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Preface

We would like to present, with great pleasure, the inaugural volume-4, Issue-5, May 2018, of a scholarly journal, *International Journal of Environmental & Agriculture Research*. This journal is part of the AD Publications series *in the field of Environmental & Agriculture Research Development*, and is devoted to the gamut of Environmental & Agriculture issues, from theoretical aspects to application-dependent studies and the validation of emerging technologies.

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

Environmental Research:

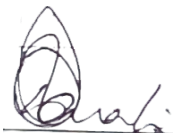
Environmental science and regulation, Ecotoxicology, Environmental health issues, Atmosphere and climate, Terrestric ecosystems, Aquatic ecosystems, Energy and environment, Marine research, Biodiversity, Pharmaceuticals in the environment, Genetically modified organisms, Biotechnology, Risk assessment,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

Agriculture Research:

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

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

Mukesh Arora
(Editor-in Chief)



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(Managing Editor)

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Agricultural Sciences	
Soil Science	Plant Science
Animal Science	Agricultural Economics
Agricultural Chemistry	Basic biology concepts
Sustainable Natural Resource Utilisation	Management of the Environment
Agricultural Management Practices	Agricultural Technology
Natural Resources	Basic Horticulture
Food System	Irrigation and water management
Crop Production	
Cereals or Basic Grains: Oats, Wheat, Barley, Rye, Triticale, Corn, Sorghum, Millet, Quinoa and Amaranth	Oilseeds: Canola, Rapeseed, Flax, Sunflowers, Corn and Hempseed
Pulse Crops: Peas (all types), field beans, faba beans, lentils, soybeans, peanuts and chickpeas.	Hay and Silage (Forage crop) Production
Vegetable crops or Olericulture: Crops utilized fresh or whole (wholefood crop, no or limited processing, i.e., fresh cut salad); (Lettuce, Cabbage, Carrots, Potatoes, Tomatoes, Herbs, etc.)	Tree Fruit crops: apples, oranges, stone fruit (i.e., peaches, plums, cherries)
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Study of the impact of Oum Azza landfill leachates on the environment of Rabat - Morocco

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Abstract— *The problem of solid household waste has arisen with great sharpness in recent decades. In particular, the management of leachates and the neutralization of their environmental impacts. The need for Morocco to meet the environmental challenge and put itself in logic of sustainable development has led to an awareness of this issue and the promulgation of the new law 28-00 on waste management and their elimination.*

The objective of this research work is the characterization of the organic and mineral load of leachate from the Oum Azza landfill and the evaluation of its environmental impacts on the city of Rabat. For this a campaign of 24 samples was undertaken in 2011.

The physicochemical characterization of leachates has revealed that these liquid discharges are:

• Very high in organic matter with mean MES = 470mg / L; Average BOD5 = 5522 mg of O2 / L and COD = 12626 mg / L;

• Very charged in mineral matter expressed in terms of electrical Conductivity (mean = 33969 µs / cm);

• Have an average temperature of 24.5 ° C and a pH of 8;

• A chloride concentration of 4289 mg / L;

• Average sodium levels in the order of 3049 mg / L;

• Average total nitrogen levels of 4090 mg / L and ammonia in the order of 3207 mg / L;

• Average level of phosphates of the order of 35 mg / L;

• Average sulphates levels of 35 mg / L.

The Rabat landfill represents a real nuisance for health and the environment because of the toxic characteristics of pollutants and bad odors. It is therefore essential to treat these liquid discharges and install a WWTP to mitigate the environmental impact of leachate.

Keywords— Oum Azza discharge, leachates, physicochemistry, Pollution, Impacts, Odors, Rabat, Morocco.

I. INTRODUCTION

In Morocco, like all the countries of the world, socio-economic activities coupled with population growth and changes in consumption patterns generate a large production of household solid waste [1].

In the face of demographic, industrial, urbanistic and tourist development, the problem of waste has arisen with great acuteness. The amount of household waste produced poses a serious threat to the environment as the current conditions for the collection, transport, disposal, recycling or destruction of such waste are inadequate [2].

The dump of Oum Azza is located a few kilometers east of the city of Rabat, on the right bank of the A5 motorway from Rabat to Casablanca and Kenitra. Initially it was a wild dump located near the urban perimeter of El Menzeh center and Ouled Mbarek Commune. The proximity to the main wind direction facilitates the spread of smoke, odors and plastic bags to nearby cities.

Like other Moroccan cities, Rabat faces an exponential increase in the amount of household waste produced by its inhabitants. Unfortunately the landfilling of waste and the accumulation of leachates in large storage ponds has contributed to the birth of a new environmental problem due to their pollutant loads and the nauseating odors that emerge [3].

The present work aims to characterize and evaluate the pollutant load of the Oum Azza de Rabat landfill by physicochemical analyzes of raw leachates collected in 2011.

II. STUDY AREA

The region of Rabat-Salé-Zemmour-Zaër, which covers an area of 18194 km^2 or 1.3% of the country is bounded (Figure 1):

• North and Northeast by the Gharb-Chrarda-Beni Hssen Region;

• In the West by the Atlantic Ocean;

• East and South-East by the Meknes-Tafilalt Region;

• South and Southwest by Chaouia-Ouardigha Region.

The population of the Rabat-Sale-Kenitra amounts to 4552585 inhabitants, or 8.07% of the total national population, of whom 3172955 in urban areas and 1379630 in rural area of the total population. Total regional population with an average density for this region of 251.8 inhabitants / km^2. The national average is 41.7 inhabitants / km^2 [4].

The landfill of Oum Azza is chosen from the large number of landfills in Morocco. It is located in Rabat-Sale. This landfill produces a leachate that is suspected of causing environmental pollution of groundwater and surface water as well as ambient air by the propagation of very bad toxic and allergenic odors [5].

The waste comes mainly from the transfer centers of Rabat, Sale and Temara. They are therefore transported by large trucks carrying 20 to 25 tons. Municipalities close to the site and some private organizations or companies bring their waste directly to the Oum Azza site. Treated waste is garbage; refuse from composting household waste and ordinary industrial waste. Production was estimated at 500 000 tons during 2011 [6].

2.1 Rainfall

The rainfall recorded by the Rabat-Sale airport weather station in the region is shown in Table 1. The water slide in Rabat during the whole of the year is about 555 mm. There are summer months (June, July, August and September) marked by very low rainfall. In contrast, November, December, January and February are marked by heavy rains [7].

TABLE 1
ANNUAL MEANS RAINFALL (mm) IN 25 YEARS.

Station	Jan	Feb	Mar	April	May	Jun	Jull	Aug	Sept	Oct	Nov	Dec
Rabat	85.1	72.6	64.9	54.6	19.8	6.5	0.48	1.05	5.5	42.3	79.5	111

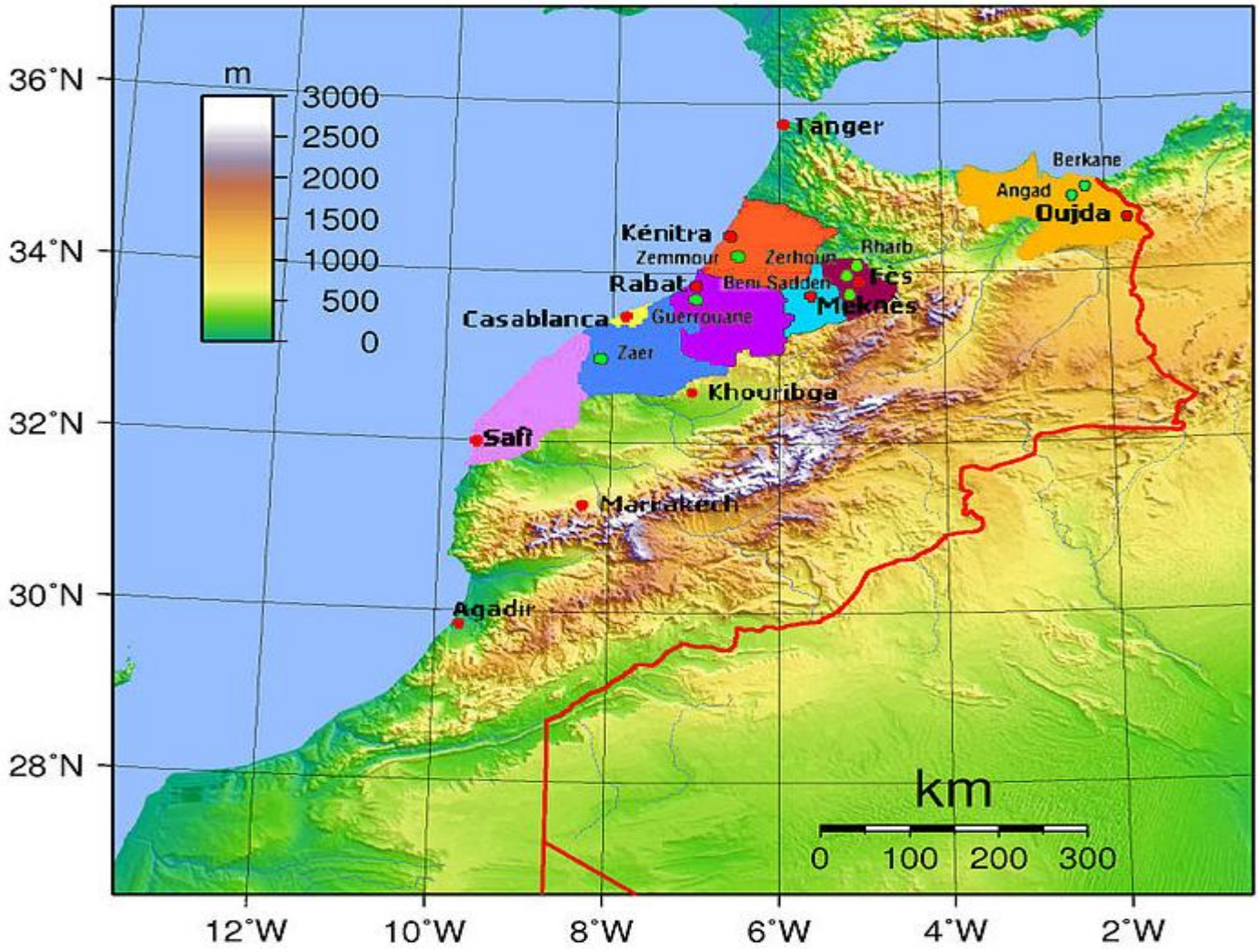

FIGURE 1: LOCATION OF THE RABAT-SALE-ZEMMOUR-ZAËR REGION (MATEE 1997)

2.2 Temperature

The analysis of monthly temperatures (Table 2) indicates that the Rabat region appears to be one of the most temperate in Morocco since the annual temperature ranges between the mean maximum temperature and the average minimum temperature are about 9.5 ° C [7].

TABLE 2
MONTHLY AVERAGE TEMPERATURES (T °C) IN RABAT.

Jan	Feb.	Mar	April	May	Jun	Jull	Aug	Sept	Oct	Nov	Dec
12,6	13,1	14,4	15,0	17,3	19,9	22,0	22,5	22.2	18,2	15,8	13,0

2.3 Wind

According to the wind rose provided by the Rabat weather station at Rabat-Salé airport, the prevailing winds in Rabat come from the western sector in winter, spring and autumn. They are followed closely by those from the North and South. Only the East sector winds play a relatively negligible role during the wet season. Note in the Atlantic region, the Gharbi, a westerly wind (actually from north-west to southwest) blows in any season on the western coast. Always fresh, it is also a source of moisture and precipitation. The desiccating Chergui is of little relevance to the Rabat region (Figure 2).

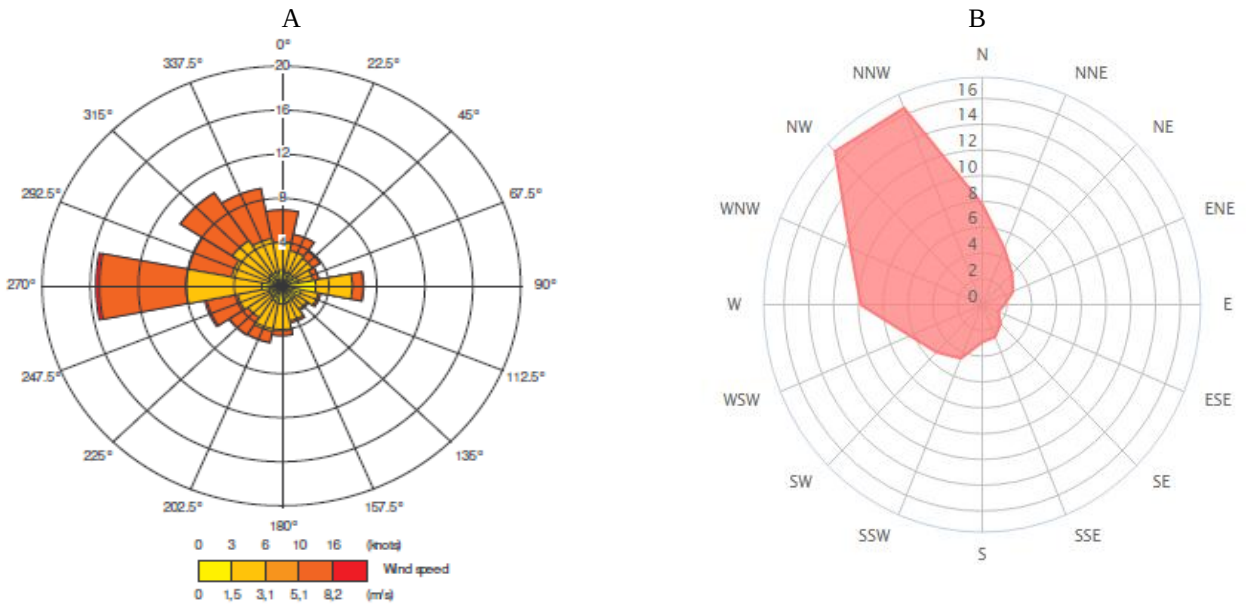

FIGURE 2: WIND FREQUENCY DISTRIBUTION (WIND ROSE) FROM 1995 TO 2004 (A) AND 2017 (B).

2.4 Landfill of Oum Azza

The wild landfill is located in the rural commune of Oum Azza on the plateau of Aïn Aouda 20 km from Rabat. It is located between the Akrach River in the west and the reservoir of the Sidi Mohamed Ben Abdellah dam in the east, at a range between 160 and 200 NGM. Its area is about 110 Ha. The purpose of the landfill is to treat household and similar waste from the 13 urban and rural communes for a population of 572717 inhabitants with a maximum annual production of about 700,000 tons per year (Figure 3-4) [8]

FIGURE 3: LEACHATE STORAGE POND AT THE OUM AZZA LANDFILL

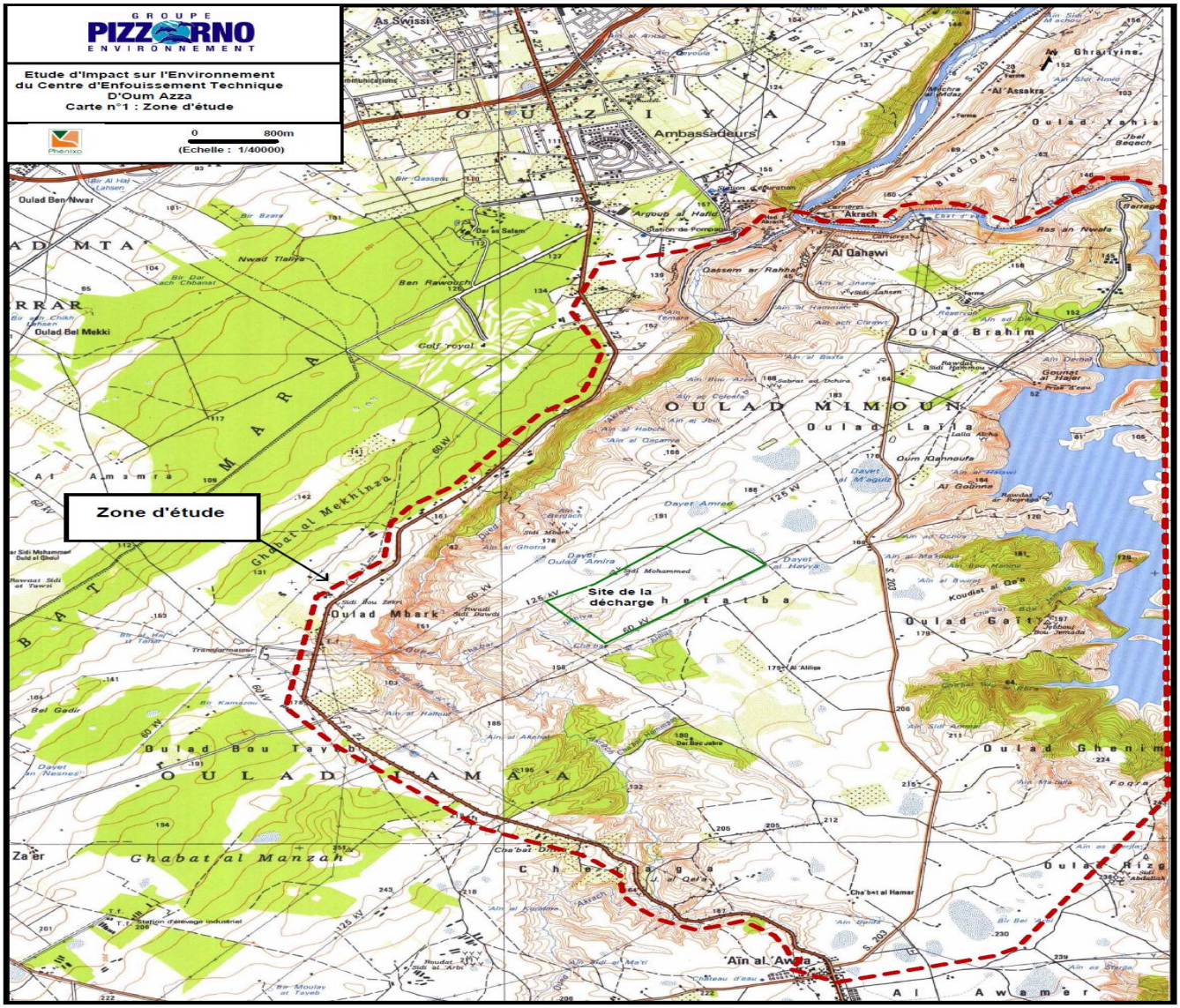

FIGURE 4: LOCATION AND ACCESS TO THE SITE OF THE OUM AZZA LANDFILL (PIZZORNO-MOROCCO)

III. MATERIAL AND METHOD

The physicochemical analyzes are carried out as follows [9-12]:

❖ **In the field:**

• The pH of the samples was measured using a Hanna pH meter;

• The temperature and the conductivity of the samples are determined by a conductivity meter of Cond315i / SET type, WTW82362;

❖ **Kenitra Faculty of Science Environmental Laboratory:**

• The chemical oxygen demand (COD) was determined using a DBC reactor;

• The biological oxygen demand (BOD5) consumed for 5 days was assayed using a DBOmeter;

• Suspended Materials MES were measured by filtration and assay;

• Ammonium and nitrogen were measured by the distillation method of Parnas and Wagner by a distiller;

• Sodium was measured using a flame photometer, type: JENWAY, CLINICAL PFP7;

• Sulfates (mg / l) were determined by the colorimetric method;

• Chlorides (Cl-) were dosed by Argentimetrie.designation.

IV. RESULTS AND DISCUSSION

The pH is an indicator of water pollution. The recorded pH values during the study period ranged from neutrality at 7.3 to a basic pH of 8.8 with an average of about 8.06.

Measurements of leachate temperature give mean values of 24.52 ° C, maximum of 40 ° C and minimum of 10 ° C (Tables 3-4). Thermal exchanges between the atmosphere and the surface of cells or compartments are balanced [13]. So the rise of temperature during the summer and its fall during the winter are in concordance with the seasonal variation of the atmospheric temperature. The maximum value of the leachate temperature is above 30 ° C, considered as limit value of direct discharges in the receiving environment [14].

At the Oum Azza landfill in Rabat-Sale, there are high concentrations of nitrogen (1204 to 5804 mg / L) and ammonia (644-4480 mg / L). Similarly, the phosphate concentration is high and varies between 8.1 and 75.8 mg / L. In the leachate of the Rabat landfill, concentrations remain high in the unloading concerned.

During the 2011 campaigns, the electrical conductivity is higher and exceeds the standard of 2700 μS / cm and varies from 20115 to 47100 μs / cm. The concentration of Na + varies from 1308 to 4630 mg / L and that of Cl- also varies from 2340 to 7100 mg / L. At the same time, the concentration of sulphates is relatively modest and ranges from 16 to 55 mg / L.

The leachate from the Oum Azza landfill shows significant concentrations of suspended solids, ranging from 88 to 1480 mg/L. These high loads generate strong measurements of BOD_5 and COD. BOD_5 varies considerably between 761 to 12976 and 48801mg / L and the COD vary from 7296 to 23789 and even reach 71880 mg / L.

TABLE 3
PHYSICOCHEMICAL PARAMETERS OF THE LEACHATE OF THE OF OUM AZZA LANDFILL.

Oum Azza	T°C	pH	CE	MES	DCO	DBO_5	Cl-	Na+	NH_4+	NTK	PT	SO_4^{2-}
S1	23,4	8,1	32800	392	13296	6126	6898	3446	1958	1960	17,8	37
S2	15	8,1	47100	211	18048	6526	3470	3010	3976	4340	8,1	47
S3	12	7,8	30100	298	18144	9638	2886	3020	2576	4844	75,8	21
S4	27	8,3	36900	662	10944	2956	4570	3200	4368	5880	40,8	45
S5	30,5	8,2	20115	567	7872	2406	4590	3020	3881	4095	46,4	42
S6	32	7,8	28000	99,5	7296	2756	5020	3470	3010	4396	19,9	34
S7	35	8,5	38500	308	11328	2876	7100	4630	4480	5908	42,9	49
S8	35	8,5	38500	308	11328	2876	4890	3450	4480	5908	42,9	54
S9	31,2	7,9	26600	161	10708	6461	5480	3680	2728	2982	22,7	55
S10	40	7,56	40200	730	23789	12301	5930	4270	3626	4480	26,7	37
S11	15,1	7,34	27060	1467	10160	12976	3028	1308	3330	3770	43,7	26
S12	18	7,9	35000	1480	7488	4800	2970	2560	3986	4410	45,2	20
S13	20,4	8,1	35720	88	7910	761	2560	2340	2499	3710	37,9	25
S14	22	7,9	35700	415	17203	7581	2800	2030	3850	4494	48,6	16
S15	10	8,8	37000	204	13065	3086	2340	2160	644	1204	16,5	22
S16	19,4	8	36725	390,8	15108	6312	4456	3169	3220	4256	35,6	37,5
S17	33,1	8,2	31279	320,6	9456	2729	5400	3643	3963	5077	38	44,8
S18	35,6	7,7	33400	445,5	17249	9381	5705	3975	3177	3731	24,7	46
S19	17,1	8	34096	730,8	11165	5841	2740	2080	2862	3518	38,4	21,8
S20	23,6	7,9	28315	813,4	9904,8	6553,8	4593,2	2803	3176,6	3443	35,2	36
S21	26,2	7,9	31860	93,8	7603	1758,5	3790	2905	2754,5	4053	28,9	29,5
S22	24,7	7,8	35333	481	19712	9840	3872	3107	3350,7	4606	50,3	24,7
S23	32,3	8,4	37967	426	11200	2902,6	5520	3760	4442,7	5899	42,2	49,3
S24	10	8,8	37000	204	13065	3086	2340	2160	644	1204	16,53	22

TABLE 4
DESCRIPTIVE STATISTICS OF PHYSICOCHEMICAL LEACHATE DATA

Variables	Observations	Minimum mg/L	Maximum mg/L	Mean	Error
T°C	24	10,00	40,00	24,52	8,77
pH	24	7,34	8,80	8,06	0,35
CE µs/cm	24	20115,00	47100,00	33969,58	5523,71
MES	24	88,00	1480,00	470,68	370,96
DCO	24	7296,00	23789,00	12626,74	4405,76
DBO_5	24	761,00	12976,00	5522,08	3375,81
Cl-	24	2340,00	7100,00	4289,51	1419,66
Na+	24	1308,00	4630,00	3049,81	781,74
NH_4+	24	644,00	4480,00	3207,60	1045,82
NTK	24	1204,00	5908,00	4090,34	1302,85
PT	24	8,10	75,80	35,24	14,63
SO_4^{2-}	24	16,00	55,00	35,06	11,97

Other analyzes were carried out on the raw and aged leachates as part of the evaluation of the remediation possibilities. The results are summarized in Table 5. The results showed that the leachate kept its pollutant load after several treatment trials [15-16].

TABLE 5
RESULTS OF LEACHATE ANALYZES OF THE OUM AZZA LANDFILL

	CE µs/cm	DCO mg/L	DBO_5 mg/L
Site 1 : Lixiviat brut	26880	71880	48801
Site 2 : Treated leachate	24600	45120	30744
Site 3: Treated leachate	25400	42320	22400
Site 4: Treated leachate	26300	38400	19368
Site 5: Treated leachate	20000	12500	1900
Site 6: Treated leachate	18000	9900	1390
Site 7: Treated leachate	17500	4830	600

The PCA Principal Component Analysis of the 24 sampling stations with the physicochemical parameters studied shows that the correlation between the temperature and the other parameters tested allowed us to note that there is a significant correlation with chlorides, sodium and sulphates with coefficients of r = 0.809; r = 0.798 and r = 0.690) (Table 6).

The PCA analysis (Figure 5) shows that the F1 axis expresses 34.45% of the variance and the F2 axis 22.52%, with 56.97% of inertia for the factorial plane F1F2 in a two-dimensional system of the twelve parameters studied. These considerations allowed us to obtain the graphical representation of the correlations between the different variables (Figures 6-8).

In the PCA analysis, the projection of the variables on the factorial plane F1-F2 (Figure 6) shows that pH, BOD5 and MES are negatively correlated with the F1 axis. On the other hand BOD5, COD, MES, Temperature, NPK, PT, NH4 + are correlated negatively with the axis F2. The analysis of the projection of the individuals on the factorial plane F1-F2 (Figure 7) allowed us to define a distribution of the stations along the axis F1 which is an increasing gradient of mineral pollution.

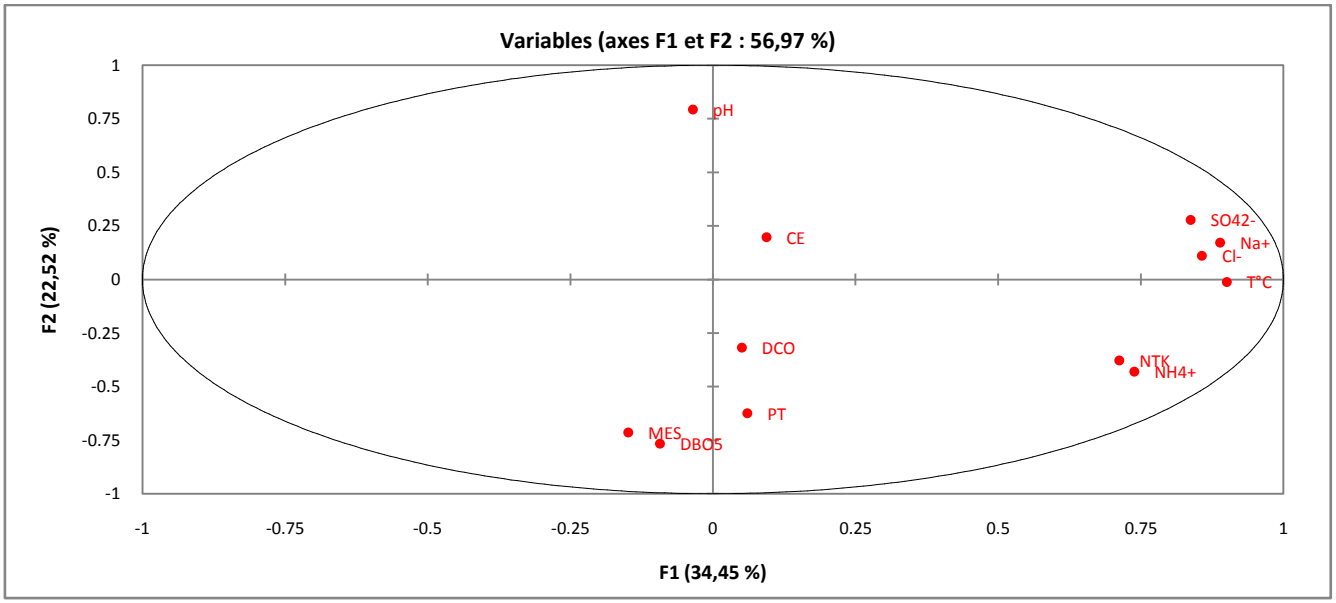

FIGURE 6: PROJECTION OF VARIABLES ON FACTORIAL F1X F2 (56.66%)

TABLE 6
CORRELATION MATRIX

Variables	T°C	pH	CE	MES	DCO	DBO_5	Cl-	Na+	NH_4+	NTK	PT	SO_4^{2-}
T°C	**1**	-0,142	-0,095	-0,125	-0,003	-0,068	0,809	0,798	0,590	0,540	-0,013	0,690
pH	-0,142	**1**	0,327	-0,443	-0,227	-0,721	-0,040	0,054	-0,231	-0,152	-0,220	0,170
CE	-0,095	0,327	**1**	-0,172	0,483	-0,016	-0,050	0,153	0,116	0,169	-0,271	0,067
MES	-0,125	-0,443	-0,172	**1**	-0,073	0,470	-0,134	-0,331	0,294	0,102	0,304	-0,244
DCO	-0,003	-0,227	0,483	-0,073	**1**	0,692	0,053	0,214	-0,025	0,007	0,014	-0,074
DBO5	-0,068	-0,721	-0,016	0,470	0,692	**1**	-0,001	-0,072	0,035	-0,045	0,174	-0,189
Cl-	0,809	-0,040	-0,050	-0,134	0,053	-0,001	**1**	0,873	0,408	0,330	-0,159	0,759
Na+	0,798	0,054	0,153	-0,331	0,214	-0,072	0,873	**1**	0,416	0,452	-0,085	0,735
NH_4+	0,590	-0,231	0,116	0,294	-0,025	0,035	0,408	0,416	**1**	0,911	0,400	0,501
NTK	0,540	-0,152	0,169	0,102	0,007	-0,045	0,330	0,452	0,911	**1**	0,554	0,411
PT	-0,013	-0,220	-0,271	0,304	0,014	0,174	-0,159	-0,085	0,400	0,554	**1**	-0,239
SO_4^{2-}	0,690	0,170	0,067	-0,244	-0,074	-0,189	0,759	0,735	0,501	0,411	-0,239	**1**

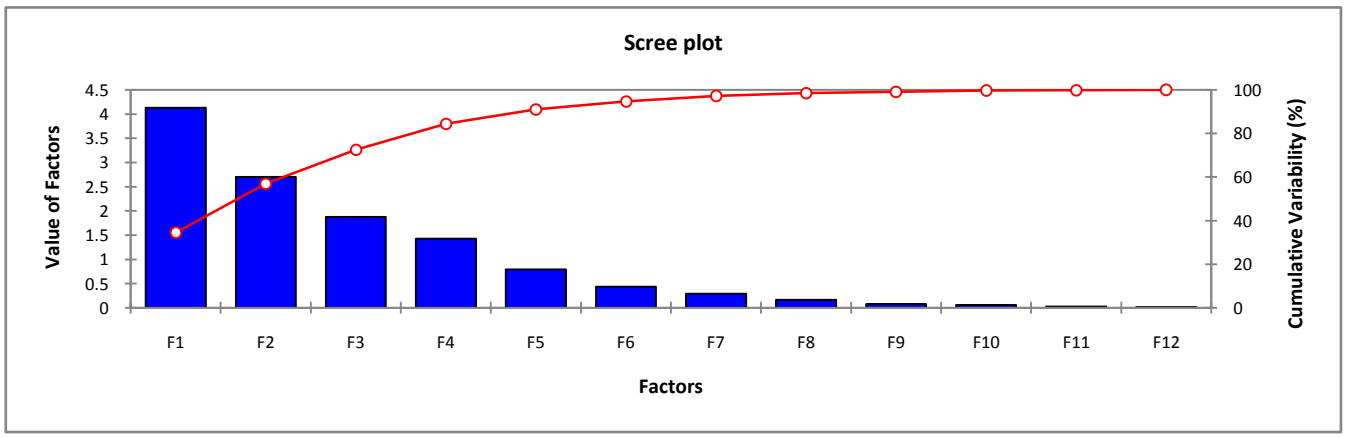

FIGURE 5: DIAGRAMS OF EIGENVALUES

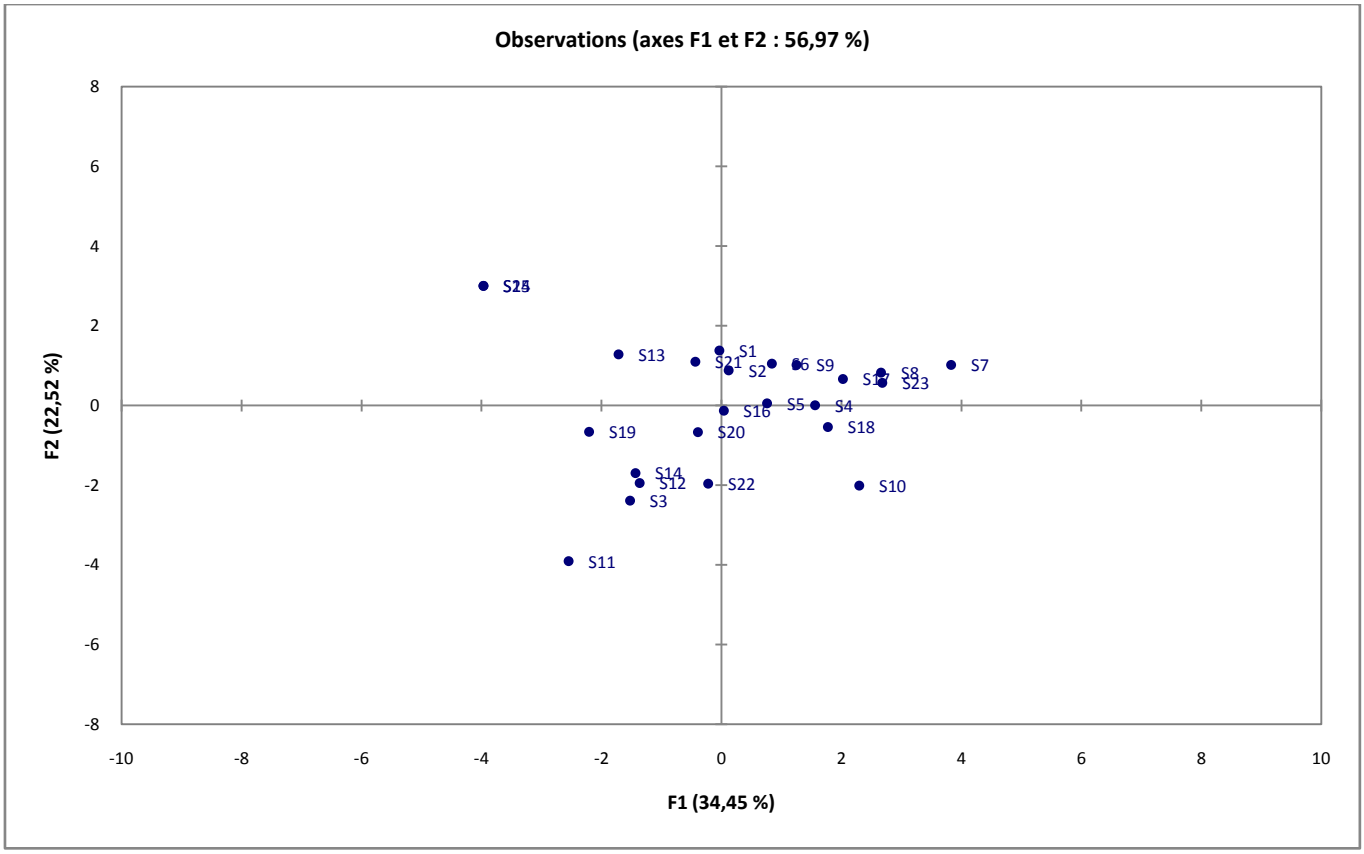

FIGURE 7: PROJECTION OF OBSERVATIONS ON FACTORIAL F1X F2 (65,31 %)

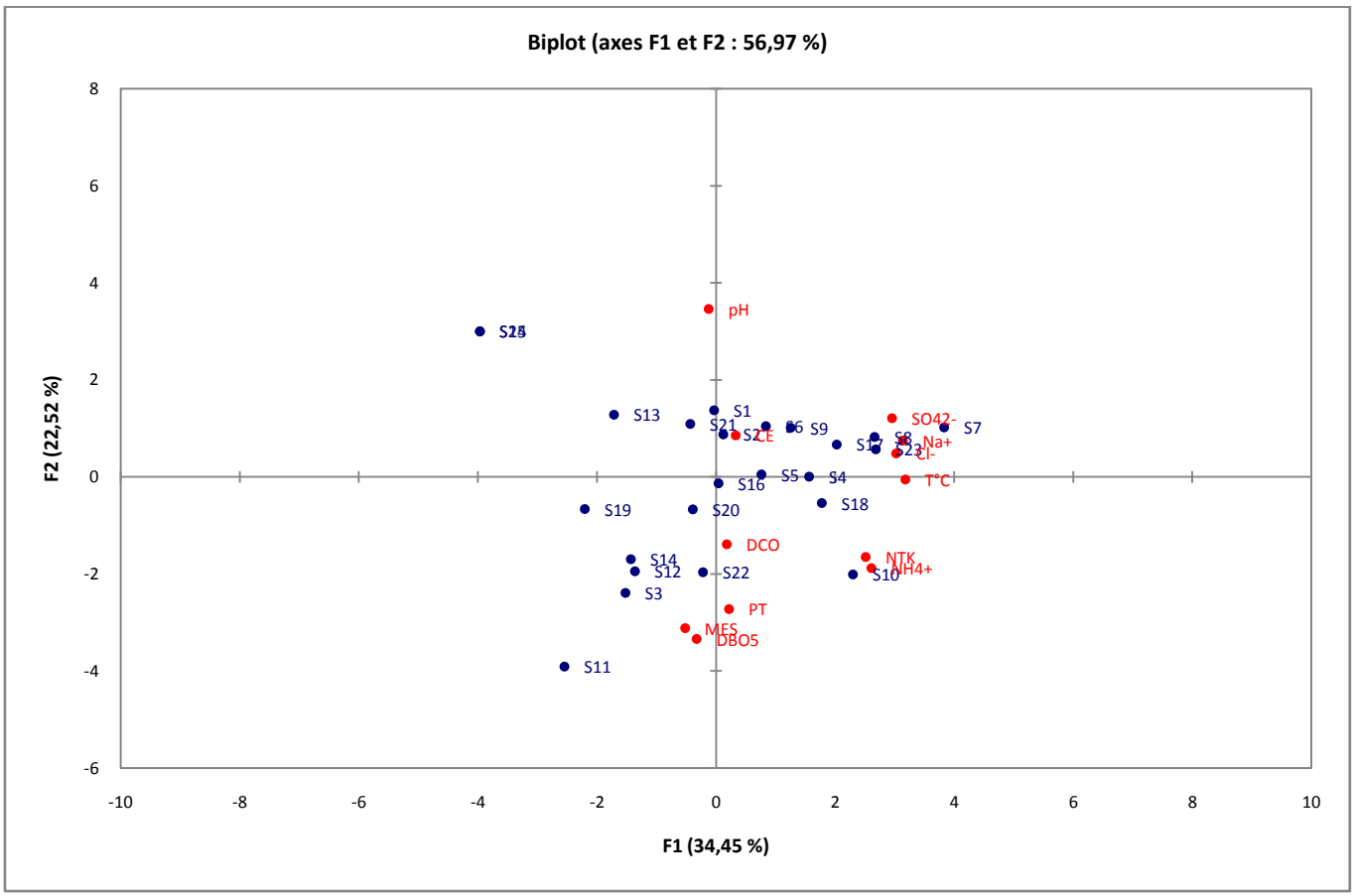

FIGURE 8: PROJECTION OF OBSERVATIONS AND VARIABLES ON FACTORIAL F1X F2 (65, 31 %)

V. CONCLUSION

The electrical conductivity reflects the total mineralization of water [9]. The average value recorded is 20115 µs / cm and is much higher than the Moroccan standards for the quality of water intended for irrigation (limit value <8700) and gives leachates a highly alkalizing power that is very dangerous for all cultivated plants [17].

The sodium has an average concentration of 3049.8 mg / L. This concentration of Na + ion is higher in leachate, and exceeds the Moroccan standard of water quality for irrigation. A large amount of sodium ions in the water affects the permeability of the soil and poses infiltration problems. This is due to the fact that the sodium present in the soil in exchangeable form replaces the calcium and magnesium adsorbed on the soil clays and causes the dispersion of the particles in the soil. This dispersion results in the alteration of soil aggregates. The soil then becomes hard and compact, reducing the infiltration rates of water and air, and consequently modifying the soil structure.

The sulphates ions (SO_4^{2-}) are sulphated compounds whose presence in water results from a contamination mainly related to the discharge of domestic and industrial effluents or a phenomenon of natural reduction of sulphates. The average value in leachate is of the order of 35 mg / L. They are the source of bad odors emanating from leachates.

The ammonium ion, NH4 +, is the reduced form of nitrogen. It comes mainly from the decomposition of natural proteins contained in phytoplankton and zooplankton. It can also be derived from the input of effluents from domestic, industrial or agricultural waste. The average value of the concentration of the NH4 + ion of the leachate of Oum Azza recorded during the study period is 3207 mg / L. According to the standard standard committee and Law 11-03, [14], ammonium does not meet discharge standards.

The chloride ions are anions of chlorine. This element is very abundant in the environment. It is present in water, soil, rocks, as well as in wastewater and leachate. The average chloride contents are 4289 mg / L. These results are consistent with those of previous studies [18-22].

In addition, the ammonium ion (NH_4 +), by nitrification is transformed into nitrites (NO_2-) and nitrates (NO_3-) and oxidized by the bacteria of the genus Nitrosomonas, then by the bacteria of the genus Nitrobacter [23]. Nitrates are very soluble in water; they migrate easily into the water table [24].

The average value of the BOD_5 of the leachates studied is 5522 mg of O2 / L and it is well above the limit value of the Moroccan standard of direct discharges which is 100 mg of O2 / L. Similarly, the COD value of 12626 mg / L exceeds the norm and can be the basis of a strong fermentation.

Bad odors systematically accompany a project of storage of household waste. Odors are due to the presence of hydrogen sulphide in the landfill gas and the decomposition of organic matter.

Landfill gas is composed of methane, carbon dioxide, oxygen, nitrogen, carbon monoxide, hydrogen, and hydrogen sulphide. Only carbon monoxide and hydrogen sulphide are likely to be toxic to humans, beyond a threshold.

Unfortunately in the project of Oum Azza, smells and fumes of gas have a real impact given the proximity of homes not far from the site of Oum Azza. This is the example of the El Manzeh housing estate about 2 km west of the project site. However, a dominance of the west sector winds closely followed by wind from the north significantly reduces the impact of odors and different gaseous emissions to this new subdivision [25-26].

This situation is exacerbated by the non-control and control of industrial, special and hazardous waste generally sent to wild dumps without pre-treatment [27-28].

In conclusion, the concentrations found in the raw leachates of Oum Azza exceed the standards for wastewater quality recommended by WHO [29] and [14]. In addition, spatiotemporal monitoring of several other factors such as pathogens, trace elements and pesticides must provide us with the true level of pollution. Thus, the area of the landfill is affected by biological and chemical pollution that puts at risk the underlying water table.

ACKNOWLEDGEMENTS

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Analysis of enterprise relationships in food industry cluster based on niche theory

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***Abstract*—** *In recent years, the Chinese government and local governments in China at all levels actively promote the construction of food industry cluster Demonstration Park. Many problems accompanied with the continuous development of industrial clusters, such as the proliferation of homogeneous enterprises and products, the lack of organic links and symbiosis relationship among enterprises, resource depletion and environmental degradation, triggering niche overlap and vicious competition. All these phenomenon leads to industry cluster an acute shortage power of competitive and innovation. In this paper, we take the livestock and poultry industry cluster as the research object, use niche theory to analyze enterprise niche inclusion, overlap and separation relationships. As well as through the neutral theory in the equilibrium state to explain the cluster of enterprises in the competition, cooperation and symbiotic relationship.*

Keywords*— *Niche Theory, Food Industry Cluster, Neutral Theory, Competition, Cooperation.

I. INTRODUCTION

Food industry cluster is composed with plenty of units, which lies in a specific geographical scope and in a particular way to rally around close neighboring communities, including government agencies, intermediary organizations, and research institutions, small and medium-sized enterprises producing food and related products and other social economic groups. In the industrial cluster, the enterprises can share the professional infrastructure, labor market and service. Relations among these units are complicated, such as complementary, cooperation, and even competing. For the important of food safety, Chinese government pays much attention to food industry development. "The 13th Five-Year Plan" of food industry and "No.1 File of 2017" clearly proposed to accelerate food industry cluster, to promote the food industry to be intensive, large-scale, so as to form a rational layout, resource conservation, modern food industry cluster. The government encourages food enterprises to strengthen cooperation and actively extend to the upstream and downstream, from the establishment of raw materials from the production of all aspects to the end of the whole industry chain. Promote the effective convergence of all aspects, to speed up the integration between the industrial chain integration, to achieve complementary advantages, information sharing, and coordinating development.

Under the guidance of government policy, most local government in China actively promotes the demonstration of food industry cluster or food park construction. And many large-scale, high levels modern food Industry Parks have been founded, such as Shandong Laiyang Food Industry Park, Chongqing Qijiang Food Industry Park, Chuzhou Green Food Industry Park and so on. At the same time, some districts are greed during food industry cluster construction, and there are some errors in their investment philosophy, leading to too much homogeneous enterprises within the cluster, and serious product homogeneity. And the lack of organic links and symbiotic relationship among enterprises within a food park, leading to depletion of resources and industrial environment deterioration, causing niche overlap and vicious competition and other issues. In particular, with the development of modern livestock production technology, animal husbandry, slaughtering, processing and logistics and other aspects is gradual getting intensive, large-scale. The fecal discharge and environmental pollution problems are becoming more and more serious, which has become a serious challenge to build a new socialist countryside and realize the coordinated development of economy and environment. In some districts, the food industry cluster has emerged the phenomena of lack of competition and excessive competition coexist, which caused the industrial cluster competitive power and innovation power is seriously inadequate, cooperation mechanism greatly reduced.

Therefore, in the food industry cluster development and upgrading process, too much competition occurs in horizontal enterprises, and lack of cooperation among vertical enterprises. In the process of industrial operation, the regionalism, incompleteness and imbalance of the competition among enterprises and the inequality of the subject status are the key problems in the development of food industry cluster.

Food industry cluster is a life-like organic whole, it has the life characteristics from emerge, development, maturity, and even to recession or other evolution. And all these behaviors are not only impacted or constrained by the environment, but also have some feedback on the environment. So the food industry cluster has obvious Ecological characteristics. Our research is

to regard the food industry cluster as a natural biological community, by using niche theory, we will analyze the niche of each unit, so as to make every enterprise in the cluster can find its own position, and all enterprises within a food park can form a "food industry ecological community". We will take the food industry park lifecycle as research object, and carry out study from its original status of less competition to the intermediate status of excessive competition and to the final status of coexistence. We will integrate niche theory to build industrial clusters collaborative evolution and sustainable development model.

II. COOPERATION AND COMPETITION AMONG ENTERPRISES IN FOOD INDUSTRY CLUSTER

Because competition and cooperation occur in different ranges and different participants, they can coexist in the same industry cluster. Therefore, proper scale competitions among enterprises in one industry cluster provide both incentives and avoid excessive competition (Porter, 1998). It can be seen that the observation of competition and cooperation in industrial clusters is based on two different perspectives of homogeneous enterprises and heterogeneous enterprises.

The competition among leading large enterprises in one industry cluster shows the greatest impact and the most obvious performance of the cluster, especially in the cluster supply chain enterprises. So, competition among leading large enterprises can be very intuitive to reflect the strong competition among them, and its heterogeneous enterprises in the cluster of mutual cooperation among the close cooperation. Because the core enterprise appears in industry cluster, especially co-existence of multiple core enterprises, the industry cluster forms a cluster supply chain, which is driven by core enterprises and a large number of collaborative enterprises co-exist parallel organization.

As shown in Fig. 1, livestock and poultry industry is the group that builds around the adjacent neighborhood residents in a specific geographical scope, including government departments, intermediary organizations, scientific research institutions, large-scale production of livestock and related products related to small and medium enterprises in a specific way. The focus of this paper is mainly on the core of animal husbandry and food industry cluster, which are shown as ①, ②, ③part in the Fig. 1. The animal husbandry food enterprises in this article is represented by Ai (i=1, 2, ---). Farmers or agricultural cooperatives, who provide raw materials for processing is represented by Bi (i=1, 2, ---), and Logistics and transport enterprises are represented by Ci (i=1, 2, ---). Besides that, government agencies and food development center, inspection and testing and certification center, checking each process of product, internationally renowned third-party testing and certification bodies, to carry out international testing and certification services, universities, research institutions, associations, trade finance and intermediary organizations, providing product innovation and enterprise financing to provide support and help, and display trading center, innovation incubator center, brand operations center, cooperation and exchange center, human resources center around the cluster, providing technical and service support, all of these mentioned units are represented by Di (i=1, 2, ---).

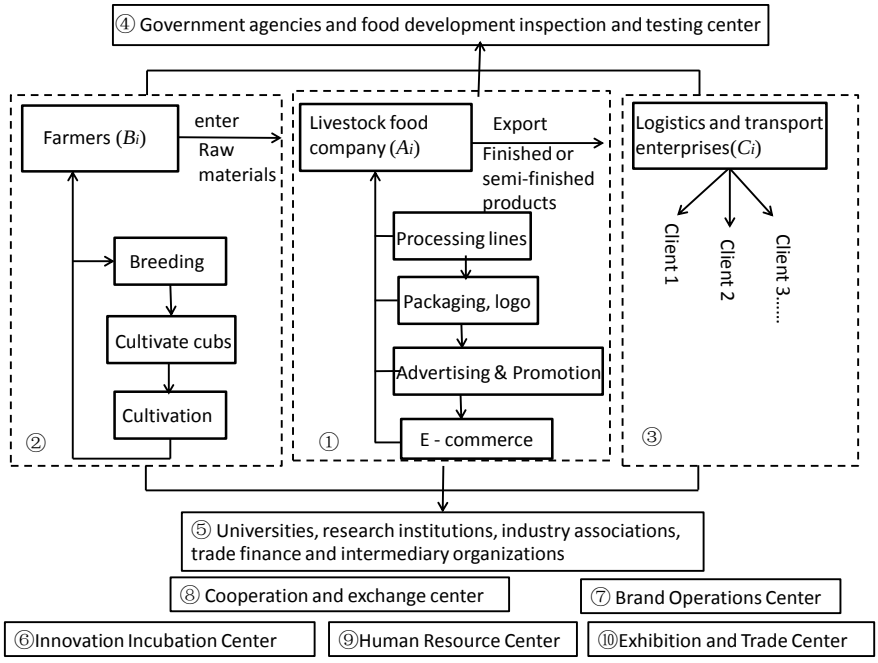

FIGURE 1: SCHEMATIC DIAGRAM OF LIVESTOCK FOOD INDUSTRY CLUSTER

With the continuous development of China's food industry cluster over the past decades, combined with the above research shows that in a relatively mature food industry cluster, there will be one or more core enterprises drive the development of other enterprises within the cluster. The following analysis is for the livestock and poultry industry cluster has two or more core enterprises situation. At the same time, due to animal husbandry food with preservation, durability characteristics, its requirements are extremely high for logistics and transportation industry. Considering the highest correlation between the two, mainly take logistics enterprises as an example.

According to existing research, the competition in the animal husbandry and food cluster can be divided into three kinds: ① competition between core enterprises; ②competition between core enterprises and related enterprises; ③Competition among related enterprises (mainly small and medium enterprises). Corresponding cooperation can also be divided into three kinds: ①cooperation between the core enterprises; ②core business and related business cooperation; ③related enterprises (mainly refers to the small and medium enterprises) competition.

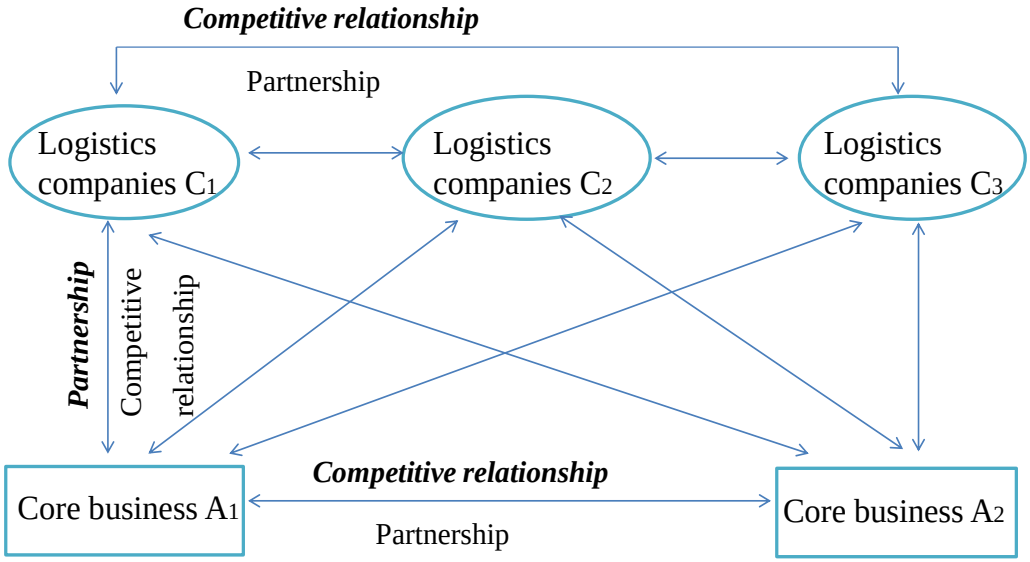

FIGURE 2: COMPETITION AND COOPERATION AMONG ENTERPRISES

Fig. 2 shows that a variety of enterprises among the competition and cooperation diagram in a livestock and poultry industry cluster, the core business A1, A2, logistics enterprises C1, C2, C3. It can be seen that competition and cooperation exist at the same time, however there might have a leading role between the two (the bold font means the dominant role of the two relations).

In food industry cluster, the interaction among enterprises is more obvious, and becomes a staggered network structure. This can be seen easily from Fig. 2 that the competition is dominated by the core enterprises A1 and A2, and between the enterprises C1 and C2 (or C1 and C2, C2 and C3, C3 and C1). Mainly due to the geographical distance is close between the core industries in the industrial clusters, more widely access to other information channels, the spread faster news, basically rely on the same geographical market, therefore the market share, product type, business publicity, management, culture and above than the general competition of enterprises are more comparable. At the same time, the two do not rule out cooperation, such as animal husbandry fresh food enterprises share cold storage, but this is a few cases. However as to cooperation, it clearly exists obviously between the core business and related enterprises, such as the core business A1 and logistics enterprises C1. The main relationship between competition and cooperation in industrial clusters can be summarized as the competition of homogeneous enterprises is greater than that of cooperation, and the cooperation of heterogeneous enterprises is greater than that of competition.

It is necessary for enterprises to give full play to their own core competitiveness, especially the core competitiveness built by a lot of money and manpower, with some other business obtained from the relevant enterprises through outsourcing and other means, making the whole production process more efficient. That is why the core business and related enterprises reached a long-term cooperation consensus. But in practice, not every food cluster in the competing relations are the same as the

theoretical analysis, there are still some situations cannot be ignored, especially obvious vicious competition and lack of competition.

As shown in Fig. 2,when enterprise A1, A2 are seeking logistics cooperation, if core companies in a food cluster is lack of competition (the core business is in a downturn and the demand for related businesses is reduced), it will lead to logistics enterprises C1, C2, C3 in a state of excessive competition. This is what the core business wants to see. In this case, it becomes the "core business market", means that the dominant power is in the hands of the core business, and related enterprises over-competition can give the core business A1, A2 bring lower transaction costs, but not conducive to the logistics enterprises C1, C2, C3 long-term development; If there is an excessive competition between the core business A1 and A2 (core business development is rapid, strong demand for related business), the logistics enterprises C1, C2, C3 have more opportunities to observe cooperation conditions offered by A1 and A2, and choose the most optimized to cooperate. In this case, it becomes the "relevant business market", means that the dominant power is in the hands of the related enterprises. Such a situation can give logistics enterprises C1, C2, C3 bring more benefits, and the core business is certainly to be affected to varying degrees in excessive competition.

No matter it is competition or cooperation, each business wants to hold its own advantage position in the cluster development process. Its development trend can be summarized as homogeneous competition is greater than cooperation; heterogeneous cooperation is greater than competition. Just like the species of the community of species, through the "survival of the fittest, discomfort eliminated" natural choice, each business is on its own ecosystem, to achieve their own and the overall niche balance, forming a stable development Community. Enterprises are just like biological individuals. To analyze the more complex relationship between enterprises is one of the main purposes of this study with niche theory.

III. NICHE ANALYSIS OF ENTERPRISE RELATIONSHIP IN ANIMAL HUSBANDRY FOOD INDUSTRY CLUSTER

Niche theory is an important theoretical concept of modern ecology. Johnson (1910) was the first person who used the word "niche". And then Grinnell (1917), Elton (1927), Hutchinson (1957), Odum (1959), Pianka (1983) and other famous scholars have been committed to the niche analysis and explore. The niche theory can be summarized into three main points: position, function and the relation of species. One of the most critical points is that species in each species spatial position to a stable adaptation. In addition it also has the function of and contact with other species. In the course of the study, scholars have given them specific numerical indicators such as niche breadth, niche overlap, niche size, and niche dimensions.

With the continuous application of niche theory in the enterprise economy, a number of domestic scholars have formed their own niche theory. Enterprise niche is the part of resources and space that can be obtained and utilized by the enterprise in the whole ecological resource space. It is an enterprise and even an industry, in the enterprise ecological environment has a definite position. The niche of the enterprise in the industry is the sign of the competitive strength of the enterprise in the industry (Liang Jiahua, GeZhenzhong, et al., 2002). It is the specific market position, location and function status of an enterprise associated with other enterprises (Lin Xiao, 2003). In the enterprise niche, enterprise in a certain market environment occupies a certain position and plays a role similar to the concept of "market orientation". But it has more ecological connotations than "market positioning" (Xu Fang, Li Jianhua, 2005).Enterprise niche refers to the relative position and function of enterprise in a certain period in the specific ecological environment actively and the environment and other enterprises in the process of interaction form (Yan An, DaQingli, 2005).

The niche of food cluster can be interpreted as those different types of food enterprises have their own stable position in the cluster. The position of each enterprise embodies its comprehensive strength in the industry. In daily production, food enterprises keep close contact with related enterprises in a particular cluster ecological environment, and give full play to their important functions in the cluster, and interact with each other.

By referring to the domestic and foreign scholars' research on niche theory and expanding the result, we can describe the abstract cluster enterprise relationship by the quantitative index mentioned above. Here mainly focus on three aspects. First, the competition between enterprises in the cluster can be expressed as niche overlap. Second, cooperation among enterprises within the cluster can be expressed as the compensation of niche. Third, symbiosis among enterprises in the cluster can be expressed as the equilibrium of neutral theory. (The third point will be analyzed in the next section.)

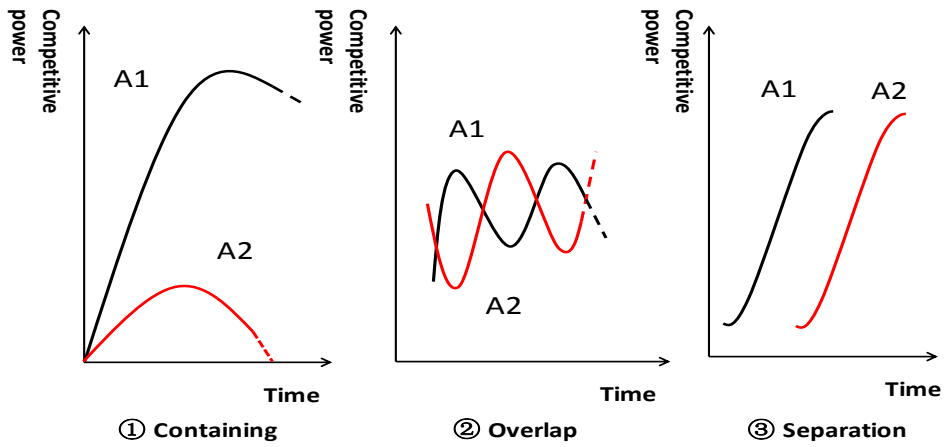

FIGURE 3: SKETCH MAP OF NICHE TYPES

Since the research object is defined as the food industry cluster, the breadth of enterprise niche can be understood as the total demand for the market resources of food enterprises. This indirectly reflects the competitive level of the food enterprises in the competition. If the resources of a food enterprise are more abundant, the niche wider, and the more generalization of the ecological niche, that's means the greater the possibilities of niche overlap. On the contrary, the niche of a food enterprise is narrower, and its niche is more specialized, which shows that the niche overlap is less likely. The above law can be divided into three types as shown in Fig. 3.

Fig. 3 shows the Enterprises A1 and A2 in the animal husbandry and food industry. The above three types indicate respectively.①containing: With the passage of time, the survival of the fittest, the enterprise A1 gradually gain higher competitiveness, gradually eliminate the enterprise A2. (It can be viewed as a special case of overlap, or indirectly as a result of the lack of competition in the enterprise A2.) ②Overlap: This situation shows that competition exists among enterprises, and enterprises A1 and A2 rise alternately in competition.③Separation: it shows that cooperation or symbiosis exists between enterprise A1 and A2. The two ecological niches are parallel, increasing and decreasing, and depend on each other.

However, in the actual situation, we should not ignore the environmental factors of the enterprise, and the related objects should also be considered. Here, we establish a multidimensional coordinate for analysis. In this paper assumes that there is three kinds of main food industry cluster study: the first category is the logistics oriented enterprises, set the X axis. Second is the resource oriented farmers, set to the Y axis. Third is for animal husbandry food enterprises, and it is as a production and processing oriented food enterprises, set to the Z axis.

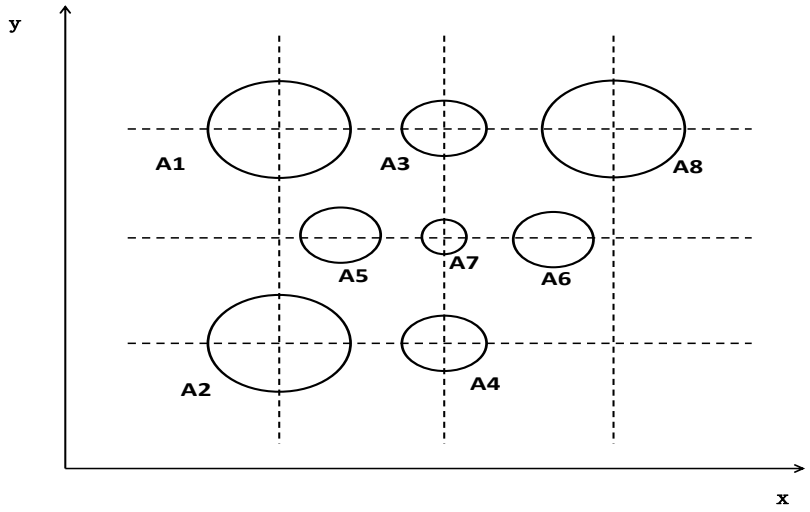

FIGURE 4: SCHEMATIC DIAGRAM OF ENTERPRISE'S TWO-DIMENSIONAL NICHE

Fig. 4 shows the niche of a livestock food industry in a two-dimensional perspective at different related objects. According to the graph above, we can see the direction of the X axis in terms of enterprise A2 and A4, enterprise A1, A3 and A8, and

enterprise A5, A7 and A6 have the same ecological niche. In the direction of the Y axis, enterprise A1 and A2, enterprise A3, A7 and A4 have the same ecological niche. That is to say, there are differences in niche in different dimensions, so we cannot ignore the angle of view of related objects in the process of research. In order to more intuitively reflect the phenomenon of niche overlap, each research enterprise can build a three-dimensional coordinate. Here, for example, the enterprise A1 and A2 of the above diagram, build the following 3D coordinates.

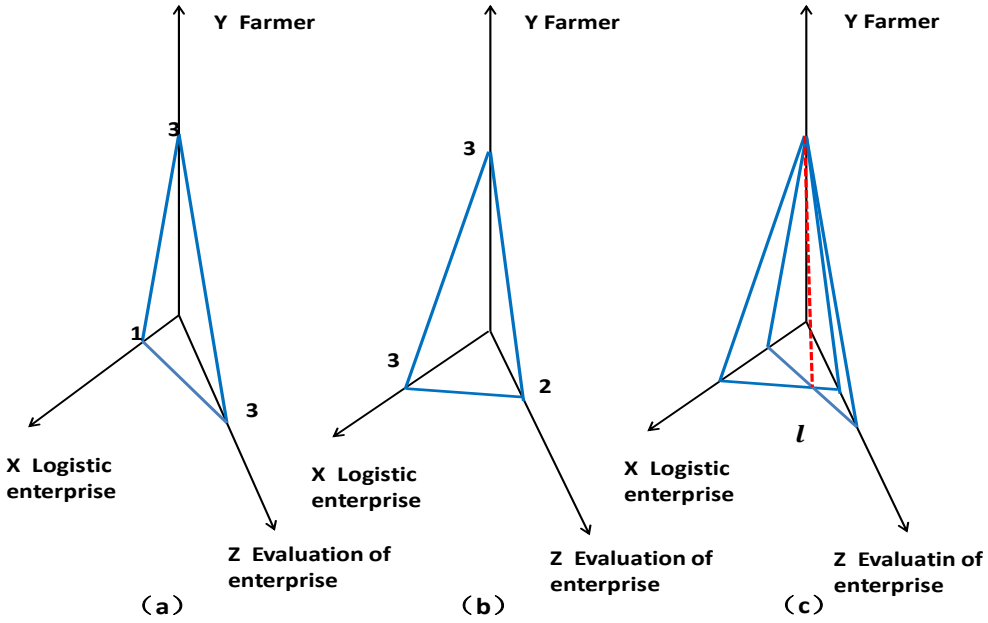

FIGURE 5: ENTERPRISE THREE-DIMENSIONAL NICHE

In Fig. 5, enterprise A1 and A2 have the same niche in the Y axis, i.e. A1 and A2 for farmers is very similar supplier. There must be a high competition between A2 and A1.Both in the Y axis is assumed to be 3. On the X axis, enterprise A1 and A2 have different ecological niche, assuming the A1 in X axis is 1, A2 is 3.The Z axis represents the enterprise's own evaluation (Of course, this evaluation needs to be adjusted after a deep understanding of the enterprises). It is assumed that A1 is 3, A2 is 2.The enterprise A1 and A2 are established in the three-dimensional space of ∆ 133 and ∆ 332.As shown in Fig. 3-C, Put the two triangles in the same coordinate system and intersect at the line l.

This shows that an enterprise and other enterprises in the axis intersect the more line and points, the more it overlaps with other enterprises in the niche, the wider the niche and the more decentralized the enterprise. On the contrary, the more specialized the enterprise is. Similarly, farmers' competitiveness evaluation of enterprises will also affect the competitiveness of farmers, because farmers will want to cooperate with more competitive enterprises. But the enterprises that are competitive with farmers are not necessarily competitive in logistics enterprises. Therefore, the competitiveness of enterprises can also lead to competition in their partner industry.

IV. EQUILIBRIUM STATE OF ENTERPRISE RELATIONS IN ANIMAL HUSBANDRY FOOD INDUSTRY CLUSTER

Niche theory shows that the difference of niche is the key to the existence of species diversity in a community after the natural selection of survival of the fittest. However, the neutral theory is the opposite of the hypothesis of niche theory. The neutral theory holds that the difference between species does not matter to the construction of ecological communities, that is to say, ecological equivalence of species. It is assumed that all individuals in the community are functionally equivalent, and in addition the number of communities is saturated in extent.

This theory of the main assumptions applied to food industry cluster can be summarized as follows: (1) each part of cluster are equivalent. That's means the Enterprises have same statistical properties, such as production, processing, and transportation efficiency. They will not change with the enterprise's changing. (2) Cluster is a saturated cluster. That is to say, once a food enterprise or other related enterprise goes bankrupt, then the cluster will be accompanied by another new random individual to come. In order to better understand the changes of the above two basic assumptions, the performance and change of enterprises and related enterprises in the neutral theory can be observed in the examples in the last section.

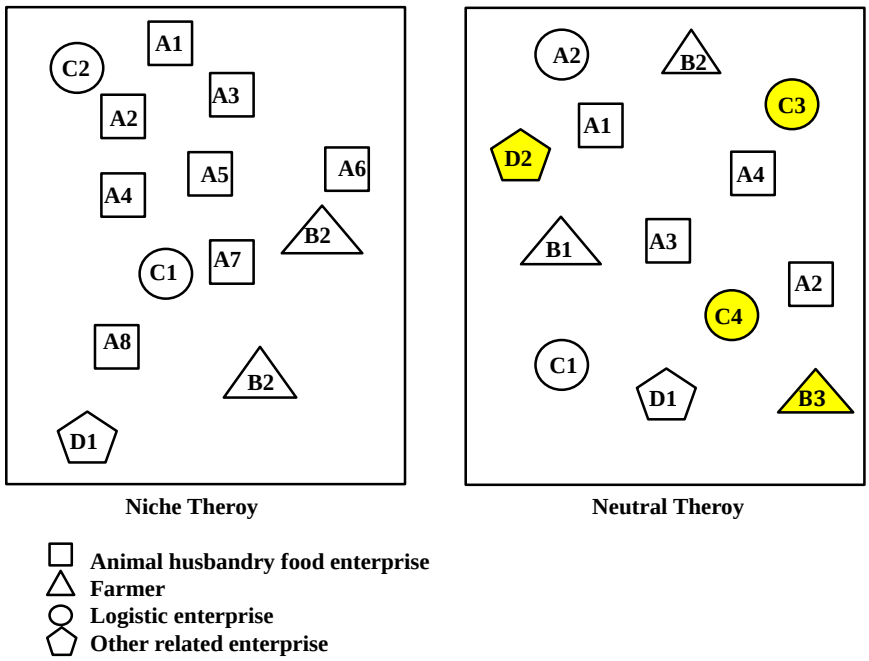

FIGURE 6: CHANGE OF NEUTRAL THEORY IN ANIMAL HUSBANDRY FOOD INDUSTRY ENTERPRISES

The diagram shows a clear change from niche theory to neutral theory. Apart from the reduction of 8 animal food industry enterprises to 4, the remaining 2 farmers increased to 3, logistic enterprises increased from 1 to 4, and 1 other related enterprises increased to 2.In the original niche, the homogeneous competition among the enterprises of animal husbandry and food industry led to the disappearance of some vulnerable enterprises. At the same time, the retained enterprises have driven the development of their cooperative enterprises.

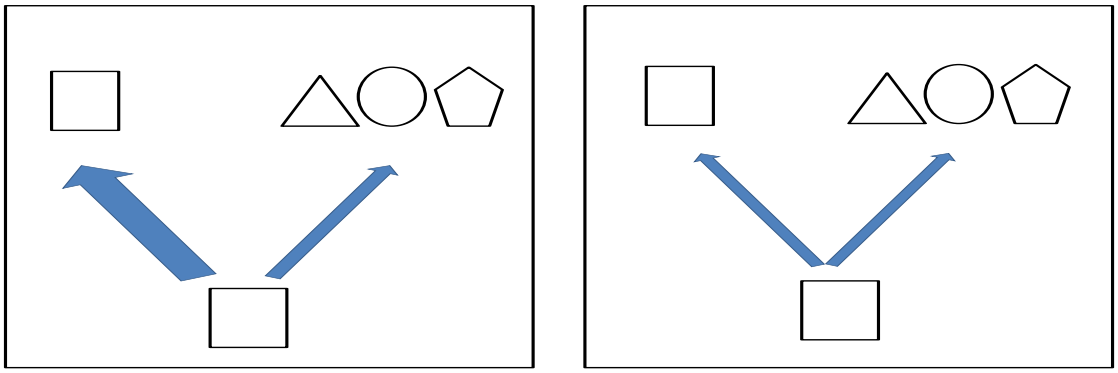

FIGURE 7: COMPETITION BETWEEN ANIMAL HUSBANDRY FOOD ENTERPRISES OF NEUTRAL THEORY

As shown in Fig.7, the size of the arrow represents the extent to which the animal husbandry food industry is more competitive with other enterprises, and the thicker the arrows, the more intense the competition. In niche theory, homogeneous enterprises inhibit each other's development to a greater extent than those of heterogeneous enterprises, mainly because cooperation between heterogeneous enterprises is greater than competition. But in neutral theory there is no extent of size and depth because each enterprise is equivalent. This law can also be represented by rates and quantity diagrams in Fig.8. Among them, l_1 andl_3 indicate the relationship between the quantity of animal husbandry food industry and the rate of development. l_2 andl_4 respectively indicate the relationship between the number of farmers, logistics enterprises and other related enterprises along with the development rate. Generally speaking, when a homogeneous population develops rapidly, it will restrain self-development and reduce the quantity. Conversely, when the homogeneous population develops slowly, its self-inhibition will decrease and the quantity will increase. Therefore, we believe that the neutral theory emphasizes a balanced state on the basis of niche theory. It is an ideal condition for enterprises in mature industrial clusters to achieve symbiotic balance through competition.

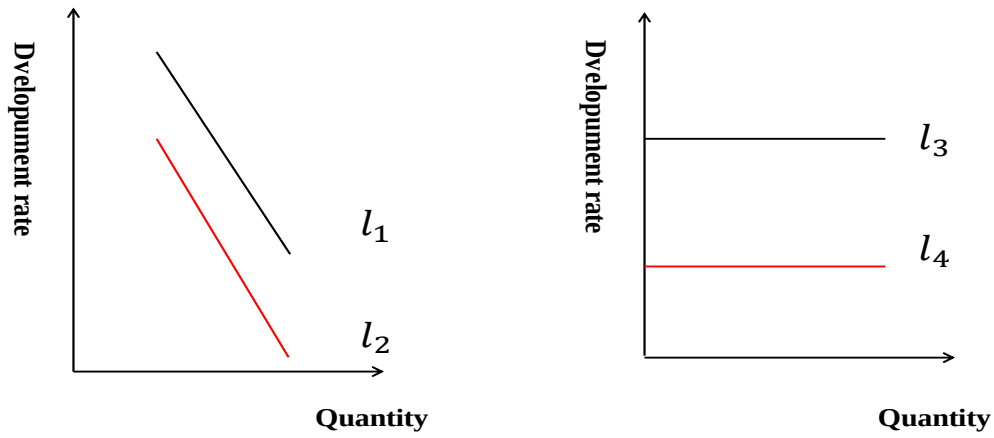

FIGURE 8: NUMBER OF ENTERPRISES OF FOOD INDUSTRY WITH THE DEVELOPMENT RATE CHANGES

V. CONCLUSION

In the research assume that the food industry cluster is a crisscross network structure, enterprises in the cluster are homogeneous, heterogeneous or complementary, reflects the relations of competition, cooperation and symbiosis among enterprises in the cluster.

Due to the particularity requirements for food safety, its operation from environment, raw material, collaboration, inspection or quarantine institutions to any participant are extremely strict, this requires enterprises, farmers, and specialized agencies set up sound relations. Our research regard the food industry cluster as a natural biological community, and to make every enterprise in the cluster find its own position, and form a "ecological community". The research aim is to build collaborative evolution and sustainable development model for food industry cluster, from the perspective of ecology.

Through the analysis of the above can be found, in animal husbandry food industry cluster, homogeneous enterprises located in the state of niche overlap, there will be a shift to the fierce competition in the spiral phase, also be a result of natural selection. In addition, with the development of the competition, the cluster tends to be balanced. In the process of equilibrium, the inhibition of competition between homogeneous enterprises is greater than that of heterogeneous enterprises, i.e. the general trend of "homogeneous competition and heterogeneous cooperation".

When the equilibrium state comes, the cluster is in a state of saturation. The demise of old businesses will be replaced by new ones. The ecological niche difference between enterprises is also played down. Each enterprise is the equivalent individual in the cluster. The speed and quantity of the enterprise will not change again. That's what we call equilibrium -- symbiosis.

Symbiosis is a special situation in a cooperative relationship and a closer relationship than cooperation. It can be simply understood as co-existence. That is to say, the symbiotic enterprise is an intimate long-term strategic cooperative partnership, which is dependent on each other and is interdependent, and the ecological niche overlap is low. The low overlap of enterprise niche will avoid the confrontation between enterprises in the cluster because they produce the same products and compete for the same market and source. Ecological niche resource complementary or ecological niche separation can promote the relationship between enterprises to seek their respective development resources. At the same time, the daily operation of the communist party will reduce the transaction cost of enterprises and improve the efficiency of production and operation. For a business, it's good to have a symbiotic relationship, and it's not good. Once a party has a major problem such as financial or even bankruptcy, the other party is bound to get involved. As businesses become more dependent on each other, once the symbiotic object goes bankrupt, the business can be severely damaged or even bankrupt.

Although the competition itself has led to cooperation, it is not what we want to see whether it is excessive competition or lack of competition. What people want is a balanced development in the cluster, when the market itself does not have a role to play, with the help of government intervention. So the other main purpose of this study is to provide a manageable, maneuverable policy recommendation series to the government level. Subsequent research will be based on the theoretical framework of this paper, and the empirical research will be carried out in detail in various animal husbandry food industry clusters, such as fresh meat, frozen meat, eggs and milk.

ACKNOWLEDGEMENTS

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Improved grey clustering method in risk zonation of mountain flash flood disaster

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***Abstract*—** Flash floods are considered one of the worst weather-related natural disasters. Flash floods are dangerous because they are sudden and highly unpredictable. Identification of the locations of high-risk areas has a major effect on the improvement of flash flood disaster control and prevention. Earlier work conducted on flood disaster risk zonation was commonly based on Digital Elevation Mode (DEM) data and statistical yearbook data and used an index, such as rainfall, topography, slope, or river distribution, with the analytic hierarchy process (AHP) method to determine the weighting. In this method, the final regional risk map was created by using ArcGIS map algebra superposition. In the present study, an improved gray clustering method is put forward to improve the comprehensive evaluation of the risk of mountain flash flood disasters by constructing the exponential whitening function and by using the information entropy weight method, which produces results that are more accurate and more reliable than those of the traditional method. This improved method can make full use of the limited information available, improving not only the resolution but also the influence of the subjective method, and produces more objective and accurate evaluation results. We obtain the risk degree by combining the information entropy weight and improved whitening function approaches in a gray clustering methodology. Additionally, a method is applied to develop models for mapping the risk grade in zones of 1436 towns and counties in Hubei Province with remotely sensed (RS) data and the ArcGIS platform. The results show that the improved approach is useful for rapidly assessing flash flood hazard and vulnerability and for completing risk assessments in mountain areas.

Keywords*— *improved gray clustering method, whitening function, information entropy, flood disaster risk, evaluation, zonation.

I. INTRODUCTION

Flash flooding is one of the major natural disasters that may hamper human development in flash flood areas. A mountain flash flood disaster is one of the most serious natural disasters. Mountain flash flood disasters occur suddenly, are considerably destructive, have short durations and cause serious harmed in the form of many casualties and considerable property loss. China is one of the countries that seriously suffer from mountain flash floods. Jonkman (2005) studied flash flood data from 1975 to 2002 and found that flash flood mortality is higher than that for other natural hazards. The potential for flash flood casualties and damages is also increasing in many regions due to the social and economic development which imply pressure on land use. Consequently, the flash flood hazard is expected to increase in frequency and severity because of the impacts of global change on climate, severe weather in the form of heavy rains and river discharge conditions. Therefore, the management of flash flood risks is a critical component of public safety and quality of life.

As one of the important and fundamental steps in flood regionalization, flood risk evaluation has general public concern. Many achievements in flood risk assessment research have been realized. Currently, the main methods of evaluating and locating flood disaster risk include geomorphologic methods (Haruyama et al. 1996), hydrology-hydraulic models, system simulation methods (Solaimani et al. 2005; Elawad et al. 2004; Smemoe et al. 2004),methods based on historical disaster data(Liu and Shi 2001;Huang et.al) and ancient flood data(Bonito et al. 1998; Bonito et al. 2003; Bonito et al. 2004), methods based on remote sensing and GIS (Sanyal and Lu 2006), and machine system analysis methods (Tang and Zhu 2005;Li et al. 2005). However, fairly few research papers have focused on mountain flash flood zonation. Even rarer are integrated analyses of flood risk that comprehensively consider flood formation mechanisms, climate, geomorphology, river water systems, and historical flood data. The research conducted in this paper is focused on comprehensive risk assessment and zonation, considering the important factors affecting flash floods: precipitation, topography, water system, vegetation, GDP, population, cultivated land and flood control capacity.

In fact, the main characteristics of natural disaster systems are the uncertainty and complexity of the system. Determination of the weights of the indicators is a problem because of the wide range of both natural uncertainty and approaches. Some in myriad sample can be solved by probability and statistics ways, and some in kenning uncertainty can be dealt with by fuzzy

mathematics. However, there also exists another category on uncertainty in less data and little sample, incomplete information and devoid of experience, which is suitable to be dealt with only by gray system theory (Deng,2005). In general terms, the uncertainty in less data and incomplete information is designated grayness. Thus, systems possessing grayness are said to be gray systems.

The gray theory provides the applications of clustering analysis, relational analysis, predication, and decision for the gray system (Deng, 1989). The so-called ‘‘gray” means that system information is incomplete, unclear, and uncertain. It is a useful method to address the problems of limited, deficient, and no rules available for data processing. Its analysis makes use of minor data and does not demand strict statistical procedures and inference rules. Recent studies have emphasized the importance of a comprehensive assessment of the flood risk using the gray system method (Liu, 2010).

To address the problem of nonadjacent domain weighted superposition failure caused by the traditional gray clustering whitening function, this paper proposes a whitening function construction method based on exponential distribution, avoiding the condition of a zero weighting, and discusses on the steps of flood risk assessment in detail.

In view of the complexity of the causes and the randomness of the occurring process of the flood disaster, we proposed a comprehensive assessment by introducing the concept of information entropy into the improved gray method and constructing a typical exponential whitening weight function. Based on the above characteristic, this method can effectively solve the zero-weight problem, make full use of the simple data and largely reserve the information implied in the clustering weight by modifying the clustering weight with the values reflected by the entropy. Finally, with data from Hubei Province, we graded the risk evaluation of 1436 towns in Hubei Province, which verifies the validity and objectivity of the method described in the paper. This illustrative example verifies that this method is simple and reasonable and can extend application range of the gray clustering in flash flood zonation.

II. GRAY CLUSTERING MODELING AND IMPROVEMENTS

2.1 Traditional gray clustering modeling and limitations

The concept of gray clustering is based on samples of every object, in accordance with the given indicators by means of the whitening function to abstract the actual sample into scales, and objects are sorted into their corresponding gray league to synthesize the priorities for selecting an appropriate alternative, which is said to be gray clustering.

As in the fuzzy approach, in this paper, different ranges of risk assessment are described as very low, low, medium, high, and very high by the so-called ‘‘whiten-weight function”. The common ones are triangular and trapezoidal functions. The trapezoidal functions are usually used and can be represented as

$$f_{\tilde{A}}(x)=\begin{cases}0 & x<a\\ \dfrac{x-a}{b-a} & a\le x\le b\\ 1 & b\le x\le c\\ \dfrac{x-d}{c-d} & c\le x\le d\\ 0 & x>d\end{cases} \tag{1}$$

where $x\in R$, $a\le b\le c\le d$, a and b are the lower limit and upper limit of $\tilde{A}$.Specifically, $\tilde{A}$ is the triangle fuzzy number when $b=c$. $\tilde{A}$ is a real number when $a=b=c=d$, as shown in Fig. 1

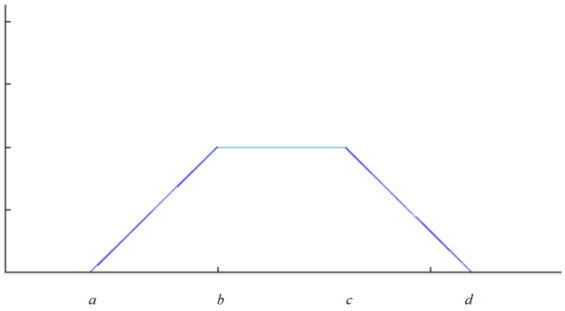

FIG. 1.TRADITIONAL LINEAR WHITENING FUNCTION

Fig. 1 shows the construction of the whitening function in the traditional gray clustering model. The whitening function is piecewise linear, and the whitening function of each rank is non-zero when it is adjacent to the interval the data belongs to, meaning that there is a relationship between only two adjacent levels in the coverage of the whitening function, and useful information is lost. In addition, the whitening function of the rank that is not adjacent to the level is zero, possibly causing a problem of zero weight.

The shortcoming of existing gray clustering methods is that the range of the linear whitening function is too narrow, especially when the distribution of the monitoring value is discrete, causing the loss of useful information. The improved gray clustering method makes better use of characteristic information in research data and calculates risk evaluation levels of the areas.

2.2 Improved gray clustering

2.2.1 Exponential whitening function

In this paper, we consider the issue that the linear whitening function in a conventional gray clustering model considers the relationship between only adjacent levels, and we construct f_{jk}, which can effectively expand the coverage of a whitening function and greatly improve the utilization of information, as shown in Fig. 2.

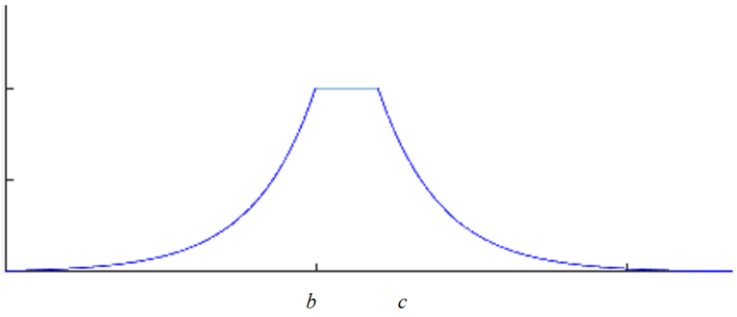

FIG. 2. EXPONENTIAL WHITENING FUNCTION

Let

$$f_{\tilde{A}}(x)=\begin{cases} e^{\frac{x-b}{b}} & x\in(0,b) \\ 1 & x\in[b,c] \\ e^{\frac{c-x}{c}} & x\in(c,+\infty) \end{cases} \tag{2}$$

If $x\in(0,b)$, the function $f_{\tilde{A}}(x)$ monotonically increases in the interval $(0,b)$, and $\lim_{x\to 0^+} f_{\tilde{A}}(x)=0$. If $x\in(c,+\infty)$, the function $f_{\tilde{A}}(x)$ monotonically decreases in the interval $(c,+\infty)$, and $\lim_{x\to+\infty} f_{\tilde{A}}(x)=0$.

The exponential whitening function can effectively overcome the limitation of the linear whitening function in the traditional gray clustering method, considering the defects between adjacent grades only, broaden the coverage of the whitening function and greatly improve the utilization of the available information.

2.2.2 Clustering weights based on entropy

It is essential to analyze the relative importance of each index in the flood risk evaluation index system. In previous research, however, flood risk evaluation index systems lacked effective methods to calculate the weights of indexes, making flood risk assessment difficult. The most commonly used method to determine index weighting is the analytic hierarchy process (AHP). However, the analytic hierarchy process is a highly subjective method that compares the indexes to each other and subjectively determines the relative importance by the judgment of individuals. The information entropy method can overcome the limitations of the subjective imprecision of the traditional AHP method, use the limited data in the weighting process and better reflect the natural conditions in the evaluation results.

The concept of entropy is derived from thermodynamics, which is used to describe the irreversible phenomena of the movement of ions or molecules. Later, entropy was introduced into information theory by Shannon (Shannon, 1948) to

measure the uncertainty, stability, and quantity of information in the system. In the actual evaluation or decision-making process, the role of the indicators and the amount of information transmitted vary. When the value of an object in a certain index is greater, the entropy value is lesser, which shows that more effective information is provided by this index; the greater the role of the comprehensive evaluation is, the greater the weight should be, and vice versa.

Anentropy-based clustering weight can avoid subjective influence, produce more reasonable and objective weights, and ensure standardization and accuracy of the flash flood evaluation and zonation. The process of information entropy is as follows.

Suppose the sample set is $\{d_1, d_2, \cdots, d_m\}$ and every sample has n indicators. The sample indicator matrix is D

$$D = \begin{pmatrix} d_{11} & d_{12} & \cdots & d_{1n} \\ d_{21} & d_{22} & \cdots & d_{2n} \\ \vdots & \vdots & \vdots & \vdots \\ d_{m1} & d_{m2} & \cdots & d_{mn} \end{pmatrix} \tag{3}$$

where x_{ij} is the j[th] indicator of sample i, i=1,2,…m, and j=1,2,…n.

Because the entropy variables range from 0 to 1, we need to address the original sample value.In this paper, the normalization method used is as follows:

$$u_{ij} = \frac{d_{ij}}{\sum_{i=1}^{m} d_{ij}} \tag{4}$$

For a j-th index in the system, the information entropy is as follows:

$$e_j = -k \sum_{i=1}^{m} u_{ij} \ln u_{ij} \tag{5}$$

where $k = 1/\ln n$ and $0 < e < 1$.

We can define the clustering weights of the j-th index as follows:

$$w_j = (1 - e_j)(m - \sum_{j=1}^{n} e_j) \tag{6}$$

Combined with the entropy weight method, the Eq. to calculate the gray clustering coefficient can be rewritten as follows:

$$\sigma_{ik} = \sum_{j=1}^{n} f_{jk}(d_{ij}) w_j \tag{7}$$

where $f_{jk}(d_{ij})$ is the whitening function of the j-th index and belongs to grade k.

III. FLASH FLOOD RISK EVALUATION MODELING FRAMEWORK

Based on abovementioned comprehensive method that includes entropy weighting and an exponential whitening function, the flash flood risk evaluation methods are conducted in the following steps. First, a reasonable evaluation index system is established. Second, the comprehensive weights of different indicators are calculated by the entropy weighting method. Finally, gray clustering analysis is carried out with the improved whitening function to achieve reasonable risk evaluations of the regions that have potential flash flood risks.

In this research, flood risk, hazard and vulnerability are determined by the so-called ‘‘whiten-weight function” and described by the following categories: very low, low, medium, high, and very high. Here, the exponential whitening functions are used, which can be represented by Eq. (2).

Detailed steps are described below.

Step 1: We calculate the improved whitenization weight function $x_{ie}^{(s)}$ for the indexes $x_i(\mathrm{i}=1,2,3,...,n)$ belonging to the gray cluster e $(e=1,2,3,\cdots,k)$. According to Table 1, whitenization weight functions are defined by boundary parameters, as shown in Eq. (2).

Then, let

$$x_i^{(s)}=\sum_{e=1}^{k} x_{ie}^{(s)} \tag{8}$$

For the data s, its evaluation indexes are $x_i(\mathrm{i}=1,2,3,...,n)$, and the gray evaluation coefficient of the data s belonging to the gray clusters e ($e=1,2,\cdots,k$) is recorded as $r_{ie}^{(s)}$. Then,

$$r_{ie}^{(s)}=\frac{x_{ie}^{(s)}}{x_i^{(s)}} \tag{9}$$

The evaluation weight matrix of the data s for different evaluation gray clusters can be determined by combining the gray evaluation weight vectors of all indexes of data s:

$$R^{(s)}=\begin{pmatrix} r_{1,1}^{(s)} & r_{1,2}^{(s)} & \cdots & r_{1,k}^{(s)} \\ r_{2,1}^{(s)} & r_{2,2}^{(s)} & \cdots & r_{2,k}^{(s)} \\ \cdots & \cdots & \cdots & \cdots \\ r_{n,1}^{(s)} & r_{n,2}^{(s)} & \cdots & r_{n,k}^{(s)} \end{pmatrix} \tag{10}$$

Step 2: We calculate the weight vector of the indicators U_R with the information entropy method with Eq. (6).

Step 3: To conduct gray clustering, the comprehensive assessment model of the data s is calculated as $B^{(s)}=U_R\cdot R^{(s)}=(\mathrm{b}_1^{(s)},\mathrm{b}_2^{(s)},...,\mathrm{b}_k^{(s)})$ (11), or Eq.(7), where U_R is the importance weight vector of the indicators. If $b_k=\max\{\mathrm{b}_1^{(s)},\mathrm{b}_2^{(s)},...,\mathrm{b}_k^{(s)}\}$ (12), then the data s belongs to the cluster k, and all data classifications are obtained.

IV. CASE STUDY

4.1 Study area

Hubei Province is located in the middle reaches of the Yangtze River. Hubei Province has a diverse range of land forms including mountains, hills and plains. Mountainous areas account for approximately 55.5% of the total area of the province; hillocks, 24.5%; and the plains and lake areas, 20%. The province covers an area of 185,900 km^{2}and has a population of 58,160 thousand people. The province is surrounded by mountains to the east, west, and north, and the middle area is primarily a flat, incomplete basin with a southern opening. The altitude of this area is generally 20~100 meters. The average precipitation is 800~1,600 millimeters with significant seasonal differences; most precipitation occurs in summer, and less precipitation occurs in winter. In Hubei Province, flash floods were recorded in 15 of the last 100 years. In the 1960s, 1980s and 1990s, this area had several flood currents and debris flows, resulting in heavy casualties and property loss. Mountain flash floods seriously threaten the safety of the people in mountainous areas across Hubei Province.

4.2 Data and indexes

Risk reflects the expected number of deaths and injuries as well as the severity of property damage and economic activity disruption due to a particular natural phenomenon, generally defined as the product of the hazard probability and its consequences. Risk can be viewed as a function of hazard and vulnerability (Maskrey, 1989).

Thus,

RISK=Vulnerability + Hazard (13)

where vulnerability is the probability of any physical, structural or socioeconomic element to a natural hazard being damaged, destroyed or lost. Vulnerability is not static but must be considered as a dynamic process, integrating changes and developments that alter and affect the probability of loss and damage of all the exposed elements.

Hazard is the probability that in a given period in a given area, an extreme potentially damaging natural phenomena occurs that induces air, earth or water movements, which affect a given zone.

Risk is the estimate of the total expected losses for a given area, and specific risk is the expected degree of loss to a given category of elements at risk as a function of hazard and vulnerability. Risk can be related directly to the concept of disaster, given that it includes the total losses and damages that can be suffered after a natural hazard: dead and injured people, damage to property and interruption of activities. Risk implies a future potential condition, a function of the magnitude of the natural hazard and of the vulnerability of all the exposed elements in a determined moment.

From the above risk model in Eq. (13), the factors that affect the flood risk can be classified into the following 2 categories: hazard factors and vulnerability factors.

Although a significant database was generated on hazard and vulnerability parameters of Hubei Province, only 15 of the parameters were used by the statistical correlation test, given that we cannot use two indicators that are strongly correlated in the same model.

A. Hazard factors

Selection of the flood hazard evaluation index is based on the flash flood analysis, considering disaster environment and disaster bearing body, combined with the index characteristics and the existing data. He BY (2002) analyzed 4 factors: precipitation, topography, river network density and historic flood frequency. Tang C and Zhu J (2005) considered 6 main factors: terrain, river network density, rainstorm occurrence, modulus of flood peak, debris flow density and comprehensive disaster degree. Jiang WG (2008) selected 7 factors as the hazard evaluation indexes: maximum 3-day rainfall, rainstorm frequency, vegetation coverage, rivernet density, standard deviation of the elevation per unit area, and old and young populations per unit area.

There are many factors that affect flooding. However, the two main factors are precipitation and underlying surface. The precipitation is determined by meteorological conditions. The precipitation characteristics including total precipitation, rainfall duration and intensity are the major factors causing hazard disaster; underlying factors including location, landform, vegetation, other environmental conditions and human activities are indirect hazard factors (Fan et al., 2008).

The topographic factor is also a parameter or index that is meaningful for the study and expression of geomorphic features. Topography is closely related to the degree of flood risk. The influence of topography on flood formation is mainly manifested in two aspects: relative elevation and terrain slope. Therefore, this study uses relative elevation and slope as topographic indicators to reflect the severity of the mountain floods, and this study uses ArcGIS software to extract the distribution of geomorphic features in Hubei Province from DEM data.

In the present study, based on the obtained data, the following parameters are selected as mountain flash flood hazard assessment indexes: precipitation, critical precipitation, water level, average gradient, relative elevation difference, river network density, and vegetation coverage.

B. Vulnerability factors.

The concept of vulnerability has been continuously widened and broadened towards a more comprehensive approach encompassing susceptibility, exposure, coping capacity and adaptive capacity, such as physical, social, economic, environmental and institutional vulnerability. Although a significant database was generated for the vulnerability parameters, only 8 of them were used.

In this paper, the vulnerability assessment indexes are divided into two categories; one category is related to natural disasters, and the other is related to socio-economic indicators. Disaster-related indicators include the current flood prevention capability, direct economic loss caused by earlier floods, and number of deaths and missing people caused by earlier floods. Socio-economic indicators include the proportion of the agricultural areas, population density, and residential housing property values in flood-controlled areas, total agricultural output and local financial revenue.

After obtaining the hazard and vulnerability, risk assessment is an easy task. According to the risk definition, risk can be determined by the following Eq., which is derived from Eq. (13).

$$R=0.8H+0.2\ V \quad (14)$$

where R is the risk assessment of a mountain flood disaster, H is the hazard assessment of a mountain flood disaster, and V is the vulnerability assessment of a mountain flood disaster.

4.3 Example

Based on this database, we selected 10 towns in Hubei Province to describe the proposed method. First, the weight coefficient of the risk evaluation indexes was obtained by the entropy method with the data of hazard indexes and vulnerability indexes from 10 towns in Hubei Province, as shown in Tables 1 and 2; these towns were selected as the research object of the flash flood disaster regions.

TABLE 1
HAZARD INDEX DATA OF 10 TOWNS

Town	Precipitation (mm)	Critical rainfall (mm)	Water level(m)	Average gradient(°)	Relative height difference(m)	River network density (km/km^2)	Vegetation coverage (%)
Longgang Town	84.66	126.90	1.84	18.18	431	0.88	80.41
Xiangkou County	61.01	72.90	0.88	28.56	610	0.86	95.28
Fengxi County	122.44	75.94	0.41	39.22	1619	0.62	93.31
Leizu Town	204.39	193.60	1.36	32.43	679	0.54	95.22
Wantan Town	105.63	178.10	34.19	20.80	1312	0.58	98.43
Chebu Town	105.88	217.25	-0.29	3.32	33.00	0.51	76.97
Sanli County	84.33	125.75	1.29	19.17	719.00	0.54	95.25
Qingtaiping Town	98.46	124.08	-3.57	23.23	982.00	0.49	95.89
Shadaogou Town	115.84	132.21	-0.05	32.23	1193.00	0.59	94.84
Rongmei Town	136.18	150.00	1.64	30.33	1205.00	0.58	97.39

TABLE 2
VULNERABILITY INDEX DATA OF 10 TOWNS

Town	Current flood prevention capability (year)	Proportion of agricultural area (%)	Population density (people/km2)	Direct economic loss by historical flash floods (10,000 RMB)	Deaths and missing in historical flash floods (people)	Residential housing property in prevention area (10,000 RMB)	Total agricultural output (tons)	Local financial revenue (10,000 RMB)
Longgang Town	1.02	0.18	301.61	120000	7	12.59	31069.86	4890.50
Xiangkou County	2.89	0.02	53.28	39966	11	21.78	14990.80	1374.00
Fengxi County	2.91	0.04	29.48	2000	16	11.24	18062.50	2375.00
Leizu Town	7.15	0.07	70.81	3100	5	28.56	17153.29	16714.29
Wantan Town	3.99	0.09	53.82	2497	45	180.63	17441.71	4157.14
Chebu Town	3.14	0.24	299.34	4950.00	0	11.79	27450.33	17193.50
Sanli County	5.80	0.26	254.61	159.00	2	43.21	38012.70	15926.00
Qingtaiping Town	31.99	0.14	144.64	8000.00	8	62.42	26683.83	4519.92
Shadaogou Town	3.04	0.07	101.33	303.00	0	25.89	24048.00	3232.00
Rongmei Town	16.32	0.08	133.95	112.00	0	40.36	19733.57	30286.29

In the process of gray clustering, the hazard and vulnerability assessments were divided into five classifications of standard risk level: I-grade, II-grade, III-grade, IV-grade and V-grade. These grades represents very high risk, high risk, moderate risk, low risk and very low risk levels, respectively, as shown in Table 3.

TABLE 3
RISK LEVEL STANDARDS

Evaluation indexes		I-grade	II-grade	III-grade	IV-grade	V-grade
Hazard	Precipitation (mm)	<10	10~70	70~00	100~150	>150
	Critical precipitation (mm)	>171.2	149.8~171.2	128.5~149.8	107.1~128.5	<107.1
	Water level (m)	<-1.4	-1.4~-0.5	-0.5~0.1	0.1~1.4	>1.4
	Average gradient (°)	<5	5~10	10~30	30~50	>50
	Relative height difference (m)	<100	100~200	200~500	500~1000	>2000
	River network density (km/km^2)	<0.5	0.5~0.7	0.7~0.9	0.9~2	>2
	Vegetation coverage (%)	>95	80~95	60~80	40~60	<40
Vulnerability	Current flood prevention capability (year)	>100	10~100	5~10	2~5	<2
	Proportion of agricultural area (%)	<0.2	0.2~0.3	0.3~0.5	0.5~0.8	>0.8
	Population density (people/km2)	0~150	150~300	300~420	420~500	>500
	Direct economic loss by historical flash floods (10,000 RMB)	<100	100~200	200~300	300~1000	>1000
	Deaths and missing in historical flash floods (people)	<2	2~5	5~10	10~30	>30
	Residential housing property in prevention area (10,000 RMB)	<10.2	10.2~16.1	16.1~51.2	51.2~80.46	>80.46
	Total agricultural output (tons)	<10000	10000~20000	20000~30000	30000~50000	>50000
	Local financial revenue (10,000 RMB)	<3000	3000~5000	5000~10000	10000~20000	>20000

Based on the categorization above (Table 3), the exponential whitening function for every index was determined. For example, the exponential whitening functions for precipitation belonging to grades I, II, III, IV and V are established as follows:

$$f_1 = \begin{cases} 1 & x \in [0,10] \\ e^{\frac{10-x}{10}} & x \in (10,+\infty) \end{cases} \quad f_2 = \begin{cases} e^{\frac{x-10}{10}} & x \in [0,10] \\ 1 & x \in (10,70] \\ e^{\frac{70-x}{70}} & x \in (70,+\infty) \end{cases} \quad f_3 = \begin{cases} e^{\frac{x-70}{70}} & x \in [0,70] \\ 1 & x \in (70,100] \\ e^{\frac{100-x}{100}} & x \in (100,+\infty) \end{cases} \quad f_4 = \begin{cases} e^{\frac{x-100}{100}} & x \in [0,100] \\ 1 & x \in (100,150] \\ e^{\frac{150-x}{150}} & x \in (150,+\infty) \end{cases}$$

$$f_5 = \begin{cases} 1 & x \in (150,+\infty) \\ \frac{x-125}{125} & x \in [0,150] \end{cases}$$

Based on the Hubei Province data in Tables 1 and 2, flash flood disaster risks in Hubei Province were evaluated according to Eq.s (8)-(12), as shown in Table 4.

TABLE 4
RISK EVALUATION RESULTS

Town	Entropy-improved gray clustering
Longgang Town	III
Xiangkou County	IV
Fengxi County	V
Leizu Town	IV
Wantan Town	II
Chebu Town	I
Sanli County	IV
Qingtaiping Town	III
Shadaogou Town	V
Rongmei Town	IV

V. RESULTS AND DISCUSSION

As an example, we calculated the degrees of risk for 10 towns in Hubei Province to compare the improved gray clustering method with the traditional gray clustering method (Liu, 2010) and fuzzy number-gray clustering method, as shown in Table 5.

TABLE 5
COMPARISON OF THE RESULTS FROM THREE METHODS

Town	Improved gray clustering	Traditional gray clustering	Fuzzynumber-gray clustering
Longgang Town	III	III	III
Xiangkou County	IV	IV	IV
Fengxi County	V	V	V
Leizu Town	IV	V	IV
Wantan Town	II	I	II
Chebu Town	I	I	I
Sanli County	IV	IV	IV
Qingtaiping Town	III	IV	III
Shadaogou Town	V	IV	V
Rongmei Town	IV	IV	IV

Using these three methods, the risk evaluation of mountain flood disaster areas in 10 towns across 10 counties is provided in Table 5. It can be seen from Table 5 that the results of the evaluation of four towns are slightly different by using the traditional gray clustering method, compared to the other two methods. This is because the range of the linear whitening function is too narrow in the traditional gray clustering method, and when the distribution of the monitoring value is discrete, it loses a considerable amount of useful information; for example, this is true for Leizu Town and Wantan Town. For all these towns, the entropy-improved gray clustering method produces risk levels that are more consistent with the actual counts of deaths and missing people as well as the direct economic loss in historical flash floods. It can be seen that the improved gray clustering method makes full use of the limited information available, more effectively reflecting the risk level of the region. The results show that the improved evaluation method produces results that are more objective and accurate.

Additionally, the Fuzzy number-gray clustering analysis and evaluation method is also used to calculate the results. The results of these two methods are analyzed, as shown in Table 5. The results in Table 5 show that the results of the two evaluation methods are both consistent with the actual disaster loss documented. The results show that the calculation results based on the improved gray clustering method can reflect the degree of the mountain flood risk more effectively and have high reliability in the field of flood disaster risk assessment and zonation.

It can be seen from Table 5 that the two methods produce identical risk levels for 10 towns in flash flood regions. The resultant risk levels of all towns basically agree with the actual number of deaths and missing people and the direct economic loss in historical flash floods. Although the number of deaths and missing people in historical flash floods in Shadaogou Town and Rongmei Town is 9, the precipitation, critical precipitation and current flood prevention capacity are weaker than other towns, significantly influencing their risk levels. In other words, the above evaluation results are reasonable and reliable for risk zonation.

In this paper, a grid-based flash flood risk zonation of Hubei Province was accomplished by using hydrometeorology, landform and socioeconomic characteristics data and the spatial data extraction, sampling, interpolation and analysis functions in ArcGIS. The distribution of risk grading was obtained from the risk grades of 1436 towns and counties in Hubei Province. Furthermore, a flash flood risk zonation map of Hubei Province was compiled (Fig. 3)

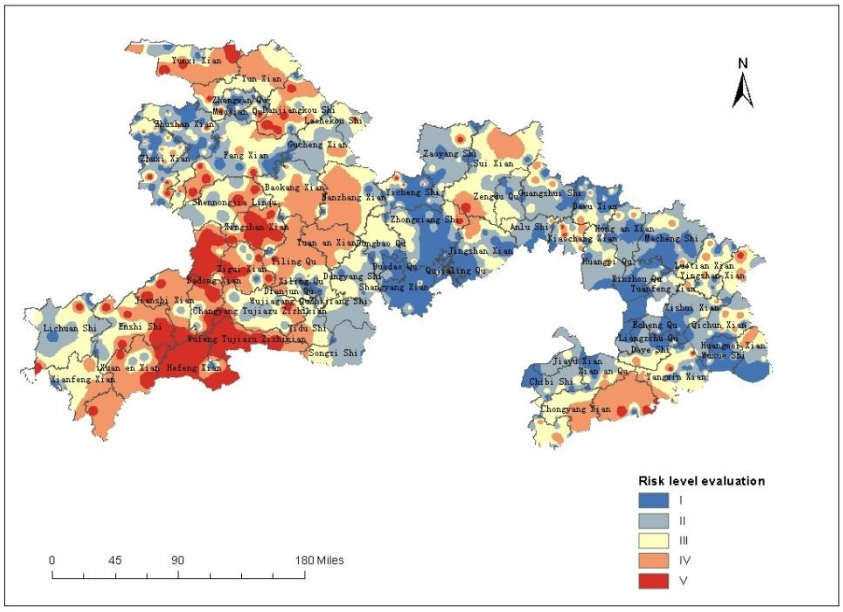

FIG. 3. FLASH FLOOD RISK ZONATION OF HUBEI PROVINCE

Furthermore, with the same method, we produced the maps of hazard and vulnerability across Hubei Province to investigate their spatial distribution, as shown in Fig. 4 and Fig. 5, respectively. The flash flood risk map identified the areas of the highest risk and the areas highly vulnerable to flash flood.

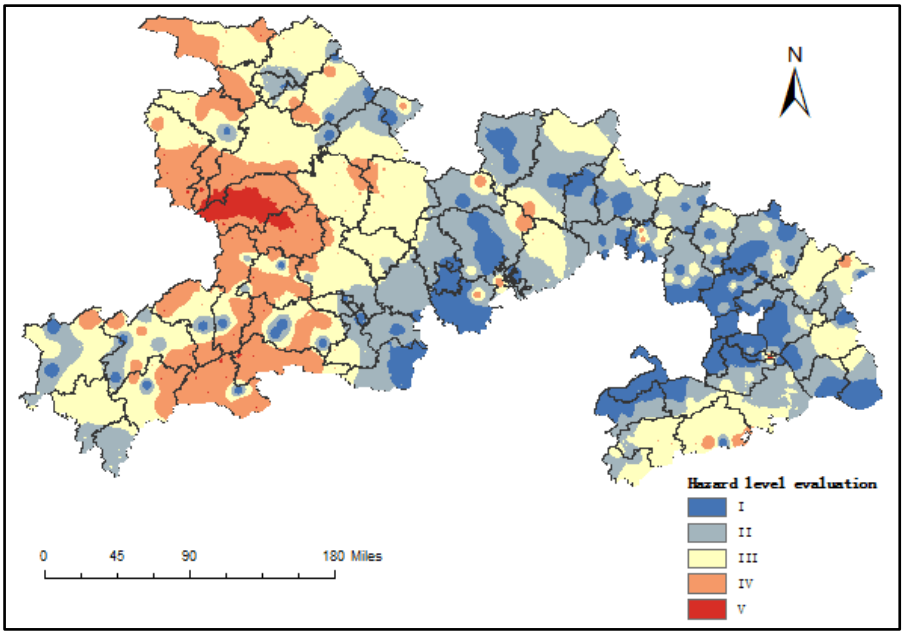

FIG. 4. FLASH FLOOD HAZARD ZONATION OF HUBEI PROVINCE

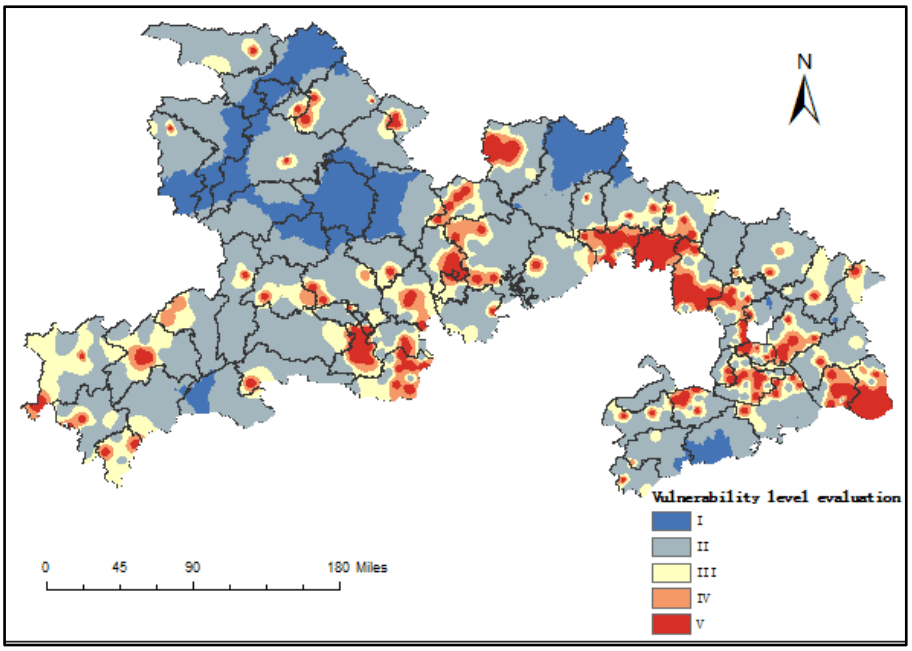

FIG. 5. FLASH FLOOD VULNERABILITY ZONATION OF HUBEI PROVINCE

VI. CONCLUSIONS

In the risk assessment of mountain floods in Hubei Province, the villages and towns are taken as the evaluation units in the study area, and the geometric calculation function is used to calculate the area proportion of different risk grades in ArcGIS, as shown in Table 6.

TABLE 6
AREA PROPORTION OF DIFFERENT RISK GRADES

risk grade	area（km^2）	area proportion（%）
Very low	43880.75	23.23%
low	16089.42	8.52%
medium	60416.60	31.98%
high	36401.06	19.27%
Very high	32123.65	17.00%

Based on the hazard map in Fig. 4, some of the southeastern regions (such as Luotian County, Xishui County, Tuanfeng County, Daye City, Tongcheng County, and Tongshan County), western regions (such as Yunxi County, Yun County, Danjiangkou City, Gucheng County, Enshi and other areas) and northern regions (such as Zaoyang City and Suizhou County) of Hubei Province pose the highest degree of flash flood hazard.

However, based on the hazard map in Fig. 5, some of the southern regions (such as Zhijiang City, Yidu City, Songzi City and Wujiagang District), northern regions (such as Laohekou City and Danjiangkou City) and eastern regions (such as Anlu City, Huangpi District, Xinzhou District, Huarong District and Jingshan County) of Hubei Province are most vulnerable to flash flood.

Based on the risk map that considers both the hazard and the vulnerability, shown in Fig. 3, Zouma Town, Sha Road Town, Five Peak Town, Xintang County, Flower County and 209 other towns and counties are exposed to the highest flash flood risk level in Hubei Province. Among the highest risk areas, Ma Zhen occupies the largest area, 1417.61 km^2, and accounts for 4.5% of the total very high-risk area.

Among 1436 towns and counties in Hubei Province, 196 towns are exposed to the low and very low flash flood risk. Juwan Town has the highest proportion of area in the low and very low flash flood risk. There are 407 towns, such as Tianjia Town, with a moderate flash flood risk, 624 towns with a high flash flood risk, and 209 towns with a very high flash flood risk.

The method presented in this paper improves the traditional gray clustering method with the following changes: constructing an exponential whitening function, effectively overcoming the shortcomings of the traditional gray clustering method that

considers the defects between only adjacent grades in the linear whitening function, broadening the coverage of the whitening function and greatly improving the utilization of the available information. Additionally, the entropy-based method for determining the clustering weight can avoid subjective influences, such as the AHP method, and produce more reasonable and objective weights and evaluations. Lastly, this research is useful for identifying the regions that are threatened by the highest risk and can be easily applied to flash flood zonation for disaster assessments.

In this paper, the issues of the existing gray clustering method are analyzed. To overcome the shortcoming of the existing gray clustering method and the method for determining weights, an improved gray clustering method that includes entropy is proposed. This improved method is used in a case study. The improved gray clustering method makes better use of characteristic information from a research database, and, compared to other methods, the improved gray clustering calculates risk levels of evaluation units more accurately and quickly. The results demonstrate that this method is simple and feasible, and the result is reasonable and accurate. It is reasonable to apply this method to the risk assessment and zonation of mountain flash floods and other disasters.

The results show that the improved approach is useful in rapidly assessing flash flood hazard and vulnerability as well as the risk assessment in mountain areas and could be adopted, with appropriate modification, elsewhere in areas with flash flooding.

Flood disaster risk assessment is very important for disaster prevention, decision-making and management, since reasonable planning and management in flood-prone areas not only reduces the flash floods loss and guarantees the safety of human lives in hilly areas but also provides disaster risk precaution information for local residents and promotes the sustained and stable development of a social economy. Flood disaster risk assessment helps to quickly determine the prevention level and complete reinforcement measured in dangerous areas, to greatly reduce the workload and to improve work efficiency, which is important to promote flash flood relief work.

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Soil Erosion Risk Assessment Using GIS Based USLE Model for Soil and Water Conservation Planning in Somodo Watershed, South West Ethiopia

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Abstract— *Soil erosion is natural phenomena and is modified by biophysical environment comprising soil, climate, terrain, ground cover and their interactions. Due to different factors, it is difficult to make watershed management successful in all areas at one time. Because of this, prioritization of sub watershed is very important for soil conservation planning and implementation. In Somodo watershed more than five years different soil and water conservation technologies were implemented and satisfactory result was not recorded. In this aspect, it is important to consider further watershed management planning., This study therefore investigated soil erosion risk assessment using GIS and USLE model for soil and water conservation in Somodo watershed southwestern Ethiopia with the aim of estimating soil erosion rate and identify soil erosion hot pot areas through prioritization of sub watershed in Somodo watershed by the help of GIS based USLE model. Both primary and secondary data sources were used for model input. These data were computed at a grid level with 30*30m resolution and then overlaid to generate mean annual soil loss by the help of raster calculator in Arc GIS tool. Results of the study showed that, the mean annual soil loss of the watershed was 18.69 ton ha^{-1} year^{-1} ranging from 0 to 131.21. More than 75% of the watershed have soil loss greater than 20 ton ha^{-1} year^{-1} and only 25% of the area have soil loss less than 10 ton ha^{-1} year^{-1}.On the bases of mean annual soil loss SW-4, SW-6 and SW-7 were under slight (0-10 ton ha^{-1} year^{-1}) erosion severity level, while the remaining SW-2, SW-3 and SW-8 were under moderate (10-20 ton ha^{-1} year^{-1}) level. And SW-1 was in high (20-30 ton ha^{-1} year^{-1}) erosion severity level, where as SW-5 and SW-9 were found in very high (>30 ton ha^{-1} year^{-1}) erosion severity level. Since large area of the watershed has soil loss more than tolerable level (11 ton ha^{-1} year^{-1}) attention should be given to identify erosion hot spot areas to minimize the on-site and off-site problems. Therefore, the study suggested that for effective watershed management and soil conservation planning, these sub-watershed priorities should be used in the watershed.*

Keywords— Soil loss, GIS, USLE, Hot spot and prioritization.

I. INTRODUCTION

Soil erosion is a major cause of land degradation that affects the physical and chemical properties of soils and resulting in on-site nutrient loss and off-site sedimentation of hydraulic structures in Ethiopia [1]. Lack of effective watershed management system and poor land use practices played significant role in land degradation in Ethiopian highlands [2]. According to the Ethiopian highland reclamation study [3], in the mid 1980's, 27 million hectare or almost 50% of the high land area was significantly eroded, 14 million hectare seriously eroded and over 2 million hectare were beyond reclamation.

Soil erosion is also a natural phenomena and modified by biophysical environment comprising soil, climate, terrain, ground cover and interactions between them. Important terrain characteristics influencing the mechanism of soil erosion are slope, length, aspect and shape. Impact of slope and aspect would play a major role in runoff mechanism. More the slope, more the runoff and thus infiltration reduces. The runoff generated from slope will find a path nearby and this would lead to erosion of soil as the velocity of the runoff increases [4]

Soil erosion models are useful to estimate soil loss and runoff rates from agricultural land, to plan land use strategies, to provide relative soil loss indices and to guide government policy and strategy on soil and water conservation. The universal soil loss equation (USLE), is one of the most popular empirical models [5] to estimate the long-term average annual rate of soil loss from small field having an average length of 22 m, a field slope of 9% based on rainfall pattern, soil type, topography, cropping system and management practices.

Most of the earlier models, such as the well-known Universal Soil Loss Equation (USLE) [5], were empirically derived. This is relatively simple technique to predict erosion, subsequently led to the application of empirical models in many parts of the world, including Ethiopia. Empirical models could be expected to be used mainly as screening tools in integrated studies,

land resource assessments would demand increased accuracy in quantification of erosion rates in a spatial and temporal context when integrated with Geographical Information System (GIS). They all consider slope steepness, slope length, vegetative cover, rainfall, soil properties and erosion control methods as parameters which influence erosion.

The efficient and optimum management and conservation of soil, land and water resources is best approached on a watershed basis. Normally, the amelioration processes are developed and applied following prioritization and landscape planning. Prioritization plays a key role in identifying areas that require attention [6]. In a watershed management program due to time and financial limitation, it is difficult to make rehabilitation, and soil and water conservation work at one time in all places. Thus it is important to study the watersheds of the area and make ordering by their risk of erosion [7]. Estimation of soil loss and identification of critical area for implementation of best management practice in watershed is central to success of a soil and water conservation program.

In Somodo watershed soil and water conservation measures was implemented to minimize soil erosion without identification of erosion hot spot areas. However, satisfactory result was not observed in the watershed for past five years through implementation of soil and water conservation technologies to the whole watershed at a time. Because of this the study was aimed to estimate erosion rate of the watershed and identify erosion hot spot areas through prioritization of the sub-watersheds for further soil conservation, and watershed management planning by the help of GIS based USLE model in the study area.

II. METHODOLOGY

2.1 Description of the study area

2.1.1 Location

The study was conducted in the upper part of Abay (Nile) river basin at Somodo watershed, Oromia regional state in the South West part of Ethiopia. It is located about about 369 kilometers to the South West of Addis Ababa, Capital City of the country. The watershed covers about 300 ha and found in between $7^0$46'00"-$7^0$47'00"N latitude and $36^0$47'00"-$36^0$48'00"E longitude with the altitude ranging from 1900 to 2075m.a.s.l

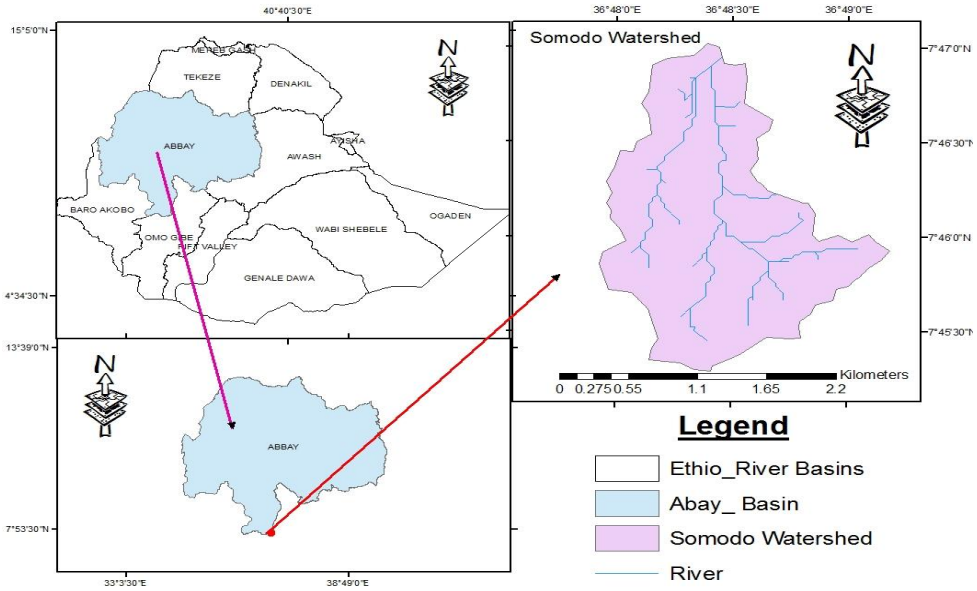

FIGURE 1. MAP OF THE STUDY AREA

2.1.2 Soil and Climate

Somodo watershed is dominated by Humic Nitosols, and percent of organic matter distribution is high at the middle part of the watershed and low near to the outlet of the watershed. The watershed found in between Jimma, Somodo, Agaro and Limu Genet meteorological stations having the mean annual rainfall of 1449.87, 1940.94, 1421.23 and 1460.92 at Jimma, Somodo, Agaro and Limu Genet respectively. The mean annual rainfall of the watershed is then 1523 mm with the mean temperature of 18.9^{o}C ranging from 13.0^{O}C and 24.8^{O}C. Fig.2. below shows that 30 years annual rainfall of all stations around the watershed [8].

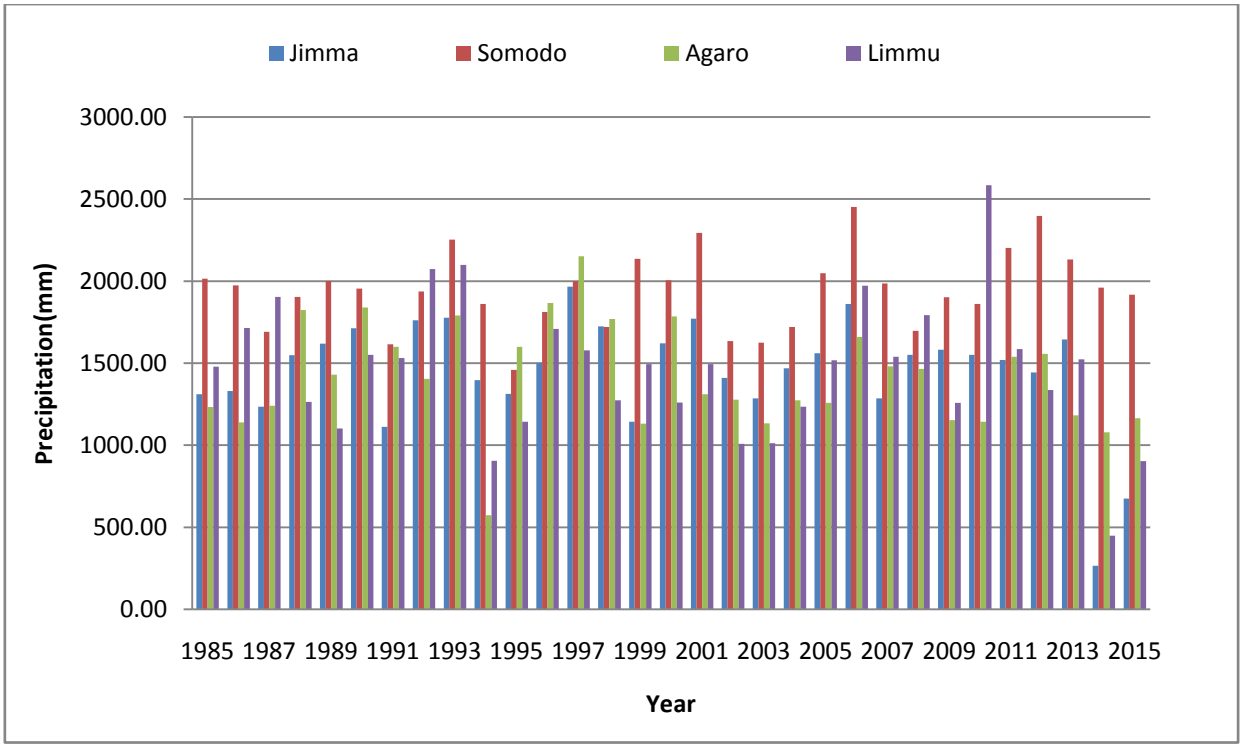

FIGURE 2. RAINFALL DATA OF STATIONS AROUND THE WATERSHED

2.1.3 Land Use of the Watershed

Coffee based farming system and agro forestry is a common practice in the watershed. Cultivation land, Forest, grazing and agro forestry are major land uses in somodo watershed. In case of this study cultivation land is used to represent for areas covered by annual crops while agro forestry stands for areas covered by perennial crops including coffee and home gardens. Agro forestry covers large area of the watershed followed by cultivation land which is about 46.97% and 21.26% respectively. About 18.51% of the watershed is covered by forest land (natural forest and plantation) while grazing land covers only 13.26% of the watershed.

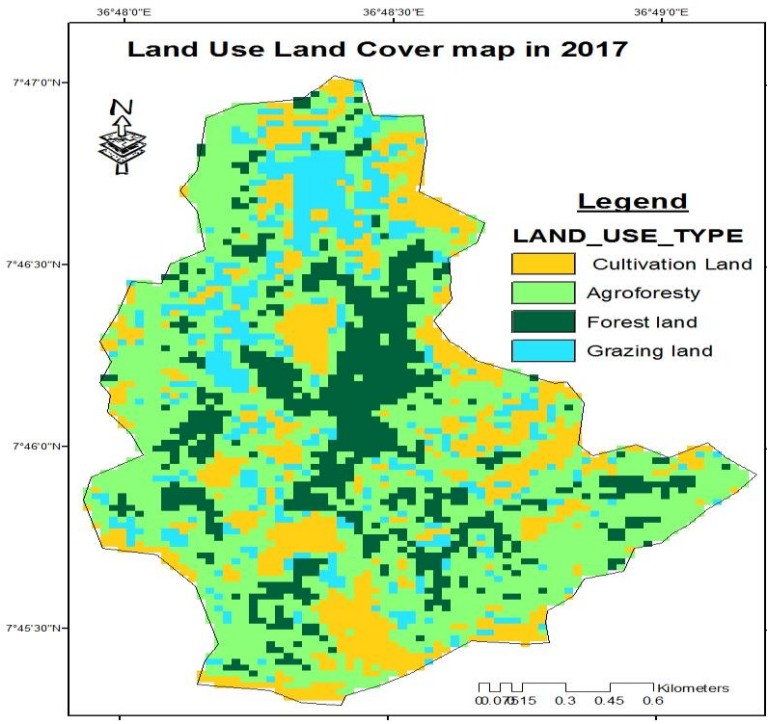

FIGURE 3. LAND USE LAND COVER MAP OF SOMODO WATERSHED IN 2017
(Source: Own data, 2017).

2.2 Determination of USLE Factors

USLE is an empirically based model, which has been developed for both natural and simulated runoff plots. Its simplicity and statistical relationships between input and output variables make it adaptable to other environments [9], [10]. The general equation of USLE is [11]:

$$\mathbf{A = R * K * LS * C * P}$$

Where A is the average soil loss (Mg/ha/year), R is the rainfall erosivity factor (MJ mm/ha/h/year), K is the soil erodibility factor (Mg h/MJ/mm), L is the slope length factor, S is the slope steepness factor C is the cover and management practice factor and P is the support practice factor.

The erosivity factor of rainfall (R) is the product of kinetic energy of the raindrop and the 30-minute maximum rainfall intensity. In Somodo watershed, 30-minute rainfall intensity data was not available and therefore, the erosivity factor R that was adapted by [12] for Ethiopian conditions based on the easily available mean annual rainfall P was used in this study.

$$\mathbf{R= -8.12 + 0.562*P}$$

30 year (1985-2015) rainfall data for four stations (Jimma,Somodo, Limmu Genet and Agaro) around the watershed were taken from National Meteorological Agency and checked for missing data, homogeneity and consistency before using it for further analysis. R-factor value was calculated for each station and spatial distribution of Rainfall runoff factor (R) was interpolated using 'Kriging' method in spatial analysis tool in Arc GIS environment.

The soil erodibility factor is based on the soil texture, structure, organic matter and permeability. Accordingly, soil survey was conducted in the watershed on the bases of slope steepness, slope aspect and soil color, and a total of 106 soil samples were collected randomly and composited into 82 composite soil samples. These soil samples were analyzed for soil textural class (*hydrometer method*) and organic matter (*walkley black method*) in Jimma Agricultural Research Center, Soil and plant tissue analysis laboratory. Therefore using the equation developed by [13] soil erodibility factor (K-value) for each soil sample was calculated and soil erodibility map was generated as a raster data through interpolation by 'Kriging' method.

$$K = [2.1\, M^{1.14} \times 10^{-4}(12 - a) + 3.25(b - 2) + 2.5(c - 3)]/100$$

where, M = particle size parameter; (percent silt + percent very fine sand) (100−percent clay), a = percent organic matter, b = soil structure code used in soil classification; (very fine granular= 1, fine granular= 2, medium or coarse granular =3, blocky, platy or massive= 4) and c = soil permeability class; (rapid= 1, moderate to rapid =2, moderate =3, slow to moderate =4, slow =5, very slow =6). Soil permeability code in relation to textural class and structural code is presented in Appendix Table.1 and 2 respectively.

The *L* and *S* factors represent the effects of slope length (*L*) and slope steepness (*S*) on soil erosion. LS-factor was calculated by Unit Stream Power Erosion and Deposition (USPED) method, which uses the raster calculation between flow accumulation and slope of watershed, [14]. The following equation was used:

LS = Power ("flow accumulation"*[cell resolution]/22.1, 0.4)*Power(Sin("slope in degree"*0.01745))/0.09, 1.4)*1.4.

Landsat image taken in 2017 was pre-processed and classified for land use land cover by the help of both ArcGIS 10.1 and ERDAS IMAGINE 2013 through supervised classification system. The watershed was classified into four major land use classes namely, cultivation land, grazing land, forest land and agro-forestry. C-values given by different scholars for different land use classes given in Appendix Table 3 were used to map and estimate the weighted C-values of the catchment, which was used in the USLE model.

The support practice affects erosion primarily by modifying the flow pattern, grade and direction of surface runoff and by reducing runoff amount and rate [15]. The P-factor values adapted for Ethiopian condition by [16] was used for this study to

determine P-value as described in Appendix Table.4. Based on the estimated P-values given for different land uses support practice factor map was generated by reclassifying land use type map by the help of spatial analysis tools in ArcGIS.

The watersheds was divided into nine sub-watersheds on the bases of hydrological response unit generated and prioritized 1 to 9 on the bases of mean annual soil loss rate of each sub-watershed, and first priority was given for sub-watershed having high mean soil loss rate while last priority was given for sub-watershed with low soil loss rate. Erosion severity class was also done using mean annual soil loss recorded in sub-watersheds. Tolerable soil loss rate is known as 11 ton ha^{-1} $year^{-1}$ [17]. On the bases of this the sub-watersheds of Somodo was categorized under four classes as mean annual soil loss ranging from 0-10 ton ha^{-1} $year^{-1}$ slight, 10-20 ton ha^{-1} $year^{-1}$ moderate, 20-30 ton ha^{-1} $year^{-1}$ high and > 30 ton ha^{-1} $year^{-1}$ very high.

III. RESULT AND DISCUSSION

3.1 Estimated Universal Soil Loss Equation (USLE) Factors

The rainfall erosivity factor (R), soil erodibility factor (K), topographic factor(LS), land cover factor(C) and land management factor (P) were resulted ranging from 990.98 to 1082.1, 0.28 to 0.42, 0 to 121.84, 0.001 to 0.15 and 0.27 to 1, with a mean weighted value of 1042.426, 0.349, 2.173, 0.036 and 0.657 respectively. Spatial distribution map of each parameters were shown in Fig. 4 below.

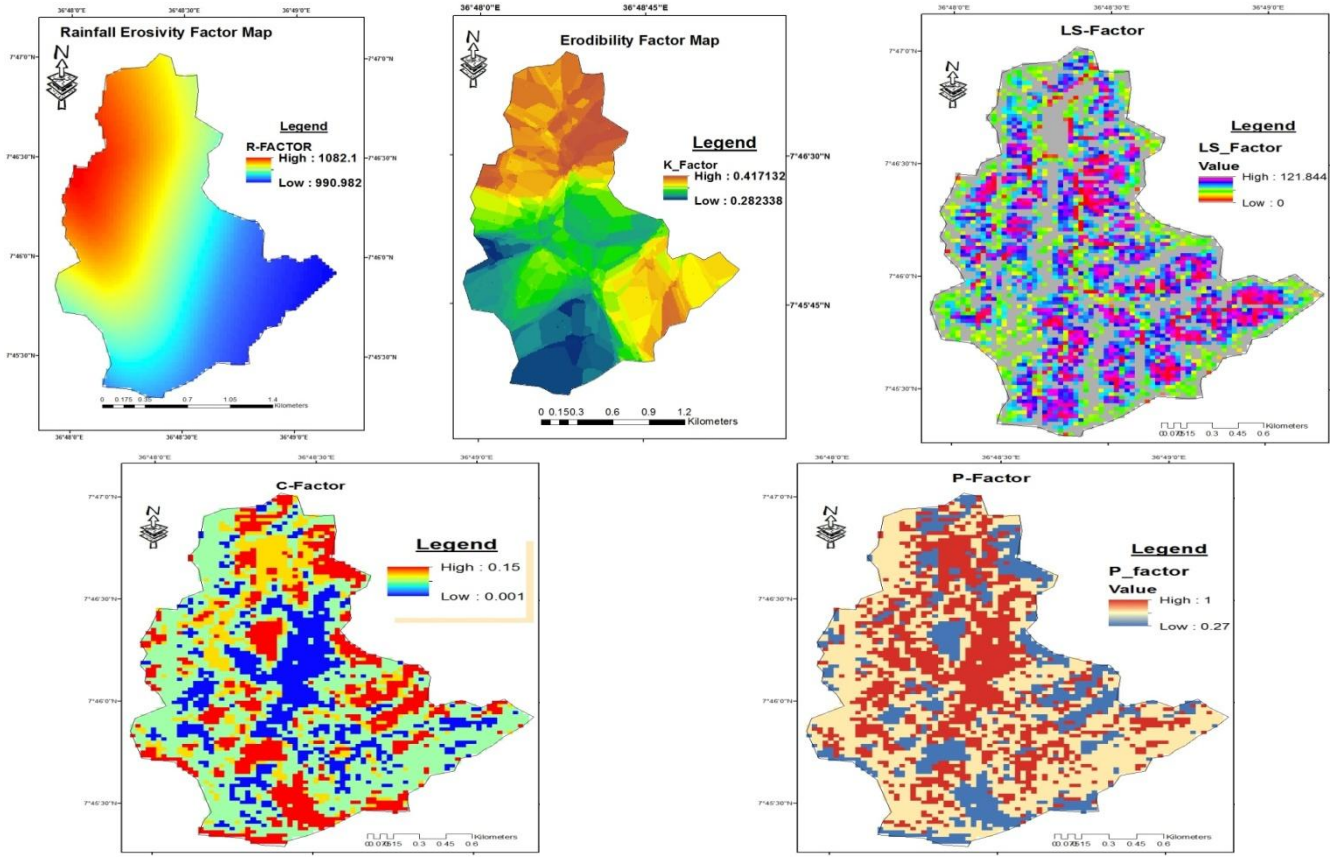

FIGURE 4. SPATIAL DISTRIBUTION OF USLE PARAMETERS

3.2 Estimated Mean Annual Soil Loss

Mean annual soil loss of Somodo watershed was resulted to be 18.699 ton ha^{-1} $year^{-1}$ ranging from 0 to 131.21 with a standard deviation of 51.05. The maximum soil loss was recorded only in one pixel area with a dimension of 30m*30m (0.09 ha). Spatial distribution of the result was shown in Fig.5. below and high soil loss value was recorded at the right side of the watershed specially nearby the outlet and upper part of the watershed at steep slopes. This may be due to high land slope and cultivation land with annual crops was the dominating land use type in these areas.

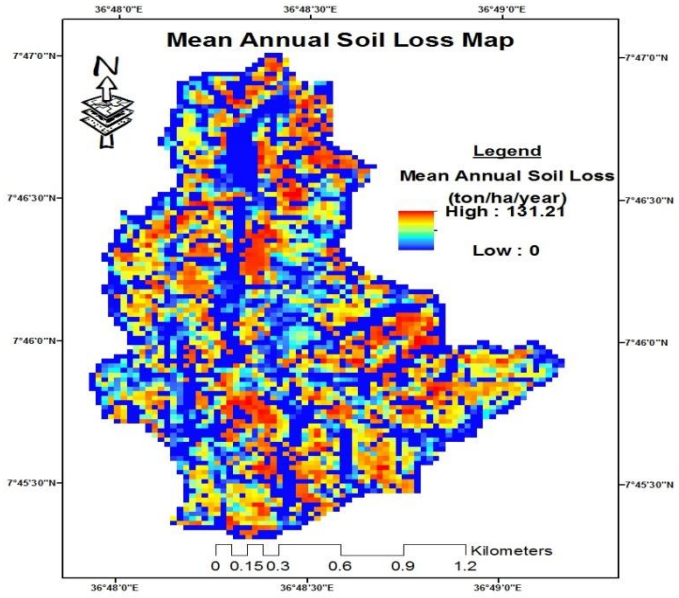

FIGURE 5. SPATIAL DISTRIBUTION OF MEAN ANNUAL SOIL LOSS IN SOMODO

The result of this study is in agreement with the findings of previous studies done in the country and around the study area on erosion rate. [18] found 16-300 ton ha^{-1} $year^{-1}$ mean annual soil loss in Ethiopia and [1] reported 3.4-84.5 ton ha^{-1} $year^{-1}$ mean soil loss in Ethiopian highlands. Similarly, [19] found soil loss rate ranging from 2.6 - 116.94 ton ha^{-1} $year^{-1}$ in Eastern Ethiopia by the help of USLE model.

In Blue Nile basin [20] reported mean annual soil loss ranging from 7 - 243 ton ha^{-1} $year^{-1}$. Since Somodo watershed is also part of upper part of the Blue Nile basin, the result of the study for Somodo watershed which is 18.69 ton ha^{-1} $year^{-1}$ is in between the range. [21] conducted a study in Jimma zone using USLE model and reported mean annual Mana Woreda, where Somodo watershed is located, had low mean soil loss rate when compared to other woredas.

[22] also found soil loss rate of 82.3, 11.4, 4.3, 9.8 and 19.4 ton ha^{-1} $year^{-1}$ from bare land, coffee, Taro, maize and Teff respectively from erosion experimental plot at Jimma Agricultural Research Center. Somodo watershed also mainly covered by coffee and forest, so the finding of this study favors with this finding.

3.3 Prioritization of Sub Watershe

Two sub watersheds (SW-9 and SW-5) were found under very high soil erosion severity level and one sub watershed (SW-1) was found under high soil erosion severity level. Similarly three sub watersheds (SW-2, SW-3 and SW-8) and the other three sub watersheds (SW-4, SW-6 and SW-7) were felt under moderate and slight soil erosion severity level respectively.

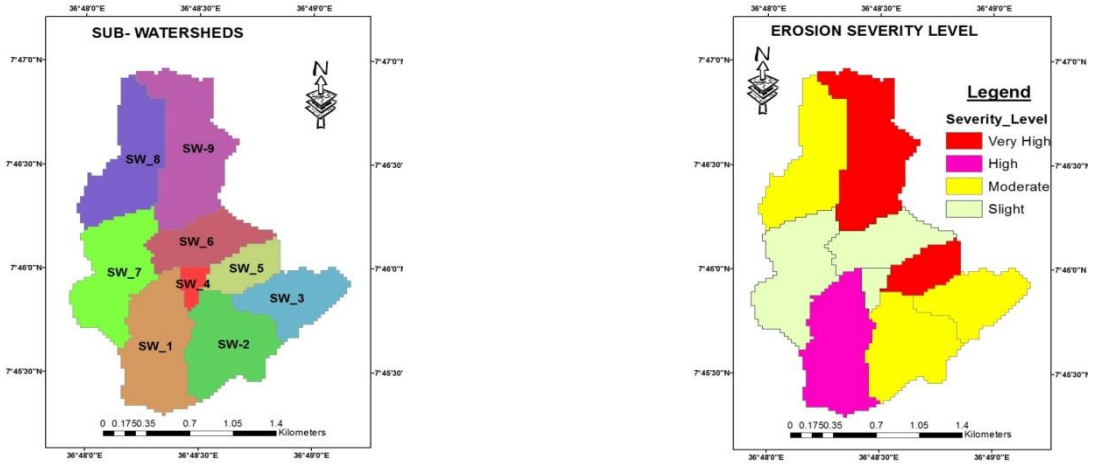

FIGURE 6. SUB-WATERSHEDS AND SOIL EROSION SEVERITY LEVEL

High area of the watershed was covered by moderate (10-20 ton $ha^{-1}year^{-1}$) soil erosion severity level followed by slight (0-10 ton $ha^{-1}year^{-1}$) severity level. Less area of the watershed was covered by high(20-30 ton $ha^{-1}year^{-1}$) erosion severity level. Slight, Moderate, High and Very high soil erosion severity levels covers 24.7%, 36.8%, 15.4% and 23.1% of the watershed respectively.

TABLE 1
AREA COVERAGE, MEAN ANNUAL SOIL LOSS, PRIORITY LEVEL AND EROSION SEVERITY CLASS OF SUB-WATERSHED

Sub Watersheds	Area(%)	Mean Annual Soil Loss	Priority level	Erosion Severity Level
SW_1	15.40	20.10	3	High
SW-2	13.34	17.28	4	Moderate
SW_3	9.19	16.32	5	Moderate
SW_4	1.66	9.07	8	Slight
SW_5	5.28	44.34	1	Very High
SW_6	8.64	7.36	9	Slight
SW_7	14.40	9.61	7	Slight
SW_8	14.28	13.84	6	Moderate
SW-9	17.83	30.26	2	Very High

This result showed that more than 75% of the watershed have soil erosion rate greater than tolerable erosion rate level, 11 ton $ha^{-1}year^{-1}$. This indicates that further soil conservation and watershed management should be planned to overcome the problem. As shown in Table 1. above SW-5, SW-9, SW-1,SW-2, SW-3, SW-8, SW-7, SW-4 and SW-6 were prioritized from 1 to 9 on the bases mean annual soil loss respectively.

IV. CONCLUSION AND RECOMMENDATION

Lack of effective watershed management system and poor land use and land management practices played a significant role in land degradation in Ethiopian highlands. Quantifying the amount of land degradation through soil erosion was difficult at a watershed or basin level for the past many years. Soil erosion models are useful to estimate soil loss and runoff rates at watershed and basin level, to plan land management strategies, to provide relative soil loss indices and guide government policy and strategy on soil and water conservation practices. Estimation of soil loss and identification of critical area for intervention of best management practices in the watershed is central for the success of soil and water conservation program. Somodo watershed also faced similar problems as other watersheds in Nile basin and then this study estimated soil erosion rate of the watershed and prioritized its sub watershed to identify erosion hot spot areas for effective soil and watershed management planning using GIS based USLE model. Accordingly, the watershed mean annual soil loss rate was found 18.69 ton ha^{-1} $year^{-1}$ ranging from negligible value to 131.21 ton ha^{-1} $year^{-1}$ with a standard deviation of 51.05.

SW-9 and SW-5 were felt in very high (>30) soil erosion level and SW-1 was under high (20-30) soil severity level. SW-2, SW-3 and SW-8 were under moderate (10-20), while the remaining SW-4, SW-6 and SW-7 were found under slight (0-10) soil erosion severity level. Therefore, for effective watershed management and soil conservation planning, these sub-watershed priorities should be used in the watershed. Further study, experimental plots or model based, in the study area are also appreciated by this study.

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APPENDIX TABLES

APPENDIX TABLE 1

SOIL PERMEABILITY CLASS AND RATING IN RELATION TO SOIL TEXTURAL CLASS

Textural class	Permeability class	Saturated hydraulic conductivity (mm/hr)	Permeability rating
Clay, Silty clay	6	<1	Very slow
Silty clay loam, sandy caly	5	1-2	Slow
Sandy clay loam, Clay loam	4	2-5	Slow to moderate
Loam, Silty loam, Silt	3	5-20	Moderate
Loamy sand, Sandy loam	2	20-60	Moderate to rapid
Sand	1	>60	Rapid

(Source: Wischmeir, et al.,1971 in Bobe Bedadi, 2004)

APPENDIX TABLE 2

SOIL STRUCTURAL CODE USED TO DETERMINE SOIL ERODIBILITY

Soil Structure	Soil structural code
Very fine granular	1
Fine granular	2
Medium or coarse granular	3
Platy or massive	4

(Source: Wischmeir, et al.,1971 in Bobe Bedadi, 2004)

APPENDIX TABLE 3

LAND COVER CLASSES AND ASSIGNED COVER (C) FACTOR VALUES BY DIFFERENT SCHOLARS

Land cover	C- value	References
Cultivated land	0.15	Hurni (1985); Bewket and Teferi (2009); Tadesse and Abebe (2014)
Forest	0.001	Hurni (1985); Reusing et al. (2000); Morgan (2005)
Open Forest	0.006	Bewket and Teferi (2009)
Grass land	0.01	Hurni (1985); Morgan (2005); Bewket and Teferi (2009); Abate (2011); Tadesse and Abebe (2014)
Built-up areas	0.09	Ganasri and Ramesh (2015)
Water body	0	Hurni (1985)

APPENDIX TABLE 4

COMPUTATION OF THE MANAGEMENT (P) FACTOR VALUE ADAPTED FOR ETHIOPIA BY NYSSEN ET AL., (2009C)

P = PC· PN· PM (on cropland);		***P = PN (on other land)***			
Ploughing and cropping practices	***PC***	***Conservation structures***	***PN***	***In situ conservation practices***	***PM***
Ploughing up and down	1	No conservation structures	1	Stubble grazing; no mulching	1
Ploughing along the contour	0.9	bunds (average condition; smaller value for new bunds and larger for older bunds)	0.3	Applying mulch	0.6
Strip cropping	0.8	Grass strip (1 m wide; slope ≤ 0.1)	0.4	Zero grazing	0.8
Intercropping	0.8	Grass strip (1 m wide; slope > 0.2)	0.8		
Dense inter cropping	0.7				

(Source: Hurni (1985), Nyssen (2001), Gebremichael et al. (2005), Nyssen et al. (2007a, b, 2008b) after Nyssen et al. (2009c)).

Chemical Constituents of Essential Oil and Cytotoxic Activity of Ducrosia asadi Alva. from Iran

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Abstract— *Hydro distillated oil of the aerial parts of Ducrosia assadi Alva. (Umbelliferae) , has been analyzed by GC/MS with two different capillary columns , HP-5MS and HP-Wax .Thirty-four compounds were identified, 94.3% of the total oils. The concentration of citronellol, chrysanthenyl acetate, decanoic acid, decanol and linalool was high in analysis of the oil with both columns . Cytotoxic activity studied on two human cancer cell lines (LS180 and MCF-7) represented moderate cytotoxic activity.*

Keywords— Ducrosia assadi, Umbelliferae, essential oil, citronellol, Chrysanthenyl acetate.

I. INTRODUCTION

The genus Ducrosia is distributed through Egypt to India. Ducrosia assadi Alva., Ducrosia anethifolia Boiss. and Ducrosia flabellifolia Boiss. grow in center parts of Iran and added to different types of food as flavoring species [1-3] .Biological activities such as antimicrobial, antibacterial effects have been reported. Phytochemical studies on essential oil of Ducrosia essential oil showed that aliphaticaldehydes and monoterpene hydrocarbons are the main constituents of these oils [5-8].We evaluated the chemical composition and cytotoxic activity [9-10] of essential oil obtained from the aerial parts of Ducrosia assadi alva.

II. MATERIAL AND METHOD

Plant material (Ducrosia assadi Alva) was collected during the flowering period from Hezar mountain, 1900 m elev. , province of Kerman , Iran ,in July 2014 . A voucher specimen has been deposited in the Herbarium of the department of Botany Faculty of Science Shaheed Beheshty University, Eveen, and Tehran, Iran. The air dried aerial parts of the plant were subjected to hydro distillation for 3 hours in a Clevenger type apparatus to give oil in 0.8% yield. The oils were dried over anhydrous sodium sulfate and stored in sealed vials at the temperature of 4°-6°C in dark for further analysis. The oil was analyzed by GC/MS using a Hewlett-Packard 5973 mass selective connected with a HP6890 Hewlett-Packard gas chromatograph. The separation by two different capillary columns, HP-5MS (5% phenyl methyl siloxane) (30 m x 0.25 mm, film thickness 0.25 μm) and HP-Wax (poly ethylene glycol) (60 m x 0.25 mm, film thickness 0.25 μm). The column temperature was kept at 60^{o}C for 20 min and programmed to 220 ^{o}C at a rate 5 ^{o}C/min, and then kept constant at 220 ^{o}C for 20 min . The flow rate of Helium as carrier gas was (1 ml / min). MS were taken at 70 eV. The identification of the volatile compounds was made by comparing their mass spectra with those given in the literature and those authentic samples. The compounds were identified by comparison of retention indices with those reported in the literature and also by comparison of their mass spectra with the published mass spectra.

Cytotoxicity assay: LS180 (human colon adenocarcinoma) and MCF-7 (human breastadenocarcinoma) cell lines were obtained from the Pasteur Institute, Tehran, Iran. Cell.LS180 and MCF-7 cells were plated at a density of 5 × 104 cells/mL (100μL per well). Control wells contained no essential oil and blank wells contained only growth medium. After incubation at 37°C, three different dilutions of the essential oils were added induplicate. At the end of incubation, the medium was removed and MTT was added to each well and to measure cell metabolic activity. The plates were incubated for 4 h at37°C.

The optical density was measured at 570 nm. The percentage of inhibition compared to control wells was calculated and IC50 values were calculated.

III. CONCLUSION

The yield of the yellow color oil from Ducrosia Asadi was 0.8% (w/w). Chemical composition of the essential oils from Ducrosia asadi are reported in table 1 in order of elution from two different capillary columns, HP-5MS (5% phenyl methyl siloxane) and, HP-Wax (poly ethylene glycol) column.

In GC/MS separation with HP-5MS column more than 90 % (29 components) of the oil, which is particularly rich in monoterpenes, was identificated: 54 % were monoterpenes and 4.74 % was sesquiterpenes. Among the monoterpenes fraction, oxygenated compounds were present in high percentage (47.5 %). Citronellol (38.2 %), Chrysanthenyl acetate (11.01%), decanoic acid (5.36 %), decanol (5.87%) and linalool (4.49 %) were found (table 1) to both major constituents. Similarly with HP-Wax column more than 75 % (28 compounds) of the oil, which is rich regard to monoterpenes, was identified: 41.6 % were monoterpenes and 3.4% ware sesquiterpenes. Among the monoterpenes fraction, oxygenated compounds were in high percentage (35.4 %). The major constituents of the volatile oil were citronellol (23.9 %), Chrysanthenyl acetate (4.34 %), decanoic acid (5.94 %), decanol (6.11 %) and linalool (2.35 %). Several aliphatic aldehydes such as n-decanal and dodecanal have been found in considerable amount in the essential oils.

The cytotoxic activities (table 2) of the essential oil from Ducrosia asadi alva on two different cancer cell lines were reported in table 2. The present study is the first report on the cytotoxic effect of this essential oil. The cytotoxic activity of Ducrosia asadi showed a lower activity on the LS180 in comparison with MCF-7. The cytotoxic activity of this oil may be attributed to the presence of monoterpenoid hydrocarbons.

TABLE 1
COMPARATIVE CHEMICAL COMPOSITION (%) OF THE OIL OF *DUCROSIA ASSADI* ALVA. WITH TWO DIFFERENT GC/MS COLUMNS

NO.	COMPOUNDS	NON-POLAR (%)	POLAR (%)	KI
1	Nonane	0.58	0.36	899
2	α–Pinene	2.25	2.75	939
3	Sabinene	0.83	0.69	976
4	Myrcene	0.78	0.81	991
5	p-Cymene	0.26	0.48	1026
6	Limonene	1.82	1.70	1031
7	cis-Linalool oxide	0.54	-	1074
8	Terpinolene	0.27	-	1088
9	Linalool	4.49	2.35	1098
10	p-Menthon	0.12	-	1098
11	Verbenol	-	0.22	1134
12	Di hydro Carveol	-	1.75	1182
13	Nonanol	1.77	3.01	1182
14	p-Cymene-8-ol	-	0.52	1183
15	Citronellol	38.2	23.98	1210
16	trans -Carvacrol	0.13	0.48	1217
17	Pulegone	0.21	-	1237

18	Ascaroide	2.63	2.61	1251
19	cis-Chrysanthenyl acetate	11.01	4.34	1262
20	Menthol	-	0.19	1163
21	Decanol	5.87	6.11	1266
22	Thymol	-	0.42	1273
23	trans-Pinocarvyl acetate	1.20	-	1297
24	α-Terpinyl acetate	0.67	1.18	1350
25	Piperitone oxide	1.47	-	1363
26	Tetradecane	5.76	6.78	1399
27	Decanoic acid	5.36	5.94	1412
28	δ-Cadinene	0.12	-	1524
29	Liguloxide	0.19	-	1531
30	Caryophyllene oxide	2.16	1.36	1578
31	Dodecanoic acid	1.23	4.03	1580
32	γ-Eudesmol	0.43	0.77	1630
32	α-Eudesmol	-	0.17	1630
33	β-Eudesmol	1.84	1.07	1649
34	Methyl Linoleate	2.10	0.46	2092
	Sum	94.29	75.5	

TABLE 2
CYTOTOXIC ACTIVITY OF ESSENTIAL OILS FROM *DUCROSIA* ASADI

Essential oil	IC_{50} (µg/mL)	
	LS180	MCF-7
D.asadi	187 ± 38	320 ± 88
Cisplatin	3.5 ± 0.8	5.0 ± 1.5

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Effect of salinity on the physiological and biochemical responses of neem

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***Abstract*—** *The present study was conducted to evaluate the plant growth, physiological and biochemical changes of neem under different salinity levels (4, 8, 10 and 12 dS/m) which have multipurpose use in agroforestry. Growth parameters, photosynthetic pigments, carbohydrate, proline and total phenol were investigated 30, 60, 90 and 120 days after treatment imposition. The results revealed that salinity caused significant differences in all the growth parameters and the maximum reduction were observed when plants were exposed to high salinity (12 dS/m) level and minimum were in control treatments. It was found that total dry matter and relative water content were reduced 72% and 40% in 12 dS/m compared to control plants at 120 DATI, respectively. By increasing saliniy 0 to 12 dS/m, the chlorophyll (the photosynthetic pigment) and carbohydrate (the photosynthetic product) content decreased, but increased the level of proline (an amino acid) and total phenol content (an antioxidant) in different days. The highest accumulation of free proline and total phenol content was recorded in 10 dS/m treatment and it was 77% and 59% greater than control plant, respectively. These findings suggest that though growth and biochemical parameters of neem were affected by salt stress, but all the plants survived in different salinity levels. Among all different salinity levels, neem can performed better up to 10 dS/m salinity level could be due to better antioxidant system of neem to cope up with oxidative damage to stressed plants.*

Keywords*— *Chlorophyll, growth, medicinal plant, neem, salinity.

I. INTRODUCTION

Salinity is a wide spread problem across the world and it has been interpreting considerate impacts on crop growth and productivity. The saline land is unfit for crop cultivation and the EC (Electrical Conductivity) value of that saline soil is more than 4 dS/m would adversely affect crop growth and productivity [1]. High content of soluble salt, usually sodium chloride causes high osmotic pressure which results reduction of absorption of water and nutrients that suppress the seedling growth and plant development [2]. These changes are also associated with decrease in chlorophyll and carbohydrate contents in leaves [3]. There is strong evidence that one of the adaptation mechanisms of plants to salinity and water deficit by accumulation of compatible solutes and proline in cytoplasm [4]. Additionally, salinity also induces osmotic and ionic imbalance and toxicity in plants that induces oxidative stress [5], which initiates antioxidant system of the plants to cope up with oxidative damage to stressed plants [6]. Many researchers explored the salt resistant plants and their tolerance mechanism. There have been prompt attempts to generate agricultural crop varieties tolerant to salinity stress. However, very few reports are available on the identification and utilization of perennial tree species, tolerant to salinity stress. Screening salt stress tolerance has been investigated in woody plant species such as olive [7], mango [8], acacia [9] and pine [10].

In Bangladesh, about 650 species have been identified as medicinal plants because of their therapeutic properties [11]. Many government and non-government organizations have had focused attention on improving the medicinal plants sector. But still the medicinal plant cultivation is in a very rudimentary stage. In order to meet the ever increasing demand for medicinal plants, for the indigenous systems of medicine as well as for the pharmaceutical industry, some medicinal plants need to be cultivated commercially. In addition, coastal area of Bangladesh is the potential area for crop cultivation. Coastal area in Bangladesh constitutes 20% of the country of which about 53% are affected by different degrees of salinity. So it seems valuable, to test the important medicinal plants for their salt tolerance capacity. Effect of salt stress has been studied in some medicinal plants such as aloe vera, golden shower, madagaskar periwinkle [12], drumstick [6]. This study is related to the effect of salt stress on our selected medicinal plants, neem.

Neem is an important multipurpose agroforestry species under the family Meliaceae, which is well-known for its medicinal value. The plant is a source of several potent botanical insecticides, soap, lamp oil, lubricants and lumber. It is a good shade tree and reduces soil erosion. Different parts like bark, leaf, seeds, root of neem have very strong medicinal value and the

seeds of this species can also be used as biological control of fungicide and pesticide of agricultural crops. Besides its use in medicine, the neem tree has great importance for its anti-desertification properties and possibly as a good carbon dioxide sinks [13]. Plantations of neem can improve the environmental condition of Bangladesh, if it can be introduced all over the country. Soil of the southern part of the country is saline affected and it is noteworthy that till date there are no reports of salt stress tolerance ability of the important medicinal plants, neem. Therefore, the objectives of the study were to understand the effect of different salinity levels on growth as well as the physiological and biochemical responses of neem.

II. MATERIAL AND METHOD

2.1 Experimental site

A pot experiment was designed at the research field of the Department of Agroforestry and Environment (24^0 09` N; $90^0$ 26` E) for a period of 5 months (April 2017 to October 2017). The minimum and maximum temperatures of the research area were fluctuated between 22 to 33^0C and 15 to 28^0C, respectively, during the experimental period. One-year-old plant was transplanted into each pot (26.5 cm in height and 27.5 cm in diameter). The pots were previously filled with soil mixture that was prepared by mixing oven-dried soil and cow dung (4:1). Each pot contained 12.04 kg of soil mixture and the soil moisture content was approximately 17% at field capacity. One-year-old neem seedlings of uniform size were collected from BRAC nursery, Gazipur, Bangladesh.

2.2 Treatments and design

After transplanting the seedlings into pots, the plants were allowed to grow for 15 days for adaptation prior to being treated with salt stress. The treatments of salinity levels were 4, 8, 10 and 12 dS/m and the control (tap water). The EC of about 4, 8, 10 and 12 dS/m were maintained by adding 4.41g, 8.81g, 11.02g and 13.22g NaCl respectively with mixing 1.7 liter of tap water. The irrigation was supplied on every 3 days interval up to 120 days. The treatments were imposed in 24 April, 2017. At first week 4 dS/m saline treatment was given to all the plants except control. At 2nd week, 8 dS/m saline treatment was given to all the plants except control and 4 dS/m. At 3rd week, 10 dS/m saline was given to the plants of 10 and 12 dS/m treatment. At 4th week, 12 dS/m saline was given only to the plants of 12 dS/m treatment. The experiment was laid out in Randomized Complete Block design (RCBD) with six replications in each treatment, and each replication comprised one plant.

2.3 Data collection

Data of growth contributing characters such as plant height, number of leaves, branches per plant and collar diameter were collected at 15 days interval up to 120 days. Growth parameters namely total fresh and dry matters (stem dry weight + leaf dry weight + root dry weight) of plants were measured after 120 days. All plants were measured for diameter over bark at collar region of above ground level by slide calipers and height was measured by meter scale. Above ground biomass or shoot dry weight was calculated by summing up the dry weights of stem and leaf of plants. Roots were washed thoroughly in tap water and dry before weighted. Soil salinity was measured by the conductivity meter (Model CD-4301). For biochemical analysis leaf samples were collected from plants of different treatment at 30 days interval up to 120 days after treatment imposition (DATI).

2.4 The relative water content (RWC)

Relative water content (RWC) was measured according to [14] and calculated as follows: RWC (%) = $[(FW-DW)/(TW-DW)]\times100$. For the determination of turgid weight (TW), leaf samples were submerged for 24 h in distilled water, then, they were blotted dry on a paper towel and weighed.

2.5 Determination of chlorophyll, proline, carbohydrate and total phenolic content

The levels of chlorophyll (Chl), proline (Pro), carbohydrate and total phenolic content (TPC) were determined following the methods described by [15], [16], [12] and [17], respectively.

2.6 Data analysis

The experiment had a Randomized Complete Block design (RCBD), and the values obtained for each plant and each variable were considered as independent replicates. The means were compared by one way analysis of variance and by using the Least Significance Difference (LSD) test at $P < 0.05$, using the Statistics 10 Software package.

III. RESULTS AND DISCUSSIONS

3.1 Effect of salt stress on plant growth parameters and RWC

3.1.1 Plant height (cm)

The results revealed that salinity had significant negative effects on the growth of neem, and the overall growth performance gradually declined upon increasing the level of salt stress for a period of 120 days (Fig. 1). The plant height of the neem was significantly influenced by different saline treatments at different DATI (Fig. 1, A). At 120 DATI, the maximum plant height (180.5 cm) was noticed in control treatment, which was statistically significant compared to different saline treatments. The tallest plant (180.5 cm) was observed in control treatment, which was statistically differed to all other saline treatments at 120 DATI. Conversely, the smallest plant (119.5cm) was noticed in 12 dS/m saline level, which was significantly differing from other treatments (Fig. 1, A). Though the smallest plant (99.5 cm) was recorded in 12 dS/m, but it was statistically identical to 10 dS/m saline treatment. At 90 DATI, the tallest (152.67 cm) plant was recorded in control treatment, while the lowest plant height (111.83) was found in 12 dS/m, which was statistically dissimilar to other three saline treatments. Relative plant height of neem seedlings gradually decreased over time and it was greater in 60, 90 and 120 DATI than 30 DATI. By increasing salinity level 0 (control) to 12dS/m the value of relative plant height were 100, 91, 78, 74 and 66 at 120 DATI, respectively. The decreasing plant height with increasing salinity was an indication of osmotic stress that was created by the pressure of saline ions. In case of some medicinal plants it has been reported that the affected growth of these plants at seedling stage as a result of salinity stress and this is considered the most essential developmental stage for plants until they establish as fully grown individuals [18]. In the present study salinity affects the plant height due to the occurring of deficit metabolism in plant cells. The negative effect of salinity on plant growth could be explained by two ways. Firstly, the plant water uptake ability reduces by the presence of high salt in the soil solution; this leads to slower growth and other one excessive amount of specific salts entering the transpiration stream which ultimately injure cells in the transpiring leaves, and this may further reduce photosynthesis and growth [19].

3.1.2 Number of leaves

In the present study, number of leaves per plant was statistically similar before treatment imposition (Fig. 1, B). Number of leaves was statistically similar between 4 dS/m and 12 dS/m; it was also similar between 8 dS/m and 10 dS/m treatments at 30 DATI. After 60 DATI, leaves of the salt stressed neem plants showed damaging symptoms such as chlorosis, necrosis, leaf burn etc. Similar observation was reported in Thai neem [20] that its leaves of salt stressed plants expressed damage symptoms such as chlorosis with patches of necrosis, leaf burn, and senescence. At 120 DATI, number of leaves of control, 4 dS/m, 8 dS/m, 10 dS/m and 12 dS/m were 171.17, 113.83, 89.83, 82.17 and 49.50, respectively. It shows that in 12 dS/m number of leaves per plant highly decreased than other saline treatments. The relative number of leaves was 67%, 52%, 48% and 29% in the plants under 4 dS/m, 8 dS/m, 10 dS/m and 12 dS/m treatments at 120 DATI, respectively compared to control plant. Similar reports were found in some other plants such as *Moringa oleifera* [6], *Mentha piperita* var. *officinalis* [21] and milk thistle [22]. The higher accumulation of sodium chloride in the cell walls and cytoplasm of the leaves could be the reason of decrease number of leaves. Concurrently, that leaves vacuole sap may not be accumulating more salt which ultimately decreases the concentration of salt inside the cells that leads to quick death of leaves [1].

3.1.3 Number of branch

Effect of salt stress on number of branches was most prominent among the growth parameters of neem plants. Number of branches of control plant was much higher than stressed plants in all dates of measurements (Fig. 1, C). At 30 and 60 DATI, number of branches varied significantly but variation was little among the plants under different salt stress. Instead of 90 and 120 DATI, number of branches was statistically different in all stressed seedlings. Similar effect on number of branches of seedlings under salt stress was reported in *Chamomilla recutita* [23], *Nigella sativa* [24] and *Withania somnifera* [25]. It was evident that formation of new buds was highly susceptible to increasing salinity and in case of neem this parameter could be a good trait to study the salinity response using other tree species.

3.1.4 Collar diameter (mm)

Collar diameter of neem seedlings was affected by different saline levels over time (Fig. 1, D). In control treatment, collar diameter was increased with time at all the measurement dates. At 30 DATI, collar diameter of the stressed seedlings was similar to each other. However, at 90 and 120 DATI, collar diameter was significantly different in control and all saline treatments. Reduction of collar diameter of neem was 48% in the highest saline treatment (12 dS/m) compared to control at

120 DATI. Affected collar diameter due to salinity is also reported in *Achillea fragratissima* [26] and *Withania somnifera* [25].

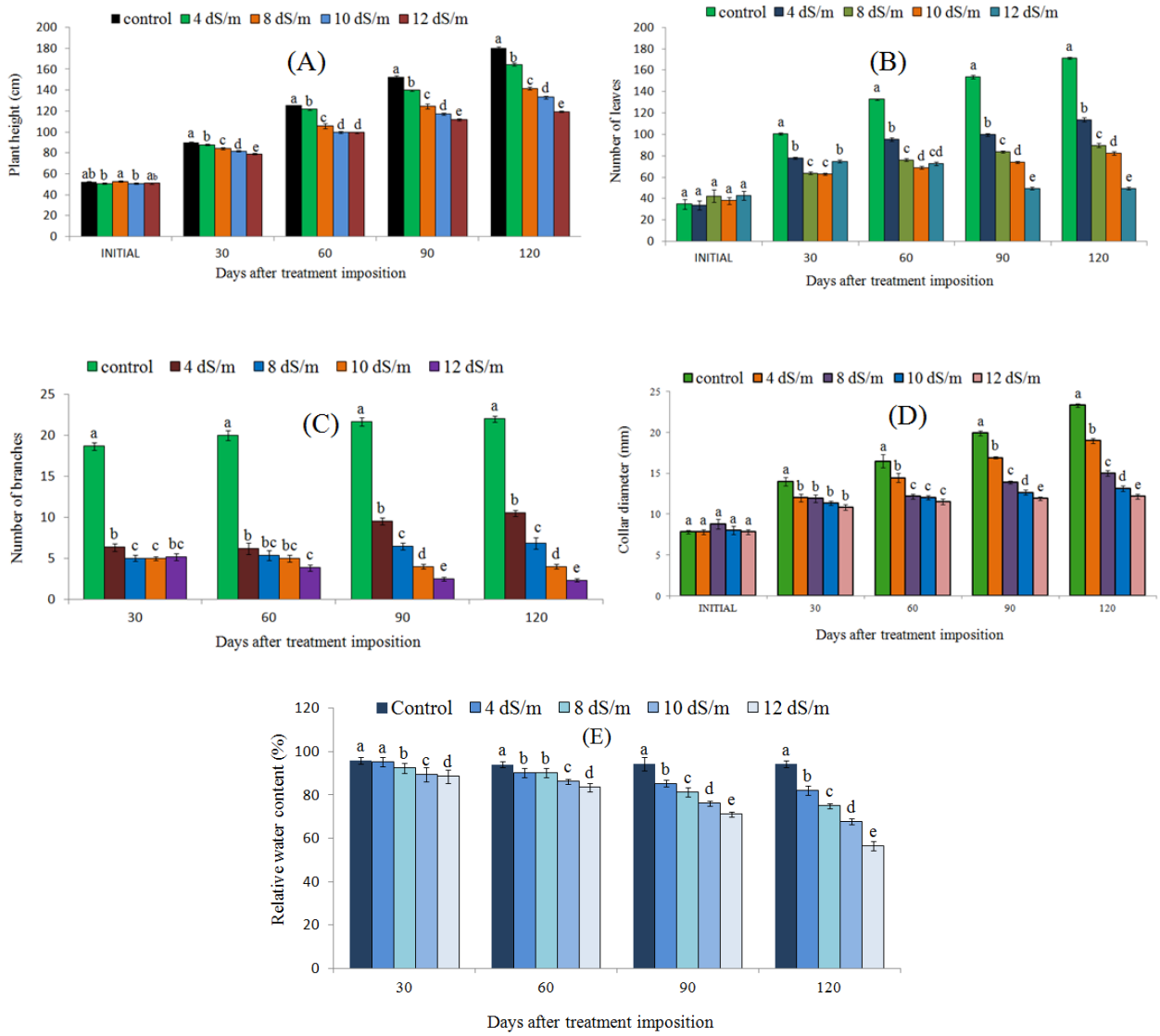

FIGURE 1. EFFECT OF DIFFERENT SALINITY LEVELS ON PLANT HEIGHT (A), NUMBER OF LEAVES (B), NUMBER OF BRANCHES (C), COLLAR DIAMETER (D) AND RELATIVE WATER CONTENT (E) OF NEEM PLANTS AT DIFFERENT DAYS AFTER TREATMENT IMPOSITION (DATI). (DATA REPRESENTS MEANS ± SE OF 6 INDEPENDENT REPLICATES AND MEANS FOLLOWED BY UNCOMMON LETTER(S) DIFFER SIGNIFICANTLY BY LSD AT 5% LEVEL.)

3.1.5 Fresh and dry weight (g)

Leaf, stem, root and total fresh weights (TFW) of neem plants were highly affected by saline treatments (Table 1). Total fresh weight was the highest (577.42 g) in control plant and the lowest (171.30 g) was noted in 12 dS/m saline level at 120 DATI. Total fresh weight of neem was reduced 70 % under 12 dS/m saline treatment as compared to control plants. Dry weights of leaf, stem and root of neem plants were also significantly diminished compared to control plant at 120 DATI (Table 2). In all cases like leaf, root, stem and total dry matter (TDM), the maximum dry weight was observed in control plant and the lowest was recorded in 12 dS/m salinity level at 120 DATI. Relative TDM of 4 dS/m, 8 dS/m, 10 dS/m and 12 dS/m was 45%, 36%, 31% and 27%, respectively. These results agreed with the findings of [27] that leaf, stem and root dry weight of *Satureja hortensis* were decreased compared to control plants in different levels of salinity. It can be explained that significant decline of total dry matter due to reducing photosynthesis, slow or less mobilization of reserve foods and suspending the cell division in plants [28].

TABLE 1
EFFECT OF DIFFERENT SALINITY LEVELS ON FRESH WEIGHT (G) OF NEEM SEEDLINGS AT 120 DAYS AFTER TREATMENT IMPOSITION

Treatment	Leaf	Stem	Root	TFW
Control	166.90 a	290.29 a	120.23 a	577.42 a
4 dS/m	110.02 b	131.71 b	64.48 b	306.21 b
8 dS/m	64.40 c	102.51 c	57.32 c	224.23 c
10 dS/m	63.98 c	87.73 d	45.33 d	197.05 d
12 dS/m	50.95 d	76.13 e	44.22 d	171.30 e
LSD(0.05)	3.9685	3.2405	2.4977	8.9432

TABLE 2
EFFECT OF DIFFERENT SALINITY LEVELS ON DRY WEIGHT (G) OF NEEM SEEDLINGS AT 120 DAYS AFTER TREATMENT IMPOSITION

Treatment	Leaf	Stem	Root	TDM
Control	45.80 a	138.53 a	45.37 a	229.70a
4 dS/m	24.60 b	57.40 b	21.67 b	103.67 b
8 dS/m	18.15 c	43.36 c	20.30 c	81.81 c
10 dS/m	17.10 c	38.32 d	15.95 d	71.37 d
12 dS/m	14.07 d	33.93 e	15.15 d	63.15e
LSD(0.05)	1.8864	1.3591	1.0691	2.4520

3.1.6 Relative water content (RWC)

The relative water content (RWC) was measured in order to evaluate the effects of salinity on the water status of neem at different DATI. At 30 and 60 DATI, a little variation was found in relative water content among stressed plants (Fig. 1, E). But at 60, 90 and 120 days after treatment imposition, the relative water content of the plant leaves under salt stress decreased significantly to each other. At 120 DATI, relative water content of control, 4 dS/m, 8 dS/m, 10 dS/m and 12 dS/m were 94.29%, 81.99%, 75.03%, 67.83% and 56.71 %, respectively. Islam, (2013) [29] observed similar results for mahagony and eucalyptus. Plant tends to cope with salt stress conditions by decreasing tissue water content (measured as RWC) which may be caused by low leaf water potential [30]. In this study, may be the decreased relative water content of neem leaf was caused by decreasing of leaf water potential. The decreasing relative water content of leaves indicate the less capacity to uptake water.

3.2 Effect of salt stress on chlorophyll and carbohydrate content

3.2.1 Chlorophyll content

Chl a, b and total Chl in leaves of neem were affected significantly under salt stress (Fig. 2). At 120 DATI, Chl a, b and total Chl in all salt stressed plants were highly decreased as compared to control plants (Fig. 2, A, B, C). However, it is also noticeable that, Chl a was more affected by salinity than Chl b. In accordance with other reports, our results also implied that Chl a is appears to be more sensitive to salinity than Chl. 'b' [6]. But Chl a is mainly responsible for photochemical phase of photosynthesis process and vital part of the light-harvesting compound, whereas Chl b as an accessory pigment acts indirectly in photosynthesis [31]. In general, decrease of these pigments under salt stress is considered to be a result of accelerated degradation and the inhibited synthesis and/or fast plastid breakdown of that pigment [32]. Rapid maturing of leaves is stated to be another reason for the decrease of Chl content under salinity [33]. So, reduction of Chl a and total Chl content in neem leaves may be one of the causes of less photosynthetic product and low biomass production of stressed neem seedlings.

3.2.2 Carbohydrate content

Data depicted in Fig. 2D, significant decrease in carbohydrate content with increasing salinity levels in all measuring dates. At 30 DATI, carbohydrate content of neem leaves were similar in all saline treatments, but it varied significantly in control. At 60 DATI, carbohydrate content was statistically similar in 4 dS/m, 8 dS/m and 10 dS/m saline treatment. But at 90 and 120 DATI, carbohydrate content slightly varied under 4 dS/, 8 dS/m and 10 dS/m (Fig. 2, D) saline treatment. According to [34] explained that increasing salinity decrease the carbohydrate content in *Foeniculum vulgare* , this can be attributed to the

reduced Chl content, nutritional imbalance due to the specific toxic effects of salinity, hyperosmotic stress and reduced photosynthesis. In this study, though reduced Chl content caused decreases in carbohydrate content but ultimately the decreasing carbohydrate content may had possitive effects in tolerance mechanism against salt stress.

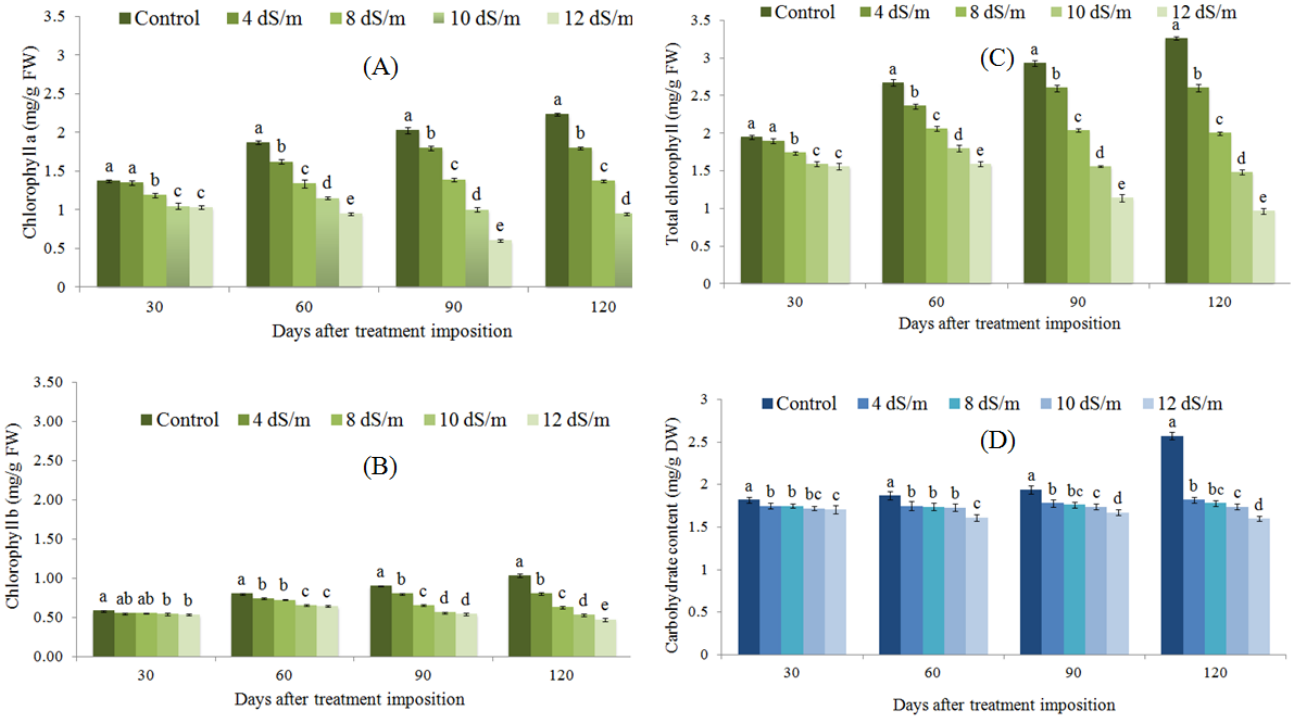

FIGURE 2. EFFECT OF DIFFERENT SALINITY LEVELS ON CHLOROPHYLL A (A), CHLOROPHYLL B (B), TOTAL CHLOROPHYLL (C) AND CARBOHYDRATE CONTENT (D) OF NEEM PLANTS AT DIFFERENT DAYS AFTER TREATMENT IMPOSITION (DATI). (DATA REPRESENTS MEANS ± SE OF 6 INDEPENDENT REPLICATES AND MEANS FOLLOWED BY UNCOMMON LETTER(S) DIFFER SIGNIFICANTLY BY LSD AT 5% LEVEL.)

3.3 Effect of salt stress on proline and total phenol content

3.3.1 Proline content

The Pro content was considerably increased in neem in response to salt stress with time. At 30 and 60 DATI, the highest Pro accumulation (10.545 and 22.29 µmol/g respectively) was found in 12 dS/m saline treatment (Fig. 3, A). But at 90 and 120 DATI, the highest Pro content (23.775 and 25.965 µmol/g respectively) was observed in seedlings under 10 dS/m salinity level. Accumulation of Pro is probably one of the most frequently reported modifications induced by salinity and water deficit in plants. In the present study, Pro content significantly increased with the increase of salinity, which agrees with previous results obtained for several medicinal plants e.g. Chamomile [35], Fennel [26] and sage [36]. The increased Pro may lead to a reduction in stress induced cellular acidification and may also act as a hydroxyl radical and singlet oxygen scavenger. Additionally, the accumulation of high Pro concentrations in the cytoplasm under stress conditions without interrupting cell structure and metabolism may be due to its zwitterion nature [37]; it is thought to be involved in osmotic adjustment of stressed tissues. This may assist plants in their adaptation to salinity stress. It has also been reported that hyperaccumulation of Pro is one of the positive indicators for the salinity resistance of plants; whereas other researchers affirm that it appeared to be a symptom of salt stress [38].

3.3.2 Total Phenol content

Accumulation of total phenol content increased with increasing salinity levels in leaves of neem plants (Fig. 3, B). At 30 and 60 DATI, the highest phenol content (4747.2 and 5678.4 mg/ 100 g dry weight, respectively) was found under 12 dS/m saline treatment, which was statistically different to other stressed plants. But at 90 and 120 DATI, the highest phenol content (23.775 and 25.965 µmol/g respectively) was observed under 10 dS/m salinity level. Similar observation was found in *Moringa oleifera* [6] and *Nigella sativa* [25]. Phenolic compounds are very important plant constituents because of their scavenging ability due to their hydroxyl groups. These compounds are also powerful chain breaking antioxidants and play a vital role in the defense against reactive oxygen species (ROS) [39]. In this study, may be the increased levels of phenols at

elevated levels of salinity induced accumulation of secondary metabolites to tolerate higher levels of salinity stress and aroused adverse conditions.

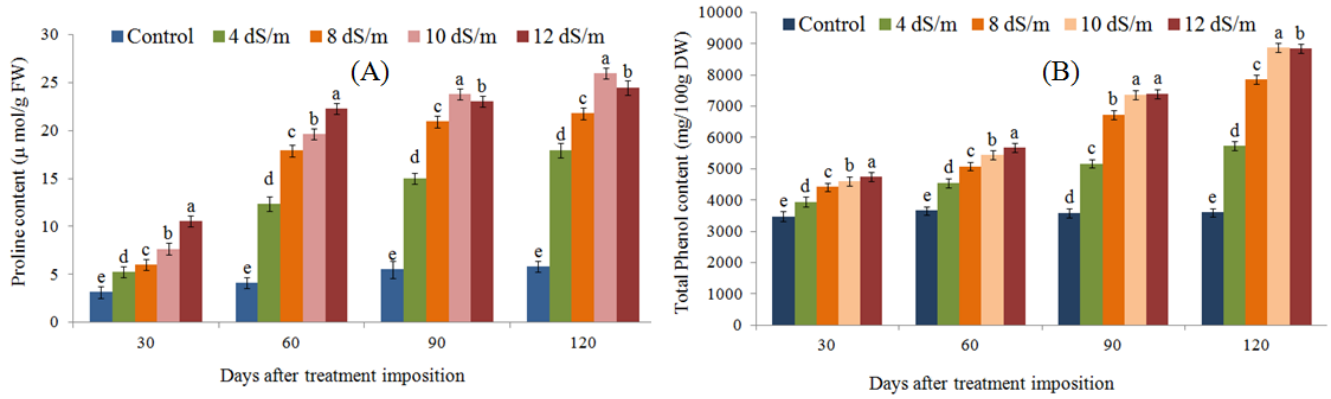

FIGURE 3. EFFECT OF DIFFERENT SALINITY LEVELS ON PROLINE (A) AND TOTAL PHENOL CONTENT (B) OF NEEM PLANTS AT DIFFERENT DAYS AFTER TREATMENT IMPOSITION (DATI). (DATA REPRESENTS MEANS ± SE OF 6 INDEPENDENT REPLICATES AND MEANS FOLLOWED BY UNCOMMON LETTER(S) DIFFER SIGNIFICANTLY BY LSD AT 5% LEVEL.)

IV. CONCLUSION

The results of this study showed that salinity stress had significant effect on growth and photosynthetic pigments of neem plants. However, salinity decreased the amount of Chl a, b, and carbohydrate contents in neem plants. During salinity stress, increasing the accumulation of free Pro and total phenol content in leaf, sustained the plants to better growth and survival under salt stress. The findings of this study show valuable information regarding plant growth and physiological performance of important medicinal tree species in different saline treatments, which may be useful to introduce neem plantation in the saline affected areas. However, based on the findings of the study it can be advocated that on-farm investigation should be conducted in real field conditions of saline prone area to confirm the performance of neem.

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Impact of Biofertilizers on Crop Seeds

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Abstract— *A laboratory experiments were conducted during the year of 2012-2014 at Annamalai University of cuddalore district under irrigated conditions to formulate site –specific nutrient management and efficiency of treatments known by comparing with rice, sunflower and black gram seeds. And the treatments includes control, 10%, 5%, 2.5% and 1% vermicompost, 10%, 5%, 2.5% and 1% phosphobacteria, 10%, 5%, 2.5% and 1% azospirillum. And the above treatments are applied to all the three seeds ie, rice, sunflower, black gram. The numbers of seeds used for the experiment are 50 seeds of paddy, 20 seeds of black gram and 10 seeds of sunflower. Highest values for plant biomass root and shoot length was noticed in 10 % all the treatments.*

Keywords— Sunflower, Nutrient management, plant biomass, root and shoot length.

I. INTRODUCTION

Nutrients play an important role in crop growth and development. Among the nutrients, N is one of the major nutrients that enhance the metabolic processes that based on protein, leads to increases in vegetative, reproductive growth and yield of the crop. Phosoporus soulubilizing bacteria possess the ability to bring insoluble phosphorus in the soil into soluble forms by secreting organic acids such as formic, acetic, propionic, glycolic, fumaric and succinic acids. And vermicompost is an established organic soil amendment produced by a non-thermophilic process in which the organic matter is broken down through interactions between earth worm and microorganisms under aerobic condition. vermicompost have been demonstrated to be valuable soil amendment that offer a balanced nutritional release pattern to plants. Providing nutrients such as available nitrogen, soluble potassium, exchangeable calicium, mangesium and phosphorus that can be taken readily by the plants (Edward, 1998 and Edwards and Fletcher, 1988). Application of vermicompost along with chemical fertilizers increase the uptake of N, P, K nutrients when compared to chemical fertilizers alone (Bhadoria and Prakash, 2003).Chaudhary *et al*.(2004) reported that apart from nutrients supply and availability, vermicompost also improves the fertilizer use efficiency by increasing the nutrient uptake of plants. Vermicomposting is a biodegradation system which stabilizes sludge and reduces its pathogenicity. Application of high analysis NPK fertilizers and very limited use of FYM cause micronutrient depletion in soils, which appears to have special role in influencing the test weight and seed filling (Tufail et al., 1990). Azospirillum reported that *Azospirillum* inoculation of increased the nitrogen uptake by sunflower (Anand, 1994). Sivakumar (1994) also stated that seed soaking in GA_3 45 ppm + *Azospirillum* increased the nitrogen uptake by sunflower. Nandhagopal *et al*. (2003) reported that inoculation of *Azospirillum* assisted in N fixation and contributed for the improved nutrient uptake of sunflower.

II. MATERIALS AND METHODS

A laboratory experiments were conducted during 2013-2014 at Annamalai university, Cuddalore located in Western Agro climatic zone of Tamilnadu to find out the impact of bio fertilizers on different crop seeds. The weather of Annamalai nagar is moderately warm with hot summer months. The mean maximum temperature is 32.2C while the mean minimum temperature is 21.5 ^{o}C with a mean relative humidity of 88 per cent. The mean annual rainfall is 1500mm of which 1000mm is received during North –East monsoon, 400 mm during South-West monsoon and 100mm as summer showers.

A laboratory experiment was under taken with varying concentrations of aqueous solutions of biofertilizers i.e., 10%, 5%, 2.5%, 1% solutions of vermicompost, *azospirillum* and phosphobacteria respectively were prepared. Seeds of paddy, blackgram and sunflower were placed in filter paper, spread in petriplates @ 50 paddy seeds, 20 black gram seeds and 10 sunflower seeds per plate over a moist filter paper dipped in water held in the cover plates kept at the bottom. Observations regarding germination count (cumulative upto 3 DAS) on 7th day were recorded and inhibition or stimulation of germination compared to control was expressed in percentage (%).

TABLE 1

IMPACT OF BIO-FERTILIZERS AT VARYING CONCENTRATIONS ON THE GERMINATION OF CROP SEEDS

FERTILIZER	CONC	PERCENTAGE OF SPROUTED SEEDS OUT OF SOWN SEEDS			SHOOT AND ROOT LENGTH AT TWO LEAF STAGE						DRY MATTER PRODUCTION (gm)		
		Paddy (50 seeds)	Black gram (20 seeds)	Sunflower (10 seeds)	Paddy (cm)		Black gram (cm)		Sunflower (cm)		Paddy (gm)	Black gram (gm)	Sunflower (gm)
					Shoot	Root	Shoot	Root	Shoot	Root			
Azospirillum	10%	100%	100%	100%	6.5	5.5	16	4.8	12	4.5	0.039	0.51	0.19
	5%	100%	95%	96%	5	4.5	15.7	4.7	11.9	3.5	0.038	0.49	0.14
	2.5%	98%	95%	70%	4.5	2.5	11	6.2	11	3.2	0.034	0.032	0.1
	1%	92%	85%	60%	4	2.3	9	4.2	9	2.5	0.02	0.01	0.1
	SEd	0.83	2.0	1.6	0.62	0.41	0.12	0.04	0.04	0.41	0.001	0.008	0.02
	CD=(p0.05)	2	5	4	1.5	1	0.3	0.1	0.1	1	0.01	0.02	0.05
Phosphobacteria	10%	100%	100%	80%	6.9	5.3	15	5	12	3.5	0.046	0.055	0.16
	5%	98%	96%	77%	6	5.2	14.5	4.5	11.8	2.5	0.028	0.053	0.14
	2.5%	96%	85%	70%	5	4.3	11.2	3	8	5.0	0.01	0.055	0.12
	1%	96%	80%	60%	4.5	4.2	6.5	2.3	6.7	2	0.02	0.035	0.09
	SEd	0.83	1.6	1.25	0.37	0.04	0.20	0.20	0.08	0.41	0.007	0.0008	0.0008
	CD=(p0.05)	2	4	3	0.9	0.1	0.5	0.5	0.2	1	0.01	0.002	0.02
Vermicompost	10%	96%	95%	82%	6	7.5	16	6	10	5.5	0.05	0.125	0.37
	5%	96%	94%	80%	5.5	6.3	13	5.5	9	4.9	0.038	0.06	0.14
	2.5%	94%	90%	70%	5.3	4.1	8	4.4	8.5	3.5	0.06	0.045	0.12
	1%	94%	85%	60%	4.5	4.0	7	2	8	3	0.02	0.04	0.37
	SEd	0.83	0.41	0.83	0.20	0.5	1.25	0.20	0.41	0.25	0.005	0.02	0.009
	CD=(p0.05)	2	1	2	0.5	1.2	3	0.5	1	0.6	0.012	0.06	0.23

III. RESULTS AND DISCUSSION

The different concentrations of bio-fertilizers compared showed varying levels of germination response of crop seeds. The magnitude of germination was increasing with increasing concentrations of bio-fertilizers and highest germination per cent was observed with increasing concentrations of bio-fertilizers i.e, 1 per cent, 2.5 per cent, 5 per cent, and 10 per cent. Hence highest performance of paddy, black gram & sunflower germination was observed to be as 100 per cent, 100 per cent and 100 per cent respectively in 10 per cent concentration of *Azospirillum* followed by 100 per cent, 100 per cent and 80 per cent with 10 per cent concentration of PSB and 96 per cent, 95 per cent, 82 per cent with 10 per cent concentration of vermicompost.

IV. CONCLUSION

The study revealed that the different concentrations of bio fertilizers compared showed varying levels of germination response of crop seeds. The magnitude of germination was increasing with increasing concentrations of bio fertilizer.

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Chloroplasts and Mitochondria: Similarities and Differences

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Abstract— *Eukaryotic cells contain two organelles originally derived from endosymbiotic bacteria: mitochondria and plastids (only plants). In eukaryotes, (owner mitochondria and chloroplast) ATP synthase complex is located in the inner membrane of mitochondria, and thylakoids membrane of chloroplast. ATP synthesis utilization and provision of both ADP and Pi need to be fine – tuned for optimal ATP synthase activity. Mitochondria and chloroplast have their DNA. The vast majority of mitochondrial and plastid proteins are encoded in the nucleus, synthesized by cytosolic ribosomes and subsequently imported into the organelles via active protein transport systems.*

Keywords— ATP synthesis, Chlororplast, Mitochondria, Protein targeting.

I. INTRODUCTION

Several proposals have been made to explain the rise of multicellular life forms. An internal environment can be created and controlled, germ cells can be protected in novel structures, and increased organismal size allows a ‘‘division of labor’’ among cell types. These proposals describe advantages of multicellular versus unicellular organisms at levels of organization at or above the individual cell. It have been focused on a subsequent phase of evolution, when multicellular organisms initiated the process of development that later became the more complex embryonic development found in animals and plants. The advantage here is realized at the level of the mitochondria and chloroplast [20].

Eukaryotic cells have chloroplast and mitochondria that both are membrane bound organelles. Prokaryotic cells, for example, bacteria have not chloroplast and mitochondria. Mitochondria occur in the cells of animals and plants but chloroplast only occur in the photosynthesising tissues of plants. These two organelles are best known for their roles in energy metabolism, notably respiration and photosynthesis [85].

Respiration occurs in mitochondria. Mitochondria were originally identified as the site of oxidative energy metabolism [13]. Mitochondria are also the host for enzymes of the Krebs cycle and β – oxidation of fatty acids. In today’s world mitochondria are known not only as the “power station” of the cell, but also for playing a vital role in the transmission of extra – and intracellular signals that activate reaction cascades leading to cellular senescence and programmed cell death (PCD) [104]. The discovery of a number of human diseases associated with mitochondrial dysfunctions once again brought mitochondria into the spotlight of biological research.

Chloroplasts are members of a class of plant cell organelles known as plastids that all originate from protoplastids. During plant development the protoplastids differentiate to form three major groups of plastids, the green chloroplasts, the colored chromoplasts and the colorless leucoplasts. The most abundant and important plastids are the chloroplasts. Chloroplasts harvest energy from sunlight to split water and fix carbon dioxide to produce sugars. This process called photosynthesis also converts harvested solar energy into a conserved form of energy: ATP and NADPH through a complex set of processes.

II. SYNTHESIS OF ATP IN MITOCHONDRIA AND CHLOROPLAST

As it has been mentioned earlier mitochondria and chloroplasts are best known for their roles in energy metabolism, notably respiration and photosynthesis [85].

It is clear that ATP synthesis is the central bioenergetic engine of all organisms and represents the smallest molecular motor, which was optimized in the course of evolution [17].

In eukaryotes, the ATP synthase complex is located in the inner membrane of mitochondria, with ATP synthesis reaction occurring on the membrane side toward matrix compartment. In plants, the enzyme is in addition localized in the thylakoid membrane of chloroplasts, with the ATP – forming – moiety facing the stroma. These topological differences between the mitochondrial and chloroplastic ATP synthases bring about two very distinct metabolic environments for ATP synthesis, where ATP utilization and provision of both ADP and Pi need to be fine–for optimal ATP synthase activity. In chloroplasts, ATP synthase receives protons from thylakoid lumen, which volume is small as compared to the mitochondrial

intermembrane space (IMS) and which pH value can drop to the values below 5 [102], while in the mitochondrial IMS it drops only slightly below 7 [11 & 12). In mitochondria, adenylates are transported through the membrane, whereas their stromal pool is self – sufficient to support chloroplastic ATP synthase; the activity of adenylate transport between chloroplast and cytosol is very low, representing _1% of activity of the triose phosphate translocator [14]. While generation of proton electrochemical potential became the central theory in the chemiosmotic concept of ATP synthase operation [84], the optimal conditions of delivery of ADP and phosphate were analyzed in the concept of thermodynamic buffering [59 & 60], underlying the importance of auxiliary buffering enzymes such as adenylate kinase (AK) and creatine kinase in provision of the stable flux of ADP to ATP synthase. This theory was extended in relation to operation of AK in the IMS of mitochondria [15]. The energy balance of photosynthetic cells is provided by equilibration of adenylate levels by chloroplasts and mitochondria (and the cytosol) and the role of AK in this equilibration appears to be important.

III. MAGNESIUM AND THE ROLE OF ITS IN ATP SYNTHESIS

Before any more explanation and commentary about mitochondria and chloroplast and their differences and similarities also ATP synthase it is better to have a statement about the role of magnesium in ATP synthase by these two important and crucial organelle.

The role of magnesium in ATP synthesis is underlined not only by the fact that MgATP is the actual product of the reaction, but also, as we show below that Mg^{2+} acts as a separate substrate in the ATP synthas reaction under physiological conditions, ADP can exist both in a free and Mg - bound state, and this dual chemical capacity determines a way that magnesium becomes a part of "energy charge". The Mg^{2+} pool is not less important than protons and it is generated (kept stable) by the AK reaction, which determines the equilibrium value of Mg^{2+} in cellular compartments [16]. This results in efficient regulation of Mg - dependent enzymes and, one such enzymeis ATP synthase. The rotation mechanism of ATP synthase was suggested by Boyer (1989) [86] and then it was demonstrated empirically [51]. The role of proton translocation consists in deforing an open catalytic site to increase the affinity for ADP and Pi, which then bind and pass through the transition state, yielding tightly bound ATP in one binding change. ADP binding appears to be a key parameter controlling rotation during synthesis, while MgADP is inhibiting. The essential role of Mg^{2+} in ATP synthase catalysis was recently established in works of scientists. Previously it was assumed that the substrate of ATP synthase was MgADP [87]. Later studies, however, have indicated that it is free ADP in the presence of magnesium which represents the real substrate. It was shown [105] that inhibition of catalysis by vanadate in the presence of MgADP could be substituted by the Mg - vanadate complex indicating that Mg^{2+} plays a pivotal role in transition state formation during ATP synthesis. This state involves the preferential coordination with Pi and the repositioning of the P – loop to bring the nonpolar alanine 158 into the catalytic pocket, which is achieved in the presence of Mg^{2+} [35]. According to these more recent data, it is correct to consider ADP_{free} rather than MgADP as a true substrate, rather than MgADP as a true substrate, while Mg^{2+} acts independently. Therefore, the substrates of ATP synthase are ADP_{free}, Pi_{free}, $Mg^{2+}{}_{free}$ and H^+, while the product is MgATP. The reaction can be presented by the following equation (one proton is the substrate, whereas other protons have catalytic function):

$$ADP^{3-} + HPO_4^{2-} + Mg^{2+} + H^+ \rightarrow MgATP^{2-} + H_2O$$

The difference in pH between matrix and IMS results in deprotonation of phosphate and of ADP in the matrix side, facilitating the Mg - dependent mechanism. Magnesium participating in ATP synthase catalysis exhibits a profound catalytic effect as shown by [10]. The activity with ^{25}Mg, which has magnetic isotopic nucleus, is two to three times higher than with ^{24}Mg or ^{25}Mg isotopes, having spinless non – magnetic isotopic nuclei. This suggests that the ATP synthesis is a spin – dependent ion – radical process. It implies a reversible electron transfer from the terminal phosphate of $ADP3^-$ to Mg^{+2} generating ion – radical pair with singlet and triplet spin states. The yields of ATP along the singlet and triplet channels are controlled by hyperfine coupling of unpaired electron in $^{25}Mg^{+2}$ ion with magnetic nucleus. The magnesium bivalent cation transforms the protein molecule mechanics into a chemical reaction [10]. Although this mechanism was suggested for the mitochondrial ATP synthase, potentially it can be generalized for all ATP sythases including the chloroplast and even for other Mg - dependent enzymes. Mg^{2+} uptake by mitochondria and its efflux are mediated by a channel or transporter responding to changes in membrane potential, in particular in pH gradient [40 & 68]. The concentration of Mg^{2+} in the mitochondrial matrix depends on Pi which interacts strongly with Mg^{+2} to decrease its concentration and, in the absence of external Mg^{+2} promotes respiration – dependent Mg^{+2} efflux and its decrease in the matrix to very low levels [41]. The uptake of Pi by respiring mitochondria converts Δ pH to $\Delta\Psi$ and provides additional Mg - binding sites permitting its large accumulations. This means that Pi, in addition to AK, buffers Mg^{+2} concentration and this buffering is important in the matrix of plant mitochondria, where AK is absent. While Mg^{2+} is an important catalyst (and substrate) of ATP synthesis and

many other processes, the changes of its content result in significant shifts in bioenergetic state of the cell. These Mg^{2+} dependent shifts strongly affect Ca^{2+} concentration in the IMS [89 & 4].

Ca^{2+} uptake by mitochondria is inhibited by Mg^{2+} via a mixed type inhibition in the process of multistate catalytic binding and interconversion, in which phosphate is also involved as a regulator [90]. A frequently observed increase in $[Ca^{2+}]$ under stress conditions is, therefore, mediated by fluctuations in Mg^{2+} and results in activation of Ca^{2+} dependent stress induced enzymes. Thus the signaling and metabolic roles of Ca^{2+} are under control of magnesium, phosphate and adenylate energy charge that establishes equilibrium Mg^{2+} concentration

IV. DNA IN MICHONDRIA AND CHLOROPLAST

The DNA in both mitochondria and chloroplasts can be extremely unstable, as illustrated by the following examples.

(i) The half – life of rat mitochondrial DNA (mtDNA), in days, is 6.7 for heart, 9.4 for liver, 10.4 for kidney, and 31 for brain [73],

(ii) In the single – celled alga Euglena, the half – lives for chloroplast DNA (cpDNA) and mtDNA are 1.6 and 1.8 cell doublings, respectively, but nuclear DNA is so stable that turnover could not be detected (56 & 80).

(iii) Two days after sowing mung bean seeds, the mtDNA in dark – grown seedlings turns over entirely in 24 hours [50].

(iv) The half – life of mtDNA in yeast is ~ 4 hours (for a mutant defective in the mtDNA polymerase gamma) [106]

(v) Light triggers the degradation of DNA in maize chloroplasts [10]. Four hours after exposing 10 – day – old dark grown seedlings to light, the leaf begins to green, and the average DNA content per chloroplast decreases to 54% by hour 6 and 9% by hour 24 .

(vi) During 6 stages of development of maize leaf tissue, the size and structural integrity of cpDNA decreases progressively from branched molecules of multigenomic size in the basal meristem of seedlings to fragments of subgenomic size in adult plants, as observed in moving pictures of individual ethidium – stained DNA molecules [36].

A similar degradative progression of individual cpDNA molecules is observed during leaf development for tobacco and the legume *Medicago truncatula* [57] and Arabidopsis [18]. (vii) In fully expanded leaves of adult plants of Arabidopsis [18 & 19] and maize, [36] more than half the chloroplasts contain no detectable DNA. How can we explain this remarkable instability of organellar DNA? It is suggested that the ROS generated during electron transport that accompanies oxidative phosphorylation and photosynthesis leads to oxidative stress and extensive damage to the DNA [20]. For Euglena, repair of the mtDNA and cpDNA is the only option because it is a unicellular organism. For dark – grown mung bean seedlings, repair again is the only option for mtDNA since respiration must provide the energy for this aerobic organism. The mtDNA is so extensively damaged that it turns over completely in one day. For a light – grown plant, however, there is another option. If some of the organellar DNA can be sequestered in quiescent germ line cells, the highly damaged organellar DNA in somatic cells can be left unrepaired; it is eventually degraded and its nucleotides are recycled for their nutritive value [23]. Similarly, oxidatively damaged mtDNA in active somatic cells can either be repaired or abandoned, as long as undamaged mtDNA is retained in quiet germ line cells. For the mesozoan *Dicyema japonicum*, mtDNA is retained in "stem" mitochondria of germ cells, but mtDNA is undetectable in most somatic cells of mature larvae and adults, a result of either dilution without replication [49] or, it is suggested that, abandonment and degradation of mtDNA [20].

4.1 DNA damage and repair in mitochondria and chloroplasts

From an evolutionary perspective, the only objective for an organism is to replicate its DNA and pass it on to the next generation. Unintended alterations in chromosomal DNA molecules can arise in various ways, including DNA polymerase errors and changes to the DNA template from internal (ROS, for example) and external (radiation, for example) sources. Only internal sources because these can be modulated during development. Changes in DNA can be perceived and acted upon as needed during development. Changes in DNA can occur as nucleotide alterations, insertions/deletions, inversions, and DNA strand breaks. Those lesions recognized as "damage" can be either repaired or removed by degrading the DNA [42].

Most information on the repair of mtDNA comes from yeast and somatic cells of mammals [57; 72; 63; 96; 54], whereas very little is known about mtDNA repair in plants or about cpDNA repair [24; 82; 25; 5]. A detected change in mtDNA is the result of both the rate of damage and the efficiency of correcting the damage. The power of genetics can sometimes be used to study each of these parameters separately in yeast. Overall, two conclusions seem generally supported. First, most DNA damage in mitochondria is due to oxidative damage, as may be expected for the site of respiration, and base excision repair

(BER) is the main way to rectify oxidative damage [72; 71; 61]. If BER fails, human mtDNA molecules containing the damage are usually degraded and base substitution (point mutation) is thus avoided [28]. Degradation of damaged DNA molecules to avoid mutation is feasible for the high – genome – copy cytoplasmic organelles, but not for the diploid nucleus. Such degradation would mask a higher rate of damage in the organelles than in the nucleus. The second conclusion is that the capacity to repair stress – induced DNA damage is lower in mitochondria than the nucleus, because mitochondria are the principal site of ROS production, employ fewer repair processes than do nuclei, or lack protective histones on their mtDNA molecules [75; 72; 61; 45; 53; 83]. Damage to organellar DNA is indicated by a rapidly increasing mutation rate (point mutations per kb of mtDNA) as mouse tissues age [71], an accumulation of mtDNA deletions with age in humans, monkeys, and rodents [75 & 62], and a decline in structural integrity of cpDNA molecules as leaves develop. Thus, it would be advantageous to shelter organellar DNA before tissues mature in the adult.

V. GENES IN MITOCHONDRIA AND CHLOROPLAST (INHERITANCE: LAWS AND MECHANISMS)

The literature on the inheritance of genes in mitochondria and chloroplasts (hereafter, organelle genes) has changed, grown, and advanced tremendously [30].

Some of the most exciting advances since the previous reviews have been made in understanding the molecular and cellular mechanisms of organelle division and distribution between daughter cells (partitioning) in yeast, animals, and plants; genetic studies of segregation and within generation selection of mitochondrial genes in mammals and Drosophila; and the controversial subject of mitochondrial bottlenecks in mammals. Other exciting discoveries dealt with the mechanisms of uniparental inheritance in Chlamydomonas and mammals, and a controversy over whether there is a low level of biparental inheritance and recombination in humans. The growing excitement about mitochondrial genetics in humans and mammals has been driven in large part by their application to human diseases caused by mitochondrial mutations, and by the widespread use of mitochondrial genes to study the population genetics and evolution of humans and other animals. These subjects have also been reviewed in the past three years, but mainly as separate subjects and not in the context of organelle heredity in general.

5.1 Chlamydomonas Chloroplasts

5.1.1 Vegetative Segregation is rapid in Chlamydomonas chloroplasts

Chlamydomonas reinhardtii has been used extensively for chloroplast genetics since the pioneering studies of Sager [93 & 94] and Gillham [77 & 78]. In contrast to the plants discussed above, Chlamydomonas cells have one chloroplast, which divides into two equal parts just before the cell divides; consequently, vegetative segregation cannot be explained by the partitioning of chloroplasts. Most data come from crosses of antibiotic – resistant by sensitive clones. Vegetative segregation can be studied in vegetative zygotes, which divide by mitosis instead of meiosis, or in the meiotic and early mitotic divisions of the small percentage of zygospores that show biparental inheritance. In either case, segregation is complete within a few cell generations. This is much too fast to be accounted for by random partitioning of the approximately 50 – 100 genomes.

5.1.2 Genome partitioning is probably stochastic but not strictly random

One possible explanation for rapid segregation is that when the two gamete chloroplasts fuse in the zygote, the plastid genomes from the parents tend to remain in different parts of the chloroplast and consequently tend to segregate together rather than strictly randomly [67]. The chloroplast genomes are grouped in about 5 – 15 nucleoids, and it is possible that the 10 or more genomes in each nucleoid tend to be replication products of one genome. In other words, genome partitioning is stochastic but not strictly random; like molecules tend to segregate together because they are joined in nucleoids and/or the nucleoids from the gametes are not completely mixed in the zygote.

5.1.3 Genome replication is stochastic

Different Chlamydomonas zygotes from the same mating give rise to clones with very different frequencies of alleles from the two parents. Some zygote clones are uniparental, with organelle genomes from only one parent or the other. Frequency distributions of gene frequencies in a large number of zygote clones bear a striking resemblance to the gene frequency distributions of Mendelian populations undergoing random genetic drift [33]. When the mitotic division of vegetative zygotes [66], or the meiotic divisions of zygospores [22], was delayed for a time by starvation, the variance in gene frequencies increased and more uniparental zygote clones were produced. These data suggested that plastid genomes continue to replicate during starvation and that replication is stochastic, with some genomes replicating more often than others by chance. The result is that gene frequencies within cells undergo stochastic changes, which is called intracellular

random drift by analogy to random drift of nuclear gene frequencies in populations of organisms [30]. Stochastic replication by itself will not completely eliminate an allele from a cell or clone, but may reduce it to a frequency too low to detect. Alternatively, there may be some degradation of organelle DNA molecules, which will then be replaced by additional replications of other molecules (turnover). Note that the stochastic replication of genomes, and the stochastic partitioning of genomes into daughter organelles when an organelle divides, can also explain how a mutant genome becomes homoplasmic in plant plastids.

5.2 Yeast Mitochondria

Much has been learned about organelle heredity from the study of another model genetic system, mitochondrial genes in budding or baker's yeast (*Saccharomyces cerevisiae*). The best markers are mutant genes conferring antibiotic resistance; respiration – deficient mutants (petites) are also used but their inheritance is strongly affected by selection. When heteroplasmic zygotes are produced by mating yeast strains that differ in one or more mitochondrial alleles, the majority of diploid progeny are homoplasmic after no more than 20 cell generations. Strictly random partitioning could only explain this rate of segregation if there were no more than 2 to 5 segregating units [30]. This is much smaller than the number of mtDNA molecules in diploid cells [approximately 100] and slightly smaller than the number of nucleoids. Mitochondria from the two parents cannot be the segregating units because they fuse in the zygote. Consequently, vegetative segregation in yeast must be explained by some combinations of the same factors that were invoked above for chloroplast genes in Chlamydomonas: (a) partitioning of genes that is stochastic but not strictly random, with similar molecules tending to remain together; (b) stochastic replication; or (c) turnover. There is experimental evidence only for the first two processes, but it is likely that all three are involved.

5.3 Mitochondrial fusion and fission

A yeast cell may contain a single large mitochondrial network, or a network plus a few small separate mitochondria, or many small discrete mitochondria, depending on its physiological state. Yeast mitochondrial genomes undergo multiple pairings with recombination in zygotes, showing that genomes from the two parents can interact extensively. Considerable progress has been made in understanding mitochondrial fusion and fission in yeast. Fission is accomplished by the dynamin system in yeast and animals [74]. The dynamin Dnmp1p localizes to mitochondria at division sites and tips and is required for normal mitochondrial morphology. Mitochondrial fusion requires the fzo1 (fuzzy onion) gene, a homologue of the fuzzy onion gene that is required for mitochondrial fusion in Drosophila. In yeast, normal mitochondrial morphology requires a balance between the activities of Dnm1p and Fzo1 [52].

5.4 Bud position effects: non random partitioning

Early models of mitochondrial gene inheritance in yeast assumed that fusion was so frequent that a cell is effectively a single population of freely interacting genomes. That this could not be strictly true was demonstrated by pedigree studies of zygotes [30; 34; 91], which showed that (a) when the first bud comes from one end of the zygote, the majority of its mitochondrial genes come from the parent which formed that end of the zygote; and (b) buds that arise from the neck of the zygote receive markers from both parents, as well as a higher frequency of recombinant genotypes. This indicates that the mixing of mitochondrial genomes from the two parents is incomplete when the first bud is formed; later buds usually include markers from both parents, indicating more complete mixing. This interpretation was verified by showing that labeled mtDNA from one parent failed to enter the opposite side of the zygote until sometime after the first bud was formed, although it did enter first center buds [58]. The mitochondrial membranes from the two parents fused quickly, so delayed mixing of mtDNA was not due to delayed mitochondrial fusion; evidently, the movement of mtDNA across the zygote involves a different mechanism from the movement of mitochondria. Mitochondrial proteins also move more quickly through the mitochondrial network than does mtDNA [88; 58; 69].

5.5 Mitochondrial movment from mother to bud

Because Saccharomyces cells bud rather than undergoing binary fission, a mechanism is required to move mitochondria and their genes from the mother into the growing bud. The experimental studies of this process have been reviewed [74]. Mitochondria are actively transported from the mother cell into the bud, where they are immobilized at the tip of the bud until cytokinesis is complete. Mitochondria probably move along actin filaments by a motor that depends on actin polymerization [103], and movement also requires intermediate filaments encoded by the MDM gene [99]. It is not surprising that a mechanism evolved which ensures that buds receive at least some mitochondria, which are required for survival, and mitochondrial genomes, which are required for respiratory competence.

5.6 Stochastic replication

As was the case for Chlamydomonas chloroplast genes, yeast cells can become homoplasmic for mitochondrial genes without dividing, owing to random genetic drift of gene frequencies within the cell [31]. This was demonstrated using delayed division experiments with both budding and fission yeast [64], analogous to those in Chlamydomonas. Birky and colleagues [32] reported that many first central buds are uniparental, producing clones with mitochondrial genes from only one parent; however, when wild – type cells were mated with ½ mutants that have mitochondria but no mtDNA, all first central buds receive mtDNA. They suggested that all first central buds probably receive mtDNA from both parents but that stochastic replication (possibly combined with turnover) eliminates genes from one parent or the other. Stochastic replication is almost certainly a major contributor to the production of homoplasmic cells during asexual reproduction in yeast, i.e., to vegetative segregation.

5.6.1 Nucleoid structure affects mitochondrial gene inheritance

It was suggested that the segregating units in yeast mitochondria might be nucleoids [30], and recent studies suggest that nucleoid structure does affect the inheritance of mitochondrial genes. The mtDNA molecules in a nucleoid appear to be held together by Holliday structures [100; 37; 38; 81], perhaps because mtDNA replication is initiated by recombination (7; 99; 44) as it is in T – even phage [46]. Mutations that affect the resolution of the Holliday structures also modify the inheritance of neutral ρ^- genomes in $\rho^- \times \rho^+$ crosses [38, 97].

VI. PROTEIN TARGETING TO MITOCHONDRIA AND CHLOROPLASTS

One of the most interesting subjects about mitochondria and chloroplasts, which should be considered earlier, is the origin of these organells. As all of us know these organells originally derive from endosymbiotic bacteria: The closest bacterial organisms to the endosymbiotic ancestors of these organelles have nearly a thousand genes (Rickettsia [98]) or several thousands (cyanobacteria [95]).

Since the endosymbiosis, many of the genes of the endosymbiotic bacteria have been lost, leaving the organelle genomes with less than a hundred proteins – coding genes each [101; 70]. The vast majority of mitochondrial and plastid proteins are encoded in the nucleus, synthesized by cytosolic ribosomes and subsequently imported into the organelles via active protein transport systems. The total number of proteins present in mitochondria and chloroplasts is thought to be about 2000 – 3000 for each of them [3]. Mitochondria originated much earlier than plastids and thus the first plastids arose in cells that already contained an efficient system for targeting cytosolically synthesized proteins to mitochondria. One might have expected evolution to have seized this opportunity to reuse the same machinery for targeting proteins to plastids, but in fact this seems not to be the case; the two protein import systems have clearly been derived independently and do not share homology. In this situation, it is thus easy to understand why protein targeting is usually highly specific. Nevertheless, it is becoming increasingly clear that despite the profound differences in the two import machineries, a certain number of proteins are efficiently recognized by both systems and are imported into both organelles [85].

6.1 Targeting protein to mitochondria

Mitochondria have two complexes of proteins called TOM proteins and TIM proteins, respectively located in the outer membrane and the inner membrane, which together form the protein import channel. The proteins that will be imported generally have a mitochondrial targeting sequence located at the N – terminus, although there are proteins that have internal or even C – terminal targeting signals. This latter case has been found only once, for a yeast mitochondrial helicase [27]. The N – terminal presequence cannot be described as a consensus sequence but contains conserved features that can be identified with more or less confidence. In plants, mitochondrial targeting sequences are generally longer than in other organisms (40 amino acids on average) [9], they have a net positive charge (rich in arginine and poor in acidic amino acids) and contain many aliphatic residues (mainly leucine and alanine). The structure adopted by the presequence is generally an amphiphilic K helix [47].

It can be noted also that plant mitochondrial targeting sequences are particularly rich in serine residues. How the translated protein is actually targeted to the mitochondria is not well understood yet. In the case of a protein targeted to the matrix of mitochondria and possessing an N – terminal presequence, cytosolic protein factors interact with the presequence. These factors are generally chaperones and can require ATP. The presequence is then transferred to the mitochondrial TOM complex proteins. These proteins, namely TOM70, TOM20 and TOM22, are generally negatively charged and can thus form

electrostatic interactions with the presequence. Once the presequence is engaged in the outer membrane channel, negative charges present on the inner membrane protein TIM23, along with the electrochemical gradient across the inner membrane (gradient created by the electron transport along the mitochondrial respiratory chain), allow the presequence to tow the protein through both the outer and the inner membrane. The last steps of protein import are carried out by mitochondrial chaperones, which literally pull the protein inside the matrix. The imported protein can then be cleaved from its import signal by specific proteases, and be refolded to carry out its function inside the mitochondria.

6.2 Targeting protein to chloroplast

Chloroplasts also possess an outer envelope protein complex called TOC, and an inner envelope protein complex, TIC, which differ in many ways from the equivalent mitochondrial complexes. Chloroplast proteins can be located in even more compartments than mitochondrial proteins. In addition to the envelope membranes and the inter membrane space and the stroma, many important chloroplast proteins (photosynthesis – related proteins) are located in the membrane and the lumen of the thylakoids. In this study will be focused only on the presequence needed for the protein to cross the double membrane envelope of the chloroplast. These targeting sequences are different from their mitochondrial counterparts but do present some similarities. They are about 50 amino acids long; rich in the hydroxylated residue serine and unlike mitochondrial presequences they do not contain many positively charged residues, especially in the first ten amino acids, and do not contain many leucine residues. However, like mitochondrial targeting sequences, they contain very few acidic amino. The structure of the presequence is somewhat less well defined than for mitochondria [48].

Proteins targeted to the chloroplast are probably also recognized in the cytosol by chaperone proteins [48], before interacting with the components of the import machinery. The major difference with protein import into mitochondria is that there is no comparable electrical gradient in chloroplasts. None of the proteins from the TOC and TIC complexes have homologues in the TOM or TIM machinery [2]. Protein import into chloroplasts largely depends on the subsequent action of different protein chaperones, the process requiring GTP and ATP. A large GTPase protein, TOC160, is one of the most cytosolic – accessible TOC proteins, and is involved in recognition of the presequence. The TOC and TIC protein complexes are in close contact with each other. TIC22 is the first protein from the inner membrane complex to interact with the presequence [9]. TIC110 is believed to form the canal through which the proteins are eventually imported into the stroma. It seems that TIC110 is also in close interaction with stromal chaperones, which could be the final motor for the import of the chloroplast – targeted protein. As in mitochondria, specific proteases can remove the presequence from the mature protein.

6.3 Dual targeting proteins to chloroplast and mitochondria

There are approximately 50 proteins in different species reported up to date to be encoded by a single gene, synthesized as one gene product, but imported into both mitochondria and chloroplasts using an ambiguous dual targeting peptide (dTP) [29; 6; 69]. The first protein shown to be dually targeted to mitochondria and chloroplasts was glutathione reductase (GR) from *Pisum sativum*. Since then, other proteins involved in many essential organellar functions, such as DNA and RNA synthesis and processing, protein folding and fate, energy metabolism, and stress response, have been shown to be dually targeted. Eighteen of the dually targeted proteins are aminoacyl – tRNA synthetases identified in *A. thaliana* [6 & 76]. The overall properties of dTPs resemble standard characteristics of mTPs and cTPs, but there are quantitative and distributional differences. Continuous identification of new dually targeted proteins gives opportunity for reinvestigating the overall properties. Determinants for dual targeting are not fully understood. It has been proposed that the information for organellar targeting can be organized in domains, as, for example, in dTPs of GR [29], RNA polymerase RpoT:2 [21], and Presequence Protease, or spread all through the targeting peptide [79] or associated with the occurrence of arginine and the properties of the second amino acid of the N – terminal sequence [28].

Moreover, expression of the 5' untranslated region (UTR) upstream of the ATG start codon has also been shown to be involved in generating a dTP [1]. Here, we have analyzed the amino acid content and distribution of 43 dual targeted proteins in *A. thaliana* using SequenceLogos and statistical methods. We have investigated targeting determinants of the dual targeting peptide of Thr – tRNA synthetase (ThrRS – dTP) studying organellar import of N and C – terminal deletion constructs coupled to GFP. Furthermore, it have been produced chemical quantities of the shortest peptide of ThrRS – dTP that was capable of conferring dual targeting capacity, ThrRS – dTP (2 – 60), and it has been studied its biochemical and biophysical properties.

VII. CONCLUSION

Chloroplasts and mitochondria are both membrane bound organelles of eukaryotic cells. They do not occur in prokaryotic cells, for example, bacteria. Mitochondria occur in the cells of animals and plants but chloroplasts only occur in the photosynthesising tissues of plants.

Chloroplasts are concerned with the process of photosynthesis whereas mitochondria are concerned with aerobic respiration. It is clear that one of the most important products of these two povital process is ATP. ATP synthesis is the central bioenergetic engine of all organisms and represents the smallest molecular motor, which was optimized in the course of evolution. In eukaryotes, the ATP synthase complex is located in the inner membrane of mitochondria, with ATP synthesis reaction occurring on the membrane side toward matrix compartment.

In plants, the enzyme is in addition localized in the thylakoid membrane of chloroplasts, with the ATP – forming – moiety facing the stroma. So we observe that there are topological differences between the mitochondrial and chloroplastic ATP synthases. Also magnesium is a important element in ATP synthesis. The role of its, is to form of Mg^{2+} which acts as separate substrate in the ATP synthas. It has also been shown that DNA mitochondria chloroplast has its own and that the DNA in both mitochondria and chloroplasts can be extremely unstable. Most information on the repair of mtDNA comes from yeast and somatic cells of mammals, whereas very little is known about mtDNA repair in plants or about cpDNA repair.

An ambiguous dual targeting peptide is a tool for importing one gene product into both mitochondria and chloplast. The first protein shown to be dually targeted to mitochondria and chloroplasts was glutathione reductase (GR) from *Pisum sativum.*

It has been proposed that the information for organellar targeting can be organized in domains.

A protein domain is a conserved part of a given protein sequence and (tertiary) structure that can evolve, function, and exist independently of the rest of the protein chain.

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Metabolomics Analysis on Antifungal Activities Produced by *Penicillium oxalicum* T3.3 Grown on Different Types of Carbon Sources

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Abstract*—In-vitro antagonist tests such as disc diffusion and minimum inhibition concentration (MIC) were conducted against C. gloeosporioides.* ^{1}*H-NMR coupled with multivariate statistical analysis was carried out to identify possible compounds produced. Glucose crude extract exhibited the highest percent inhibition of radial growth (PIRG) with 75% and the lowest MIC value with 78 µg mL*$^{-1}$*. For metabolomics, different metabolites produced were clustered according to the carbon sources used and gave a representative impression of the metabolites produced by P. oxalicum T3.3. The study has shown the potential of using a combination of* ^{1}*H-NMR spectroscopy and multivariate statistical analysis and their correlation with MIC in differentiating the effect of carbon sources used based on the identification of possible metabolites contributing to their differences. Findings from this work may potentially provide the basis for further studies on both antimicrobial activities against plant pathogen and elucidation of the metabolite compounds produced by P. oxalicum T3.3.*

***Keywords*—*Colletotrichum gloeosporioides, metabolomics, Partial Least Square (PLS), Penicillium oxalicum*.**

I. INTRODUCTION

Colletotrichum gloeosporioides, known as one of the world's most plant pathogenic fungi can cause a serious damage to most parts of plants including stems, fruits, roots, leaves, and flowers but are often highly specific to individual tissues (Bailey *et al.* 1992). This pathogenic fungus attacked an extremely wide range of plants growing in both temperate and tropical environments. In Korea, *C. gloeosproriodes* had been identified as the cause of anthracnose disease that attack tulip trees as the necrotic lesions became black as the spots expanded on the leaves of that trees (Choi *et al.* 2012). The first report of anthracnose of *Pisonia alba,* commonly called lettuce tree was reported in India described the *C. gloeosproriodes* as the fungus that produced white mycelia, which became dark grey with later formation of numerous salmon pink colored spore masses (Vidyalakshmi and Divya, 2013).

In Malaysia, the first report on the occurrence of anthracnose disease in dragon fruit (*Hylocereus* spp.) caused by this fungus was reported by Masyahit *et al.* (2009). The infected stem and fruit had reddish-brown lesions or black spots symptoms where it can expand and merge to cover the whole affected area. At present, the great potential health benefit (Ching and Yusof, 2005), physic-chemical characteristics (Chuah *et al.* 2008) and nutritional value (Ariffin *et al.* 2009; Rebecca *et al.* 2008) of dragon fruit had been a great interest among the researchers, however the exploitation of natural organism as biological control and the potentialities of these microorganisms in production of bioactive metabolites and bio-control agents in controlling the pathogenic fungi has not receive any further investigations yet.

There is no agreement on which media are the optimal for metabolite production. However, according to Mathan *et al.* (2013), the growth media and incubation conditions have a very great influence on secondary metabolites production. Some of the physical and chemical parameters like pH, temperature, carbon and nitrogen sources play a major role on fungal growth and production of bioactive compounds and antimicrobial agents (Gunasekaran and Poorniammal, 2008; Mathan *et al.* 2013). The availability and type of carbon and nitrogen source gives effect on polyketide production whereby carbon

source such as glucose and sucrose have been found to increase the fungal growth and sporulation along with the high aflatoxin production (Keller *et al.* 2002).

Metabolomics can be described as a comprehensive quantitative and qualitative analysis of holistic metabolites present in a biological organism, which are the end-products of its gene expression (Van der kooy *et al*. 2009). There are many tools that can be used to analyze large number metabolites simultaneously such as nuclear magnetic resonance (NMR), gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS) and Fourier transform infrared spectroscopy (FTIR). Some of them rely on chromatographic separation step and others do not require any in which represent a global view of the sample (Ulrich-Merzenich *et al*. 2007). Among these tools, NMR method is able to provide a wide range of many molecular classes including sugars, amino acids, organic acids, alcohols or polyols, amine and ketones (Wishart, 2008).

P. oxalicum T3.3 had shown a promising antagonism in producing antifungal secondary metabolites that could inhibit the growth of *C. gloeosporioides*. Thus, the aim of this study was to determine the effect of different types of carbon sources for the production of antifungal compounds from *P. oxalicum* T3.3 against *C. gloeosporiodes*. Metabolomics approach was employed in identifying the possible compounds produced by theT3.3 strain exposed to the different carbon sources.

II. MATERIAL AND METHOD

2.1 Fungal strains and culture conditions

P. oxalicum T3.3 was obtained from the Industrial Biotechnology Laboratory culture collection. *C. gloeosporiodes*, a plant pathogenic fungus tested in this study was obtained from MARDI Serdang, Selangor. Stock cultures were maintained on potato dextrose agar (PDA) slant and stored at 18^{o}C. *P. oxalicum* and pathogenic fungi *C. gloeosporioides* were cultured on PDA plates and incubated at 30^{o}C.

2.2 Preparation of fungal inocula

To produce spores of *P. oxalicum,* a volume of 10 mL sterile distilled water was added to a 5-day culture of strain T3.3 on a PDA plate. A spore-suspension was made with a sterile glass rod to collect the spores and was diluted serially before the concentration was counted using a haemocytometer.

2.3 Submerged fermentation in different growth media

The spore suspension was inoculated into 500 mL Erlenmeyer flasks containing 250 mL sterilized medium. There are five types of medium were used in this study which are Richard medium, Czapek dox medium, Malt extract broth, Sabouraud dextrose medium and Potato dextrose broth. The cultures were grown at 30^{o}C, 120 rpm in rotary incubator shaker (Lab companion/S-971R) for 12 days. The samples were withdrawn at regular intervals of one day (destroy samples). The biomass was separated by the filtration through Whatman No 1 filter paper and the cell-free culture broth was extracted using organic solvent for further used in disc diffusion test.

2.4 Dry cell weight of mycelia

The samples of fermentation for each medium were harvested every day until the twelve day of the fermentation. The weight of Whatman filter paper was recorded before the filtration process. Then, the fungal mycelia were filtrated using the vacuum filtration. The filter papers containing the cells were dried at 60^{o}C overnight. Then the weight of the dried filter paper containing the cells was recorded.

2.5 Biolog FF microplate analysis

Biolog identification and carbon utilization test was done according to the method described by Tosiah, 2013. FF Microplate with 95 wells prefilled with different carbon sources and a single well prefilled with water as control were used to identify the carbon utilization of strain T3.3. A volume of 100 µL of spore suspension with inocula turbidity of 75%±2% at A590 nm was dispersed into each the microplate well and incubated at 26^{o}C. The mycelia growth based on turbidity and the change of optical density were measured at dual wavelength 490nm and 750nm using microlog 3 software. After 72 h of incubation, the mycelia growth and metabolic reaction of *P. oxalicum* T3.3 were measured at 490 nm and 750 nm respectively.

2.6 Cultivation with different carbon sources

A volume of 250 µL of *P. oxalicum* spores suspension (10^4 spores mL^{-1}) was inoculated into fresh medium containing: KNO_3 0.25 g, KH_2PO_4 0.125 g, MgSO4 0.0625 g, $FeCl_3$ $2.5x10^{-4}$ g and carbon source 7.5 g. The fermentation was carried out in

500 mL Erlenmeyer flasks containing 250 mL medium on rotary shaker at 120 rpm, 30°C for 10 days, and the culture filtrates were used for the extraction process. The carbon sources used; glucose, maltose, sucrose, xylitol, starch and *U. pinnatifida*, edible seaweed, were substituted into fermentation media.

2.7 Extraction of metabolites

After 10 days of fermentation, the fermented broths were filtrated to separate the cells by using 90 mm Whatman filter paper. The supernatant was then centrifuged at 10000 x g for 15 min before extracting with organic solvent, ethyl acetate. An equal volume of ethyl acetate was used for liquid-liquid extraction of the chemical compounds from *P. oxalicum* T3.3 supernatant. The suspension was vigorously mixed by using magnetic stirrer. After 1 h, the suspension was poured into a separatory funnel with an equal volume of ethyl acetate as the solvent and the organic layer was collected. The organic layer was dried and concentrated using a rotary evaporator (Rotavapor R-3, BüCHI, Switzerland). The extraction process was repeated twice to maximize the extraction of the organic compounds. The pooled fractions were dried, weighed and stored before subjected to antagonist test and NMR analysis.

2.8 In-vitro susceptibility test against *C. gloeosporioides*

2.8.1 Disc diffusion test

Ten mg of the dried samples from previous extraction was dissolved with 1 mL ethyl acetate. A sterile Whatman filter paper with diameter 1 cm was dipped into the sample solution and was dried in sterile condition for 1 hour. Then, seven days old 5 mm disc of pathogen will be cut near the periphery of the colony using sterile cork borer and place on one side of the PDA plate. Similarly, the filter paper impregnated with the sample solution be place on other side of PDA plate and was kept at a distance of 1 cm at an angle of 180°. Disc impregnated with ethyl acetate was act as a negative control while disc impregnated with cyclohexamide (commercial antibiotics) was act as a positive control. All PDA plates were incubated at 30 ± 1°C for seven days and the inhibition zone around the disc was observed and measured. The percentage of inhibition was measured according to this formula:

Percent inhibition of radial growth (PIRG): (R1-R2)/R1

Where, R1 = radial growth of pathogen in control plate

R2 = radial growth of pathogen in opposed plate

2.8.2 Spores suspension preparation

Inoculum suspensions of filamentous fungi were prepared by the method of NCCLS M38-A (NCCLS, 2002). Briefly, *C. gloeosporioides* were grown on a PDA at 30°C for 5 days. The five-day-old colonies were covered with approximately 10 mL of sterile 0.85% saline, and suspensions were made by gently probing the colonies with the tip of Pasteur pipette. The resulting mixture of conidia and hypha fragments was withdrawn and transferred to a sterile tube. After heavy particles were allowed to settle for 3 to 5 min, the upper homogenous suspensions were collected and mixed with a vortex mixer for 30 s. The concentration of the conidia was count using the counting chamber, haemocytometer.

2.8.3 Minimum Inhibition Concentration (MIC)

Individual MICs was determined following the broth microdilution method recommended by NCCLS, approved standard M38-A (NCCLS, 2002), as modified by Espinel-Ingroff *et al.* (2002) and Santos *et al.* (2006). The broth microdilution tests were performed using sterile and disposable 96-well flat bottomed microtitration plates. Each microdilution well containing 100 µL of the two-fold antifungal concentration was inoculated with 100 µL of medium of the diluted of inoculum suspension. For each test plate, two antifungal free controls were included, one with medium alone and the other with 100 µL of medium plus 100 µL of inoculum suspension. Each type of inoculums suspension was treated with cyclohexamide (Sigma, USA) and T3.3 fungal extracts. The concentration assayed ranged from 0.039 to 10 mg mL^{-1}. The plates were incubated at 30°C and the endpoints were read visually after 5 days incubation. Assays were always run in duplicate (Rukayadi and Hwang, 2007).

2.9 Nuclear magnetic resonance analysis

Ten milligrams of each dried fraction was dissolved with 0.6 mL of deuterated acetone containing 0.05% Tetramethylsilane (Merck, Germany) as the reference peak. The samples were sonicated and centrifuged for 3 minutes and 10 minutes, respectively before directly transferred into 5 mm NMR tubes and subjected to ^{1}H-NMR analysis. ^{1}H-NMR spectra were

acquired at 25^0C on a Varian Unity INOVA 500 MHz spectrometer (Varian Inc, CA). All spectra were manually phased and baseline corrected. For each sample, 64 scans were recorded and the spectral width was adjusted to a range between 0.00 and 10.00 ppm.

2.10 Data processing and analysis

The ^{1}H-NMR spectra were automatically reduced to ASCII format file using Chenomx software (v. 5.1, Alberta, Canada). Spectral intensities were scaled in reference to TMS and binned into regions of 0.04 ppm width for the spectral region of 0 to 10 ppm. The signals between δ2.04-2.1 were excluded from the analysis since this due to residual signal from the deuterated acetone. The binned ^{1}H-NMR data were then subjected to PCA and PLS, performed using SIMCA-P+ version 12.0.1.0 (Umetrics AB, Umeå, Sweden) in unit Pareto scaling was applied in all analyses.

III. RESULTS AND DISCUSSION

3.1 Dry cell weight and antifungal activity of *P. oxalicum* T3.3

Based on Fig. 1, the highest dry cell weight was achieved in Richard medium on day 10 of fermentation compared to other media. Meanwhile, Malt extract broth and Sabouraud medium recorded lowest dry cell weight until the end of fermentation. The antifungal activities of the crude extracts of *P. oxalicum* T3.3 grown in different media were not detected during the early fermentation day. It is interestingly to note that the antifungal activity through disc diffusion test was only detected for Richard and Czapek dox medium on 10^{th} day of fermentation and decreased on subsequent day. Solvent fractions from both media showed the highest antifungal activity with 20% PIRG value during tenth and eleventh day of fermentation period. In contrast, Santamarina *et al.* (2002) reported that extracts from isolates of *P. oxalicum* Corrie and Thom cultured in PDB was more suitable for the production of antibiotic compounds. It can be deduced that the antifungal production was directly proportional to the biomass produced as the antifungal activity showed highest activity when the biomass was at the highest weight. Since the minimum antifungal activity of this strain was detected during 10^{th} day of the fermentation using Richard medium, this medium was hence selected in maximizing the production of antifungal compound in different carbon sources.

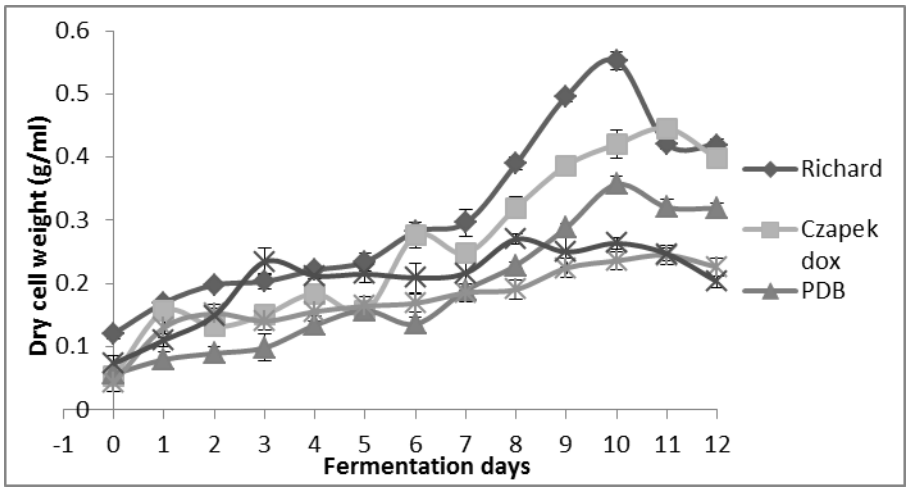


FIG. 1: DRY CELL WEIGHT OF *P. OXALICUM* T3.3 GROWN IN DIFFERENT FERMENTATION MEDIA AT 30^oC AND PH 5.5. ERROR BARS REPRESENT STANDARD DEVIATION FROM THREE REPLICATES

3.2 Biolog FF Microplate Analysis

Based on Biolog FF Microplate analysis, the growth of *P. oxalicum* T3.3 at 72 hour on 95 carbon sources and water as a control had shown variation. In this study, glucose, maltose, sucrose and xylitol were selected from the Biolog FF Microplate analysis and were used as the carbon sources in the fermentation medium (Table 1). This finding proved that *P. oxalicum* T3.3 is a versatile filamentous fungus as it able to utilize wide range of carbon sources including monosaccharides, disaccharides, polysaccharides and sugar alcohols. However, there are some of the carbon sources which are not suitable for the growth of this strain. According to Singh (2009), the growth of the cultures used has shown strong correlation between substrate utilization, antifungal activity and presence of the responsible secondary metabolite produced. However, some of the substrates tested did appear to support growth but not the production of the antimicrobial metabolite. Papaspyridi *et al.* (2011) supported that substrate utilization fingerprint obtained from Biolog FF Microplate analysis was useful in the

optimization of media components for maximum biomass as well as secondary metabolite production for the various cultures (Singh, 2009).

TABLE 1
CARBON-BASED SOURCES CONSUMPTION BY STRAIN T3.3 IN BIOLOG FF MICROPLATE ANALYSIS AFTER 72H

Well	Carbon	Turbidity (+)*
C11	Maltose	+++
E7	Sucrose	+++
E11	Xylitol	+++
C12	Maltotriose	+++
B12	α-D-glucose	++
A12	D-cellobiose	++
B5	D-fructose	++
E9	D-trehalose	++
A3	N-acetyl-D-galactosamine	-
E5	L-sorbose	-

'+++' represent the turbidity of Strain T3.3 is high, '++' means that turbidity is moderate, and '-' means no turbidity can be observed. Turbidity is referring to the biomass concentration of Strain T3.3.

3.3 In-vitro antagonist tests against *C. gloeosporioides*

3.3.1 Disc diffusion test

Disc diffusion methods are widely used to investigate the antimicrobial activity against certain fungi or bacteria using the Kirby-Bauer method (Bauer *et al*. 1966) with a minor modification. These assays are based on the use of discs as reservoirs containing solutions of substances to be examined (Bartner *et al.* 1994). A filter paper disc impregnated with sample fraction was placed on the agar in which the chemical constituents in it would be diffused from the disc into the agar around the disc. The solubility of the components and their molecular sizes determined the circumference of the area around the disc. Once *C. gloeosporioides* was placed on the agar, after the incubation period, it would not grow in the area around the disc when it was susceptible to the components. This area of no growth around the disc is known as a "zone of inhibition". The findings from this study revealed that, crude extracts of *P. oxalicum* T3.3 in all carbon sources shows a significant reduction in colony radial growth against *C. gloeosporioides* as showed in Fig. 2. Glucose crude extract of *P. oxalicum* T3.3 exhibited the highest antifungal activity with 75% inhibition against *C. gloeosporioides.* This was followed by sucrose crude extract and maltose crude extract which exhibited 40% to 30% inhibition, respectively. Xylitol and *U. pinnatifida* crude extracts, less than 25% antifungal activity were recorded (25 to 20% inihibition), respectively. Starch crude extract of *P. oxalicum* T3.3 shows the lowest reduction in colony radial growth with only 10% inhibition against *C. gloeosporioides.*

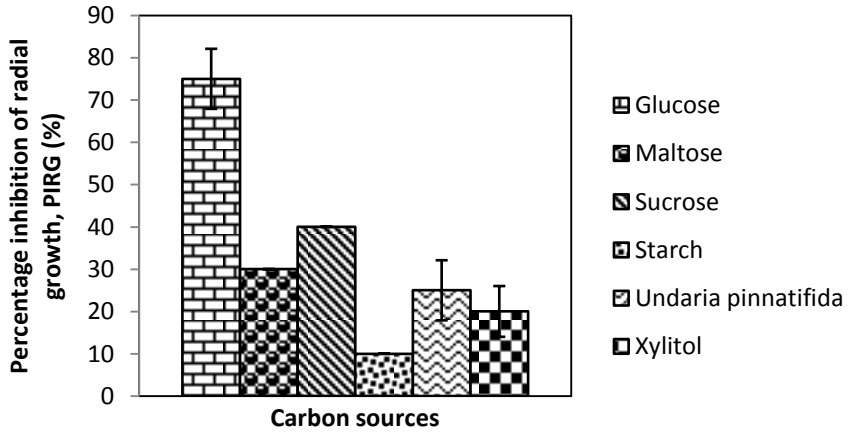


FIG. 2 PERCENTAGE INHIBITION OF RADIAL GROWTH, PIRG (%) FOR DIFFERENT CARBON SOURCES USED

Glucose extract of *P. oxalicum* T3.3 was found to be the most effective against *C. gloeosporioides* with more than 70% inhibition of radial growth (7.5 mm) compared to other carbon sources. Carbon sources played an important role in the onset and intensity of secondary metabolites produced (da Silva *et al.* 2012). Specificity in the production of antifungal compound towards *C. gloeosporioides* related to the carbon sources might be suggested as the reason why *P. oxalicum* T3.3 could not

give a high antifungal activity in all carbon sources. The finding in this study is in total agreement with Gebreel *et al.* (2008), wherein reported a higher production of antifungal activity was observed to be at the highest when glucose was used as the carbon sources. As reported by Lucas *et al.* (2007), the isolation of bioactive compounds such as pencolide, sclerotiorin and isochromophilone VI from the cultivation of *Penicillium sclerotiorum* in a liquid medium rich in glucose has shown a diameter of inhibition zone ranges from 10-16.75 mm against various bacteria species.

The utilization of glucose as a sole carbon source is preferable compared to others because glucose is the simplest sugar (monosaccharide) where it can be easily consumed by the cells as a source of energy and metabolic intermediate without any breakdown process. The inhibition zone produced by the crude extracts of *P. oxalicum* T3.3 was considered higher since the metabolites are in a mixture form where the crude did not undergo any isolation or fractionation of the bioactive compounds. This proved that the inhibitory action of the crude extracts showed a clear indication that the crude extracts contained active components that have antifungal properties. However, the efficiency of substituting starch as carbon source was not efficient enough since the starch extract of *P. oxalicum* T3.3 shows the lowest inhibition zone against *C. gloeosporioides.* This result was in total agreement with Mathan *et al.* (2013), where starch was the least utilized carbon compound by *Aspergillus terreus* KC 582297and produce lowest inhibition zone (6mm) whereas starch crude extract of *P. oxalicum* T3.3 producing 1 mm inhibition zone.

3.3.2 Minimum Inhibition Concentration (MIC)

Minimum inhibition concentration (MIC) of extracts of glucose, sucrose, maltose, xylitol, starch and *U. pinnatifida* were examined and recorded (Table 2). Minimum inhibitory concentration of glucose crude extracts the most active with the lowest MIC value with 78 µg mL^{-1}, followed by sucrose crude extracts with 156 µg mL^{-1}. Both maltose and *U. pinnatifida* crude extracts resulting on a MIC value of 313 µg mL^{-1}. In contrast, both crude extracts of xylitol and starch were much less active for all the tested strains with the highest MIC value, 1250 µg mL^{-1}.

TABLE 2
MIC VALUES FOR *P. OXALICUM* T3.3 EXTRACTS AGAINST *C. GLOEOSPORIOIDES*

Carbon sources	MIC (µg mL^{-1})
Glucose	78
Maltose	313
Sucrose	156
Starch	1250
Xylitol	1250
U. pinnatifida (seaweed)	313

The MIC of the glucose extract in this study is 78 µg mL^{-1} which is comparable with the MIC recorded for the metabolites produced by *P. sclerotiorum* isolated from Brazilian's cerrado soil samples (Lucas *et al.* 2009). Both Pencolide (I) and Isochromophilone VI (III) showed MIC values 64 µg mL^{-1} against *C. albicans*. Meanwhile, the sucrose extract with 156 µg mL^{-1} against *C. gloeosporioides* obtained from this study was considered higher when compared with Sclerotiorin (II) against *S. aureus* with MIC 128 µg mL^{-1}, metabolite produced from the same fungus (Lucas *et al.* 2007). According to Petit *et al.* (2009), there are three new naphthalenoids has successfully characterized from a *Penicillium* sp. isolated from Brazillian cerrado soil which consumed glucose as their carbon source in fermentation media. The corresponding minimum inhibition concentrations were determined and natural secondary metabolite methyl 6-acetyl-4-methoxy-5,7,8-trihydroxynaphthalene-2-carboxylate showed to be the most active compound against *Candida albicans* with MIC 32 µg mL^{-1} meanwhile against *Listeria monocytogenes* and *Bacillus cereus* with MIC value 64 µg mL^{-1} for both. Since there were no any isolation and purification steps for the crude extracts, the compounds responsible for the susceptibility test cannot be determined. However, from this test we can conclude that the metabolites produced by *P. oxalicum* T3.3 were able to suppress the growth of the pathogen, *C. gloeosporioides.* There are many secondary metabolites and chemical constituents produced by *P. oxalicum* extracts that may contribute to the observed positive antifungal effects. The MIC value obtained in this study explains that *P. oxalicum* T3.3 extracts act as antifungal agent against *C. gloeosporioides.*

3.4 Metabolomics of different carbon sources used using PCA and PLS data analysis

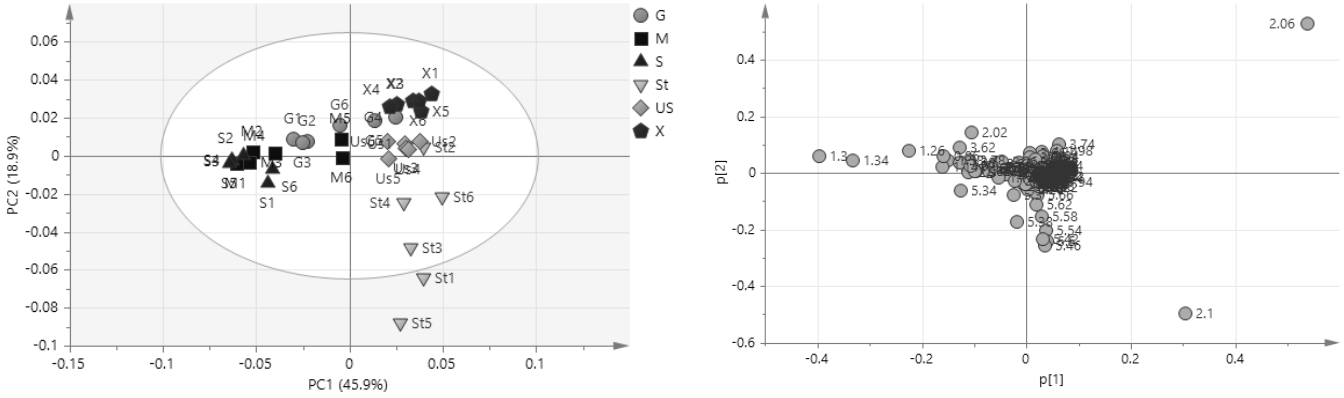
3.4.1 Principal component analysis (PCA)

Visual inspection of the ^{1}H NMR spectra of the six different carbon sources used showed the presence of different classes of metabolites which included fatty acids, amino acids, organic acids, alcohols, and sugars. Principal component analysis (PCA)

was used for the clustering of the six samples of different carbon sources and the metabolites contributing to the variability. The application of PCA in multivariate analysis by disclosing the samples to different principal components (PCs) is to identify the pattern and cluster of the samples depending on their variance (Mediani *et al.* 2012). All assignments of the ^{1}H NMR signals were accomplished by comparison with ^{1}H NMR spectra of reference compounds from Chenomx database and Yeast Metabolome Database (YMDB, http://www.ymdb.ca/) as shown in Table 3.

TABLE 3
LIST OF ^{1}H-NMR CHEMICAL SHIFTS AND VIP VALUES OF THE MAJOR COMPOUNDS CONTRIBUTING TO THE CLASSIFICATION IN THE PLS MODEL

^{1}H NMR chemical shift (δ)	Compound	VIP value	References
2.06	Acetic acid	6.56	Kim *et al.* (2011)
2.1	Methionine	4.97	Chenomx database
1.3	Threonine	4.77	Seung *et al.* (2012)
1.34	Lactate	3.62	Chenomx database
1.26	Isopropanol	3.02	Chenomx database
1.14	2,3-Butanediol	2.2	Georgiev *et al.* (2011)
3.74	Glycerol	2.06	YMDB
3.66	Leucine	1.98	Chenomx database
0.86	Butyrate	1.78	Chenomx database
5.42	Sucrose	1.72	Seung *et al.* (2012); Georgiev *et al.* (2011)
2.3	Valine	1.67	Chenomx database
2.46	*N*-carbamoylaspartate	1.62	Chenomx database
1.58	Butyrate	1.61	Chenomx database
3.94	Serine	1.25	Chenomx database
3.98	Serine	1.24	Chenomx database
0.9	Valine	1.23	YMDB
3.58	Threonine, fructose	1.17	Chenomx database
2.78	5-aminolevulinate	1.16	Chenomx database
6.58	Cinnamate	1.15	Chenomx database
4.18	*o*-Phosphoserine	1.13	Chenomx database
4.14	Uridine	1.07	Chenomx database
3.7	Leucine,glucitol	1.02	Chenomx database
7.46	Cytosine	1.01	Chenomx database



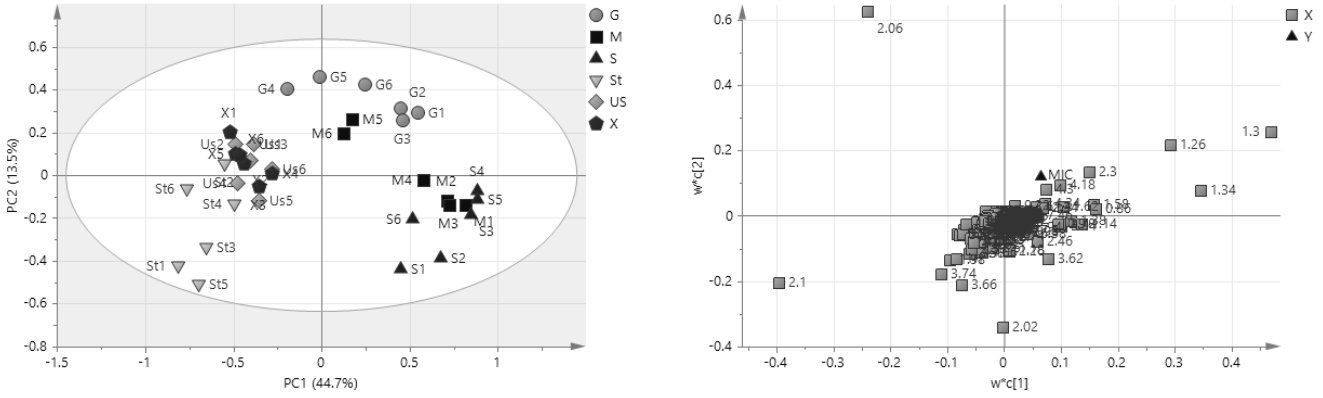
(A) **(B)**

FIG. 3: SCORE SCATTER PLOT (A) AND LOADING SCATTER PLOT (B) OF PCA SEPARATED BY PC1 AND PC2 OF THE STRAIN T3.3 EXTRACTS. SCORE PLOT (A) SHOWS THE DISCRIMINATION OF SIX CARBON SOURCES USED. LOADING PLOT (B) INDICATE THE 1H-NMR SIGNALS OF COMPOUNDS THAT ARE RESPONSIBLE FOR THE SEPARATION OF THE SIX CARBON SOURCES. (A) (●): glucose, (■): maltose, (▲): sucrose, (▼): starch, (♦): *U. pinnatifida*, (): xylitol

In this study, initial PCA which is an unsupervised analysis method was performed on the NMR data of the *P. oxalicum* T3.3 grown in different carbon sources to determine the primary observation between the clustering samples and to evaluate the variance in their metabolite content and concentration. The score plot was implemented to evaluate the variation within the processed samples with regard to the six carbon sources used, whereas the loading plot indicated the metabolite signals that may contribute to cluster differentiation as shown in Fig. 3(A) and (B). From the PCA score scatter plot (Fig. 3A), six samples were separated into two clusters between the carbon sources along PC1 with two notable outliers (St1 and St2). PC1 showed the most sample variation, followed by PC2. An eigen value of about 64.8% was described by the first two PCs wherein the first component PC1 contributed 45.9% of the variance, meanwhile by PC2 with 18.9%. The loading plot of the carbon sources (Fig. 3A) indicated the important chemical shifts responsible for discrimination in the score plots. The accumulation of the signals at the centre of the PC1 and PC2 axes indicating the consumption of these carbon sources might produce similar compounds. However, the intensity and concentration of each compound produced may differ and can be determined from the NMR spectra. The loadings illustrated the correlations between the original variables (chemical shifts) and the new variables which are the PCs (PC1, PC2, etc.). The signals in ppm on the loadings plot that fall within the same quadrant represent specific NMR spectral regions and the location of clusters in a score plot situated in the same quadrant/dimension in the loading plot is remarkably influenced by these variables (Khatib *et al.* 2011).

3.4.2 Partial least square (PLS)

The previous MIC results showed *P. oxalicum* T3.3as having the ability in producing metabolites that could inhibit the growth of *C. gloeosporioides*. Thus, in order to have further investigation between the correlation of MIC and the impact of the six carbon sources used, a supervised analysis which is known as Partial least square (PLS) was applied using a validation model with a degree of over fit between the variables and the responses (Maisuthisakul *et al.* 2008). PLS expands a regression of PCA and uses class data to maximize the separation between groups of observations. This frequently used classification approach is categorical which pronounces the class membership of the statistical units (Eriksson *et al.* 2006; Barker and Rayens, 2003). Furthermore, PLS was performed to acquire a more precise view on the correlation between the MIC activity and the carbon sources used. PLS has been reported as a better statistical model in linking DPPH with phytochemicals and also serves as a model for prediction (Kim *et al.* 2011). According to Maisuthisakul *et al.* (2008), the PLS model could illustrate the correlation between chemical and DPPH data, which used in predicting variations among dried samples. The determination of a correlation between anti-radical activity ($1/IC_{50}$) and phytochemical composition of extracts from different drying effects samples also has been done by using PLS evaluation (Mediani *et al.* 2012).



(A) **(B)**

FIG. 4: PLS-DERIVED SCORE PLOT (A) AND PLS-DERIVED LOADING PLOT (B). (A) (●): glucose, (■): maltose, (▲): sucrose, (▼): starch, (♦): *U. pinnatifida*, ⬟): xylitol. (B) (■):^{1}H-NMR signals of compounds that are responsible for the separation of the six carbon sources, (▲): MIC value as the Y-variable

In this study, PLS was performed using the ^{1}H NMR data of the extracts/fractions and the data were scaled using Pareto (par) by the SIMCA-P 12.0 software. In the PLS analysis, samples were well separated and showing a significantly obvious discrimination between the carbon sources along PC1 (Fig. 4A). Meanwhile, the monosaccharide and disaccharide sugar samples were strongly correlated with MIC rather than complex sugar samples as shown in Fig. 4B.The metabolites produced by the extracts from monosaccharide and disaccharide group of sugars, glucose, maltose and sucrose were well separated along PC1 from those extracts of the polysaccharide and complex sugar of starch, xylitol and *U. pinnatifida*. The

spectral data of the glucose and sucrose samples were relatively close with those of maltose and xylitol, while *U. pinnatifida* and starch samples were found to partially overlapped with each other.

The monosaccharide and disaccharide sugar samples were strongly correlated with MIC. Meanwhile the complex sugars were projected in the negative side of the plot far away from MIC. The compounds contributed to this separation by PC1 were identified as threonine (δ1.3), 2-heptanone (δ1.26), lactate (δ1.34), valine (δ2.3), butyrate (δ1.58 and δ0.86), and *o*-phosphoserine (δ4.18). All of these compounds were located closer to monosaccharide, disaccharide samples and MIC activity rather than complex sugar samples as shown in Figure 4B. However, the complex sugar samples were separated from the others by their high content of acetic acid (δ2.06), methionine (δ2.1) and leucine (δ3.66). This finding confirmed that the MIC, which showed that the extracts from glucose and sucrose had the highest positive impact on the in-vitro susceptibility test against *C. gloeosporioides.*

PLS model validation was carry out by checking the Q^2 and R^2 accumulative means after cross validation and permutation test (Mediani *et al.* 2012). The goodness of fit of the PLS model was performed and represented by R^2Y, where R^2 determines the model fitness, while the predictive ability was indicated by Q^2Y in which, Q^2 provides the predictive quality of the model (Barker and Rayens, 2003). Generally, R^2Y varies between 0-1 and 1 indicates a model with a perfect fit. Meanwhile models with Q^2Y values >0.5 and >0.9 are considered to have excellent and good predictability. As shown in Table 4, R^2Y and Q^2Y values for PLS model in this study are 0.830 and 0.687, respectively which are a good fitness and predictive abilities. Furthermore, permutation test was performed with 20 permutations to validate the PLS model. According to Eriksson *et al.* (2006), permutation test provides the statistical significance of the estimated predictive power of models by comparing R^2Y and Q^2Y values from the original model with the values for a reordered model, which was created newly whenever Y-data was permutated at random. It was noted that R^2Y-intercept should not exceed 0.3-0.4 and that Q^2Y-intercept should not exceed 0.05. The R^2Y-intercept and Q^2Y-intercept values for the PLS model in the present study were 0.449 and -0.408 respectively as shown in Table 4. Even though the R^2Y-intercept value is slightly higher than the validation value, with proper Q^2Y-intercept value that was under recommended cut-off, it is suggested that the PLS model in this study was successfully validated by permutation testing.

TABLE 4
PLS MODEL PARAMETERS DERIVED FROM THE DATA SAMPLES

PLS parameters	Values	Validation values
R^2Y	0.830	0-1
Q^2Y	0.687	0.5-0.9 and 0.9-1.0
R^2Y-intercept	0.449	0.3-0.4
Q^2Y-intercept	-0.408	<0.05

IV. CONCLUSION

In conclusion, among 6 carbon sources used, glucose crude extract showed the highest inhibition zone with 75%. PIRG and exhibited the lowest MIC and MFC values with 78 and 2500 µg/mL, respectively. Meanwhile, from PLS analysis, it was observed that the sugar crude extracts which are glucose, maltose and sucrose extracts contained more threonine, 2-heptanone, lactate, valine, butyrate, *o*-phosphoserine whereas the complex sugar extracts which are starch, *U. pinnatifida* and xylitol have higher acetic acid, methionine and leucine. The results obtained in this study gave a representative impression of the metabolites present in *P. oxalicum* T3.3 with different carbon sources.

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Pretreatment with 1-methylcyclopropene (1-MCP) reduced the flower abscission in *Phaleonopsis* cut orchid

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Abstract*—Phaleonopsis cut orchids were pretreated for 6 hours at 25 °C with or without 1-MCP. Treated cut orchids were exposed to 800 ppm of 1- methylcyclopropane (1-MCP). Then, all cut flowers were treated with ethylene for 15 hours and after that were held in flask containing flower food individually at 25 °C to follow abscission. It was observed that, 20–30% of the floral buds and flowers abscised within 4 days in untreated sample. However, in treated sample, the 1-MCP pretreatment reduced the bud and petal abscission and the cut orchids were still maintained acceptable until day 7 before starting to abscise between 10-14 days of storage period. Result also showed that the ethylene production was inhibited and ACC oxidase activity was decreased in samples treated with 1-MCP. Thus, 1-MCP pretreatments prolong the shelf life of cut orchids from 4 days in control up to 10 days in treated samples, both displayed in 25 °C.*

***Keywords*—ornamental, ACC oxidase activity, ethylene production, quality, shelf life.**

I. INTRODUCTION

The critical criteria of cut flowers are the quality and appearance at the retail and consumer level. Once the flowers are cut from their mother plant, they are gotten to be perishable and sensitive to the environment. They are sensitive to ethylene, agaseous plant hormone that significantly influences their development and growth. A few detrimental consequences of ethylene exposure are premature wilting, flower or petal drop and abnormal opening (Nur Azlin et al., 2013). So, to expand market, the cut orchid needs to have a long vase life, consequently it gets premium price.

In numerous studies to expand the shelf life of ornamental plants, inhibiting the reaction to ethylene is a proficient procedure. Some ethylene inhibitors for example aminoethoxyvinylglycine (AVG), silver ions and aminooxyacetic (AOA) have been assessed for their impacts on ethylene responses. Other than that, the revelation of cyclopropenes, specifically 1-methycyclopropene (1-MCP) is successfully inhibiting ethylene reactions (Sisler, E.C. and Serek, M. 1997, 2003). Application of 1-MCP detailing its advantageous effect against ethylene such as in chrysanthemum and carnation (Hassan and Gerzson, 2002), rosa hybrid (Serek et al., 1994) and many other ornamental species.

Extending the storage life of cut orchids would able them to be distributed in further markets thus gives more prominent opportunity for growers and exporters. Extending the storage life would also result in greater flexibility and efficiency in dealing with cut orchids. Premium Malaysian cut orchid's quality package with long shelf life, all of these will benefit in setting up a premium price thus move forward the economy and develop Malaysian cut flowers industry. Therefore, this study was aimed to evaluate the potential of 1-MCPpretreatment in reducing flower abscission in *Phaleonopsis* cut orchids.

II. MATERIAL AND METHOD

2.1 Plant material

Export grade *Phaleonopsis* cut orchids grown in southern Malaysia were purchased from a commercial farm. The flowers were harvested at commercial maturity stage (50% blooming flowers) and transported on the day of harvest to the Postharvest Laboratory at MARDI, Malaysia. The flowers were sorted for the shape, uniformity of size and defects. Flowers (n=12) were randomized into two treatment groups:1. without 1-MCP or 2. with 1-MCP.

2.2 1-MCP pretreatment

The cut orchids were placed and remained sealed in a plastic chamber at 25°C for 4 h. The amounts of 1-MCP sachet (EthylBloc^{TM}, Floralife, SC, USA) were determined to supply a 10ppm 1-MCP concentration as recommended by manufacturer. Before sealing the chamber lid, 15ml of distilled water was added into the container that contained the

EthylBloc^{TM} sachet. Immediately after addition of water, a proportion of 1-MCP gas was released. Cut orchids that placed in other identical chambers without 1-MCP treatment were set as control.

2.3 Ethylene exposure

After 4 h of 1-MCP treatment, all cut orchids were treated with 1ppm ethylene gas (one injection) for 15 hours at 25 °C. Then, all cut orchids were individually held in 250 ml conical flask containing flower food and displayed in laboratory at ambient temperature of 25 °C.

The bud and flower abscission's rate were observed weekly. When 50% of the flowers had abscised, the shelf life of a cut flower was considered terminated. The ethylene production and capacity to convert ACC to ethylene in vivo were also evaluated during observation.

2.4 Ethylene Production

The ethylene gases produced by cut orchids were measured every 3 days. The method used in this study was similar to method described by Nur Azlin et al.(2013). Following sampling, lids were removed from each container; samples were determined every 2 days for 7 days.

2.5 ACC oxidase Activity

The determination of ACC oxidase activity was based on method described by Fernandez-Maculet and Yang (1992) with modification. Briefly, 8mm in diameter and 10mm in thickness discs were excised from petals. The discs were extracted in 10mM ACC buffer for 30 minutes to measure in vivo ACC oxidase activity. The ethylene gas produced during the incubation was measured using gas chromatography as described by Nur Azlin et al. (2013).

2.6 Statistical Analysis

The results were statistically analyzed using Analysis of Variance (ANOVA). Means separation for each variable was performed using Least Significant Difference (LSD). The means of the main effect are presented in tables (SAS 9.4, Cary, North Carolina).

III. RESULTS AND DISCUSSION

3.1 Rate of flower and bud abscission

Flowers (n=12) from each treatment were evaluated every 4 and 7 days of storage. In white *Phaleonopsis* cut orchids, results showed that buds (20%) and flowers (30%) were abscised within 4 days in control. Fig. 1 showed the flower abscission of white *Phaleonopsis* cut orchids upon arriving, day 4 and day 7 stored in 25 °C. Cut orchids pretreated with 1-MCP maintained fresh with all buds and flowers still intact for 7 days of storage period. The same effect of 1-MCP was also been reported in Pelargonium cut flowers (Cameron and Reid, 2001).This result was also in line with study from Jones et al., 2001, who reported that pretreatment of Geranium inflorescence with the 1-MCP was effective at reducing petal abscission in all the cultivars.

FIG. 1: FLOWER ABSCISSION OF WHITE *PHALEONOPSIS* CUT ORCHIDS UPON ARRIVING, DAY 4 AND DAY 7, ALLDISPLAYED IN 25 °C.

In *purple Phaleonopsis* cut orchids, result showed that bud (30%) and flower (20%) were abscised within 4 days in control as shown by Fig. 2. The abscission rate in control was higher in day 7 and the samples were totally discarded. The 1-MCPtreated samples maintained intactup to day 10 and started to abscise at day 14 with 30% of the buds and 20% of the flower abscised. Bud and flower abscission of white and purple Phaleonopsis cut orchids at day 7 stored in 25 °C were shown in Fig. 3. Thus, pretreatment with 1-MCPcould extend their shelf life from 4 days in control to 10 days in 1-MCP treated samples.

FIG. 2. FLOWER ABSCISSION OF PURPLE PHALEONOPSIS CUT ORCHIDS UPON ARRIVING, DAY 4 AND DAY 7, ALLDISPLAYED IN 25 °C

(A) **(B)**

FIG. 3. BUD AND FLOWER ABSCISSION OF WHITE AND PURPLE *PHALEONOPSIS* CUT ORCHIDS, (A) CONTROL & (B) 1-MCP TREATMENT, BOTH AT DAY 7DISPLAYED IN 25°C

3.2 Ethylene Production

Ethylene production of white *Phaleonopsis* cut orchids treated with 1-MCP was decreased, 0.761µl/kg/hour in control compared to 0.725µl/kg/hour in treated samples. In purple *Phaleonopsis* cut orchids, 1-MCP treatment reduced the ethylene concentration by 0.798µl/kg/hour in control to 0.735µl/kg/hour in treated samples. However, it was no significant difference ($p>0.05$) between control and treated sample in both white and purple *Phaleonopsis* cut orchids for ethylene production (Table 1 and 2).

1-MCP treatment slow down the ACC oxidase activity for both white and purple *Phaleonopsis* cut orchids. The ACC oxidase activity was higher in control sample (15.774µl/kg/hour) and significantly ($p<0.05$) reduced to 10.293µl/kg/hour in treated white *Phaleonopsis* cut orchids. In purple *Phaleonopsis* cut orchids, the ACC oxidase activity in control sample was 17.481µl/kg/hour and reduced to 14.338µl/kg/hour in treated samples. This reduction affected the conversion of ACC to

ethylene and as a result, the ethylene production in treated samples also reduces. This finding was in line with Ketsa et al. (2007) that reported the same result in Dendrobium orchids treated with 1-MCP.

TABLE 1
ACC OXIDASE ACTIVITY AND ETHYLENE PRODUCTION OF WHITE *PHALEONOPSIS* CUT ORCHIDS AFTER PRETREATMENT WITH 1-MCP AND STORAGE AT 25°C.

Treatments	ACC oxidase activity (µl/kg/hour)	Ethylene production (µl/kg/hour)
Without 1-MCP	15.774a	0.761a
With 1-MCP	10.293b	0.725a

Means within a main factor followed by the same letter in the column are not significant (p<0.05).

TABLE 2
ACCO ACTIVITY AND ETHYLENE PRODUCTION OF PURPLE *PHALEONOPSIS* CUT ORCHIDS AFTER PRETREATMENT WITH 1-MCP AND STORAGE AT 25 °C.

Treatments	ACCO activity (µl/kg/hour)	Ethylene production (µl/kg/hour)
Without 1-MCP	17.481a	0.798a
With 1-MCP	14.338b	0.735a

Means within a main factor followed by the same letter in the column are not significant (p<0.05).

IV. CONCLUSION

In conclusion, 1-MCP pretreatment extend the shelf life and reduce the abscission rate of buds and flowers in *Phaleonopsis* orchids. The 1-MCP seems to reduce ethylene production in cut orchids by limiting the ACC to convert to ethylene exhibit by decrease in ACC oxidase activity.

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