1
SR	0.215
SFW	-0.995
SL	-0.993
RL	-0.992
SDW	-0.991
RFW	-0.988
RDW	-0.981
CR	-0.792

TABLE 7
PRINCIPAL COMPONENTS (PCS) FOR 8 CHARACTERS OF 30 WHEAT GENOTYPES IN T_3 80% SOIL MOISTURE CONDITIONS.

Traits	PC1
Eigen value	7.022
Proportion of variance	7.022
Cumulative variance	87.775
Eigen vectors	
	PC1
RFW	-0.996
SDW	-0.994
RDW	-0.993
SFW	-0.988
RL	-0.979
CR	-0.948
SL	-0.936
SR	-0.585

TABLE 8
PRINCIPAL COMPONENTS (PCS) FOR 8 CHARACTERS OF 30 WHEAT GENOTYPES IN T_4 100% SOIL MOISTURE CONDITIONS.

Traits	PC1
Eigen value	6.778
Proportion of variance	6.778
Cumulative variance	84.731
Eigen vectors	
	PC1
RFW	-0.991
SFW	-0.988
RDW	-0.986
RL	-0.981
SL	-0.979
SDW	-0.931
CR	-0.926
SR	-0.446

3.2 Cluster analysis

Using ward's linkage clustering method experimental data was analyzed by cluster analysis. In treatment T140% the dendogram classified the thirty wheat genotypes into five clusters. The genotype 11903 present in cluster 5 and showed dissimilarity with rest of the genotypes under T1 conditions, which showed genetic differences between 11903 and other genotypes under limited water conditions. But under T4 moisture conditions 11903 showed similarity with 11935 which showed expression of different genes under different environmental conditions. Similarly, genotypes explained less variation under T4 100% water conditions because they were grouped in 4 clusters but under limited moisture condition genotypes were grouped in 5 clusters which showed variation among genotypes under different water regimes.

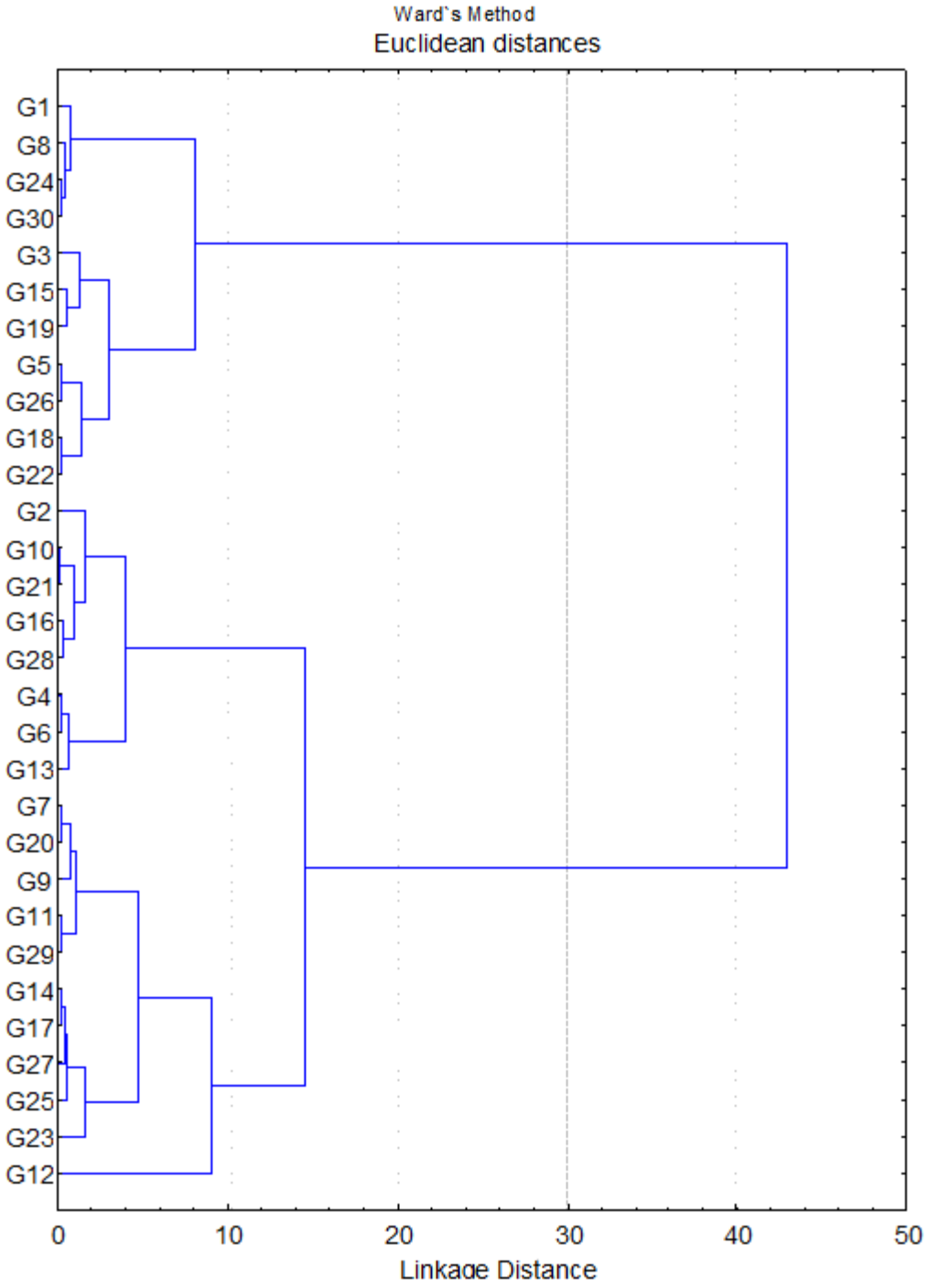


FIGURE 1: DENDOGRAM RESULTING FROM CLUSTER ANALYSIS OF 30 WHEAT GENOTYPES IN T_1 40% SOIL MOISTURE CONDITIONS

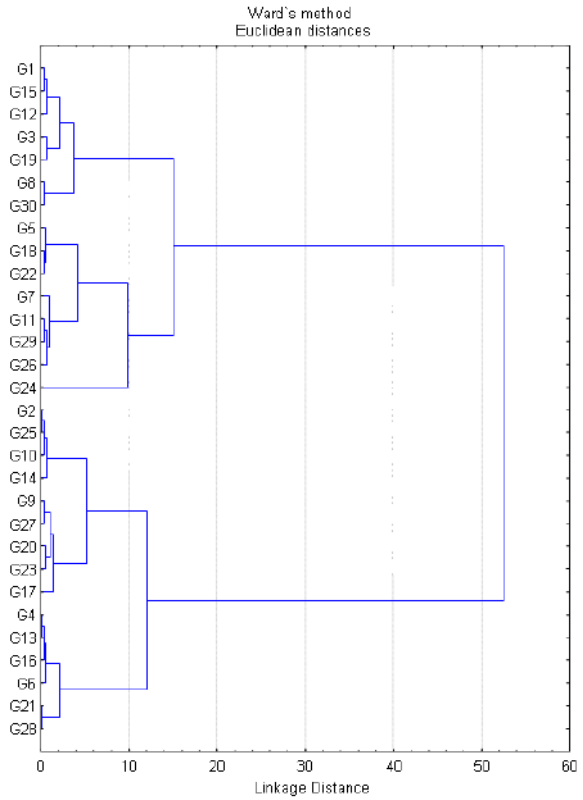
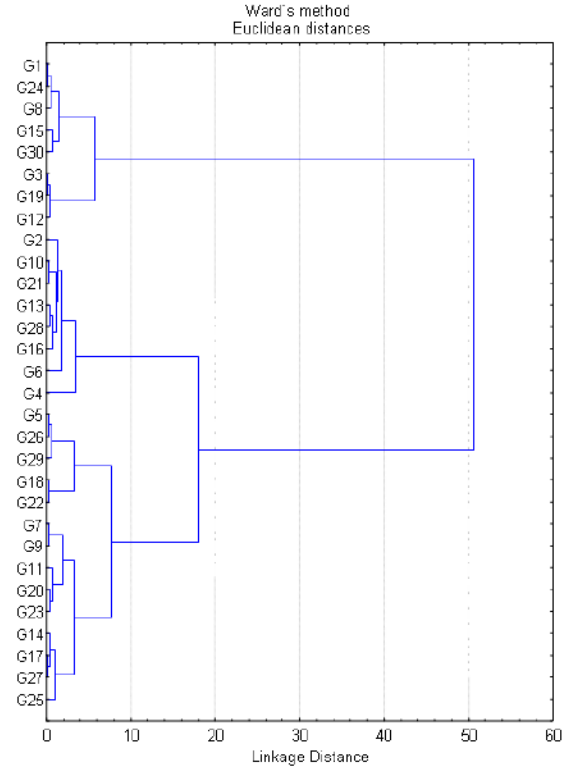


FIGURE 2: DENDOGRAM RESULTING FROM CLUSTER ANALYSIS OF 30 WHEAT GENOTYPES IN T_2 60% SOIL MOISTURE CONDITIONS.



DENDOGRAM RESULTING FROM CLUSTER ANALYSIS OF 30 WHEAT GENOTYPES IN T_3 80% SOIL MOISTURE CONDITIONS.

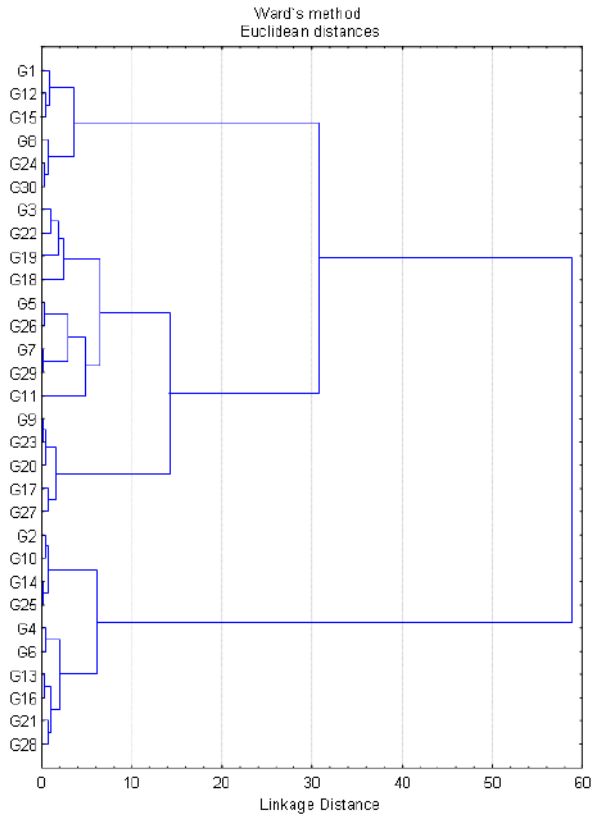
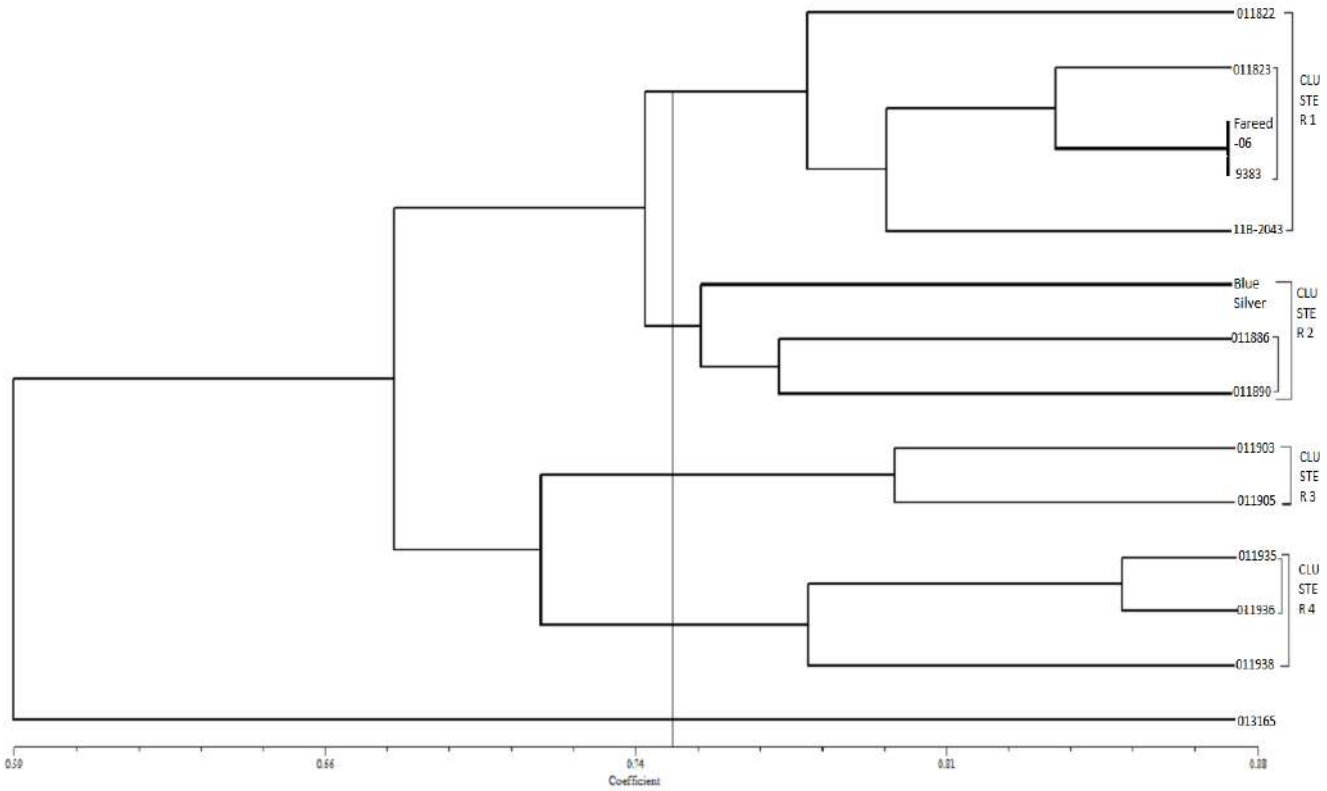


FIGURE 4: DENDOGRAM RESULTING FROM CLUSTER ANALYSIS OF 30 WHEAT GENOTYPES IN T_4 100% SOIL MOISTURE CONDITIONS.

To distinguish varieties from one another at molecular level DNA finger printing can be used. Among wheat varieties similarity values showed substantial differences (Table 7). The genetic similarity ranged from 0.53 to 0.88 with an average of 71%. High genetic similarity was observed between 9383 and Fareed-06 (0.88). The low genetic similarity between 013165 and 011936 (0.53) was observed. To observe the genetic association among genotypes based on ISSR marker analysis. A dendogram classified the 14 wheat genotypes into 4 clusters (Fig.2). The first cluster contain genotypes 011822, 011823, Fareed-06, 9383 and 11B-2043. The genetic similarity between genotypes 011822, 011823 and Fareed-06, 9383 were 0.76 and 0.74. Second cluster include 011886, 011890 and Blue silver. The genetic similarity between 011886 and 011890 was 0.77. Third cluster contain 011930 and 011905 with genetic similarity 0.80. Cluster four contains genotypes 011935, 011936 and 011938.



3.3 Relation between the phenotypic characterization and ISSR loci data:

Cluster analysis was performed separately for each treatment and for markers data to study the genetic diversity in wheat. The dendogram constructed on the basis of phenotypic data showed maximum similarity between 9383 and Fareed-06 genotypes. The dendogram generated on the basis of molecular markers data also grouped the Fareed-06 and 9383 in the same cluster which showed their genetic closeness.

The genotype 11903 (Iran) showed no association with rest of the genotypes under T1 conditions but showed maximum similarity with 11935 (Japan) under T4 conditions. But molecular data showed association between 11903 and 11905 genotypes both have Iranian origin. Most notable is the location of genotypes 11903 and 11935 which are located in the nearby clusters on the basis of molecular fingerprinting. This may be concluded that both these genotypes which belong to different geographical regions may share some parents having similar allelic combinations that express only under normal conditions as represented here by phenotypic and molecular characterization.

Molecular characterization grouped the genotypes into 4 clusters similarly phenotypic evaluation under normal conditions also allocated genotypes into 4 clusters whereas under T1 (moisture stress) conditions genotypes were grouped into 5 clusters this may be due to varying degree of drought tolerance in different genotypes. The observed similarity among the dendogram between phenotypic data and molecular data give an evidence for the presence of relationship between seedling traits under different water regimes and molecular data.

Results obtained by principal component analysis also resembled with the cluster analysis which showed that under T1 conditions all the traits such as, shoot fresh weight, root dry weight, shoot length, chlorophyll, root fresh weight, shoot dry weight, root length and survival rate contributed for diversity. Cluster analysis showed 5 clusters under T1 conditions, so more genes are involved under drought conditions. PCA under T2 was only related to SR which also showed less heritability and genetic advance values, therefore no selection should be carried out under T2 conditions.

TABLE 11
ISSR MARKERS USED AMPLIFIED PRODUCTS AND ANALYSIS OF GENETIC DIVERSITY OF WHEAT GENOTYPES.S

Primer	Total amplified band	No. of monomorphic band	No. of polymorphic bands	Percentage of polymorphic bands	Polymorphism information content (PIC)
UBC-807	10	-	10	100	0.55
UBC-808	11	3	8	72.7	0.55
UBC-809	4	-	4	100	0.38
UBC-810	10	1	9	90	0.77
UBC-811	5	-	5	100	0.50
UBC-812	7	1	6	85.7	0.75
UBC-813	6	1	5	83.3	0.32
UBC-815	8	2	6	75	0.81
UBC-816	6	-	6	100	0.55
UBC-817	7	-	7	100	0.46
Total	74	8	66		
Minimum	4	1	4	72.7	0.32
Maximum	11	3	10	100	0.81
Average	7.4	0.8	6.6		

IV. DISCUSSION AND CONCLUSION

Dwindling environmental conditions and rapid increase in world's population has created serious threats to world food security. To combat with hunger and diminishing water resources is a greatest challenge being faced by scientists today. Decreasing water resources has created alarming situation to sustainable food production. Wheat is the leading cereal crop being consumed by humans across the globe. Limited water supply may decrease wheat yields upto 90% (Dhanda et al. 2004). Different morpho-physiological traits can be studied to evaluate the performance of plants under limited water conditions (Inou et al. 2004). Understanding of the genomic regions controlling these important traits will contribute in the genetic improvement of wheat to cope with number of stresses particularly low moisture (Frova et al. 1999). Moreover, the association among different plant traits should be determined either it is genetic or phonetics, heritable or non heritable.

In the following study wheat genotypes were evaluated under different water regimes. The study showed significant variation among genotypes and treatments (different water levels) which demonstrated the contribution of genetic attributes (Birsin 2005). Heritability values were higher than 70% for all the parameters except SR. Awan et al. (2007) and Haidar et al. (2012) also observed significant differences among genotypes and higher values of heritability. The traits SL, RL, SFW, SDW, RFW, RDW, CR showed greater magnitudes of heritability along with higher values of genetic advance were under the control of additive genetic effects. Heritability also provides the estimation of genetic advance, either the selection under certain environment is heritable or non heritable. Magnitude of heritability determines the simplicity of selection (Khan et al. 2008). To undertake selection in succeeding generation, heritability should accompany substantial amount of genetic advance, which is the indicative of potential to which the trait can be improved under certain environment, therefore higher values of heritability and genetic advance in this study provides an opportunity to breeders to fix these traits with full strength and ease in coherent selection programs (Eid 2009). Lower values for coefficient of variation also demonstrated higher precision levels of the study. Noorka et al. (2007) also observed lower values of coefficient of variation.

Sardana et al. (2007) demonstrated that high heritability may not always lead to high genetic gain, unless sufficient genetic variability existed in the germplasm. Therefore, to account variation among genotypes principal component analysis was performed (Panthee et al. 2006). As the results indicates that the first PC accounted maximum variation for the studied traits

such as SFW, RDW, CR, SL, RFW, SDW, RL and SR but other PCs have not played an important role in accounting variation**.** Mohammadi and Prasanna (2003) explained that if there is high correlation among the data set then first few PCs expresses maximum variation but it decreases with the decresae in correlation among original data set. Gulnaz et al. (2012) observed four significant PCs in a set of seven PCs. Similarly results were also reported by Ahmad et al. (2012). Most of the variation has been accounted by first PC so other PCs were not given due importance in the following study. Eigen values showed continuous decrease, which exhibits that major amount of variation has been accounted by the first few principle components (Leilah and Al-Khateeb 2005). High positive association among root and shoot parameters as depicted by this study provide an opportunity to breeders to breed for these traits at the same time. Furthermore, genetic control of these traits should be identified to enhance breeding accuracy.

To explore diversity at genetic level, 14 most diverse genotypes were selected on the basis of accession component score which were further analyzed with ISSR markers. The PCR results showed characteristics differences among genotypes. Assessment of genetic diversity in wheat has been carried out by different molecular marker systems. Najaphy et al. (2012) observed that for evaluating genetic diversity of wheat genotypes ISSR markers provide sufficient polymorphisms and reproducible fingerprint profiles. Sofalian et al. (2003) reported high level of polymorphism of wheat landraces based on ISSR markers as compared to other markers. The amplified DNA fragments were ranged from 4 (UBC-809) to 11 (UBC-808). Carvalho et al. (2009) observed 12.9 polymorphic bands per primer using 8 ISSR primers in 48 wheat accessions. Nagaoka and Ogihara (1997) found that 3.7 polymorphic bands per ISSR primer. Presence of high polymorphism in wheat genotypes using ISSR markers indicates high efficiency of this marker technique. The lowest level of polymorphisms (72.7%) was represented by ISSR primer UBC-808 (Table 6). Abou-Dief et al. (2013) identified 112 amplified DNA fragments, of which 17 were monomorphic (15.2%) and 95 fragments showed polymorphism (84.8%). PIC values were ranged from 0.32 to 0.81. PIC index has been widely used to explore genetic diversity among genotypes (Tatikonda et al. 2009; Talebi et al. 2010; Thudi et al. 2010).

In self pollinated crops like wheat genetic variation is vital for stress tolerance. Joshi et al. (2004) observed genetic diversity between parents is essential to derive transgressive segregants from a cross. To start a wheat hybridization program in which parents have high heritability along with high molecular diversity, cluster analysis should be carried out to exclude similar parents from the breeding material. Therefore, PCA should be followed by cluster analysis so that genotypes can be grouped in similar and distinct groups (Ahmad et al. 2012). Ayed et al. (2010) demonstrated that cluster analysis is a successful strategy for selection of genotypes to initiate a wheat hybridization programme on the basis of certain morphological traits. Using ward,s linkage clustering method experimental data was analyzed by cluster analysis. Ahmad et al. (2012) identified 2 clusters and 3 subclusters by ward,s linkage clustering method.

Rana and Bhat (2005) estimated 74% genetic similarity by cluster analysis. Similarly, Aliyu and Fawal (2000) highlighted the efficiency of cluster analysis to identify and group crop accessions on the basis of genetic similarity using dendrogram. Multivariate analysis is a valid system to study germplasm collection (Ghafoor et al. 2001; Ahmad et al. 2012). Ijaz and Khan, (2009) classified the 63 genotype into three clusters. Salem et al. (2008) showed the cluster analysis of seven wheat varieties into two major clusters and three sub cluster. The dendrogram represents a number of dissimilar groups. Within the same cluster individuals are similar but there have significant differences with other cluster (Finsten 1996).

In the following study some genotypes occupy different clusters under different water conditions which showed expression of different genes under different environmental conditions. Similarly, under T4 100% water conditions genotypes were grouped in 4 clusters except 5 as under different environments which showed variation among genotypes under different water regimes. Moisture stress induces the expression of large number of genes (Shinozaki and Yamaguchi-Shinozaki, 2007). Drought tolerance is a veryd trait which is controlled by many genes and their expressions are influenced by various environmental elements. As these traits are controlled by different QTLs so it may be due to the response of different QTLs to different environments. On the other hand it may be due to the pleiotropic effect by the co-location of QTLs for different traits at a single locus or cluster of closely linked genes (Landjeva et al. 2008).

The following study has depicted the influence of different moisture regimes on the trait expression. Molecular and phenotypic characterization also explored the genetic differences among genotypes. Moreover the genetic diversity dissected in this study using ISSR markers should be explored with SSR or SNP markers to identify QTLs controlling these important traits. Because the seedling growth in wheat is under the control of many loci as concluded by Landjeva et al. (2008) while studying on the International Triticeae Mapping Initiative (ITMI) recombinant inbred population, and find QTLs located on different chromosomes.

The results of the following study have demonstrated the involvement of different genetic components which are controlling seedling traits. Traits which showed high heritability and genetic advance should be given due importance to start a breeding program. We conclude that only one level of moisture deficit is not a suitable strategy to breed for drought tolerance. As the study depicted that different plant traits are influenced by different water levels. So, phenotypic evaluation should be done at different water levels to select best genotypes having drought tolerance.

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