IJOEAR

International Journal of Environmental & Agriculture Research

www.ijoear.com

ISSN
2454-1850

Volume-4, Issue-1, January 2018

Preface

We would like to present, with great pleasure, the inaugural volume-4, Issue-1, January 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:

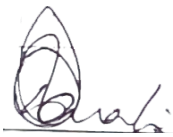
Environmental science and regulation, Ecotoxicology, Environmental health issues, Atmosphere and climate, Terrestric ecosystems, Aquatic ecosystems, Energy and environment, Marine research, Biodiversity, Pharmaceuticals in the environment, Genetically modified organisms, Biotechnology, Risk assessment,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



Dr. Bhagawan Bharali
(Managing Editor)

Fields of Interests

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

Agricultural Input Products	
Crop Protection Chemicals	Feed supplements
Chemical based (inorganic) fertilizers	Organic fertilizers
Environmental Science	
Environmental science and regulation	Ecotoxicology
Environmental health issues	Atmosphere and climate
Terrestric ecosystems	Aquatic ecosystems
Energy and environment	Marine research
Biodiversity	Pharmaceuticals in the environment
Genetically modified organisms	Biotechnology
Risk assessment	Environment society
Theoretical production ecology	horticulture
Breeding	plant fertilization

Board Members

Dr. V K Joshi

Professor V.K.Joshi is M.Sc., Ph.D. (Microbiology) from Punjab Agricultural University, Ludhiana and Guru Nanak Dev University, Amritsar, respectively with more than 35 years experience in Fruit Fermentation Technology, Indigenous fermented foods, patulin ,biocolour ,Quality Control and Waste Utilization. Presently, heading the dept. of Food Science and Technology in University of Horticulture and Forestry, Nauni-Solan (HP), India.

Mr. Aklilu Bajigo Madalcho

Working at Jigjiga University, Ethiopia, as lecturer and researcher at the College of Dry land Agriculture, department of Natural Resources Management.

Dr. Vijay A. Patil

Working as Assistant Research Scientist in Main Rice Research Centre, Navsari Agricultural University, Navsari. Gujarat- 396 450 (India).

Dr. S. K. Jain

Presently working as Officer Incharge of All India Coordinated Sorghum Improvement Project, S. D. Agricultural University, Deesa, Gujarat.

Dr. Salvinder Singh

Presently working as Associate Professor in the Department of Agricultural Biotechnology in Assam Agricultural University, Jorhat, Assam.

Dr. Salvinder received MacKnight Foundation Fellowship for pre-doc training at WSU, USA – January 2000- March 2002 and DBT oversease Associateship for Post-Doc at WSU, USA – April, 2012 to October, 2012.

Mr. Anil Kumar

Working as Junior Research Officer/Asstt. Prof. in the dept. of Food Science & Technology in Agriculture & Technology, Pantnagar.

Table of Contents

The Effect of Different Seeding Rates on Grain Yield and Yield Components in SomeFlax (*Linumusitatissimum L.*) Varieties

Yasemin ERDOĞDU[1], Seviye YAVER[2], Fadıl ONEMLI[3]

Department of Field Crops, NKU University, TEKIRDAG-TURKEY

***Abstract*—** *This study was carried out during the growing seasons of 2014/2015 and 2015/2016, in the Application and Experiment Fields of Namık Kemal University, Faculty of Agriculture, Department of Field Crops, according to the Split-Plot Experiment Design in Randomized Blocks with 3 replications. In the study, it was aimed to determine the effect of three different amounts of seeds (30-50 and 70 kg/ha) applied to the unit area on the grain yield and yield components in five varieties of flax (Selection, York, Nekoma, Pembina, Neche). In the first year of the experiment on these genotypes, the plant height was 70.91-77.46 cm, the number of axillary branches was 6.50-10.24, the number of capsules was 48.46-81.87, the thousand grain weight (TGW) was 5.51-5.85 g and the grain yield was 1260.00-1863.30 kg/ha; while in the second year, the plant height was 74.61-82.25 cm, number of capsules was 14.50-27.52, the TGW was 5.51-5.83 g and the grain yield was 1715.50-2071.10 kg/ha. Due to the increase in the sowing norm, the number of plants increasing in the unit area has also significantly increased the grain yield in both years. In addition, it was observed that the number of axillary branches, the number of capsules, and the weight of thousand grains significantly decreased with the increasing plant density, although there was no difference in plant heights between the two years in accordance with the increase in the number of seeds. While the number of seeds in the capsule was not affected by the number of seeds in the first year of the experiment, it decreased significantly in the second year when the number of seeds used in the unit area increased.*

***Keywords*— oilseed flax, seeding rate, grain yield, number of capsules, thousand grain weight.**

I. INTRODUCTION

Flax (*Linumusitatissimum L.*) is a multi-purpose annual industrial plant, whose seeds are used and fiber is obtained from its stems. The flax's fibers obtained from its stems, its seeds, oil, and residue have been used in different sectors. The oil, which is approximately found in the rate of 35-46% in the flaxseed, has been used in chemical industry areas such as paint and varnish production due to having high amount of linoleic (16-75%) and alpha-linolenic (17-59%) acids in it and due to being a polyunsaturated oil (M. Zuk et al., 2015).

The need for the oilseeds has been increasing every year throughout the world, and the flax has also been contributing to the production of the oilseeds in addition to the produced major oil plants. The flax, which is produced approximately 2.5 million tons in the world, is mostly cultivated in Canada, Kazakhstan, China, Russia, USA and India (FAO 2017). Considering the existence of agricultural areas in the world, since the horizontal growth is not possible, vertical growth should be considered; and selection of the plant types and agronomic applications should be emphasized to increase the yield in the unit area.

There are three major components of yield in the production of flaxseed, namely, number of capsules, number of seeds in capsules and seed weight (Lafond 2001). Knowing the amount of seed is one of the most important facts of the agronomic applications (Casa et al., 1999). It has also been confirmed in scientific studies that the grain yield varies depending on agronomic applications and environmental factors, i.e. while the yield was 3,310-4,360 kg/ha in England (Turner 1991), it was 950-2,795 kg/ha in Germany (Diepenbrock and Parksen 1992).

Studies have shown that the amount of seeds is important in flax and that the flax varieties can react differently to the plant density (Gabiana 2005). Studies on the number of seeds to be sowed in the unit area in the oilseed flax found the ideal rate as 200-400 plants/m^2 for the grain yield (Turner 1991; Diepenbrock and Parksen 1992; Gubbles and Kenaschuk 1989; Lisson and Mendham 2000) and reported that this plant density increased the photosynthesis capacity of the plant as a result of the length of the green leaf area during capsule and seed development (Diepenbrock and Parksen 1992). On the other hand,

Hassan and Leitch (2000) reported that when the plant density increased, plants entered into competition with each other, resulting in fewer and shorter branches with smaller leaves, limited plant growth and decreased number of capsules. In another study, it was observed that high sowing norm increased plant height, leading to the problem of leaning in plants (Lisson and Mendham 2000). Leitch and Sahi (1999) reported that when the plant density in the unit area increased, the individual plant size decreased, while the total dry matter ratio obtained from the unit area increased. In some studies on flax, the difference between the numbers of plant parts in the unit area did not make a difference in both dry matter and grain yield as a result of plants' denser branching and filling the existing gaps due to increased plant size (Khan and Bradshaw, 1976).

It is observed that the effects of climate and soil factors are great on the difference found in terms of the effects of plant density on grain yield and yield components in the conducted researches. It is important to determine the most suitable plant frequency by carrying out studies in all of the ecologically different production areas. In this study, it was aimed to determine the ideal amount of seeds in Tekirdağ conditions, considering the yield and yield components of different flax varieties.

II. MATERIAL AND METHOD

This study was carried out during the growing seasons of 2014/2015 and 2015/2016, in the Application and Experiment Fields of Namık Kemal University, Faculty of Agriculture, Department of Field Crops. The total rainfall was 519.4 mm, the average temperature was 12.01°C during the growing season of 2014/2015, while the relative humidity was 78.8%, the total precipitation was 324.4 mm, the average temperature was 13.35°C, and the relative humidity was 79.2% during the growing season of 2014/2015, when the experiments were carried out. When the average of the flax growing seasons of the region for a long period of time, the season average of precipitation in 2014/2015 was the same as the average rainfall for many years, while, in 2015/2016 the precipitation was about 200 mm lower. When the temperature averages are examined, the figures are close to each other and the average temperature in 2015/2016 is found high. Regarding the relative humidity values, there was no difference between the average relative humidity values for a long period of years and no difference was found between the years when the tests were conducted.

In both years, the soil of the test site was found to be neutral, unsalted, with very little lime, poor in organic matter, and sufficient in terms of phosphorus and potassium as the result of the soil analysis.

Selection, York, Nekoma, Pembina and Neche varieties were used in the research. The experiments were carried out with 3 replications in the Split-Plot Experiment Design in Randomized Blocks, with the main parcels formed by the varieties and the seed quantities (30, 50 and 70 kg/ha) forming the sub-parcels. Each sub-parcel consisted of 5 rows with a length of 5 m. In sowing, the distance between rows was kept constant at 20 cm. The experiments were conducted manually on October 21, 2015 in the first year and on November 7, 2016 in the second year.

The test sites were given 20-20-0 compound fertilizer with 60 kg/ha of pure nitrogen and 60 kg/ha of pure phosphorus in both growing years. In addition, during the flowering period of the plants, urea (46%) fertilizer was given in the form of pure nitrogen of 30 kg/ha. Mechanical intervention was applied against weeds.

In the harvest carried out in June 11, 2015 in the first year and in June 20, 2016 in the second year, 50 cm of margins were left on the edge rows of each parcel, at the beginnings and the ends of the rows. In the research, plant height, number of axillary branches, number of capsules, number of seeds per capsule, thousand grain weight and grain yield were examined.

For the data obtained from the experiments, variance analysis was performed by dividing the years according to the Split-Plot Experiment Design in Randomized Blocks. The statistical significance of differences between the averages was determined according to the LSD test, and JUMP program pack was used during the evaluations.

III. RESULT AND DISCUSSION

Average values and significance groups of seeding rate and variety × seeding rate interaction, plant height, number of axillary branches, number of capsules, number of seeds per capsule, thousand grain weight and grain yield are given in Table 1 and Table 2.

TABLE 1
AVERAGE VALUES AND SIGNIFICANCE GROUPS OF PLANT HEIGHT, NUMBER OF AXILLARY BRANCHES, NUMBER OF CAPSULES

Plantheight (cm)					
Year	**Varieties**	**Seedingrates kg/ha**			
		30	**50**	**70**	**Ort.**
2014-2015	Seleksiyon	70.76 de	69.40 e	76.45 ab	72.2bc
	York	71.00 de	70.13 e	71.60 cde	70.91 c
	Nekoma	75.70 abc	69.73 e	74.84 bcd	73.41 b
	Pempina	79.20 a	79.70 a	73.48 b_e	77.46 a
	Neche	71.20de	76.86 ab	75.83 abc	74.63 b
	Ort.	73.57	73.16	74.43	73.72
	LSD %: V= 2.47** S.R= ns V × S.R= 4.28** C.V. % = 3.47				
2015-2016	Seleksiyon	74.83	73.80	75.20	74.61 b
	York	77.90	74.23	74.90	75.67 b
	Nekoma	81.36	77.90	80.46	79.91 a
	Pempina	82.00	81.96	82.63	82.20 a
	Neche	82.40	82.03	82.33	82.25 a
	Ort.	79.70	77.98	79.10	78.93
	LSD %: V=2.46** S.R= ns V × S.R= ns C.V. % = 3.23				
Number of AxillaryBranches(perplant)					
Year	Varieties	Seedingrates kg/ha			
		30	50	70	Ort.
2014-2015	Seleksiyon	10.22 ab	6.59 efg	7.75 cde	8.19 b
	York	10.83 a	9.06 bc	10.83 a	10.24 a
	Nekoma	8.16 cd	6.80 d_g	5.60 g	6.85 c
	Pempina	6.55 efg	6.76 efg	6.20 fg	6.50 c
	Neche	7.62 de	7.07 def	5.65 g	6.78 c
	Ort.	8.67 a	7.26 b	7.21 b	7.71
	LSD %: V=0.79** S.R=0.61** V× S.R=1.38** C.V. % = 10.71				
2015-2016	Seleksiyon	6.60	4.75	5.15	5.50
	York	5.70	5.56	5.34	5.53
	Nekoma	5.44	4.82	5.30	5.18
	Pempina	5.98	4.85	4.63	5.15
	Neche	5.75	4.77	4.43	4.98
	Ort.	5.89 a	4.95 b	4.97 b	5.27
	LSD %: V=nsS.R=0.94 ** V× S.R=ns C.V. % = 10.75				
Number of Capsules (perplant)					
Year	Varieties	Seedingrates kg/ha			
		30	50	70	Ort.
2014-2015	Seleksiyon	68.37 c	51.70 ef	45.87 fgh	55.30 c
	York	98.97 a	63.25 cd	83.40 b	81.87 a
	Nekoma	77.30 b	81.33 b	34.25 ı	64.29 b
	Pempina	56.43 de	47.60 fg	41.35 ghı	48.46 d
	Neche	63.90 cd	59.75 d	38.86 hı	54.17 c
	Ort.	72.99 a	60.72 b	48.74 c	60.82
	LSD %: V=4.32** S.R=3.35** V × S.R=7.49** C.V. % = 7.36				
2015-2016	Seleksiyon	21.26 de	15.74 fgh	14.73 fgh	17.24 bc
	York	35.71 a	21.73 cd	25.13 bc	27.52 a
	Nekoma	17.93 ef	17.53 fg	14.40 gh	16.62 c
	Pempina	27.01 b	14.12 h	15.23 fgh	18.78 b
	Neche	17.99 ef	12.82 h	12.70 h	14.50 d
	Ort.	23.98 a	16.39 b	16.44 b	18.93
	LSD %: V=1.96** S.R= 1.52 ** V× S.R=3.40 ** C.V. % =10.73				

significant at p<0.05 probability level, ** significant at p<0.01 probability level ns: not significant, LSD: Least Significant Difference, C.V.: Coefficient of Variation

TABLE 2
AVERAGE VALUES AND SIGNIFICANCE GROUPS NUMBER OF SEEDS PER CAPSULE, THOUSAND GRAIN WEIGHT AND GRAIN YIELD

Number of SeedsPer Capsule					
Year	**Varieties**	**Seedingrates kg/ha**			
		30	**50**	**70**	**Ort.**
2014-2015	Seleksiyon	9.33 ab	9.00 b	9.06 b	9.13
	York	9.40 ab	8.93 b	9.16 ab	9.16
	Nekoma	9.36 ab	9.16 ab	9.36 ab	9.30
	Pempina	9.63 a	9.30 ab	9.40 ab	9.44
	Neche	9.20 ab	9.40 ab	9.26 ab	9.28
	Ort.	9.38	9.16	9.25	9.26
	LSD %: V=ns S.R.= ns V× S.R=0.51* C.V. % = 3.30				
2015-2016	Seleksiyon	8.93	8.70	8.83	8.82
	York	8.80	9.46	8.53	8.93
	Nekoma	8.90	8.83	8.76	8.83
	Pempina	9.13	8.63	8.43	8.73
	Neche	9.26	9.06	8.96	9.10
	Ort.	9.00 a	8.94 a	8.70 b	8.88
	LSD %: V=ns S.R=0.22* V × S.R= ns C.V. % = 3.42				
ThousandGrainWeight (g)					
Year	Varieties	Seedingrates kg/ha			
		30	50	70	Ort.
2014-2015	Seleksiyon	5.78 cde	5.57 g	5.51 gh	5.62 c
	York	5.51 gh	5.53 gh	5.50 gh	5.51 d
	Nekoma	5.72 ef	5.69 f	5.47 h	5.63 c
	Pempina	6.00 a	5.74 ef	5.81 c	5.85 a
	Neche	5.88 b	5.81 cd	5.75 def	5.81 b
	Ort.	5.78 a	5.67 b	5.61 c	5.68
	LSD %: V=0.037** S.R=0.029** V × S.R=0.065** C.V. % = 0.68				
2015-2016	Seleksiyon	5.64 e	5.54 fg	5.47 h	5.55 c
	York	5.52 fg	5.51 gh	5.50 gh	5.51 d
	Nekoma	5.71 cd	5.67 de	5.53 fg	5.64 b
	Pempina	5.94 a	5.81 b	5.75 c	5.83 a
	Neche	5.74 c	5.64 e	5.57 f	5.65 b
	Ort.	5.71 a	5.63 b	5.56 c	5.63
	LSD %: V=0.027** S.R=0.021** V × S.R=0.048** C.V. % = 0.51				
GrainYield (kg/ha)					
Year	Varieties	Seedingrates kg/ha			
		30	50	70	Ort.
2014-2015	Seleksiyon	1760.00 c	1200.00 g	2043.30 a	1667.70 c
	York	906.60 h	1310.00 f	1563.30 d	1260.00 d
	Nekoma	1783.30 c	1746.60 c	2060.00 a	1863.30 a
	Pempina	1456.60 e	1643.30 d	1910.00 b	1670.00 c
	Neche	1610.00 d	1786.60 c	1786.60 c	1727.70 b
	Ort.	1503.30 b	1537.30 b	1872.60 a	1637.70
	LSD %: V=5.53** S.R=4.28** V × S.R=9.56** C.V. % = 3.49				
2015-2016	Seleksiyon	1840.00 de	1243.30 h	2123.30 bc	1735.50 b
	York	1893.30 d	1403.30 g	1850.00 de	1715.50 b
	Nekoma	2223.30 b	1610.00 f	2380.00 a	2071.10 a
	Pempina	1753.30 e	1446.60 g	2060.00 c	1753.30 b
	Neche	2160.00 bc	1880.00 d	2150.00 bc	2063.30 a
	Ort.	1974.00 b	1516.60 c	2112.60 a	1867.70
	LSD %: V=7.25** S.R=5.63** V × S.R=12.59** C.V. % = 4.03				

significant at p<0.05 probability level, ** significant at p<0.01 probability level ns: not significant, LSD: Least Significant Difference C.V.: Coefficient of Variation

3.1 Plant Height

According to the results of variance analysis, plant height differences between the varieties in both years were statistically significant at 0.1% level. The plant height values of varieties varied between 70.91 and 77.46 cm during the growing season of 2014-2015 (Table 1). In this winter growing season, the highest plant height was obtained from the Pempina variety while the lowest plant height was obtained from the York variety. During the growing season of 2015-2016, the plant height values of the varieties ranged from 74.61 to 82.25 cm and the highest plant height was obtained from the Neche variety. This variety was followed by Pempina and Nekoma varieties with an insignificant difference. The lowest plant height was measured in the selection range. The varieties studied in the experiment showed similar performance in both trial periods. The varieties with the highest plant height in both years were Pempina and Neche while the varieties with the lowest plant height were the Selection and York varieties. When the average plant height performances according to years are examined, the plant height of the varietiesis measured higher in the growing season of 2015-2016. This may be due to the difference in climate factors between years.

As the result of the variance analysis, the effect of the seeding rates on the plant height was found to be statistically insignificant in both growing season. Plant height values according to seed amount varied between 73.16-74.43 cm in the growing season of 2014-2015 and 79.10-79.70 cm in the growing season of 2015-2016 (Table 1).These results are similar to those of Gubbels and Kenaschuk (1989), R. Casa et al. (1999) who reported that the amount of seed and the size of the plant did not change. However, our study does not correspond with the studies of Dillman and Brinsmade (1938), Albrechtsen and Dybing (1973), Gubbels (1977), Lafond (1992), Agegnehu and Honermeier (1997), and Gabiana et al. (2005), which have shown that the plant height varies significantly according to the amount of seeds.

Regarding the plant height, variety ×seeding rate interaction was statistically significant in the 2014-2015 growing season. The lowest plant height was obtained with application of 50 kg/ha seeding in Selection variety and the highest plant height was obtained with application of 30 kg/ha seeding in Pembina variety. In the 2015-2016 growing season, the variety x seeding rate interaction was insignificant and the plant height values were measured as 73.80-82.63 cm (Table 1).

3.2 Number of Axillary Branches

According to the results of the variance analysis, the difference between the varieties in terms of the number of axillary branches were found to be statistically significant in the 2014-2015 growing season, and insignificant in the 2015-2016 growing season. When the average values of the varieties were examined, the highest number of the axillary branches in the first year was obtained in York variety with 10.24 pieces and the lowest number of axillary branches was in Pembina variety with 6.50 pieces (Table 1). In the second year, the average number of axillary branches was lower than the previous year. This may be due to the difference in climate factors between years.

The difference between the seeding rates in terms of the number of axillary branches was statistically significant for both of the growing seasons. In the first year, the highest number of axillary branches was obtained as 8.67 using 30 kg/ha seeds and the lowest number of axillary branches was obtained using 70 and 50 kg/ha seeds respectively. In the second year, the highest number of axillary branches was seen as 5.89 in the seed amount of 30 kg/ha, while the lowest number of axillary branches was determined in applications of 50 and 70 kg/ha seeds respectively (Table 1). When the seeding rates were evaluated according to years, the highest number of axillary branches was obtained from the amount of 30 kg/ha seeds in both years of the experiment and there was no significant difference in the number of axillary branches in 50 and 70 kg/ha seed application. These results show similarity to the findings of Dillman and Brinsmade (1938), Gubbels (1977), Gubbels and Kenaschuk (1989), Diepenbrock and Pörksen (1992), Stevenson and Wright (1996), Agegnehu and Honermeier (1997), Gabiana et al. (2005), who have stated that the number of axillary branches decrease significantly with the increase in the number of seeds.

When the varieties × seeding rates interaction was examined in terms of the number of axillary branches, statistically significant differences were found in 2014-2015, while the difference in the 2015-2016 growing season was not significant. As can be seen in Table 1, the highest number of axillary branches (10.83) in the variety × seeding rate interaction during the

2014-2015 growing season and the lowest number of axillary branches (5.60) in the application of 30 and 70 kg/ha of York variety seed, while the lowest number of axillary branches was seen in 70 kg/ha seeding rate application of Nekoma variety.

3.3 Number of Capsules

When the variance analysis results regarding the numbers of capsules were examined, the difference between the varieties was significant for both growing seasons. When the variety average was examined in 2014-2015, the highest number of capsules was determined in the York variety with 81.87 and the lowest number of capsules was found in the Pembina variety with 48.46. In 2015-2016, the highest number of capsules was obtained in the York variety with 27.52 and the lowest number of capsules in the Neche variety with the number of 14.50. With regard to the number of capsules, the average of varieties decreased in the second year of the experiment. In both years, the highest number of capsules was determined in the York variety, while the lowest number of capsules was obtained in Pembina in the first year and in Neche in the second year (Table 1). For flax plants, the number of capsules is one of the most important characters in terms of grain yield (Lafond 2001). The fact that the capsule numbers of the varieties handled in the experiment are different may be due to the different genetic structure of the varieties and their different responses to the applied seeding rate.

The effects of the different seeding rates on the number of capsules were significant for both growing seasons. According to 2014-2015 data, the most capsules were counted as 72.99 in the application and 30 kg/ha seeds, followed by 60.72 capsules for 50 kg/ha seeds, 48.74 capsules for 70 kg/ha seeds, respectively. In the growing season of 2015-2016, the highest number of capsules was obtained as 23.98 from the application of 30 kg/ha seeds, and the lowest number of capsules was obtained as 16.39 capsules from the application of 50 kg/ha seeds (Table 1). In both years of the experiment, the highest number of capsules was obtained from the application of 30 kg/ha seeds. When the two-year results of the experiment were evaluated together, the number of capsules decreased with the increasing amount of seeds. This is due to the fact that plants are not able to make enough use of the sunlight because of the increase in the number of seeds, resulting in less capsule growth (Diepenbrock and Parksen, 1992) and a decrease in the number of leaves and leaf size when the plant density is high and the branches are shorter (Hassan and Leitch 2000). The results of this study show similarity with the results of the studies of Dillman and Brinsmade (1938), Albrechtsen and Dybing (1973), Elsahookie (1978), Diepenbrock and Pörksen (1992), Agegnehu and Honermeier (1997), Casa et al. (1999), and Gabiana et al. (2005), which stated that the number of capsules decreased significantly with the rise in the number of seeds, in addition to this our result differed from the study of Gubbels (1977), who stated that there was no significant difference in the number of capsules after an increase in the number of seeds.

When the results of variance analysis were examined, the varieties × seeding rates interaction was statistically significant for both growing seasons. The highest number of capsules (98.97) obtained from the varieties × seeding rates interaction during the 2014-2015 growing season was determined in the application of 30 kg/ha seeding rate in York variety and the lowest number of capsules (34.25 pieces) was determined in application of 70 kg/ha seeding rate in Nekoma variety. In the growing season of 2015-2016, 35.71 capsules were obtained from the application of 30 kg/ha seed in York variety, and the lowest number of capsules was taken as 12.70 pieces in Neche variety with the application of 70 kg/ha seeds (Table 1).

3.4 Number of Seeds in the Capsule

According to the results of the variance analysis, the differences between the varieties were statistically insignificant for both of the growing seasons (Table 2). When the seed numbers in the capsules of varieties were examined, a slight decrease was observed in the second year. This may be due to the fact that the second year was rather dry compared to the first year.

In terms of the number of seeds in the capsule, the differences between the seeding rates appeared insignificant in the first year of the experiment, whereas in the second year it was statistically significant. In the period of 2015-2016, the maximum number of seeds was obtained as 9.00 seeds with 30 kg/ha seed application, and the lowest value with 8.70 seeds with 70 kg/ha seed application (Table 2). In flaxseed production, the number of seeds in the capsule is an important criterion (Lafond 2001). Findings from the first year were similar to the studies of Albrechtsen and Dybing (1973), Gubbels (1977), Elsahookie (1978), and Casa et al (1999), which reported that the number of seeds in the capsule did not change after a change in the amount of seeds. The findings of the second year were similar to those of Diepenbrock and Pörksen (1992), Agegnehu and Honermeier (1997), and Gabiana et al. (2005), which reported that the number of seeds in the capsule changed significantly

after a change in the amount of seeds. Despite the fact that there was no significant change in the first year of the experiment, the significant change in the second year indicates that this character was affected by climate factors, especially drought.

In terms of the number of seeds in the capsule, the varieties × seeding rates interaction was found to be insignificant in the first year of study and significant in the second year. The highest number of seeds in the capsule (9.63) in the varieties × seeding rates interaction was found in the application of 30 kg/ha seeding rates of the Pembina variety, and the lowest seed number in the capsule (9.00) was found in the application of 50 kg/ha seeding rate of the Selection variety in 2014-2015 (Table 2).

3.5 Thousand Grain Weight

As can be seen in Table 2, the difference between one thousand grain weights of the varieties used in the study was statistically significant for both growing seasons. In both growing seasons, the highest seed weight was found in the species of Pembina (5.85 g in the first year, 5.83 g in the second year) and the lowest seed weight was found in the species of York with 5.51 g for both of the growing seasons.

According to the results of variance analysis, the differences in the amount of seeds regarding the thousand grain weight were significant for both growing seasons. In the period of 2014-2015, the highest thousand grain weight was determined as 5.78 g in the application of 30 kg/ha seed, the lowest thousand grain weight was determined as 5.61 g in the application of 70 kg/ha seed. In the period of 2015-2016, the highest thousand grain weight was obtained as 5.71 g in the application of 30 kg/ha seed and the lowest thousand grain weight was obtained as 5.56 g in the application of 70 kg/ha seed (Table 2). When the seeding rates are evaluated, the increase in the amount of seed per two years decreases the thousand grain weight. The thousand grain weight in flax seed production is an important yielding factor (Lafond 2001) and it is desirable to be high. As in our research, findings from previously conducted studies indicate that the thousand grain weight is a highly influenced character affected by the genotype as well as the cultivating techniques such as the genotype and seeding rates (Gubbels 1977). These findings show similarities with the findings that were obtained by Gubbels and Kenaschuk (1989) and Diepenbrock and Pörksen (1992); while this study does not correspond with the findings of Albrechtsen and Dybing (1973), Elsahookie (1978), and Casa et al (1999), who reported that the thousand grain weight did not change after a change in the amount of seeding.

According to the results of the variance analysis on the thousand grain weights, the varieties × seeding rates interaction was found to be statistically significant. When the varieties × seeding rates interaction obtained in the 2014-2015 growing season was examined, the highest thousand grain weight was found in 30 kg/ha seed application of Pembina as 6.00 g, and the lowest thousand grain weight was found in 70 kg/ha seed application of York as 5.50 g. When the varieties × seeding rates interaction obtained in the growing season 2015-2016 was examined, the highest thousand grain weight was found in 30 kg/ha seed application of Pembina as 5.94 g, and the lowest thousand grain weight was found in 70 kg/ha seed application in the species of Selection as 5.47.g (Table 2).

3.6 Grain Yield

In both years of the study, the differences between the grain yields of the flax varieties were statistically significant. In 2014-2015, the highest grain yield was obtained in the Nekoma variety with 1863.30 kg/ha, while the lowest grain yield was obtained in the York variety with 1260.00 kg/ha. In the period of 2015-2016, the highest grain yield was determined in the Necoma with 2071.10 kg/ha and Neche varieties with 2063.30 kg/ha, being in the same statistical group; while the lowest grain yield was respectively in York species with 1715.50 kg/ha, in Selection species with 1735.50 kg/ha and in Pembina species with 1753.30 kg/ha, again being in the same statistical group (Table 2). When the two-year results of the experiment were evaluated together, the highest grain yield was obtained in the Nekoma variety and the lowest grain yield was obtained in the York variety in both years. When the results were examined, it was seen that the average of varieties is higher in the growing season of 2015-2016. This may be due to the difference in climate factors between years.

According to the results of the variance analysis, the differences between the amounts of seeds were found to be significant for both years, with regard to the grain yield. When the grain yields of the year 2014-2015 are examined, the highest grain yield was obtained by applying 1872.60 kg/ha with 70 kg/ha of seed and the lowest grain yield was obtained by applying

1503.30 kg/ha with 30 kg/ha of seed and by applying 1537.30 kg/ha with 50 kg/ha of seed, which are in the same statistical significance group. In 2015-2016, the highest grain yield was obtained from the application of 70 kg/ha seed with 2112.60 kg/ha and the lowest grain yield was obtained from the applying 1516.60 kg/ha with 50 kg/ha of seed (Table 2). When the two-year results were evaluated together for the amounts of seeds, the highest grain yield was obtained at a rate of 70 kg/ha seed for both of the years. In the production of flaxseed, there are three major yield components, namely the number of capsules, the number of seeds in the capsule and the seed weight (Lafond 2001). In our study, the highest grain yield was obtained from the application of 70 kg/ha seeds, while the highest number of capsules, number of seeds in capsule, and the thousand grain weight were obtained from 30 kg/ha seed application. This can be explained by the large number of plants in the unit area. Findings obtained were similar to those of Lafond (1992), Agegnehu and Honermeier (1997), Gubbels and Kenaschuk (1989), Diepenbrock and Pörksen (1992), which reported that the yield increased with seeding rate. However, Gabina (2005) reported that the yield decreased with the increase in the seeding rate, while Albrechtsen and Dybing (1973), Elsahookie (1979), Stevenson and Wright (1996), Casa et al (1999), and Easson and Molloy (2000) stated that the yield was not affected by changes in the amounts of seeds.

According to the results of variance analysis of the grain yield, the varieties × seeding rates interaction was found to be statistically significant for both of the years. When the varieties × seeding rates interaction for the 2014-2015 growing season was examined, the highest grain yield was 2060.00 kg/ha from the application of Nekoma seeding rate with 70 kg/ha and the lowest grain yield was 906.60 kg/ha from the application of York seeding rate with 30 kg/ha. In the 2015-2016 growing season, the highest grain yield was 2380.00 kg/ha from the application of Nekoma seeding rate at 70 kg/ha and the lowest grain yield was taken as 1243.30 kg/ha from Selection variety with 50 kg/ha seed application (Table 2).

IV. CONCLUSION

The research findings show that the genotype has a significant effect on the yield and yield components of the flax plant, and the highest grain yield was obtained with a seeding rate 70 kg/ha, which was the highest seeding amount during the experiments. When we look at the interactions between the two factors, it was seen that the varieties reach the highest yields with the application of 70 kg/ha of seed. Although the measured yield components were adversely affected by the increase in plant density, there has been a significant increase in yield statistics due to the increase in the number of plants in the unit area. If we apply new interrow spaces such as twin rows which will ensure that the yield elements are minimally affected, it may be possible to reach even higher yields. In this respect, it is deemed necessary to investigate new seed norms which can reduce the competition between plants and increase the unit field yields with new interrow applications by taking the application of 70 kg/ha of seed, which is determined as the optimal norm in our research, as the lowest sowing norm.

REFERENCES

[1] Albrechtsen R.S. and Dybing C. D (1973). Influence of Seeding Rate Upon Seed and Oil Yield and Thei rComponents in Flax. Crops ScienceVol. 13

[2] Casa R. Russell G., LoCascio B., Rossini F (1999). Environmental effects on linseed (*Linumusitatissimum* L.) yield and growth of flax at differentst and densities. European Journal of Agronomy11:267-278

[3] Diepenbrock W.and Pörksen N. (1992). Phenotypicplasticity in Growth and Yield Components of Linseed (*Linumusitatissimum* L.) in Response To spacing and N-nutrition. Journal of Agronomy and Crop Science 169. 46-60

[4] Dillman A.C. and Brinsmade J. C (1938). Effect of Spacing on The Development of the Flax Plant. American Society of Agronomy Vol:30 No:4

[5] D. L. Molloy R. M (2000). A study of the Plant Fibre and Seed Development in Flaxand Linseed (*Linumusitatissimum* L.) Grown at a Range of Seed Rates. Journal of Agricultural Science. Cambridge 135: 361-369

[6] Elsahookie M. M. (1978). Effects of Varying Row Spacing on Lin seed Yield and Quality. Can. J. Plant Sci. 58: 935-937

[7] Gabiana C.,McKenzie B. A.,Hill G. D. (2005). The Influence of Plant Population Nitrogen and Irrigation on Yield and Yield Components of Linseed. Agronomy N.Z. 35

[8] Gubbels G. H. (1977). Interaction of Cultivar and Seeding Rate on Various Agronomic Characteristics of Flax. Can. J. Plant Sci. 58:303-309

[9] Gubbels G. H.,Kenaschuk E. O. (1989). Effect of Seeding Rate on Plant and Seed Characteristics of New Flax Cultivars. Can. J. Plant Sci. 69: 791-795

[10] Hassan F. U.and Leitch M. H. (2000). Dry Matter Accumulation in Linseed (*Linumusitatissimum* L.). Journal of Agronomy and Crop Science 186:83-87

[11] Khan M. A. And Bradshaw A. D. (1976). Adaptation toHeterogeneous Encvironments. II Phenotypic Plasticity in Response to Spacing in Linum. Australian Journal of Agricultural Research 27:519-531

[12] http://www.fao.org/statistics/en/ 2017-10-27

[13] Lafond G. P. (1992). The Effects of Nitrogen. Row Spacing and Seeding Rate on the Yield of Flax Under a zero-till Production System. Can J. Plant Sci. 73:375-382

[14] Lafond G. P. (2001). How is Yield Determined in flax? FlaxFocus. 14. 6-8

[15] Lafond G. P.,Irvine B., Johnston A. M., May W. E., McAndrew D. W., Shirtliffe S. J., Stevenson F. C. (2008). Impact of Agronomic Factors on Seed Yield Formation and Quality in Flax Canadian Jornal of Plant Science 485-500

[16] Leitch M H. Snd Sahi F. (1999). The effect of Plant Spacing on Growth and Development of Linseed. Annals of Applied Biology 135:529-534

[17] Lisson S. N. And Mendham N. J. (2000). Agronomic Studies of Flax (*Linumusitatissimum* L.) in a South-eastern Australia. Australian Journal of Experimental Agriculture 40:1101-1112

[18] Stevenson F. C. And Wright A. T. (1996). Seeding Rate and Row Spacing Affect Flax Yield and Weed Interference. Canadian Journal of Plant Science76:537-544

[19] Turner J.A (1991). Linseed Plant Population Relative to Cultivar and Fertility. Aspects of Applied Biology 28. 41-48

[20] M. Richter D.,Matula J., Szopa J (2015). Linseed. The Multi Purpose Plant. Industrial Crops and Products75:165-177.

Assessment of irrigation water pollution by oil waste at Jalo-Libya.

Aboubaker Mohamed Alzwi Saad[1]*, Farid Hamdaoui[2], Ayoub EL ATMANI[3], Ali Alemad[4], Mohamed Saleh Almagbari[5], Salem Aguila Elsakran[6], Ghizlane ZTIT[7], Ahmed Hassan[8], Abdelsalam Ipeda[9], Mahde Aljadidi[10], Khadija El kharrim[11], Driss Belghyti[12*]

[1,2,3,4,7,11,12]Biology, University Ibn Tofail, Kenitra, Morocco
[5]Geology, University Benghazi, Libya.
[6]Laboratory of Gestion et Managment,University Benghazi, Libya.
[8]Society Arabic Gulf of Oil, Champ Nafora, Gallo, Libya.
[9]Society of Development, Sabha, Libya
[10]Academic attached of Ambassy Libya, in Rabat- Maroc.

Abstract— *The petrochemicals wastes and pollutants are dumped without treatment in the environment, lakes and soil in Libya. The objective of our present research is to study the impact of waste from crude oil extraction on the environment of the Libyan region of Ajdabiya. The monitored physicochemical indicators are: Temperature, pH, electrical conductivity (CE), TDS, CL- , NO_3-, SO_4^{2-}, HCO_3-, Na+, Mg^{2+}, K+, Ca^{2+}, Salinity, Total Hardness (TH). The mineralization faithfully follows the rates of dissolved salts. The electrical conductivity varies from 7880 to 46700 µs/cm and far exceeds the Libyan irrigation standards (>2700µs/cm). Concerning the nitrate their contents range from 230 to 1210 mg/L and clearly reflect the crude oil pollution origin. The Piper diagram and Wilcox-Riversade projections shows that the waters associated with crude oil have a chloride-sodium and potassium or sulphated sodic and slightly bicarbonated sodium or potassium facies. Moreover, the hydrophysico chemical plot shows that the quality of this water is poor and above all a degraded quality.*

Keywords— Agriculture, Irrigation, Hydrochimestry, Pollution, Ajdabiya, Libya.

I. INTRODUCTION

Oil is a natural resource that is an important resource for many countries in the Gulf, Africa, Asia and America [1-2]. Unfortunately the history of oil in the African region is fraught with problems identified by the Extractive Industries Assessment Report (EIR) which has highlighted social and environmental problems [3-4]. Several marine, coastal and continental ecosystems have been damaged by oil activities around the world and especially Libyan cities, such as the city of Ajdabiyia [5-8].

Our work evaluates the degree and the modalities of pollution generated by the waste of oil in Jalo-Libya. Thus, to provide decision-makers with scientific and technical support to initiate a continuous decision-making dynamic aimed at protecting the environment and waters for irrigation that is vital for the whole country.

Indeed, the present study proposes to validate a physicochemical monitoring [9-11] intended to describe and evaluate the nature, the quality and quantity of pollutants generated by the oil extraction in the Libyan region of Jalo. In fact, the waters associated with the oil production are highly polluted and have an environmental impact on groundwater, surface water and oceans. We will also look for ways to reduce their consequences [12-13].

II. MATERIAL AND METHODS

2.1 Field of study

Ajdabiya is the capital of Al Wahat district, located in north-eastern Libya. It is located about 160 km south of Benghazi, on the coastal highway leading to Tripoli in the Gulf of Sirte. It was from 2001 to 2007 the capital of the district of the same name, Ajdabiya which has about 76968 inhabitants (**Fig.1**) [14].

2.2 Water sampling and analysis

For all sampling area (**Tab.1),** the parameters studied and the methods used are as follows:

*Temperature (T °C) is measured on site by a thermometer probe;

*The pH was measured directly after sample collection using a Model 3310 pH meter;

*The electrical conductivity of the water samples was measured directly after sample collection using a Conductimeter Model Consort. Electrical conductivity EC is expressed in ds / m or µs / cm at 25 °C;

*Total Dissolved Solute TDS in mg / L and Salinity in g / L ;

*Estimate of calcium and magnesium (Ca^{+2}, Mg^{+2}). Calcium and magnesium ions were estimated by plating the EDTA solution, which is a stable compound with calcium and magnesium ions using the Eriochrome black T reagent, Murexid [15-16] ;

*Determination of chloride (Cl-). Chlorides are measured by the method of Mohr (AFNOR T90-014). The chlorinated water samples were calibrated with 0.014M silver nitrate using potassium chromate as a reagent in a neutral or alkaline medium [17].

*Determination of sulfates SO_4^{2-}. The method used was based on the fact that the sulfate ions are deposited in the 1: 1 HCl acid medium in the presence of barium chloride due to the formation of barium sulfate in the form of single crystals of barium sulfate. Absorption can be measured by UV. V is Spectrophotometer [18];

*Determination of carbonate and bicarbonate (HCO_3^-, CO_3^{2-}). Carbonates and bicarbonates were estimated by the concentration of HCL (0.05 N) [19] ;

*Determination of sodium and potassium (Na^+, k^+). Each element was estimated to have distinct radii when excited by a flame (photovoltaic) using a flame photometer [20].

*Total hardness TH, calcium or magnesian hardness, alkalinity, bicarbonate and carbonates are measured by volumetric method of hydrochloric acid (0.05N) titration method (AFNOR T90-036).

FIGURE 1: MAP OF THE STUDY AREA AJDABIYA-JALO, LIBYA

TABLE 1

GEOLOCATION OF WATER SAMPLING STATIONS ASSOCIATED WITH CRUDE OIL IN AJDABIYA-JALO.

Stations	Latitude	Longitude		Stations	Latitude	Longitude
Well 2	27°51'18.12"N	21°56'49.03"E		Well SEP	28°59'44.44"N	21°34'29.73"E
Well 3	27°10'52.51"N	21°34'55.18"E		Well 249	28°59'12.19"N	21°22'32.48"E
Well 4	27°47'56.12"N	20°31'54.67"E		Well 256	28°45'50.37"N	22°12'26.00"E
Well 5	29°10'45.26"N	21°17'56.09"E		Well D44	29°15'50.42"N	19°12'27.11"E
Well 6	28°59'44.44"N	21°34'29.73"E		Well G128	28°56'47.46"N	19°42'23.98"E
Well 7	28°59'12.19"N	21°22'32.48"E		Well G144	29°50'29.70"N	19°46'38.06"E
Well 8	28°45'50.37"N	22°12'26.00"E		Well G36	30°20'30.24"N	19°35'34.61"E
Well 9	29°15'50.42"N	19°12'27.11"E		Well tank	30°45'44.17"N	20°14'22.06"E
Well 10	28°56'47.46"N	19°42'23.98"E		Well S1	27°51'18.12"N	21°56'49.03"E
Well 11	29°50'29.70"N	19°46'38.06"E		Well S2	27°10'52.51"N	21°34'55.18"E
Well 12	30°20'30.24"N	19°35'34.61"E		Well S3	27°47'56.12"N	20°31'54.67"E
Well 13	30°45'44.17"N	20°14'22.06"E		Well S4	29°10'45.26"N	21°17'56.09"E

FIGURE 2: SPREADING AND STORAGE AREAS FOR CRUDE OIL WASTE IN LIBYA

FIGURE 3: METHODS OF ASSAYING AND ANALYZING PETROLEUM WASTE

III. RESULT AND DISCUSSION

In the initial production of oil fields, the oil is not associated with water, but after a period of production, begins the emergence of water with oil extracted. The amount of water is gradually increased due to the upward water creep and in the final phase of the field operation, the proportion of produced water can reach 90% and more [25-26].

In the tanks, there is always water that is below the tank is the water associated with the oil. The water that accompanies the oil is characterized by a huge amount of dissolved mineral salts. It is salt water; see very salty ranging from a few hundred thousand to more than 600000 ppm. The salinity varies in Libya from 25 to 117,5 g/L and comes from a salt-laden oil of 25000 to 117500 mg / L (**Tab. 3**). Nitrates (234 to 609 mg / L); sulphates (278 to 2609 mg/L); Total Hardness (572 to 9820 mg / L) are present at levels exceeding acceptable standards [27].

TABLE 2
DESCRIPTIVE STATISTICS OF PHYSICOCHEMICAL CRUDE OIL WASTE OF AJDABIYA.

Variables	Observations	Minimum	Maximum	Mean	Standard deviation
T°C	42	21	46	33,3714	6,71
pH	42	6,2	7,96	7,2195	0,41
CE µS/cm	42	12654	66925	36855,5476	19135
TDS mg/L	42	8225	48432	24075,0952	12649
TH mg/L	42	572	9820	4095,9762	2435
Na^+ mg/L	42	723	37320	9941,3786	10894
Mg^{2+} mg/L	42	219	1009	622,5048	208
Ca^{2+} mg/L	42	464	5820	1894,0714	1434
K^+ mg/L	42	19,5	1140	403,3238	373
CL- mg/L	42	2800	70421	27537,1667	17119
SO_4^{2-} mg/L	42	278	2609	1238,2333	640
NO_3- mg/L	42	234	609	402,1190	108
HCO_3- mg/L	42	410	6561	799,1024	923
$CaCO_3$ mg/l	42	28,8	4032	1829,5262	1121
Salinity g/L	42	25	117,50	76,1429	19

The pH does not show significant variations and the waters are generally acidic to slightly basic ranging between 5.57 and 7.86 (**Tab.2**) following their contamination by petroleum residues.

The electrical conductivity is measured in µs / cm and varied from 12654 and 66925 µs/cm (**Tab.2**). When pure water is free of salts, bases and acids, it increases the electrical conductivity in the water [15]. This fact makes it possible to introduce the quantity of salts into the water. Any water has an electrical conductivity, but the removal of the ionic concentration of the water decreases its conductivity.

The Total dissolved solutes (TDS) is an important indicator of the suitability of water for various uses. The more soluble salts, the less soluble is water. If the water contains less than 1 mg / liter, the water is unacceptable and this water is not valid. For many uses, the concentration of dissolved salts in water varies considerably from one region to another [28]. In water polluted by oil it varies from 8225 and 48432 mg/L and is far from norms.

Total Hardness TH varied from 572 to 9820 mg/L and is linked to calcium and magnesium concentrations.

Calcium and magnesium with bicarbonate and carbonate and sulphate or silica components are insulation materials for heat in boilers and in household and industrial appliances. But combined with fatty acid ions give undesirable deposits lead to distortion of basins and walls in bathrooms and toilets. The high level of magnesium also causes intestinal diarrhea, especially for new users who do not know this water.

Chlorides with a concentration greater than 100 mg / L for salt water, lead to physiological complications and various diseases. The food industry usually requires less than 250 mg / L and the textile, paper and synthetic rubber industries require less than 100 mg / L of chloride. With 2800 and 70421 mg / L of chlorides the waters associated with oil exceed the norms of

agricultural irrigation.Sulphates also combine with calcium to be an adhesive that limits the thermal conductivity in the tubes. Therefore, it is prohibited for certain industries such as sulphate level higher than 250 mg / L. The sulphate level of 500 mg / L or more gives the water a bitter taste. Water containing more than 1000 mg / L of sulphates causes damage to physical health. The waters studied had between 278 and 2609 mg / L of sulphates and are widely polluted.Bicarbonate when heated with water vapor gives carbon dioxide and carbonate and the latter combines with alkaline earth elements and the head of calcium and magnesium and forms a crust composed of Calcium and magnesium carbonate leads to reduced thermal conductivity through the walls of the conduction tubes and reduces the flow of fluid in these tubes and sometimes clogged completely. For many industries, the level of carbonate, bicarbonate or alkalinity is generally high. Concerning the nitrate contents (**Tab.2**), the values oscillate between 234 mg / L and 609 mg/L and clearly reflect a nitrogen pollution of petroleum origin [27].

The Piper diagram (**Fig.4**) shows that globally the waters associated with petroleum have a chloric sodium facies and potassium or sulphated sodium and slightly bicarbonated sodium or potassium. Moreover, the projection of hydrophysicochemical data in the Wilcox diagram and Riversade (**Fig.4**), shows that the water quality associated with Ajdabiya Libya oil is poor and above all a deteriorated quality at because of the alkalizing power of sodium (SAR). These waters are classified in group C4S4 and outside this grid and are unfit for irrigation.

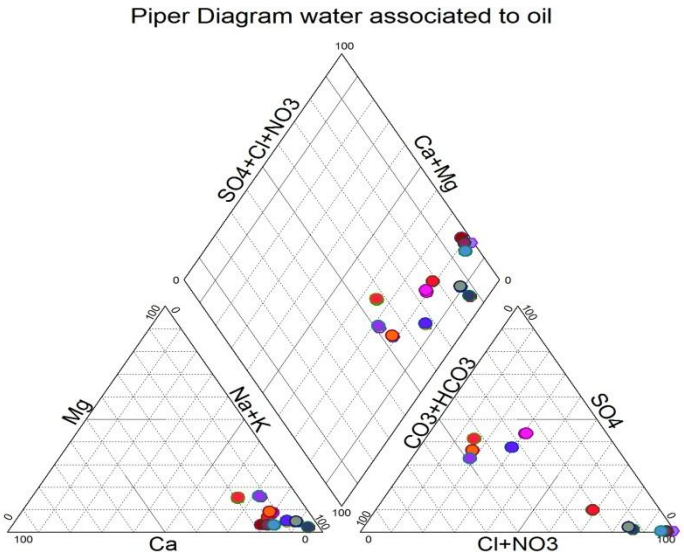


FIGURE 4: HYDROCHEMICAL FACIES OF WATER ASSOCIATED TO CRUDE OIL IN AJDABIYA, LIBYA

IV. CONCLUSION

All water studies and research have identified water needs in light of Libya's population increase, which is expected to reach about 14 million by 2025. Total freshwater resources are around 4.5 billion cubic meters and the total needs of this population are about 7.6 billion. The expected deficit is about 1.3 billion cubic meters. To compensate for this deficit, groundwater resources must be studied and rationalized. This study has explore and analyze water resources in order to assess their quality and validity [33]. Clouds are not well distributed and precipitation is random [34].

Large-scale oil pollution has led to a deterioration of water quality. In addition to increased salinity of the waters due to several factors such as the intrusion of salt water from layers carrying salt water to fresh rolling classes or the entry of seawater or salt water to proximity. The water associated with oil "or" productive water "is estimated at 44 million barrels of water a day, assuming an average rate of four million barrels of water per million barrels of oil, with a production rate of only 11 million barrels a day.

In conclusion of this study it is proposed to develop WWTPs for the treatment of water produced with the extraction of oil and its purification and re-injection into waterways. Thus, further reduce the amount of metal ions present in the treated water and the removal of metal ions and also reduce sulphate ions, nitrates, salinity and electrical conductivity [36-39].

Most countries already involved in offshore oil development have developed their own laws and standards at national and regional level. Instead of presenting final policy recommendations, we prefer to put in place tools and build a strong normative, regulatory and legal framework to protect the environment in Libya.

REFERENCES

[1] F.C Waddans, 1980. The Libyan oil industry. Croom Helm, London. 338p.

[2] J Gurney, 1996. Libya; the political economy of oil. Oxford Institute of Energy Studies. Oxford University Press: 24Ip. p.203-206.

[3] T.H Moller, Molloy, F.C., H.M. Thomas, 2002. Oil spill Risks and the State of Preparedness in the Regional Seas. Discussion paper of the International Tanker Owners Pollution Federation Limited, London, UK. 9p.

[4] UNEP, 2002. Africa Environment Outlook: Past Present and Future Perspectives. UNEP, 400p.

[5] J.H Teas, Lessard, R.R., Canevari, G.P., Brown, C.D., Glenn, R., 1993. Saving oiled mangroves using a new non-dispersing shoreline cleaner. In: Proceedings, Conference on Assessment of Ecological Impacts of Oil Spills. American Institute of Biological Sciences, Washington, D.C., pp. 147-151.

[6] M.B Baegi, H.S Assaf, and K.M Hangari, 1991. Al Awaynat surface uranium mineralisation - a new approach to its origin. Third Symposium on the Geology of Libya, vol. 7 (eds. M.J. Salem and M.T. Busrewil and A.M. Ben Ashour), Elsevier, Amsterdam, p. 2619-2626.

[7] P Stanislav, 1999. Environmental impact of the offshore oil and gas industry, EcoMonitor Publishing East Northport, N.Y. 425p.

[8] NRC, 2002. National Research Council of the National Academies. Oil in the Sea III. Inputs, Fates and Effects, The National Academies Press, Washington D.C. 265 pp.

[9] A., Ibeda. Ph.D. Evaluations de la Salubrité Microbiologique et Physicochimique et de la pollution des Eaux de la Nappe Murzuq (Sabha - Libye). Université Ibn Tofail, Faculté des Sciences Kénitra Maroc, 2014.

[10] A Ibeda, M.F Abosith, A Alemad, K El Kharrim, D Belghyti, "Physicochemical quality of murzuq groundwater Sabha – Libya" WIT Water and society, Vol 178, WIT Press, 2013, ISSN 1743-3541, doi. 10.2495/WS130191.

[11] A Ibeda. et al., 2014 : Evaluation physicochimique des eaux souterraines de la nappe Murzuq Sabha, Libye. Journées Méditerranéennes des Systèmes d'Information de l'eau. WIS 189, 19-21 Mars 2014 Rabat Maroc.

[12] Québec, 2003: Centre d'expertise en analyse environnementale du Québec MA. 408-IdePet 1.0 *Identification des produits pétroliers*, 2003, p.10-22.

[13] Metrohm, 2012 : Analyse pétrochimique, l'assurance qualité des produits pétroliers, Mise en page Ecknauer + Schoch ASW, imprimé en Suisse par Metrohm AG, CH-9100 Herisau 8.000.5080FR – 2012-11.

[14] O Salem. Management of Shared Groundwater Basins in Libya. In, Policies and strategic options for water management in the Islamic countries. Symposium, Tehran, Islamic Republic of Iran, 15-16 dec. 2003, UNESCO, pp.89-97. IHP-IV Series on Groundwater n°73.

[15] J Rodier. L'analyse de l'eau: eaux naturelles, eaux résiduaires, eau de mer : physico-chimie, bactériologie et biologie », Ed. Dunod, Paris, France, 8, 1996. p1383.

[16] J Rodier et C Graude. détermination de la dureté dans les eaux par la méthode au complexon III. Bull. Institut hygiène du Maroc, (1952), N.S. XII, (3-4), 275.

[17] APHA, AWWA, et WEF, (1998), Standard methods for the examination of water and wastewater. American Public Health Association, American Water Works Association et Water Environment Federation, 20^{e} edition.

[18] J Rodier: L'analyse de l'eau : Eaux naturelle, eaux résiduaires, eaux de mer, 7ème Edition, Dénod, 1986. Paris, p.1383.

[19] A Alemad, M Nagi, A Ibeda, R Nasser, Alwathaf Y., Elrhaouat O., Elkharrim K., Babaqi A., Belghyti D., (2013): The impact of sana'a solid waste on the quality of groundwater in Yemen, 2nd Intemational Conference on Water and Society, 4 - 6 September 2013, New Forest, UK, PaperDOI: 10,2495/WS130151.

[20] D Belghyti, Daifi H, Alemad A, Elkharrim K, Elmarkhi M, Souidi Y, Benelharkati F, Joti B, Elmoukrifi Z, Ibeda A, Azami-Idrissi Y, Baroud S, Elkhayyat F, Elrhaouat O, SadeK S, Y. Taboz, H. Sbai, R. Naser, H. Chigger & N. Groundwater management for sustainable production of drinking water quality in Maâmora. Water and Society II 241. WIT Transactions on Ecology and The Environment, Vol 178, © 2014 WIT Press. www.witpress.com, ISSN 1743-3541.

[21] J Beens et U.A.T Brinkman: The role of gas chromatography in compositional analyses in the petroleum industry. TrAC, Trends in Analytical Chemistry, 19:260– 275, 2000.

[22] M Rondon. PhD Influence de la formulation physico-chimique et des propriétés interfaciales sur la stabilité des émulsions asphaltènes-eau-huile. Application à la déshydratation du pétrole, Université, 2006.

[23] J Laxalde. Analyse des produits lourds du pétrole par spectroscopie vibrationnelle. HAL Id. 2012.

[24] N Jaffrezic-Renault 2003 : développements analytiques: micro capteurs électrochimiques pour le suivi in-situ des contaminants, Laboratoire IFOS, Ecole Centrale de Lyon, 69134 ECULLY Cedex (France) Reçu le, 06 Janvier 2003, Accepté le 16 Mai 2003.

[25] *E & P Forum/ UNEP technical Publication, (1997). Environmental Management in oil & gas. Exploration and Production: An overview of issues and management approaches. UNEP IE/PAC Technical Report 37.*

[26] A FUTYAN, and AH. JAWZI. The hydrocarbon habitat of the oil and gas fields of North Africa with emphasis on the Siit Basin. First Symposium on the Sedimentary Basins of Libya, Geology of the Sirt Basin, vol. 2. (eds. M.J. Salem., A.S. El-Hawat and A.M. Sbeta), Elsevier, Amsterdam, 1996. p. 287-308.

[27] H Abouzid et A Outair. Les Nitrates dans les eaux, 7ème Congrès Mondial des ressources en eau, Rabat, Maroc, 13-18 Mai 1991, Volume 2.

[28] FAO. Comparing water requirements for agricultural production in the Libyan Jamahiriya and twenty other countries. Rome, 2004.

[29] CSS, Committee Standards and Standard, Department of Environment- Morocco, Folds back, 1994.

[30] MEM, « Moroccan Standards, official Bulletin of Morocco, » Ministry of Environment of Morocco, n° 5062. 2002, Rabat.

[31] IAEA. Regional Shared Aquifer Diagnostic Analysis For the Nubian Sandstone Aquifer System, Second Draft Report, International Atomic Energy Agency Vienna, Austria, 24 September 2010.

[32] I Merdrignacet, D. Espinat. Physicochemical characterization of petroleum fractions: the state of the art. Oil & Gas Science and Technology - Rev.IFP, 62(1): 7–32, 2007.

[33] Organisation mondiale de la santé (OMS), (1984). Directives de qualité pour l'eau de boisson. Vol. 2. Critères de santé et autre information justificative. Genève.

[34] World Health Organization (WHO), (eds). Guideline for Drinking Water Quality, Health Criteria and Other Supporting Information: 2nd Edition, Vol.2, Geneva, pp. 940-949, 2004.

[35] Salem O. et Pallas P., 2002. The Nubian Sandstone Aquifer System (NSAS), In, Appelgren B. (ed.). Managing Shared Aquifer Resources in Africa (ISARM Africa), Procedeedings of the International Workshop, Tripoli (Lybia), 2-4 june 2002. Paris, UNESCO, pp. 19-21. IHP-VI, Series on Groundwater n°8.

[36] D Mara. Guidelines for the safe use of wastewater and excreta in Agriculture and Aquaculture: Measures for public health protection, World Health Organization, Geneva, 1989, 187p.

[37] UNESCO. 1972. Étude des ressources en eau du Sahara septentrional. Nappe de la Djeffara. Projet ERESS.Unesco presse, 2002. Eau: les ressources cachées de l'Afrique [en ligne]. Paris, UNESCO. Communiqué de presse N° 2002-35. Disponible sur Internet,http://portal.unesco.org/fr/ev.phpURL_ID=3026&URL_DO=DO_TOPIC&URL_SECTION=201.html.

[38] UNESCO, 2006, Ressources en eaux et gestion des aquifères transfrontaliers de l'Afrique du nord et du sahel: Paris.

[39] UNESCO, *Vers une Gestion Concertée des Systèmes Aquifères Transfrontaliers. Constat Préliminaire Partie I:* Académie de l'Eau, BRGM, OIEau, UNESCO, Paris, Août 2011.

Approaches for Enhancing Nitrogen Use Efficiency in some Upland rice *(Oryza sativa L.)* Genotypes under Water Stress Conditions

Larbeen Teronpi[1], Bhagawan Bharali[2]

Department of Crop Physiology, Assam Agricultural University, Jorhat-13

***Abstract*—** *Water stress causes serious yield loss of upland rice under water stress condition due to reduction in various physiological parameters, more particularly nitrogen use efficiency (NUE). Therefore, a pot experiment was conducted to evaluate six indigenous upland rice genotypes (viz., Mairen Ake-er, Soksu Ajoha, Soksu Abara, Chubok Abara, Bijor Soksu, and Inglongkiri) from North Hill zones (i.e. Karbi Anglong) for higher NUE and yield potential under different water regimes (Full irrigation & No water + 5000ppm of 6000PEG spray at tillering and heading stages). Real Time Nutrient Management (RTNM) approaches were used to determine the optimum rate of nitrogen for maximum yield and higher NUE under physiological drought condition. As such, amount of nitrogen fertilizer received by each of the varieties during growing period was 130 kgN/ha (1300ppm=1.3%) irrespective of water regimes. The genotypes showed differences in grain yield, plant height, chlorophyll content, proline, nitrate reductase, N-content and N-uptake. The variety Inglongkiri with the highest score corresponding to total N-uptake (88.85%), NUE (25.78%) and HI (43.51%), and the lowest reduction in grain yield (1.6%) has emerged as suitable genotype under water stress condition as compared to the irrigated one. Inglongkiri, a developed variety of Assam (RARS, Diphu), was found physiologically efficient among the varieties tested. This variety possesses the adaptive traits, especially higher N use efficiency, higher yield and attributes under physiological drought condition. Therefore, Inglongkiri may be taken as a donor in breeding programme for direct seeded upland limited moisture condition, and can be grown suitably under agro climatic conditions of elsewhere in Assam during Ahu season.*

***Keywords*— Chlorophyll, grain yield, leaf area, plant height, proline, rice, water productivity.**

I. INTRODUCTION

Rice is a semi-aquatic (mesophyte) plant which is commonly grown under submerged conditions. Submersed rice occupies about half of the cultivating areas (79 million hectares) in the world. Alternatively, rice is also grown in upland conditions. The yield of upland rice is reduced to some extent by scarcity of water, so called drought. Drought is defined as a period of no rainfall or no irrigation that affects crop growth (Hanson *et al*., 1990). Rice is the principal food crop for North Eastern region of India accounting for more than 80 per cent of the food grain production. The crop is extensively cultivated (72 per cent of the total cultivated area) in upland, lowland and deep water conditions. On an average 3,869 km^2 areas are put under shifting cultivation every year. The productivity of upland rice in N.E. India is very poor (0.9 tonnes per hectare) as compared to the national average (i.e. about 1.9 tonnes per hectare) (Singh 2002)

Water stress or drought is one of the most important abiotic constraints in rice. The effect of varying soil water regimes during different growth phases on rice yield. They reported that the soil water stress applied at any of the growth phases reduced rice grain yield, compared to the continuous flooding irrigation. The ripening phase appeared to be most sensitive as compared to the other phases. Soil water stress during the earlier growth phases (vegetative) reduces the production of effective tillers which lessens grain yield ultimately. Water stress during the later growth phases (reproductive) appeared to affect the reproductive physiology by interfering with pollination, fertilization and grain filling. As a result, there is reduction of grain yield in rice crop (Jana *et al.* 1971).

Nutrient availability might be further reduced by the often alternating soil water regimes and soil chemistry. Low soil fertility and the limited use of fertilizers contribute considerably to the low productivity of rain fed rice based systems (Haefels and Hijmans, 2007; Wade *et al.,* 1999; Pandey, 1998). Increased yield from fertilizer application even under water limited conditions were reported repeatedly, but it is often assumed that the economic return to applied fertilizer decreases with increasing drought stress (O'Toole and Baldia, 1982).

Indigenous rice genotypes grown in different water regimes may vary in nutrient use efficiency. Genotypic differences in nutrient use efficiency have been reported when they were mostly grown in well water intensive lowlands (Broadbent *et al.*, 1987; De Datta and Broadbent, 1990). It is, therefore, one of the major considerations to identify the critical steps controlling plant N use efficiency (NUE). Moll *et al*. (1982) defined NUE as being the yield of grain per unit of available N in the soil (including the residual N present in the soil and the fertilizer). According to Ladha *et al.* (1998), desirable cultivars with high nitrogen use efficiency (NUE) should produce large yields at low N supply. This seems even more important in upland environment where no nitrogen rates are applied. Several studies have addressed the optimization of fertilization and the improvement of NUE of crops to achieve high yields with reduced N fertilization rates, and limited environmental side effects related to N leaching (Agostini *et al.*, 2010; Burns, 2006; Neeteson and Carton, 2001; Rahn, 2002). Species and cultivars are expected to play a primary role: the genotype affects both the N uptake and the use of absorbed N, because every genotype has its own morphological and functional characteristics for roots, leaves, etc. (Schenk, 2006; Thorup-Kristense and Sørensen, 1999; Thorup-Kristensen and Vander Boogard, 1999). However, the same genotype can show different NUEs when subjected to different levels of N availability. New technologies in nutrient management in rice have been developed to increase nutrient use efficiency in recent years. Site-specific nutrient management (SSNM) such as Real-Time Nitrogen Management (RTNM) and Fixed-Time adjustable-dose Nitrogen Management (FTNM) were developed to increase the N use efficiency of irrigated rice (Peng *et al*., 1996 and Dobermann *et al*., 2002). In RTNM, N is applied only when the leaf N content is below a critical level. In this approach, the timing and number of N applications vary across seasons and locations, while the rate of each N application is fixed. The leaf N content is estimated non-destructively with a chlorophyll meter (SPAD: Soil Plant Analytical Development value) or Leaf Color Chart, commonly known as LCC (Tao *et al*., 1990, Peng *et al.*, 1996, Balasubramanian *et al*., 1999 and Yang *et al.*, 2003). In FTNM, the timing and number of N applications are fixed, while the rate of each N application varies across season and location. There is paucity of information on the responses of upland indigenous rice genotypes from North Hill zones (i.e. Karbi Anglong) to varying levels of water stress conditions. Moreover, management of Nitrogen in upland rice crop based on SPAD values under water stress conditions is lacking. The experiment was conducted to evaluate upland rice genotype(s) for higher nitrogen use efficiency (NUE) and yield potential using the Real Time Nutrient Management (RTNM) approaches, nitrogen use efficiency and productivity under water regimes and nitrogen levels.

II. MATERIALS AND METHODS

The investigation was carried out in 2015 at the vicinity of stress physiology laboratory under the Department of Crop physiology during Ahu season as a part of the M.Sc (Agri) degree program in Assam Agricultural University, Jorhat. A pot experiment was carried out with six upland (Ahu) rice varieties of same medium duration. The study was carried out by keeping the pots inside a poly house only during drought treatment periods in vegetative and reproductive stages, and then in the open field conditions for exposure to more sunlight so that crop does not suffer from low light situations during its developmental periods. A mixture of sandy loamy soil with FYM was used to fill in one pot (capacity: 6.5 Kg soil). FYM @5t/ha (≈.50g/pot) was applied initially to each pot. The whole amount of P&K @ 20:20 in the form of SSP (Single Super Phosphate) and MoP (Muriet of Potash) were applied as basal. N was applied based on RTNM method (Section 3.7). In here, N @ 20-150 Kg Nha^{-1} was applied according to the demand of the crop based on the SPAD (Soil Plant Analytical Development) values measured at different growth stages of the crop. In RTNM, a certain rate of N- fertilizer was applied when leaf N content was below a critical level (Peng *et al.*, 1996) as follows: First dose: 30 KgN/ha was applied at 10 days after sowing, Second dose: 40 kgN/ha was applied at tillering (SPAD value <33), Third dose: 40 kgN/ha was applied at panicle initiation (SPAD value <33), Fourth dose: 20 kgN/ha at heading (SPAD value <33). A constant water supply (2-3cm) was ensured from transplanting till seven days before harvesting except the periods of drought in treated pots. The soil of the experimental field was sandy loam with acid in reaction (pH= 5.6), available N, P and K was 257.2 kg/ha, 24.6kg/ha and 106.3 kg/ha respectively. The total rainfall received during the crop growth period was 1314.6 mm in the open field conditions. The temperature (31-25 ºc and 33-27 ºc) relative humidity (69-66 % and 72-69 %) and light intensity (2050 and 1685 lux) was also maintained inside the polyhouse in the vegetative and reproductive stage. Grain yield and straw yield was calculated.

TABLE 1 (A)
METEOROLOGICAL DATA DURING THE CROP SEASON (2015)

Months	Temperature (ºC) (Monthly mean)		Relative Humidity (%) (Monthly mean)		Monthly total Bright Sunshine (Hours)	Monthly total Rainfall (mm)
	Max.	Min.	Morn.	Even.		
March	29.8	16.1	90	55	154.6	42.7
April	27.4	19.0	93	73	115.7	293.3
May	30.1	22.5	92	77	97.4	298.0
June	31.6	24.4	94	80	78.7	335.8
July	34.0	25.3	90	72	161.1	344.8
Total						1314.6

Source: Meteorological observatory, Agricultural Meteorological Department, Assam Agricultural University, Jorhat

TABLE 1(B)
METEOROLOGICAL DATA DURING DROUGHT TREATMENTS INSIDE THE POLY HOUSE

Duration of withholding water and Polyethylene Glycol (PEG) treatments	Temp. (ºC) (Mean of 7days)		Relative Humidity (%) (Mean of 7days)		Light intensity (Reading x10) Lux (Mean of 7days)
	Max.	Mini.	Morn.	Eve.	
7 Days (11^{st} – 20^{th} April) + apply PEG 6000 (5000ppm)	31	25	69	66	2050
7 Days (13^{th} May – 22^{nd} May) + apply PEG 6000 (5000ppm)	33	27	72	69	1685

III. RESULTS AND DISCUSSION

The crop was subjected to water stress by withholding irrigation for seven days plus spraying with PEG-6000 (5000ppm) both at maximum tillering and heading stages. The crop was deprived of natural precipitation during these periods inside a poly house except supply of live saving water while soil tensiometer auto-fixed its readings at 80 centibars, and crop wilted visually. All the plants received a range of temperature (25-33°C), Relative humidity (66-72%) and light intensity (1685-2050 Lux) during the drought treatments in the months of April and May. The crop experienced same weather conditions outside the poly house during the rest of the growth periods. As the soil was strongly acidic in nature (pH 5.64), and N, P, K were in the lower ranges, fertilizer SSP and MoP were applied at recommended doses (20:20) as basal, but N was supplied based on SPAD values at different growth stages to get rid of crop starvation and to contribute in crop growth. Interestingly, all the plants, irrespective of drought and irrigation treatments, demanded equal amount of N (i.e. 130 kg/ha) throughout the growth periods (10days after sowing to tillering, panicle initiation and heading). As such, soil moisture remained as the only variable factor during the period of treatments, and its impacts on physiological changes were recognised subsequently.

3.1 Growth characteristics

Water and nitrogen significantly influenced average plant height and leaf area. Plant height and leaf area increased with nitrogen application in the well-watered treatment, but excessive nitrogen inhibited their growth. The variety Inglongkiri has the highest percent reduction and Soksu ajoha has the lowest percent reduction in plant height among the varieties at harvest. The reduction in plant height was either as a result of water stress imposed at tillering stage or might be due to its genetic behaviour x environment interaction, which inclined it towards high yielder at harvest. Bhattacharjee *et. al.*, (1973) and De Datta (1973) found significant reductions in tillers and panicles numbers as well as plant height and grain yield when water stress was imposed at tillering stage. Water stress resulted to decrease in plant height, number of tillers per plant, total biomass and grain yield (Tantawi and Ghanem, 2001; Tuong *et. al.*, 2005). The water deficit in rice caused a larger reduction in leaf area demonstrating the greater sensitivity of leaf enlargement to water stress (Gloria *et. al.*, 2002).

Specific leaf weight (SLW), characteristic features of plants which could be used as a selection criterion for abiotic stress factor (Bharali and Chandra 1996). The variety Inglongkiri had the highest per cent reduction (19.7 %) of SLW at heading stage as compared to tillering stage among all the other varieties, illustrated in (Fig. 3) Stress leaves had a lower SLW, suggesting that these leaves were thicker or had more densely packed mesophyll cells with less intracellular air space.

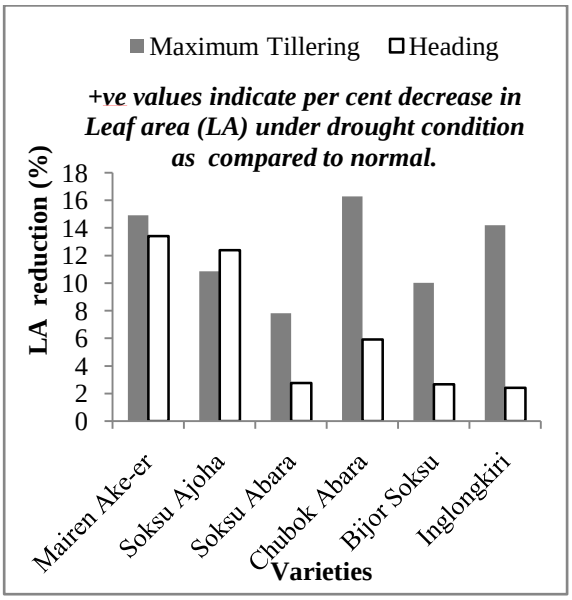


FIG. 1. CHANGES OF LEAF AREA (LA) UNDER DROUGHT AS COMPARED TO IRRIGATION

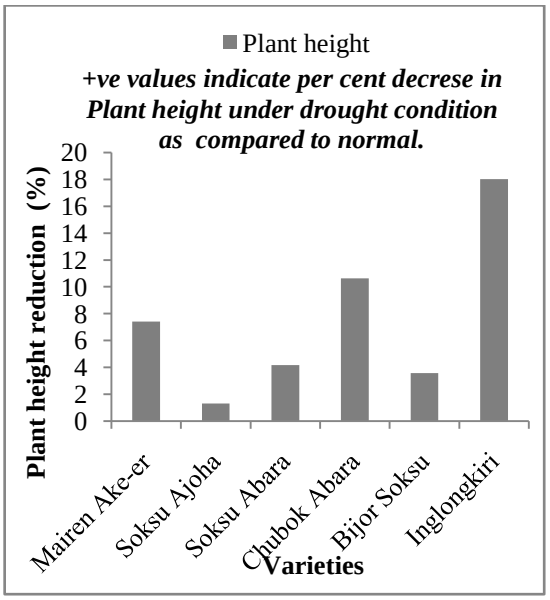


FIG. 2. CHANGES OF PLANT HEIGHT UNDER DROUGHT AS COMPARED TO IRRIGATION

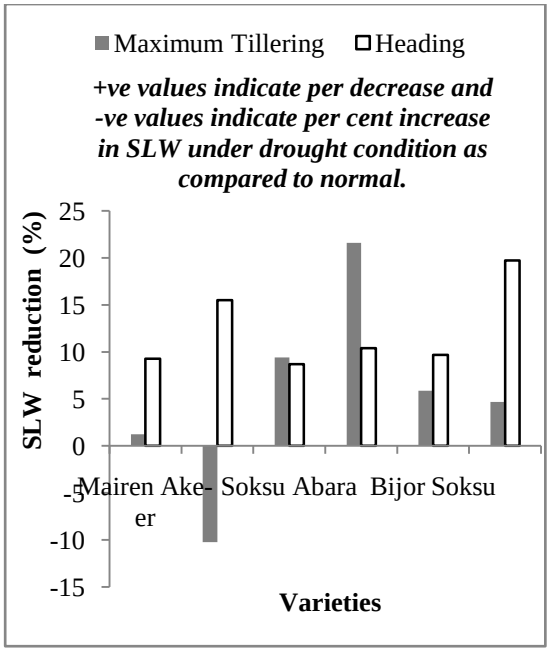


FIG. 3. CHANGES OF SPECIFIC LEAF WEIGHT (SLW) UNDER DROUGHT AS COMPARED TO IRRIGATION

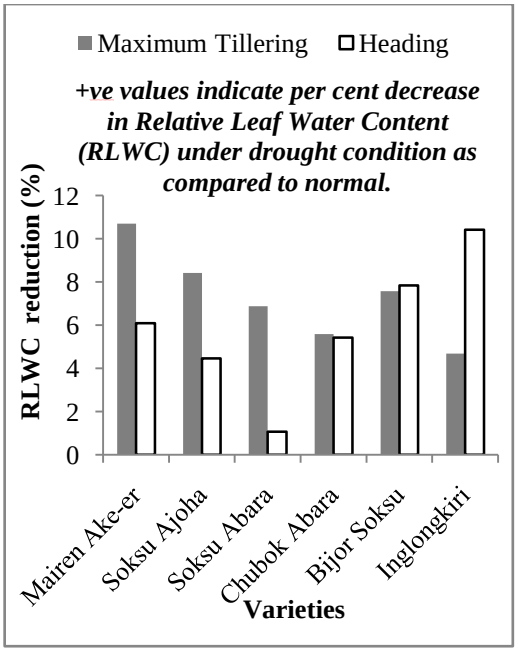


FIG. 4. CHANGES OF RLWC UNDER DROUGHT AS COMPARED TO IRRIGATION

Relative leaf water content (RLWC) of all the genotypes decreased significantly under drought condition (Fig. 4). There was more reduction in RLWC at tillering stage of all the varieties except Inglongkiri. Although at heading stage, Inglongkiri had the highest RLWC reduction (10.4%) among the varieties. Khan *et. al.,* (2007) concluded that water deficit stress results in a considerable decline in RLWC (18-68%). The variety Soksu abara with 1 % reduction contained the highest relative leaf water at heading stage: it might possess a drought tolerant tendency. Schonfeld *et. al.,* (1988) suggested that cultivars with high RLWC are likely to be drought resistant.

In the present investigation there was a fluctuate per cent reduction of chlorophyll content at tillering and heading stage under drought as compared to irrigation. The variety Bijor (40.4 %) had the highest and Soksu Ajoha (6.06%) had the lowest per cent reduction at heading stage (Fig. 5). Mohan *et. al.,* (2000) stated that the chlorophyll content is an indication of stress tolerance capacity of plants, and its high value means that the stress did not have much effect on chlorophyll content of tolerant plants. It is apparent from the (Fig. 6) that there were significant increase in proline contents both in tillering (upto 47.35 % in Soksu Ajoha) and heading stages (17.38%) of the varieties under water stress condition. Under water stress, accumulated proline might act as a compatible solute regulating and reducing water loss from the plant cell during water deficit (Yokota *et. al.,* 2006). Proline accumulates under stress also supplies energy for survivor and growth, and thereby helps the plants to tolerate stress condition (Kumar *et al*., 2011). Chubok Abara had the highest nitrate reductase activity reduction (37.46%) at tillering whereas Bijor soksu (40.6%) had the highest nitrate reductase activity at heading stage. There was a significant decline of NR at maximum tillering (1-37%) and heading (12-41%) stages in the varieties. Polyethylene glycol induces stress resulting in free amino acids as well as reduction in nitrate reductase activity in pearl millet (Hanson *et al.,* 1981; Hanson *et al*., 1982). Water stress induced decline in nitrate reductase activity has also been reported by Sarkar *et al*. (1991). In the study, changes of (0-0.71%) and (0-0.43%) were found at the two subsequent growth stages of rice varieties respectively. As such, the highest per cent changes were observed in Soksu ajoha (0.71%) at tillering and Soksu abara (0.43%) at heading stage. This means, Soksu Abara contained the highest amount of leaf nitrogen at reproductive stage under drought as compared to irrigated, indicating better ability of this genotype in acquiring N either from soil or applied nitrogen, and in remobilizing N under favorable water regime (normal) conditions. The results of Ghanbari *et al.* (2013) were also in confirmatory with the present finding. According to Janadhan and Murty (1980) nitrogen present in all plant parts decreases at harvest when compared with that of flowering stage. There was higher changes in carbohydrate contents in Mairen ake-er (1.32%) at maximum tillering stage followed by Soksu Ajoha (1.0%) under drought as compared to irrigated one. Whereas, at heading stage Chubok Abara (1.17%), Mairen ake-er (1.15%) and Bijor soksu (0.98%) had experienced some changes in carbohydrate content. Inglongkiri (1.03%) followed by Soksu abara (0.98%) and Soksu ajoha (0.70%) had higher changes in carbohydrate contents at this stage. It indicates that these varieties could carry out the process of photosynthesis well, particularly at maximum tillering stage under physiological drought stress. Dubey and Singh (1999) also reported that the sugar contents increases more in the sensitive than in the tolerant rice cultivars. Nakayama (1974) suggested that reduce transport of carbohydrate from the source to the developing grain might be a casual factor for impaired grain filling in rice.

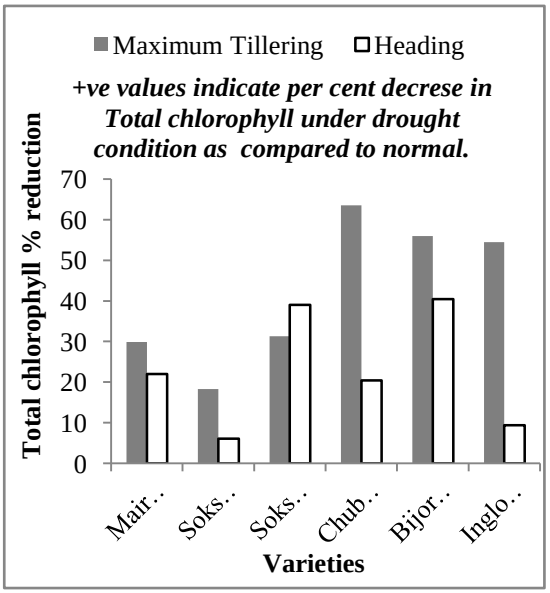


FIG. 5. CHANGE OF TOTAL CHLOROPHYLL UNDER DROUGHT AS COMPARED TO IRRIGATION

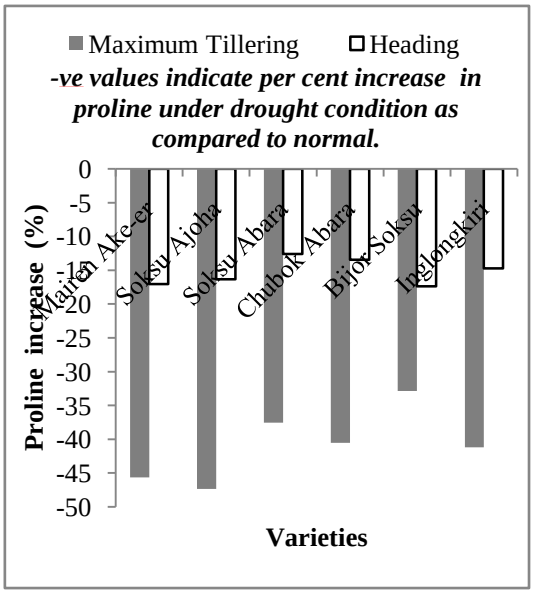


FIG. 6. CHANGES OF PROLINE UNDER DROUGHT AS COMPARED TO IRRIGATION

Photosynthesis is the main metabolic process determining crop production, and is affected by drought stress. Drought reduces photosynthetic rate of rice crop (Ji *et al.*, 2012; Lauteri *et al.*, 2014; Yang *et al.*, 2014). The lowest per cent reduction was seen in Chubok abara (14.14%) at tillering, and in Mairen ake-er (26.95%) at heading under drought as compared to normal (Fig. 10). The lowest per cent reduction under drought as compared to normal indicates the increase in photosynthetic rate in rice leaves. The increase in leaf photosynthetic rate is important to increase the yield potential of rice (Hirasawa *et al.*, 2010), because the photosynthetic rate of individual leaf which form the canopy, affects dry matter production via photosynthesis within the canopy. There were insignificant differences in Root: shoot among the varieties (Fig. 11a), and increase in root biomass (Fig. 11b) under drought as compared to normal. The highest changes in root: shoot was observed in Chubok Abara (0.072%) followed by Soksu Abara (0.065%) under drought as compared to irrigated. The highest increment in root biomass was found in Chubok Abara (41.2%) under drought. O'Toole and Chang (1979) suggested that upland variety should have a relatively high root-shoot ratio in order to yield efficiently under soil moisture stress conditions. Root dry mass and length are good predictor of rice yield under drought (Fageria and Moreira, 2011; Feng *et al*., 2012).

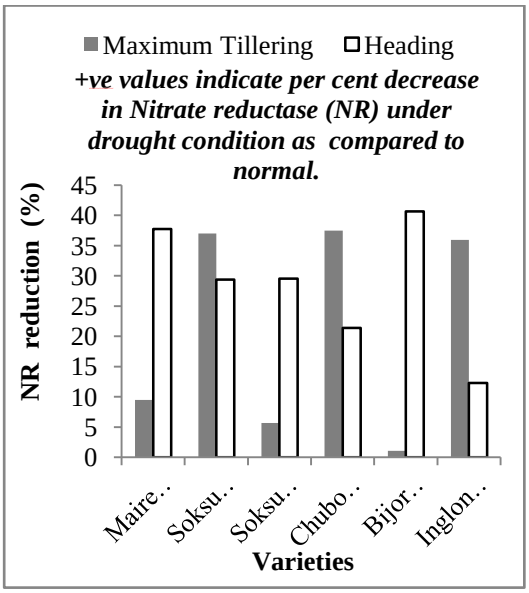


FIG. 7. CHANGES OF NITRATE REDUCTASE (NR) UNDER DROUGHT AS COMPARED TO IRRIGATION

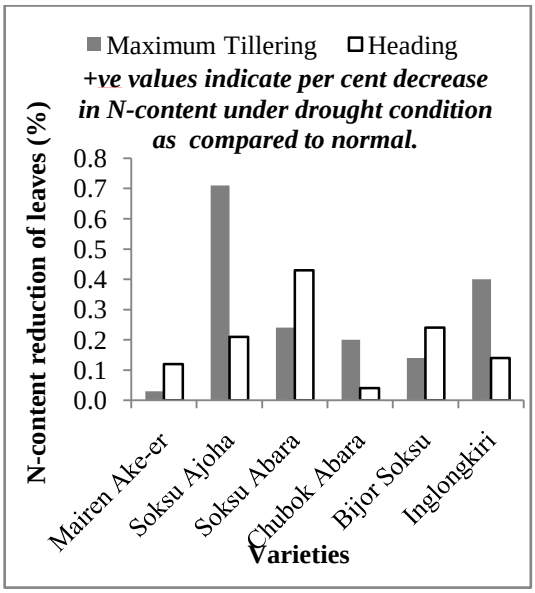


FIG. 8. CHANGES OF N-CONTENT OF LEAF TISSUES UNDER DROUGHT AS COMPARED TO IRRIGATION

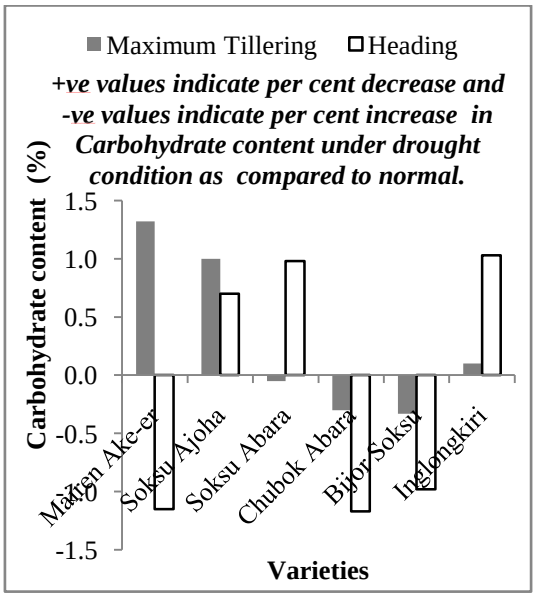


FIG. 9. CHANGES OF CARBOHYDRATE CONTENT OF LEAF TISSUES UNDER DROUGHT AS COMPARED TO IRRIGATION

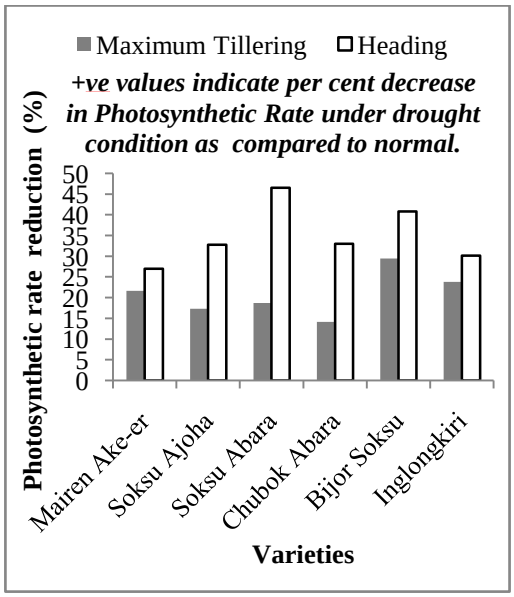


FIG. 10. CHANGES OF PHOTOSYNTHETIC RATE UNDER DROUGHT AS COMPARED TO IRRIGATION

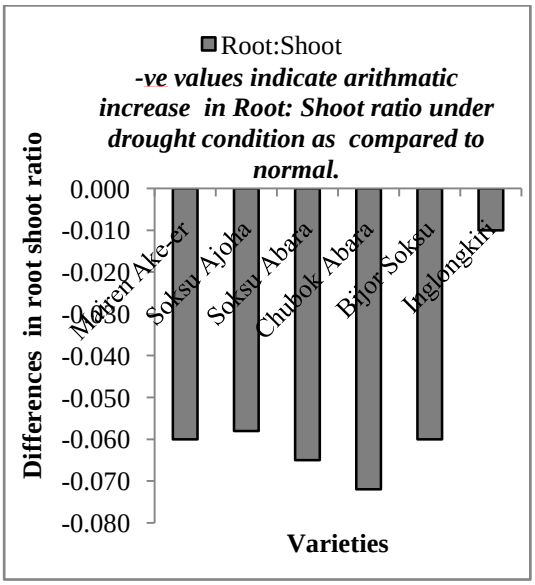


FIG. 11(A). CHANGES OF ROOT: SHOOT UNDER DROUGHT AS COMPARED TO IRRIGATION

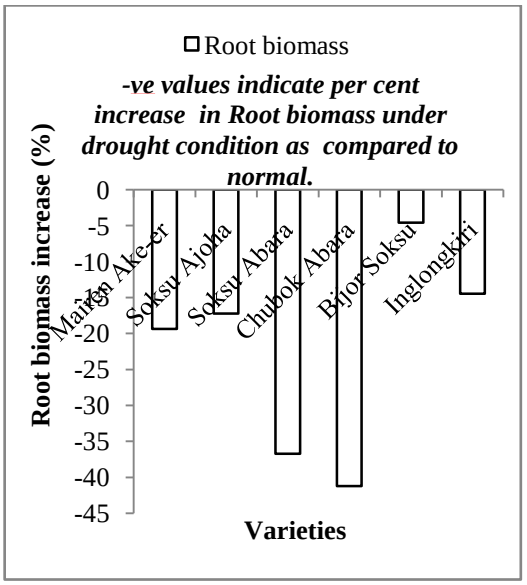


FIG. 11(B). CHANGES OF ROOT BIOMASS UNDER DROUGHT AS COMPARED TO IRRIGATION

3.2 Yield and yield attributing characters

There were significant variations among the varieties and between the treatments with respect of grains per panicle, which is illustrated in (Fig. 12). The highest per cent reduction of number of grains per panicle was observed in Soksu abara (8.37%) under drought as compared to irrigated one, and the lowest reduction was in Bijor soksu (1.10%). The lowest per cent reduction indicates increase in number of seeds per panicle under drought. However, grains per panicle are genetically controlled yielding component. This finding supports the result of Chaunhan *et al.* (1989). They reported that grains per panicle was a varietal character, and varied according to cultivars. Thousand grain weights (Test weight) differed significantly among the varieties, but the water regimes had no significant effects on it. The highest per cent reduction (Fig. 12) of test weight was found in Chubok Abara (1.4%), and the lowest was in Bijor soksu (0.61%). This is in support of the findings of Schonfeld *et al.* (1988) who were also in the opinion that kernel weight of wheat cultivars decreased under stress.

It is apparent from the (Fig. 13) that there was significant difference in economical yield among the varieties under drought as compared to irrigation. The highest per cent reduction in economical yield under drought was observed in Soksu ajoha (21.06%), whereas the lowest per cent reduction in grain yield was maintained in Inglongkiri (2.27%). This indicates that Inglongkiri behaves as one of the drought tolerant varieties. This finding is in support with Pandey *et al*., (2014). They reported that rice crop under water stress markedly reduces the grain-filling percentage and grain weight, resulting in a significant decrease of grain yield. Biological (straw) yield of rice showed significant difference among the varieties under stress condition. There is much reduction of straw yield in rice under moisture stress condition in some varieties which have higher grain yield (Fig. 13). The highest reduction per cent in straw yield was in Bijor soksu (29.78%) > Soksu abara (24.64%) > Chubok abara (23.7%) and Inglongkiri (21.19%). These varieties have higher grain yield and lower straw yield. The lowest per cent reduction was observed in Soksu ajoha (2.21%), which indicates the highest straw yield in the variety under drought as compared to irrigated condition. These results are in accordance with the findings of Radford (1986), Kalamian *et al.* (2006), Jasso *et al.* (2002), who also showed decreasing biological yield because of drought stress. It is evident from the (Fig. 13) that there were significant varietal variations in HI in rice crop in the study. Among the varieties, Soksu ajoha (14.67%) had the highest per cent reduction of HI under drought as compared to normal. The physiological drought condition did not affect HI in Soksu Abara, Chubak Abara, Bijor Soksu and Inglongkiri at all; rather there were increases (8-20%) in HI in these varieties under drought condition. Water stress at flowering and grain filling resulted in lower HI than water stress at tillering stage. Sharma *et al.*, (2003) observed higher HI in well irrigated genotypes compared to that of the genotypes which were grown under water stress condition. They reported that water stress at flowering and grain filling caused lower HI than water stress at tillering stage.

It is seen from the (Fig. 14) that the moisture stress exerted significant effect on nitrogen uptake by grain, straw and total N uptake among the varieties under different water regimes. The nitrogen uptake into grain, straw and total uptake decreased significantly with water stress. The highest per cent reduction of grain N uptake was seen in Soksu ajoha (31.05%), and the lowest was in Inglongkiri (1.93%). The highest per cent reduction in N uptake into straw was in Bijor soksu (47.75%), and

the lowest was seen in Mairen ake-er (13.92%). In case of total N uptake, the highest per cent reduction was recorded in Bijor soksu (32.6%), and the lowest was obtained in Mairen ake-er (9.3%) under drought as compared to irrigated. The increase in uptake of nitrogen at higher moisture regimes due to cumulative effect of increase in grain and straw yield as well as increased nitrogen content in grain and straw. Increase in uptake of nitrogen at higher moisture regimes have also been reported by Murthy and Reddy (2013) and Sandhu and Mahal (2014).

3.3 Nitrogen Use Efficiency

The (Fig. 15) clearly indicates that there was significant variation among the varieties in respect of nitrogen use efficiency (NUE). However, it was observed that Soksu Ajoha (31.06%) had the highest NUE per cent reduction, while Inglongkiri (1.9%) exhibited the lowest per cent reduction of NUE under drought as compared to irrigated one. Haefele *et al.* (2008) observed that water stress lowered the NUE in rice plants. This parameter remained high in tolerant cultivars that presented greater production. In the current study, as equal amount of fertilizer was demanded by all the varieties at different stages based on the RTNM method of N application. There was an exception with the variety Inglongkiri, which showed the lowest per cent reduction of NUE, and produced more yield. This might be due to varietal character or the variety has an adaptive mechanism under the stress condition as evidenced from its higher yield. Campbell and Davison (1979) suggested that, inefficient use of N is associated with excessive vegetative growth. Part of the decrease in NUE can be attributed to decrease in light Intensity or increase in evapotranspiration that could result from excessive vegetation (Pearman *et al*., 1977).

3.4 RTNM Technique

In use of the Real Time Nitrogen Management (RTNM), a certain amount of N- fertilizer is applied only when leaf N content is below a critical level (Peng *et al.,* 1996). Therefore, N status in leaf (Tao *et al.,* 1990) determines the timing, number of Nitrogen applications and doses of N in crop conditions. Feeding with N nutrition at different successive growth stages of rice crop following the N management technique for higher NUE of the genotype under physiological drought condition is in the heart of the present investigation. So, the RTNM approaches were followed for application of N-fertilizer in splits to increase NUE and corresponding yield of the varieties under different water regimes (physiological drought and full irrigation). Although each of the varieties equally received an amount 130 kg N during their period of growth, a few of them could maintain higher N uptake in grains, boost NUE, and exhibit higher yield in response to the whole dose of applied N. Therefore, such varieties(s) viz., Inglongkiri might have some special molecular features (i.e. QTL) for higher NUE and yield potential under physiological drought condition. This possibility has not been explored in the current study, and needs serious attention in future frontier research goal.

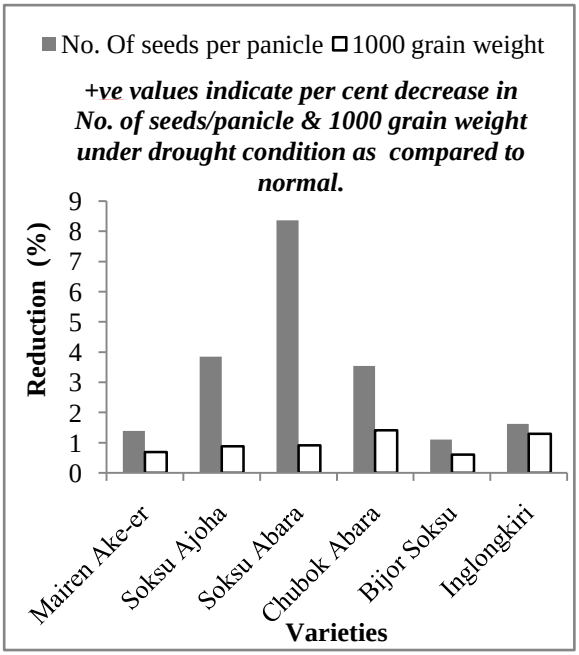


FIG. 12. CHANGES OF NO. OF SEEDS/PANICLE & 1000 GRAIN WEIGHT UNDER DROUGHT AS COMPARED TO IRRIGATION

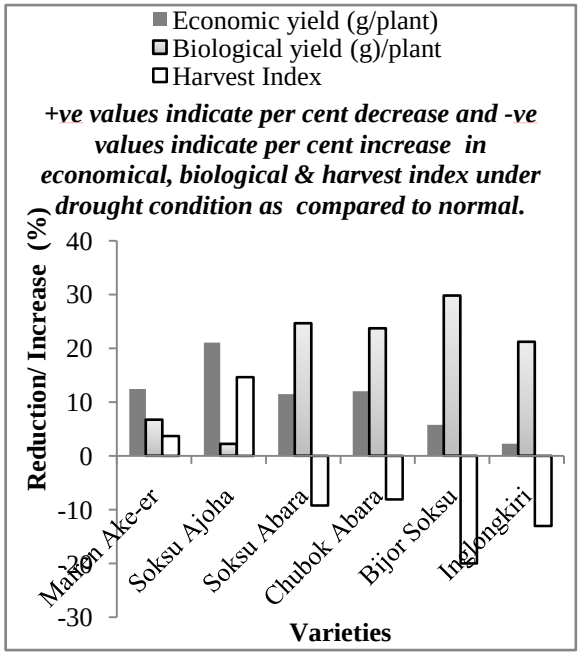


FIG. 13. CHANGES OF ECONOMICAL, BIOLOGICAL YIELD AND HARVEST INDEX UNDER DROUGHT AS COMPARED TO IRRIGATION

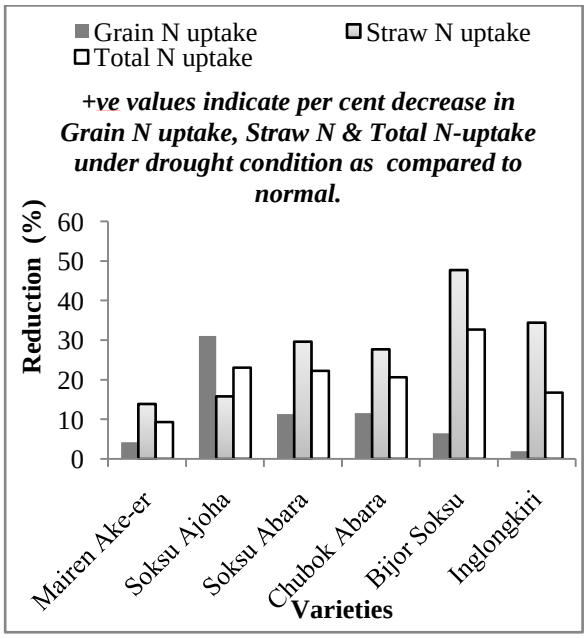


FIG. 14. CHANGES OF GRAIN, STRAW AND TOTAL N-UPTAKE UNDER DROUGHT AS COMPARED TO IRRIGATION

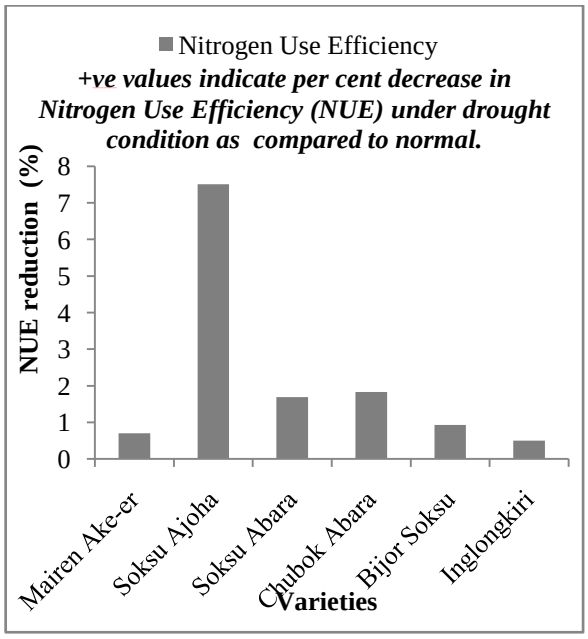


FIG. 15. CHANGES OF NITROGEN USE EFFICIENCY (NUE) UNDER DROUGHT AS COMPARED TO IRRIGATION

IV. CONCLUSION

In present study, the reduction in plant height was observed highest in Inglongkiri (18.03%) under drought as compared to irrigated. The highest per cent reduction in economical yield under drought was observed in Soksu ajoha (21.06%), whereas the lowest per cent reduction in grain yield was maintained in Inglongkiri (2.27%). This indicates that Inglongkiri behaves as one of the drought tolerant varieties. Inglongkiri (1.93%) processes the lowest per cent reduction of grain N uptake. The lowest per cent reduction of NUE under drought as compared to irrigated was exhibited in Inglongkiri (1.9%). Thus in this series of experiment, it could be concluded that Inglongkiri, a developed variety of Assam (RARS, Diphu), was found physiologically efficient among the varieties tested. This variety possesses the adaptive traits, especially higher N use efficiency, higher yield and attributes under physiological drought condition. Therefore, Inglongkiri may be taken as a donor in breeding programme for direct seeded upland limited moisture condition, and can be grown suitably under agro climatic conditions of elsewhere in Assam during *Ahu* season. Furthermore, to achieve an optimum yield, the cumulative dose of nitrogen as envisaged in the RTNM approaches, may be applied in splits up to 130 kg/ha based on the SPAD values of upland *Ahu* rice crop under physiological drought condition.

ACKNOWLEDGEMENTS

The Author wishes to thank Assam Agricultural University, Jorhat for furnishing all the necessary field and laboratory facilities for accomplishing the experiment in the Department of Crop Physiology, AAU, Jorhat and also like to thank Dr. Bhagawan Bharali, HoD of Department of Crop Physiology for their kind operation and valuable guidance.

REFERENCES

[1] Agostini, F.; Tei, F.; Silgram, M.; Farneselli, M.; Benincasa, P.; Aller, M.F. (2010). Decreasing N leaching in vegetable crops through improvements in N fertiliser management, Genetic engineering, biofertilisation, soil quality and organic farming. *Sustainable Agr. Rev.* **4**: 147-200.

[2] Balasubramanian, V.; Morales, A.C.; Cruz, R.T. and Abdulrachman, S. (1999). On-farm adaptation of knowledge-intensive nitrogen management technologies for rice systems. *Nutr. Cycl. Agroecosyst.,* **53**: 59-69

[3] Bharali and Chandra, K. (1996). SLW, a selection criterion for exploiting potential yield in rice (*Oryza sativa* L.) under low light condition. *J Envt. Biol.* **17**: 1-71.

[4] Bhattacharjee, D.P.; Krishnayya, G.R. and Ghosh, A.K. (1973). Analyses of yield components and productive efficiency of rice varieties under soil moisture deficit. *Indian J. Agr*. **16**: 314-343.

[5] Broadbent, F.E.; de Datta, S.K. and Laureles, E.V. (1987). Measurement of nitrogen- use efficiency in rice genotypes. *Agron. J.* **79**: 786-791.

[6] Burns,I.G. (2006). Assessing N fertiliser requirements and the reliability of different recommendation systems. *Acta Hort*. **700**: 35-48.

[7] Campbell, C. A. and Davidson, H R. (1979). Effect of temperature, nitrogen fertilization and moisture stress use by Manitou spring wheat. *Canadian Journal of Plant Science*, **59**: 603-626.

[8] Chauhan, J.S.; Chaunhan, V.S.; Sinha, P.K. and Prasad, K. (1989). Analysis of insitu variability for some panicle and grain characters in native germplasm of rice. *Oryza* **26**: 243-249.

[9] De Datta, S.K. and Broadbent, F.E., (1990). Nitrogen use efficiency of 24 rice genotypes on an N deficient soil. *Field Crops Res.* **23**(2): 81-92.

[10] De Datta, S.K.; Abilay, W.P. and Kalwar. (1973). Water stress effect on flooded tropical rice. *Water management in Philippines irrigation system research and operation*. 16-36.

[11] Dobermann, A.; Witt, C.; Dawe, D.; Abdulrachman, S.; Gines, H.C.; Nagarajan, R.; Satawathananont, S.; Son, T.T.; Tan, P.S. and Wang, G.H. (2002). Site-specific nutrient management for intensive rice cropping systems in Asia. *Field Crop Res.* **74**(1): 37-66.

[12] Dubey, R.S. and Singh, A.K. (1999). Salinity induces accumulation of soluble sugars and alters the activity of sugar metabolising enzymes in rice plants. *Biologia Plant.* **42**: 233.

[13] Fageria, N K. and Moreira, A. (2011). The role of mineral nutrition on root crop growth of crop plants. *Adv Agron*, **110**: 251–331.

[14] Feng, F J.; Xu, X Y.; Du, X B.; Tong, H H.; Luo, L J. and Mei, H W. (2012). Assessment of drought resistance among wild rice accessions using a protocol based on single-tiller propagation and PVC-tube cultivation. *Aust J Crop Sci*, **6**: 1205–1211.

[15] Ghanbari, A.A.; Shakiba, M.R.; Toorchi, M. and Choukan, R. (2013). Nitrogen changes in the leaves and accumulation of some minerals in the seeds of red, white and Chitti beans (*Phaseolus vulgaris)* under water deficit conditions. *AJCS* **7**(5): 706-712.

[16] Gloria, C.S.; Ito, O.; Alejar, A.A. (2002). Physiological evaluation of responses of rice (*Oryza sativa* L.) to water deficit. *Plant Sci.* **163**: 815-827.

[17] Haefele, S.M. and Hijmans, R.J. (2007). Soil quality in rice-based rainfed lowlands of Asia: characterization and distribution. In: *Science, Technology, and Trade for Peace and Prosperity. Proceedings of the 26th International Rice Research Conference, October 9–12, 2006, New Delhi, India.* Aggarwal, P.K.; Ladha, J.K.; Singh, R.K.; Devakumar, C.; Hardy, B. (eds.). IRRI/ICAR and NAAC, Los Banos, Philippines/New Delhi, India, pp. 297–308.

[18] Haefele, S.M.; Jabbar, S.M.A. and Siopongco, J.D.L.C. (2008). Nitrogen use efficiency in selected rice (*Oryza sativa* L.) genotypes under different water regimes and nitrogen levels. *Field Crop Res*. **107**: 137-146.

[19] Hanson, A.D.; Peacock, W.J.; Evans, L.T.; Arntzen, C.J. and Khush, G.S. (1990). Drought resistance in rice. *Nature* **234**: 2.

[20] Hanson, I.E.; Alagarswamy, G.A.; Mahalakshmi, V. and Biolenger, F.R. (1982). Diurnal changes of endogenous abscisic acid in leaves of pearl millet (*Pennisetum americanum*) under field conditions. *J. Exp. Bot.* **33**: 416-425.

[21] Hanson, I.E.; Mahalakshmi, V.; Biolenger, F.R. and Alagarswamy, G.A. (1981). Stomatal response of pearlmillet (*Pennisetum americanum* L.) genotypes in relation to abscisic acid and water stress. *J. Exp. Bot.* **32**: 1211-1221.

[22] Hirasawa, T.; Ozawa, S.; Tayraran, R.D. and Ookawa, T. (2010). Varietal differences in photosynthetic rates in rice plants with special reference to the nitrogen content of leaves. *Plant Prod. Sci.* **13**: 53-57.

[23] Jana, R.K. and Ghildyal, B.P. (1971). Effect of varying soil water regimes during different growth phases on the yield of rice under different atmospheric evaporative demands. *Il Riso Anno* 31-37.

[24] Janardhan, K.V. and Murty. (1980). Effect of low light during vegetative stage on photosynthesis and growth attributes in rice: *Indian J. Pl. Physiol* **23**(2): 156.

[25] Jasso, D.R.D.; Phillips, B.S; Garcia, R.R. and Angulo, S.J.L. (2002). Grain Yield and fatty acid composition of sunflower seed for cultivars developed under dry land conditions. *Agron*, **25**: 132-142.

[26] Ji, K X.; Wang, Y Y.; Sun, W N.; Lou, Q .;, Mei, H W.; Shen, S H. and Chen, H. (2012). Drought-responsive mechanisms in rice genotypes with contrasting drought tolerance during reproductive stage. *J Plant Physiol*, **169**(4): 336–344.

[27] Kalamian, S.; Modares sanavi, S A M. and Sepehri, A. (2006). Effect on of water deficit at vegetative and reproductive growth stages in leafy and commercial hybrids of maize. *Agri. Res. Winter*, **5**(3): 38-53.

[28] Khan, H.R.; Link, W.; Hocking, T.J. and Stoddard, F.L. (2007). Evaluation of physiological traits for improving drought tolerance in faba bean (*Vicia faba* L.) *Plant and Soil.* **292**: 205-217.

[29] Kumar, R.R.; Karajol, K. and Naik, G.R. (2011). Effect of polyethylene glycol induced water stress on physiological and biochemical responses in pigeon pea (*Cajanus cajan* L. Mill sp.). *Recent Res. Sci. Tech*. **3**(1): 148-152.

[30] Ladha, J.K.; Kirk, G.J.D.; Bennett, J.; Peng, S.; Reddy, C.K.; Reddy, P.M. and Singh, U. (1998). Opportunities for increased nitrogen-use efficiency from improved lowland rice germplasm. *Field Crops Res.* **56**: 41-71.

[31] Lauteri, M.; Haworth, M.; Serraj, R.; Monteverdi, M C. and Centritto, M. (2014). Photosynthetic diffusional constraints affect yield in drought stressed rice cultivars during flowering. *PLoS One*, **9**(10): e109054.

[32] Mohan, M.M.; Laxmi, N.S. and Ibrahim, S.M. (2000). Chlorophyll stability index (CSI): its impact on salt tolerance in rice. *International Rice Research Notes* **25**: 38-39.

[33] Moll, R.H.; Kamparth, E.J. and Jackson, W.A. (1982). Analysis and interpretation of factors which contribute to efficiency of nitrogen mobilization. *Agron J.* **74**: 262-264.

[34] Murthy, K.V.R. and Reddy, D.S. (2013). Effect of irrigation and weed management practices on nutrient uptake and economics of production of Aerobic rice. *J. Agri. Vet. Sci.* **3:**15-21.

[35] Nakayama, H. (1974). Panicle senescense in rice plant. *Bull Hokurika Nall Exp. Sta* No. **16**: 15-57.

[36] Neeteson, J.J. and Carton, O.T. (2001). The environmental impact of nitrogen in field vegetable production. *Acta Hort.* **563**: 21-28.

[37] O'Toole, J.C. and Baldia, E.P. (1982). Water deficits and mineral uptake in rice. *Crop Sci.* **22**: 1144-1150.

[38] O'Toole, J.C. and Chang, T.T. (1979). Drought resistance in cereal rice, A case study in stress physiology of crop plants. Mussel, H. and Staples, R.C. (eds.). Wiley Inter Science, New York, pp. 375-405.

[39] Pandey, A.; Kumar, A.; Pandey, D.S. and Thongbam, P.D. (2014). Rice quality under water stress. *IJPR.*

[40] Pandey, S. (1998). Nutrient management technologies for rainfed rice in tomorrow's Asia: economic and institutional considerations. In: Rainfed lowland rice: advances in nutrient management research. Ladha, J.K.; Wade, L.; Dobermann, A.; Reichhardt, W.; Kirk, G.J.D. and Piggin, C. (eds). IRRI, Los Baños, Philippines, pp. 3-28.

[41] Pearman, I., Susan, M.; Thomas and Thorne, G N. (1977). Effects of nitrogen fertilizer on growth and yield of spring wheat. Annal of Botany, 41: 93-108.

[42] Peng, S.; Garcia, F.V.; Laza, R.C.; Sanico, A.L.; Visperas, R.M. and Cassman, K.G. (1996). Increased N-use efficiency using a chlorophyll meter on high yielding irrigated rice. *Field Crops Res.* **47**: 243-252.

[43] Radford, P J. (1986). Genetic variability in sunflower cultivars under drought. II. Growth and water relations. *Aust. J. Res*, **37**: 583-598.

[44] Rahn, C. (2002). Management strategies to reduce nutrient losses from vegetables crops. *Acta Hort.* **571**: 19-25.

[45] Sandhu, S.S and Mahal, S.S. (2014). Performance of rice under different planting methods, nitrogen levels and irrigation schedules. *Indian J. Agron.* **59:** 392-397.

[46] Sarkar, R.K.; Saini, J.P. and Dubey, C.D. (1991). Testing of soybean (*Glycine max*) genotypes for drought tolerance. *J. Agric. Sci.* **61**: 369-373.

[47] Schenk, M.K. (2006) Nutrient efficiency of vegetable crops. *Acta Hort.* **700**: 25-38.

[48] Schonfeld, M.A.; Johnson, R.C.; Carver, B.F. and Mornhinweg, D.W. (1988). Water relation in winter wheat as drought resistant indicators. *Crop.Sci*. **28**: 526-531.

[49] Sharma, K.D.; Pannu, R.K.; Tyagi, P.K.; Chaudhary, B.D. and Singh, D.P. (2003). Effect of moisture stress on plant water relations and yield of different wheat genotypes. *Indian J. Plant Physiol.* **8**: 99-102.

[50] Singh, M P. (2002). Rice productivity in India under variable climates. *IARI*

[51] Tantawi, B.A. and Ghanem, S.A. (2001). Water use efficiency in rice culture. Agricultural Research Center, Giza (Egypt). *CIHM-Optin Mediterraneennes*, **40**: 39-45.

[52] Tao, Q.N.; Fang, P.; Wu, L.H. and Zhou, W. (1990). Study of leaf color diagnosis of nitrogen nutrition in rice plants. *Soils* **22**: 190-193.

[53] Thorup-Kristensen, K.; Sørensen, J.N. (1999). Soil nitrogen depletion by vegetable crops with variable root growth. *Acta Agri. Scand., Sect. B – Soil Plant Sci.* **49**: 92-97.

[54] Thorup-Kristensen, K.; Vander Boogard, R. (1999). Vertical and horizontal development of the root system of carrots following green manure. *Plant Soil* **212**: 145-153.

[55] Tuong, T.P.; Bouman, B.A.M. and Mortimer, M. (2005). More rice, less water-integrated approaches for increasing water productivity in irrigated rice-based systems in Asia. *Plant Prod. Sci.* **8**: 229-239.

[56] Wade, L.J.; Amarante, S.T.; Olea, A.; Harnpichitvitaya, D.; Naklang, K.; Wihardjaka, A.; Sengar, S.S.; Mazid, M.A.; Singh, G. and McLaren, C.G. (1999). Nutrient requirements in rainfed lowland rice. *Field Crops Res*. **64**: 91-107.

[57] Yang, P M.; Huang, Q C.; Qin, G Y.; Zhao, S P. and Zhou J G. (2014). Different drought-stress responses in photosynthesis and reactive oxygen metabolism between autotetraploid and diploid rice. *Photosynthetica*, **52**(2): 193–202.

[58] Yang, W.H.; Peng, S.; Huang, J.; Sanico, A.L.; Buresh, R.J. and Witt, C. (2003). Using leaf colour charts to estimate leaf nitrogen status of rice. *Agron. J.* **95**: 212-217.

[59] Yokota, A.; Takahara, K. and Akashi, K. (2006). Physiology and molecular biology of stress tolerance in plants. In: Madhavarao, K. Raghavendra and K. Janardhanreddy (eds.). Springer, pp. 15-40.

Development of Food Security through Integrated Bio-Cycles Farming System in Manokwari, Papua, Indonesia

Ambar Pertiwiningrum[1], Cahyono Agus[2], Supriadi[3], Arief Fahmi[4], Yudistira Soeherman[5]

[1,5]Faculty of Animal Science, Universitas Gadjah Mada Yogyakarta, Indonesia
[2.4]Faculty of Forestry, Universitas Gadjah Mada Yogyakarta, Indonesia
[3]Ministry of Villages, Development of Disadvantaged Areas and Transmigration, Indonesia

Abstract— *Indonesia's Law No. 18/2012 defines food security as the condition in which all people, in all households, at all times have sufficient food in both quantity and quality to enable them to live healthy, active, productive and sustainable lives, and that the food is safe, diverse, nutritious, equitably distributed and affordable, and does not conflict with religion, beliefs or culture. According to the Food Security and Vulnerability Atlas, Manokwari District in Province of West Papua was categorized as priority 2, strongly priority for improvement. The aim of this program was developing integrated bio-cycles farming system to improve their level of food security. The program was focused in Mansuburi and Wariori village, Masni Sub-district, Manokwari District, West Papua, Indonesia, from August 2015 for multi-years activities. Program was developed in cooperation between Kemendes PDTT (Ministry of Village, Development of Disadvantaged Areas and Transmigration); UGM Yogyakarta and local goverment of Manokwari District.*

The availability of food especially the production of rice, maize, cassava in Manokwari were relatively less developed. Based on the indicators of Normative Consumption per Capita Ratio (NCPR), the ratio of consumption to production in Manokwari was relatively high deficits (> 1.50) due to the limited rice area. Percentage of villages with no access to land and natural fresh water is quite low (<10%)., because of large area and high presipitation in Papua. The poverty was high (25-35%) causes a low access to food. About 30-40% of households have no access to clean water directly. Land conversion from forest area to Sawit estate area around Wariori river caused annual flooding that destroyed 60 ha of agricultural land during rain season since 2014, but the dried effect of El Nino phenomen in 2015 was strongly affecting agricultural production and food security.

Development of master plan for food security 2015-2019 through (i) capacity building of human resources, (ii) natural resource management (iii) business management, would give smart and futuristic perpective program for food security. Facilitating menu 1 (improved seed and fertilizer), menu 2 (infrastructure of check dam, technical irrigation), menu 3 (hand-tractor, handsrayer, cultivator, transplator, composting unit), and menu 4 (rice milling unit, power threser, post-harvest machines) would be very usefull for food security, although delayed in execution. Technical assistance by expert from UGM Yogyakarta improved technical capability in managing natural resource for improvement of food security. The role of 2 assistants that were staying in the village was very important for community empowerment to support food security.

Keywords: community empowerment, Top of Form, food security, integrated farming, master plan, world food program.

I. INTRODUCTION

The population of Indonesia is about 240 million. This is a very large number of people and it is the government's responsibility to ensure their quality of life. However, Indonesia is ranked 107 out of 177 countries in terms of Human Development Index. Food insecurity and poverty are still the major problems in Indonesia, and both are closely related. Food security is defined by the WHO as existing "when all people at all times have access to sufficient, safe, nutritious food to maintain a healthy and active life" (UNHCR, 2013). Conversely, groups that experience food insecurity consume insufficient and/or poor quality food, and may engage in socially undesirable activities to obtain food. Food insecurity (FI) is a major cause of poor nutritional status in populations globally De vriese (2006). Serious short- and long-term health implications (Foley et., al, 2010) include poor physical, mental, and social health (Strauss and corbin, 1994). FI can be chronic and persistent, generally caused by extreme poverty, or acute, which is transitory and often triggered by violent conflict or forced migration (Hadley, 2007).

The data of Dewan Ketahanan Pangan (National Food Security Council) in 2006 showed that most people have protein-energy malnutrition because they consume less than the recommended daily intake of calories (2000 kcal per capita) and protein (52 grams per capita). A total of 127.9 million people or 60 percent of the Indonesia's total population consume as much as 1322 to 1998 kcal/day. The impact of food insecurity is the malnutrition that can occur at any age. The BPS data in

(2006) showed that more than half of the districts/cities in Indonesia had over 25 percent prevalence of malnutrition among children under five years old. The results of Basic Health Research in 2013 conducted by the Ministry of Health showed that nationally, the prevalence of low weight-less in 2013 was 19.6 percent, composed of 5.7 percent malnutrition and 13.9 percent undernutrition. When compared to the national prevalence rate in 2007 (18.4 percent) and in 2010 (17.9 percent) there had been an increase. The prevalence of malnutrition changed from 5.4 percent in 2007, 4.9 percent in 2010, to 5.7 percent in 2013, while the prevalence of undernutrition rose by 0.9 percent from 2007 to 2013.

For Millennium Development Goals in 2015, the government had set the target of only 15.5 percent nutritional deficiencies. The root of these malnutrition problems is insufficient intake of nutrition among children under 5 years of age. The Indonesian government pays serious attention to the problem of food security and in 2012 had issued a specific regulation regarding food, namely Law No. 18/2012 on food. The law is intended as a legal basis for the implementation of food safety programs which include food planning, food availability, food affordability, food and nutrition consumption, food safety, food labeling and advertising, food supervising, food information systems, food research and development, food institution, community participation, and investigation. Indonesia's food production itself has some issues, namely: (i) the continued conversion of agricultural land to non-agricultural use; (ii) the decrease of the quality and fertility of the land due to environmental degradation; (iii) the more limited and uncertain availability of water for food production due to forest destruction; (iv) the destruction of approximately 30 percent of water infrastructure, where it should be rehabilitated twice in the last 25 years; (v) competition in the utilization of water resources by industrial and residential sectors; (vi) the damage caused by drought and flooding due to the greatly reduced natural protective functions; (vii) the still high proportion of yield loss in the production process, in harvest handling, and in post-harvest processing, which remains an obstacle that decreases the ability to supply the food in high proportion; (viii) climate change; and (ix) the competition between food and biofuel production.

The objectives of the Food Security Master Plan are as follows; (1) Knowing the causes and factors that affect the vulnerability and food insecurity in each predetermined region; (2) Conducting assessment for the region's potentials to develop a resilient food system; (3) Developing a strategy to increase food security in each predetermined region. The completion of the Food Security Master Plan is expected to bring several positive impacts, namely: (1) the existence of a focused policy at regional level on how to handle the vulnerable areas; (2) the management of vulnerable areas in a systematic, sustainable, and measurable way; (3) the changes from vulnerability to food security; (4) the improving conditions of national food security.

Food Security Council and the World Food Programme 2015 has published Food Security and Vulnerability Atlas of Indonesia in 2015. Based on the mapping, 37 percent of children under five years of age were stunted, three quarters of districts had a surplus of cereals, poverty in Indonesia had been reduced but still high, and 34 percent of households do not have access to clean water (WFP[1], 2015). In 2015, the government, through the Food Security Council and supported by the World Food Programme (WFP[2]), published a Food Security and Vulnerability Atlas. The food insecurity in Manokwari District is showed as follows on (tabel 1). Manokwari District was number 2 on priority list, so it could be concluded that the district was vulnerable to food insecurity. This vulnerability was based on the composite food security index. For the first parameter, which was the prevalence of stunting among children under 5 years of age, the percentage of stunted children under five in Manokwari was considered very high: more than 40 percent. It means that for this parameter, Manokwari district was in emergency situation. For the second parameter, which was the ratio of per capita normative consumption towards net cereal production (rice, maize, sweet potato, and cassava), the ratio of consumption to production in Manokwari was a high deficit: more than 1.5. Still in this parameter, concerning the village with inadequate physical access, the condition in Manokwari was good, only less than 10 percent of the villages there had no access to roads or waterways. The definition and measurement used was the proportion of villages that were not accessible by four-wheeled vehicle or water transport. In the third parameter, which was the population living below the poverty line, the percentage of poor people in Manokwari district was bad: about 25 to 35 percent of the population live below the poverty line. The definition and measurement used was the total expenditure per capita monthly (in Rupiah) to meet the minimum level of consumption (food and non-food) needed by an individual to have a decent life. For the forth parameter, which was the households without access to clean water, Manokwari district was still in good condition: about 30 to 40 percent of the villages do not have access to clean water. Clean water was defined as bottled water, refilled water, plumbing, protected spring, protected well, and pumped well – at least 10 meters from the nearest septic tank.

TABLE 1
THE STATUS OF FOOD INSECURITY IN THE STUDIED AREA BASED ON FSVA ATLAS 2015

District (*Kabupaten*)	Cases					Key Issues of Food Insecurity in the District Based on FSVA
	P1	P2	P3	P4	P5	
Manokwari	> 40 %	> 1.5 %	< 10 %	30 – 40 %	25 - 35 %	P1, P2, P5

P1: Prevalence of stunting among children under 5 years of age.
P2: Ratio of per capita normative consumption towards net cereal production.
P3: Population living below poverty line.
P4: Households without access to clean water.
P5: Poor families

II. METHODS

2.1 Data Collection

The primary and secondary data is used to attain the purposes of the study. The primary data collection uses two techniques (figure 1), namely: 1) observation: data collection by directly observing the object, 2) interview: the collection of data by requesting information through a structured questionnaire. The secondary data is collected by using recording techniques; it is conducted by writing down the data that is already exist and belongs to the agencies or institutions involved in this research. The secondary data are obtained from several publications, among others from the National Statistics Center (BPS), Regional Planning Agency (Bappeda), and other agencies concerned. The primary data is taken with interviews, questionnaires, and focus group discussion (FGD). This data is the main focus of the analysis, while the secondary data is used to complete the analysis of the primary data. The primary data is obtained from direct observation and interviews with respondents based on a structured questionnaire. Respondents are divided into two groups: the respondents from local community and the respondents from the government agencies. The respondents from local community are asked for information about; (a) community characteristics, including sources of livelihood and income, economic activities, food and economic assets, conditions of the area/village associated with food needs; (b) evaluation of the government's food program, programs that have been undertaken and the follow-up, community participation in the program, relevance of the program to the needs of the community, aspiration for future program to overcome food insecurity.

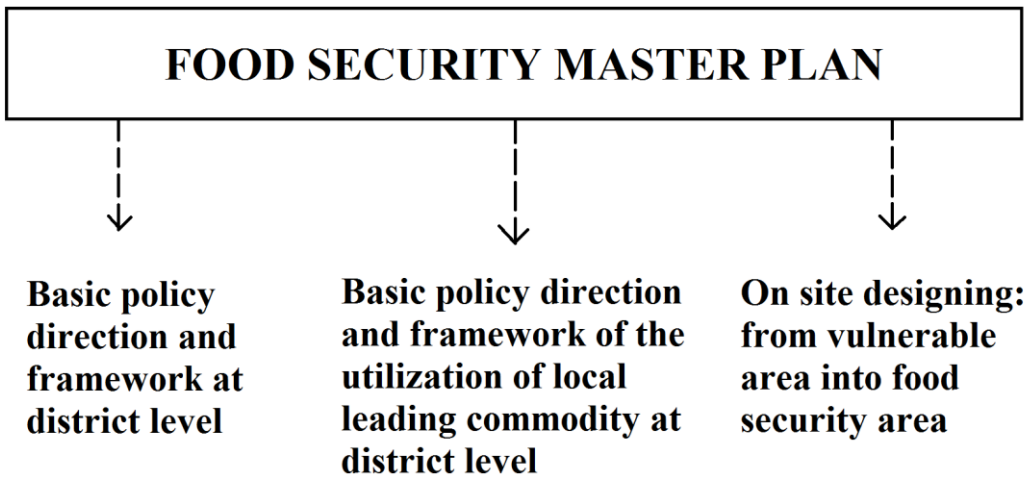


FIGURE 1. THE OUTPUTS OF FOOD SECURITY MASTER PLAN

The respondents from government agencies will be asked for data on the implementation of the government's food programs. The collection of data and information from them is conducted with interviews using variable list and keywords. Meanwhile, a semi-structured questionnaire is used to gather the data from community leaders in groups, while a structured question list is used for the interviews with the households.

2.2 Data Analysis

The results of the data collection are analyzed from the perspective of the needs for policy direction and program intervention to make the changes from vulnerability to food security. While doing this, the conditions and typology of the agroecosystem,

the existing socio-economic conditions, and the variations of local food resources in each area are also taken into account. This research was designed by qualitative approach and using descriptive analysis method in generating research phenomenon to know some aspects of a research topic (Donald and Schlinders, 1998). Data analysis by Tadjoer (2004) was designed with the following steps, namely: 1) data collection; 2) data reduction; 3) data display; 4) verification and affirmation of conclusions.

III. RESULT AND DISCUSSION

3.1 Food Productivity in Manokwari District

The quantity of various agricultural products continues to rise. Rice as a strategic and political commodity has experienced an increase in production. The average area harvested in the last 5 years was 4,432 ha with the harvest reached 16,399 tons of dry-mill unhusked rice (*gabah kering giling*). The amount of the dry-mill unhusked rice is equal to 13,119 tons of rice. With the population of 187,591 inhabitants and the assumption that the rice consumption per capita per year is 118 kg, it can be said that the need for rice in Manokwari district is 22,135 tons per year. Thus, there is still a shortage of rice supply as much as 9,016 tons. The increasing productivity of land is expected to reduce the food deficit so Manokwari can become an area with resilient food system. In addition to paddy, Manokwari regency also has the potential to develop other cereal and horticulture production. The superior commodities are corn, soybeans, green beans, peanuts, cassava, and sweet potatoes. The superior local food diversification is a good source of food for local communities. However, the productivity of the crops in Manokwari's vulnerable areas is still very low compared to national average: rice (3 tons/year/ha), sweet potatoes (1 ton/season), cassava (1 ton/season) and maize (1.5 tons/season/ha). The prices of those commodities are as follows: rice: Rp. 10,000/kg, ubi: Rp. 4,000/kg, cassava: Rp. 4,000/kg, maize: Rp. 7,000/kg, sago: Rp. 20,000/pack, banana: Rp. 5,000/bunch, fish: Rp. 20,000 – Rp. 30,000/stack, chicken: Rp. 50,000 – Rp. 65,000/whole chicken, beef: Rp. 90,000 – Rp. 100,000/kg, other meats (deer, pork, etc.): Rp. 75.000, eggs: Rp. 2,000/egg.

As for business analysis to produce rice in Manokwari, every 1 ha of land needs: 50 kg of seed, 150 kg of urea (fertilizer) that costs Rp. 95.000/50kg, 50kg Ponskha (fertilizer) that costs Rp. 120,000/50 kg, 50kg KCl (fertilizer) that costs Rp. 150,000/50kg, and chemical pesticides (Detan, Antrakol, Demolis, Kolakron, Dutbran, Prefaton, Bambu Hijau, Grentonik, Gramason, Round Up, Top Kill, Supremo). The labor needed to prepare the soil: 14 persons with wage of Rp. 100,000/day; tractor rent: Rp. 1,000,000/ha; the labor for planting: 16 persons with wage of Rp. 900,000/ha; the cost of care: Rp. 300,000, and the harvesting costs can be paid with the harvested crop. The production per hectare (in the form of dried unhulled rice harvest/*gabah kering panen*) is 3 tons/year at the price of Rp. 8,000/kg, while the production per hectare in the form of rice is 1.8 ton/year. The ownership of paddy land is 1 hectare per person, and there is no cooperation pattern between farmers and landowners. There is also no straw utilization since the farmers simply burn them. The paddy cultivated is a local variety named Cigilis, which is ready for harvest about 3 months after the seeds are planted. The average number of tractors per village is 8 units, managed by the group, rented with operating costs, salaries for the operators, and donations for the group. They use a threshing machine in the harvesting process, and they have to deal with planthoppers or sometimes snails as constraints or problems in paddy cultivating.

The cattle ranch is still run in traditional way with the ownership of cattle per household is 7 to 20. The cattle are fed with grass and allowed to graze freely on at least 10 hectares pasture fields that belongs to the village of Wariori. The field consists of uncultivated land, village land and oil palm plantation. Cows are not confined in cages because people of the village cannot afford to make them, and why bother to gather the forage grass and carry it to the cages while there is still extensive field that provides ample grass to feed their livestock. The average cattle price is 6 to 7 million rupiah. The constraints for cattle production in this village are the maintenance management that is still conducted in traditional way, and the people's low awareness.

3.2 Mansaburi Village

Mansaburi village is located on the shoreline of Pacific Ocean, 3 km from the capital of Masni district, which is the nearest market for agricultural products. Less than 5 percent of Mansaburi village has slope area, and its height is between 0-2 meters above sea level. The type of its soil includes clay, sandy clay and alluvial. The thickness of the topsoil is 10-20 cm. Mansaburi village has a quite good fertility rate. It can be seen in the use of land that mostly take advantage from the soil's fertility for farms and plantations. Another type of the soil is red-yellow podzolic latosol and litosol, while the types of the rocks are sedimentary rock and metamort. Quaternary age sediments only appear in a few places, while Resen (Qa) sediments only fills the valleys where today a big river and its tributaries flow. The climatic conditions in Mansaburi are

generally erratic, showing that the wet weather is more dominant than the dry months. This is in accordance with rain frequency and intensity that is rather high. The village is in the category of zone 5 with average annual precipitation ranges between 3000-4000 mm.

The land use in Mansaburi village are as follows; (1) 10 ha of primary forest area; (2) 6 ha of settlement and yard; (3) 2 ha of common facilities; (4) 65 ha of oil palm plantations; (5) 2 ha of shrubs area; (6) 3 ha of grazing area for livestock. Mansaburi's land where it is possible to grow crops is usually around the houses, which covers 0.25 hectares including a part of it to build a house. Mansaburi people do not use the land for agriculture but for crops, fruits, and vegetables. In Red Kali village, there is no farmland; only yard area around the houses alone. Their regular yard area is planted with cassava and maize (0.8 ha), vegetables and fruits (with 1.5 ha of banana plantation). Mansaburi people have natural food sources such as taro that is taken from nature. The fields were later turned into palm plantations. The commodities of Mansaburi village include sweet potato, cassava, corn, taro and horticultural plants such as spinach and squash in a very small area. The types of commodities that are widely grown are bananas (1.5 ha), coconut (0.8 ha) and cocoa (0.5 ha). However, its food processing is still undeveloped and very simple, for example: the abundant amount of coconuts is processed into copra and the extract can be made into coconut sugar. Also, cacao seeds can be dried to make raw material for chocolate, and in addition, bananas are also processed into chips or simply fried. While working as farmers, Mansaburi people also look for a way to diversify their farm. The livestock and poultry options are chickens, pigs, cows, and duck. The forage and fodder (*Hijauan Makanan Ternak*) were obtained from yards and fields. Free-range chickens are sold for their eggs and meat. Chickens, pigs, cows, and ducks are usually sold directly to the market. The plantations that are most widely developed in Mansaburi village are bananas and coconuts. Both are primary commodities that serve as a variation of local food. In addition, people also grow mango, citrus, areca nut, rambutan and durian as additional crop.

3.3 Wariori Village

Wariori is one of the villages in Masni District with the population of 1,298 inhabitants. The village has great agricultural potential since it has vast tracts of land. The commodities that are cultivated on this land are rice (that has a productivity of 12 tons/ha/year from an area of 300 ha), and corn (that has the productivity of 1.8 tons/ha/year from an area of 10 ha). Based on that data, it can be seen that the total rice production is 3,600 tons/year, and maize production is 18 tons/year. Some other plants that are also cultivated are: cassava with total area of 10 ha, sweet potato with a total area of 5 ha, and coconut with total area of 15 ha. Commodities such as rice, maize, and cassava are usually self-consumed and partly sold to wholesaler. There is no further food-processing in Wariori village other than making corn into milled corn. Aside from these commodities, another natural resource in the village comes from livestock and poultry. The existing potential are: 1,335 chickens, 145 pigs, 4 goats, and 404 cows. Fishery potential is not yet developed and only a few people grow fish in the ponds for food. Fish farming can be a good source of protein that can be developed in Wariori river with floating fish cages, if people can solve the flooding problems.

3.4 Design of a Region with Integrated Resilient Food System

The increased capacity is needed to ensure the improvement of labor's skills that is environmentally sustainable through; (a) The development of human resources through a mental revolution with independent human resource development and the improvement of management, technical, and financial capacity through training, mentoring, and empowering the actors in the field; (b) The mentoring by "TROOPS OF FOOD SECURITY" coordinated professionally by Unit Coordinator and Sector Coordinator, involving a combined team of students, scholars, agents of influence, volunteers, army, facilitators, community leaders, teachers, environmental student group, and field workers, all in intensive, integrated, and sustainable way for the empowerment of natural and human resources; (c) The development of "PRODUCTIVE ECONOMY & BUSINESS", which is the development of integrated agricultural enterprises as the source of food and family income through productive-conservative programs and creative industries based on local resources that are featured, integrated, and comprehensive, from "upstream" to "downstream"; (d) The organization of "HEALTHY MANAGEMENT & ORGANIZATION" to strengthen community and institution empowerment through management and rehabilitation of natural resources based on local wisdom and region with integrated resilient food system, involving marginalized local communities, women's groups, youth groups and junior high school students, to facilitate the village and school's plant nursery, neighborhood trainers, etc.

The development of resilient food system with refinement and application of integrated farming concept such as;(a) The innovation of "INTEGRATED AGRICULTURE" through application of IBFS concept (Integrated Bio-cycles Farming System) which consists of the management of the plant (Integrated Crop Management), soil moisture (Integrated Moisture Management), nutrients (Integrated Nutrient Management), pest and disease (Integrated Pest Management), from "upstream"

to “downstream” through 5A (Agro-production, Agro-technology, Agro-industrial, Agro-business, Agro-tourism) in the integrated and sustainable empowerment of natural resources for the improvement of land productivity in conservative way, based on society; (b) To facilitate "AGRO-PRODUCTION" through management of community land along with innovations in integrated featured agriculture to synergize the sectors of agriculture, livestock, forestry, veterinary, agricultural technology, in an integrated and comprehensive manner from “upstream” to “downstream”; (c) To facilitate "AGRIBUSINESS" through business management so the business doer can participate in modern way, not getting stuck in traditional system which is the subsystem. It also makes business-doer more prosperous, keeping them from being manipulated by other economic sectors; (d) To facilitate “AGRO-TECHNOLOGY” through efficient technology and suitable biotechnology to create a new revolution in the fulfillment of many people’s lives; (e) To facilitate "AGRO-TOURISM" through educational tours so that every living being can enjoy and contribute in fulfilling the needs of life and environmental improvement; (f) To facilitate ”AGRO-CONSUMPTION” through diversification of food and local food processing; (g) To facilitate “AGRO-DISTRIBUTION” through storage, distribution, and trading systems; (h) To facilitate “FOOD PRODUCTION’S MEANS AND FACILITIES“ by increasing the quantity and quality of the means and infrastructure of integrated food production, such as tractors, harvesters, fertilizer processing unit, etc; (i) To facilitate “FOOD INFRASTRUCTURE” in the form of ponds, dams, irrigation canals, farm roads, new fields, green cottage, nursery farm, productive-conservative land, green meeting rooms, etc; (j) The revitalization of "SELF-ENERGY" through rehabilitation of biomass-biogas energy by making use of waste in the form of animal manure, agricultural waste, and other waste in biogas installations with various types of dome, pipe, and material; (k) The development of "GREEN FERTILIZER" through composting and the use of organic matter as a source of nutrients to improve productivity and land rehabilitation; (l) The development of “FOOD SAVINGS & FELLOWSHIP" through the cultivation of animals and plants to fund education, scholarships for children and green trainers by planting fast growing plants and raising animals to pay students' school fees; (m) DEVELOPMENT OF FOOD SCIENCE RESOURCE to support the activities to develop a region with Resilient Food System by facilitating the collection, application, and dissemination of knowledge related to development, production, consumption and distribution in that region; (n) Survey, inventory, data analysis, mapping and information systems in the database of natural resources in integrated FOOD INFORMATION SYSTEMS; (o) Researches that are applicative-collaborative, innovative-inventive, as well as multi-, inter-, and intra-disciplinary with the theme “RESEARCH & SOCIAL SERVICES IN FOOD SECTOR” concerning local natural resources, conducted by reliable researchers from universities and research organizations supported by undergraduate and postgraduate students; (p) The development of "FOOD PUBLICATION & COMMUNICATIONS” through training, in-house and field training, workshops, focus group discussions, teleconferences, seminars, seminar publications, national-international journals, the improvement of vulnerable and marginal people’s skill, as well as dissemination of the results of programs and research; (q) The development of strategic information systems and integrated information dissemination concerning land resources, biodiversity, and environment, through online technology system of “google drive”, web, electronic media, virtual media, printed media, leaflets, booklets, social media, books, newsletters, writing competitions about green knowledge, etc; (r) Knowledge Management development done sustainably by conducting the transfer of information, knowledge, and experience to be equally implemented and developed during the program.

KISS ME (coordination, integration, synchronization, synergism, Monitoring and Evaluation) will be conducted through; (a) Routine coordination and consolidation Meetings between the implementation team and all stakeholders involved; (b) Integrating activities and policies based on the database; (c) Synchronization of planning and field implementation based on the latest database; (d) Synergism between internal and external program to have greater leverage in achieving the optimal results of performance; (e) Monitoring the implementation of activities in the field by internal and external team (in actual or virtual way). The monitoring activities are carried out by observing carefully the circumstances or conditions, including the conduct or activities of the program. The aim is to make all input data or information obtained in the observation as the basis for making decisions needed for further action. The purpose of this monitoring is to observe/determine the development and progress, to identify the problems and the anticipation/attempted solution; (f) Evaluation by internal and external team (in actual or virtual way). The evaluation of the program aims to see the success rate of activity management through the study of management and the output of its implementation, as well as the problems faced. All of the information will be used to evaluate the performance of the next program and activities. The form of the evaluation is the assessment of management and the output of its implementation, as well as the problems faced; (g) Written and oral report; (h) Progress and activity’s follow-up for continuous quality improvement

The performance indicators are measured with the input, the process, and the output of the implementation of the program which include: time management, financial planning, program input, program implementation, implementation process, the

achievement of program targets, the precision in planning and execution, program product, and so on. Food Security Master plan for Manokwari District described by (table 2).

TABLE 2
FOOD SECURITY MASTER PLAN FOR MANOKWARI DISTRICT

Focus on Issues Based on FSVA (*Food Security and Vulnerabi-lity Assess-ment*)	Region's Agro-ecosystem condition	Resume of Socio-Economic Conditions	Region's Policy Direction	Food Security Master Plan	
				The Choice of Program Interventio n	Supportin g Activities
•Prevalence of children stunting is high. • The level of cereal consumption is low. • Poor family	1. **The main food commo-dities**: the major food source for the community is cultivated rice in the fields. 2. **The availability of land for food production**: Potential vast tracts of land, and part of it have not been managed /utilized. 3. **Pasture and potential grazing land**: Pasture still vastly available, and grazing po-tential is very high 4. **Water potential for cultivation**: natural potential of the river flow is high enough, but not all used for irrigation system 5. **Rainfall**: Very high, around 2008.38 mm / year 6. **Variation of food sources**: the community's staple food is rice, with food sources variation of corn, soybeans, green beans, groundnuts, cassava, and sweet potatoes. **Problems**: • Conversion of forest to oil palm plantations without considering environmental issue. Annual flood damage food land area • The land for food production is limited to the yard **Risk of Disaster**: earthquake and tsunami, because it is located in the vicinity of the fault	1. **Social cohesion**: social life in the observed villages is quite well, there are no obstacles related to social conflicts or conflicts of interest 2. Motivation and work ethic of the community to progress are quite well, but the independence still needs to be improved. 3. **Village organization and farmer's group**: Village governmental institutions function well in serving the community. Farmer's group has been formed long and capable of functioning. 4. **Education**: the lack of education, skills and human resources are the causes of the high unemploy-ment. Trainings for specialized skills are needed to improve the quality of human resources. 5**. The technical ability to cultivate**: People have been using the method of intensive rice cultivation. Livestock is still cultivated by traditional grazing systems. Intensive broiler farms have been cultivated on a small scale (population: hundreds) 6. **Agricultural technology**: people are already quite familiar with the use of modern agricultural equipment such as tractors, manual threshing and milling machine. 7. **Potential commodities**: besides the staple food, there are potential for rubber plantations, coffee and oil palm plantation.	1. To improve the quality of food/nutriti-on intake for children, mothers, and nursing mothers. 2. To improve the quality and quantity of food production facilities and infrastructure 3. To deve-lop and increase the consumption of local food varieties. 4. To increase family access to sources of clean water. 5. To build installation of clean water to meet the needs. 6. To maintain the pattern of local food consumption. 7. To increase the productivity of paddy field. 8. To develop an institutional system that can perform the functions of coordination and synergy across sectors	To improve family's ability in producing and processing nutritious food, based on local food resources To improve agricultural infrastructur e To develop processed products made from local potential	1. To use the houses' backyard to sustain family nutrition 2. To increase the capability of nutritious food processing . 3. Protein production on household scale 1. To developof tertiary irrigation canal. 2. To manage farm road constructi on 1.Facilitati ng the means of production 2. Facilitatin g the certificatio n and marketing

IV. CONCLUSION

There are four main issues concerning vulnerability to food insecurity in Papua region, as follows: the prevalence of stunting children under five, the ratio of per capita normative consumption towards net cereal production, households without access to clean water, and poor families. The toddlers stunting is caused by insufficient nutritional intake by the toddlers themselves and by pregnant women. Several factors are associated with the stunting problems, including the lack of energy and protein, chronic disease that often occurs, insufficient feeding practices and poverty. Those problems also need to be responded to enhance the ability of families in consuming nutritious food.

To cope with this aspect of malnutrition, it is recommended to implement the Program of Family's Capacity Improvement in Producing and Processing Nutritious Food Based on Local Food Resource. This program needs to be supported by a wide range of household-scale productive activities such as backyard farming to produce vegetables for the family, and raising free-range chickens on "mini" scale to produce eggs as a source of supplemental protein for the family. The "mini" scale (5-10 chickens) aims to reduce the burden of maintenance costs to zero. The needs for poultry feed in such volume of business can be provided by the household's food waste and other sources.

To overcome the problems concerning the ratio of per capita normative consumption towards net cereal production and the preservation of local food, local crops should be grown, primarily sweet potatoes and cassava. A strict monitoring should also be conducted to control the conversion of agricultural land to grow food into oil palm fields. To deal with the problems concerning poor families, a program should be implemented: The Improvement of Rural Poor Families' Welfare through Optimization of Integrated Land Resources Intensively. The program is based on the finding that there are still a lot of land resources in the studied locations that have not been utilized optimally. Regarding clean water issues, the efforts to apply appropriate technology should be encouraged to meet the community's needs for clean water and drinking water.

REFERENCES

[1] Badan Ketahanan Pangan. 2006. *Pedoman Umum Program Aksi Desa Mandiri Pangan* (MAPAN). Depatemen Pertanian.

[2] BPS. 2006. *Produk Domestik Regional Bruto Kabupaten Semarang*. BP. Kabupaten Semarang.

[3] BPS. 2013. *Proyeksi Penduduk Indonesia 2010-2035*. Badan Perencanaan Pembangunan Nasional, Badan Pusat Statistika Indonesia. Jakarta.

[4] De Vriese, M. 2006. Refugee Livelihoods: A Review of the Evidence. Evaluation and Policy Analysis Unit. UNHCR: Geneva.

[5] Donald, R.C., dan Schlinders. 1998, Business Research Methods 6th ed. Illinois: Richard D. Irwin, 1998, handbook.

[6] Foley, W., Ward, P., Carter, P., Coveney, J., Tsourtos, G., and Taylor, A. (2010) 'An Ecological Analysis of Factors Associated with Food Insecurity in South Australia, 2002-7'. Public Health Nutrition 13(2), 215-221.

[7] Hadley, C., Zodhiates, A., and Sellen, D.W. 2007. Acculturation, Economics and Food Insecurity among Refugees Resettled in the USA: A Case Study of West African Refugees'. Public Health Nutrition 10(4), 405-412.

[8] Riset Kesehatan Dasar 2013. *Pedoman Pewawancara Petugas Pengumpul Data*. Jakarta: Badan Litbangkes, Depkes RI.

[9] Strauss, A. and Corbin, J. 1994. Grounded Theory Methodology: An Overview. In Denzin, N.K. and Lincoln, Y.S. (eds) Handbook of Qualitative Research. London: Thousand Oaks: Sage, pp. 273-285.

[10] Tadjoer, R. 2004. Metode Bricolage dalam Penelitian Sosial, dalam Burhan Bugin (eds.). "Metodelogi Penelitian Kualitatif" Aktualisasi Metodelogis ke Arah Ragam Varian Kontemporer, Edisi 1, Cetakan III. PT. Raja Grafindo Persada, Jakarta.

[11] UNHCR (United Nations High Commissioner For Refugees). (2013) UNHCR Global Appeal 2014-2015 – Ecuador. UNHCR: Geneva.

[12] WFP[1]. 2015. Peta Kerentanan dan Ketahanan Pangan Papua. World Food Programme www.wfp.org. (Accessed 06.11.2017).

[13] WFP[2]. 2015. Peta Kerentanan dan Ketahanan Pangan Indonesia. World Food Programme www.wfp.org. (Accessed 06.11.2017).

Morphological Characterisation of Harumanis Mango (*Mangifera indica* Linn.) in Malaysia

Mohd Asrul Sani[1#], Hartinee Abbas[2], Mahmad Nor Jaafar[3], Mohamad Bahagia Abd Ghaffar[4]

[1,3]School of Bioprocess Engineering, University Malaysia Perlis, Jejawi 3 Academic Complex, 02600 Arau, Perlis, Malaysia
[2]Horticulture Research Centre, MARDI Sintok, 06050 Bukit KayuHitam, Kedah, Malaysia
[4]Rice Research & Innovation Centre, MARDI SeberangPerai, 13200 Kepala Batas, Penang, Malaysia

Abstract*—Harumanis is a very popular green eating mango variety which has been planted commercially in the State of Perlis, Malaysia. However, several variations such as fruit shape and tree architecture which are apparent on field have caused confusing to the farmers as well as consumers of the 'genuine' Harumanis. This study was designed to observe the variation among Harumanis population at several locations in Malaysia. Samples were classified into two treatments based on their tree architecture: droop Harumanis and erect Harumanis. The samples were collected from six farmers' plot in Perlis, Kedah and Johor. Samples were subjected to fruit quality and branch strength analysis. Fruit quality characterisations and branch strength analysis revealed significant variation among treatments. Fruits harvested from droop Harumanis tree was bigger than erect Harumanis. Droop Harumanis fruit samples recorded higher value for relative water content, moisture content and modulus of elasticity while erect Harumanis recorded higher value for fresh-weight basis density and modulus of rupture. This study indicated that morphological characterisations play an important role in determining phenotypic variation in inter-variety population including Harumanis.*

***Keywords*—branch strength analysis, fruit quality analysis, Harumanis, Mangifera indica L., modulus of rupture.**

I. INTRODUCTION

The mango (*Mangiferaindica* L.) is among the widely grown tropical and subtropical fruit of the world including Malaysia and is a diploid fruit tree with 2n = 40 chromosomes[1], [2]. Until 2016, there was about 5,816.4 hectare of mango cultivation in Malaysia with 17,429.7 metric tonne of production worth more than RM57 million [3]. There are several varieties of mango grown in Malaysia; the better known cultivars are Golek (MA 162), Masmuda (MA 204), Maha 65 (MA 165), Chok Anan (MA 224), Nam Dok Mai (MA 223), Sala and Harumanis. Harumanis among them, the most popular clone, which was registered as MA128 by the Department of Agriculture, Malaysia [4], [5], yielding at 2.69 tonne/ha in 2014 [6]. As described by JabatanPertanian Malaysia (2015), the fruit shape of Harumanis is oblong, has a prominent beak, the skin colour is green with a little bit of glossy and will turn to yellowish green when ripen, the fruit size varies ranging from 300 to 650 gram, has 16-17°Brix and the flesh is orange colour and sweet. Harumanis is very suitable for the export market as it has desirable colour and sweetness and good eating quality with good aroma [7].

Harumanis was believed to be originated from Indonesia and being domesticated in Malaysia ever since it was registered by the Department of Agriculture, Malaysia on May 28, 1971 [4]. Like many other cultivars derived from South East Asia, Harumanis is polyembryonic, i.e. the seeds have nucellar embryos that are genetically identical to the mother plant [8]. Because of this, polyembryonic cultivars have generally been propagated by seed and Harumanis is no exception to this rule. A viable zygotic embryo is also present in the seed of some polyembryonic mango cultivars [8]. As reported by Schnell & Knight (1992), the number of zygotic off types in seedling populations varied and can be as high as 64% in the cultivar Golek and as low as 0% in the Israeli cultivar 13-1.

Harumanis is well accepted by Malaysians and were planted by many as well as by commercial planters. In the state of Perlis alone, 1,037 hectare of land were planted with Harumanis [10]. However, a few reports by the growers of Harumanis in Perlis indicated that there are variations in term of fruit size, flesh colour and number of fruits per panicle (Mohd Azhar Hassan, personal communication, October 6, 2017). There are some cases where the growers found that the flesh colour of matured Harumanis fruits are yellow instead of orange. This phenomenon is not new in mango cultivation. There were reports of identification of intra-cultivar variation in mango such as in Sala [5], Chok Anan [11], Indian mango [12] and Kensington mango [8]. Khan, Ali, & Khan (2015) also reported that extensive differences exist among mango genotypes of the similar clones in any particular orchard, specifically with the respect to fruit shape, size, colour, aroma, flavour eating quality and texture in which, usually caused by either outcrossing or natural mutations. The presence of fruits in the

evaluation of morphological variations in fruit crop is crucial. Nevertheless, in off fruiting season, breeders still need to identify dissimilarity among mango varieties/cultivars. Therefore, other morphological characteristics namely differences in tree architecture type need to be defined. A basic knowledge of the strength properties of mango wood such as moisture content, bending strength, elasticity and etc. are essential and interesting to explore. There is a need to study the variations in Harumanis population whether the variations, if any, could lead to economic and social importance, as well as to the scientific values. Morphological characters evaluation is the first step that should be carried out before advanced biochemical or molecular studies are carried out. Hence, justify the objective of this study.

Generally, morphological characterisation is an evergreen methodology that uses the ocular estimate method with the help of several qualitative and quantitative measuring tools to score and distinguish tropical fruits including mangoes. The characters include tree height, growth habit, flowering pattern, disease resistant, inflorescence shape, colour and type of flowers, fruit shape and shape, shoulder position and type of seeds i.e. monoembryonic or polyembryonic.

II. MATERIAL AND METHOD

2.1 Plant Materials and Fruit Quality Analysis

Throughout this experiment, Harumanis plants were classified into two main category based on its tree architecture namely; droop and erect type (Fig. 1) as described by IPGRI (2006). Samples were collected from six farmers' plots in Perlis, Kedah and Johor. This experiment was divided to two sub-experiments which required two different types of samples. Sub-experiment 1 was conducted to evaluate fruit quality of selected Harumanis. Meanwhile in sub-experiment 2, the branch strength of selected Harumanis plants were studied.

FIGURE 1: TREE ARCHITECTURE OF TWO DIFFERENT TYPE OF HARUMANIS I.E. DROOP HARUMANIS (LEFT), AND ERECT HARUMANIS (RIGHT).

Matured fruits were harvested and artificially ripened by using calcium carbide prior to evaluation. Five fruits per plant were collected randomly and were analysed in the laboratory for various physico-chemical traits such as fruit weight (g), skin weight (g), flesh weight (g), fruit length (mm), fruit diameter (mm), fruit length-to-diameter ratio, flesh recovery (%) and total soluble solids (TSS) (°Brix) following standard analytical procedures. Fruit length to diameter ratio was defined by the length of the fruit divided by the diameter of the fruit. Flesh recovery was calculated by the weight of the flesh divided by total weight of the fruit multiplied by 100.

2.2 Branch Strength Analysis

Two separate sets of samples were obtained for static bending properties and basic density test. Samples were collected from various farmers' farms in Perlis, Kedah, and Johor. Both tests were done with the actual form of stems i.e. no moulding to the samples. For basic density test, 20 cm in length with diameter ranging from 15 to 20 mm test pieces, were harvested from selected live trees from different places for both types of tree architecture. Cut ends of the stems were coated with wax to prevent moisture loss. Moisture content and specific gravity of the test pieces was measured based on ASTM D1037-99 (1999). Specific gravity was computed from the weight and dimensions of the test pieces on a dry-weight basis. Moisture content and specific gravity were calculated as follows:

$$M = 100\left[\frac{(W-W_f)}{W_f}\right]. \quad (1)$$

where M is the moisture content (%), W is the initial weight (g) of the harvested branch measures on-site, and W_f is the weight of branch measures before the static bending test.

$$sp\ gr = \frac{KF}{L(\pi r^2)} \quad (2)$$

where F is the final weight when oven dry (g), L is the length of test specimen (mm), r is the radius of test specimen (mm) and K = 1, when SI units of weight and measurement are used.

Other parameter calculated in basic density test was relative water content, RWC, as mentioned by Kirkham (2014) and the formula is as follows:

$$RWC = \left[\frac{(fresh\ weight - dry\ weight)}{(turgid\ \ weight - dry\ weight)}\right] \times 100 \quad (3)$$

Static bending properties was tested according to ASTM D 1037-99 (1999). Modulus of rupture (MOR) and modulus of elasticity (MOE) were determined in a static bending test on 20 cm in length and 15 to 20 mm diameter of fresh branches using Instron 5569 50kN Universal Testing Machine at a loading rate of 6.1 mm min^{-1}[16]. The test pieces were loaded to failure in a three-point loading over a span of 100 mm.

The MOR were calculated using Equation (4):

$$\sigma = \frac{3PL}{2(\pi d^3)} \quad (4)$$

The MOE was calculated using Equation (5):

$$E = \frac{0.5PL^3}{\pi d^3} \quad (5)$$

where P is the maximum load (N), L is the distance (mm) between the support span, and d is the branch diameter (mm).

Density for static bending test was calculated on a fresh-weight basis using formula as follows [17]:

$$\rho = \frac{m}{V} \quad (6)$$

where m is the mass of test specimen (g) and V is the volume of test specimen (cm^3).

2.3 Statistical Analysis

Data were analysed by using the SAS® University software version 9.4 and were subjected to one-way analysis of variance (ANOVA) and means were separated by Student-Newman-Keuls (SNK).

III. RESULTS AND DISCUSSION

3.1 Fruit Quality Analysis

All fruit samples from both type of droop and erect Harumanis were subjected to physico-chemical traits i.e. fruit weight, skin weight, flesh weight, fruit length, fruit diameter, fruit length-to-diameter ratio, flesh recovery and TSS. The analysis of variance showed no significant different between the treatments with fruit weight, skin weight, flesh weight, flesh recovery and TSS. However, significant differences were observed between treatments for fruit length and fruit length-to-diameter ratio (Table 1).

TABLE 1
MEAN SQUARES FROM ANOVA FOR FRUIT QUALITY ATTRIBUTES IN HARUMANIS POPULATION

Sources	Df	Fruit Weight (g)	Skin Weight (g)	Flesh Weight (g)	Fruit Length (mm)	Fruit Diameter (mm)	Fruit Length-to-Diameter Ratio	Flesh Recovery (%)	TSS (°Brix)
Variants	1	8847.90	206.08	5557.13	3598.57 **	1.95	0.48 **	1.51	2.68
Rep	25	6248.51	180.84	4175.30	165.18	17.23	0.02	7.27	2.80
Mean		478.02	89.21	344.39	126.88	83.19	1.53	71.96	19.38
CV		14.53	17.94	14.59	13.41	5.58	12.11	4.07	8.86

*****Significantly different at P≤0.01***

Results on mean of studied pyhsico-chemical traits are shown in Table 2. It shows that there was variation in fruit size of Harumanis. Based on description by IPGRI (2006), fruits from droop Harumanis is in obovoid shape while fruits from erect

Harumanis is roundish shape (Fig. 2) as reflected from the findings of fruit length and fruit length-to-diameter ratio. Fruit length-to-diameter ratio was used as a referral to fruit shape. Droop Harumanis' fruits are longer (135.20 mm) than fruits from erect type (118.56 mm). The most prominent feature of Harumanis mango is the formation of a slight projection which develops laterally at proximal end of the fruit, usually known as the "beak" by the locals. Both droop and erect Harumanis have this beak (Fig. 2). The topic of fruit growth and development may be influenced by genes, proteins as well as agronomic practices, climate and other mechanical processes that specify or affect the fruit formation and development. Plants compromised in photosynthesis, phloem transport, floral initiation and development, or male or female fertility either cannot produce fruit or are abnormal in their fruit production i.e. parthenocarpic fruit, reduced fruit size, or reduced fruit load [18]. Khan et al. (2015) also reported that even if a variety of mango being grown in the same region, its quality will be affected by different environmental.

TABLE 2

MEANS OF FRUIT WEIGHT, SKIN WEIGHT, FLESH WEIGHT, FRUIT LENGTH, FRUIT DIAMETER, FRUIT-LENGTH-TO-DIAMETER RATIO, FLESH RECOVERY AND TSS IN HARUMANIS POPULATION

Tree Architecture Type	Fruit Weight (g)	Skin Weight (g)	Flesh Weight (g)	Fruit Length (mm)	Fruit Diameter (mm)	Fruit Length-to-Diameter Ratio	Flesh Recovery (%)	TSS (°Brix)
Droop	491.06a	91.20a	354.73a	135.20a	83.39a	1.62a	72.13a	19.16a
Erect	464.97a	87.22a	334.06a	118.56b	82.99a	1.43b	71.79a	19.61a

Means within column followed by the same letters are not significantly different at p=0.05.

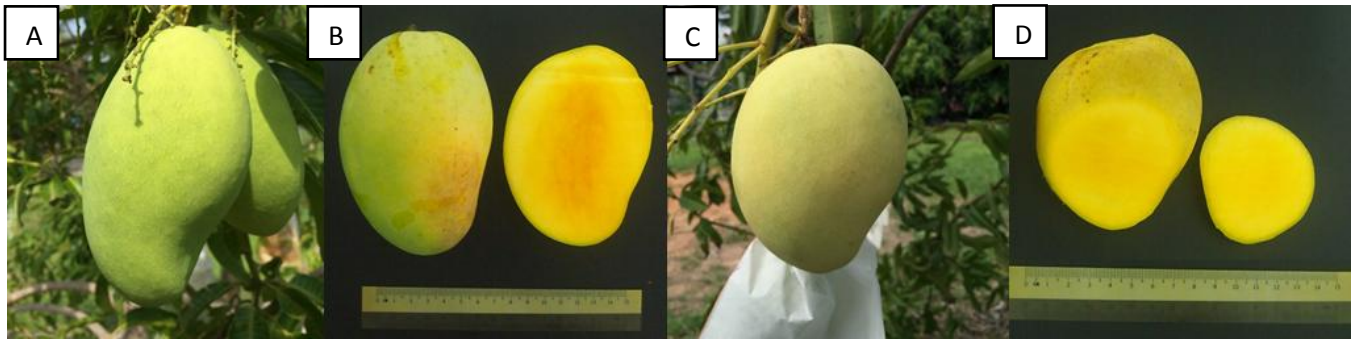


FIGURE 2: FRUIT FROM TWO DIFFERENT TYPE OF HARUMANIS I.E. DROOP HARUMANIS (A) & (B), AND ERECT HARUMANIS (C) & (D).

3.2 Branch Strength Analysis

Stem traits emerge as important plant functional traits, even for fruit trees, because of their role for stability, defence, architecture, hydraulics, carbon gain and growth potential, hence justify this experiment. Two sub-experiments were conducted to study branch strength of droop and erect Harumanis i.e. basic density test and static bending properties. Branch strength analysis were divided into those sub-experiments because they involved destructive analysis.

Data from ANOVA showed that for basic density test, there was no significant different for stem diameter and specific gravity, but significant differences were observed between treatments in relative water content (RWC) and moisture content (Table 3). Results on mean of stem diameter, RWC, specific gravity and moisture content are shown in Table 4. Droop Harumanis recorded higher percentage of RWC and moisture content (17.23% and 177.87% respectively) than erect Harumanis (17.05% and 151.31% respectively). RWC compares the water content of a stem with the maximum water content at full turgor and take into consideration the quantity of water in the plant. However, two different species may have the same RWC values with different amounts of water in their leaves (Ceccato et al.,2001). Malavasi et al. (2016) noted that variations in wood stem water content are generally related to environment such as rainfall factor as well as agricultural practices. Zziwa et al. (2016) and Malavasi et al. (2016) reported that the biological properties of biological materials such as wood and mechanisms of internal water influx regulation, vary within and among tree and species. This is attributed partly to differences in phenotypic and genetic composition.

TABLE 3
MEAN SQUARES FROM ANOVA FOR STEM DIAMETER, RWC, SPECIFIC GRAVITY AND MOISTURE CONTENT OF SELECTED HARUMANIS ACCESSIONS.

Sources	df	Stem Diameter (mm)	RWC(%)	Specific Gravity	Moisture Content (%)
Treatments	1	2.28	172.01**	0.0019	5641.61**
Rep	15	0.68	19.45	0.0011	217.28
Mean		16.34	91.23	0.51	164.59
CV		5.83	4.17	13.28	11.89

***** Significantly different at p≤0.01***

TABLE 4
MEANS FOR STEM DIAMETER, RWC, SPECIFIC GRAVITY AND MOISTURE CONTENT OF SELECTED HARUMANIS ACCESSIONS.

Treatment	Stem Diameter (mm)	RWC(%)	Specific Gravity	Moisture Content(%)
Droop	16.61a	17.23a	0.52a	177.87a
Erect	16.08a	17.05b	0.50a	151.31b

Means within column followed by the same letters are not significantly different at p=0.05.

Zziwa et al. (2016) noted that wood density is a measure of the cell wall material per unit volume and there it is a very good indicator of the strength properties. However, density alone is not a reasonable basis for estimating strength properties of wood, hence MOR and MOE needed to be examined. For static bending test properties, results from ANOVA showed that significant differences were observed between treatments and density, modulus of rupture (MOR) and modulus of elasticity (MOE) (*Table 5*). The results of Student-Newman-Keuls tests indicated significant differences ($P< 0.05$) in density, MOR and MOE of droop and erect Harumanis. Erect type of Harumanis showed higher value in density and MOR but less elasticity than droop type of Harumanis (*Table 6*). The mean density and MOR for erect Harumanis was 1.33 g/cm^3 and 58.92 MPa while 1.24g/cm3 and 48.28 MPa were recorded for droop Harumanis' density and MOR. Interestingly, droop Harumanis showed higher value for MOE (7926.50 MPa) compared to erect Harumanis (5723.75 MPa). Malavasi et al. (2016) reported that plants with high wood density have xylem conduits less susceptible to cavitation. The extent in which wood plant species can conduct water and resist xylem cavitation in the stem is determined by vessel adaptation. Failure of the conductive tissue to resist high negative pressures can result in collapse of the conduit walls resulting in cavitation. Joly & Zaerr (1987) in their report said that increased in cell-wall water content may modify the viscoelasticity properties of cell wall explaining our findings on droop and erect Harumanis. Furthermore, the elastic modulus of the wood decreases with water content, such that excessive water withdrawal from the stem could affect mechanical stability [22].

TABLE 5
MEAN SQUARES FROM ANOVA FOR DIAMETER, DENSITY, WEIGHT TO DIAMETER RATIO, MODULUS OF RUPTURE, AND MODULUS OF ELASTICITY OF SELECTED HARUMANIS ACCESSIONS.

Sources	df	Stem Diameter (mm)	Density (g/cm^3)	Weight to Diameter Ratio	Modulus of Rupture (MPa)	Modulus of Elasticity (MPa)
Treatments	1	2.63	0.07*	0.10	9059194.08*	38816992.70**
Rep	15	4.81	0.03	0.28	984060.10	7505152.30
Mean		18.61	1.29	2.82	53.61	6825.13
CV		13.35	9.55	17.38	25.53	34.60

**** Significantly different at p≤0.05***

TABLE 6
MEANS FOR DIAMETER, DENSITY, WEIGHT TO DIAMETER RATIO, MODULUS OF RUPTURE, FLEXURE STRESS AND MODULUS OF ELASTICITY OF SELECTED HARUMANIS ACCESSIONS.

Treatment	Stem Diameter (mm)	Density (g/cm^3)	Weight to Diameter Ratio	Modulus of Rupture (MPa)	Modulus of Elasticity (Mpa)
Droop	18.90a	1.24b	2.77a	48.28b	7926.50a
Erect	18.32a	1.33a	2.88a	58.92a	5723.75b

Means within column followed by the same letters are not significantly different at p=0.05.

IV. CONCLUSION

A conclusion section must be included and should indicate clearly the advantages, limitations, and possible applications of the paper. Although a conclusion may review the main points of the paper, do not replicate the abstract as the conclusion. A conclusion might elaborate on the importance of the work or suggest applications and extensions.There are rooms for Harumanis improvement through breeding as there are a good collection of variants that could be used as parents or shall be introduced as new variant in Harumanis cultivation. Although identification and evaluation of mango varieties/cultivars is quite possible with morphological characterisation, but the markers also have certain limitation especially in such cases when the cultivars are differentiated on the basis of growth habit, panicle, fruit characteristics [13]. In this study, significant differences were observed for fruit length, fruit-length-to-diameter ratio, stem RWC and moisture content, fresh-weight basis stem density, stem MOR and MOE. Morphological characters alone cannot really define whether the variation among cultivars/varieties exist at DNA level as phenotypic traits may affected by different environmental and growing conditions. In conclusion, droop and erect Harumanis showed a little variation phenotypically. However, thorough evaluation with modern approaches such as the use of Chroma meter to record flesh and skin colour, and molecular markers to differentiate variants at DNA level should be used in future.

ACKNOWLEDGEMENTS

Appreciation is extended to the Director and Deputy Director of Horticulture Research Centre, MARDI as well as the MARDI Kedah State Director for supporting this study.

REFERENCES

[1] D. N. Kuhn *et al.*, "Genetic Map of Mango: A Tool for Mango Breeding.," *Front. Plant Sci.*, vol. 8, p. 577, 2017.
[2] S. K. Mukherjee, "Origin of mango (*Mangifera indica*)," *Econ. Bot.*, 1972.
[3] Department of Agriculture, *Fruit Crops Statistic of Malaysia*. Putrajaya, Malaysia, 2016.
[4] Jabatan Pertanian Malaysia, *Senarai klon buah-buahan yang didaftarkan oleh Jabatan Pertanian Semenanjung Malaysia*. Kuala Lumpur, Malaysia: Jabatan Pertanian Malaysia, 1995.
[5] J. Sarip, "Pembaikan mangga (*Mangifera indica*) var. Sala dari populasi pokok pendebungaan terbuka," in *National Horticulture Conference*, 1999, pp. 123–131.
[6] Jabatan Audit Negara Malaysia, "Laporan Ketua Audit Negara," Putrajaya, Malaysia, 2014.
[7] M. F. Mohd Mokhtar, "Mango (Harum Manis) Quality Grading System," Tun Hussein Onn University of Malaysia, 2014.
[8] I. S. E. Bally, G. C. Graham, and R. J. Henry, "Genetic diversity of Kensington mango in Australia," *Aust. J. Exp. Agric.*, vol. 36, no. 2, pp. 243–247, 1996.
[9] R. J. Schnell and R. J. Knight, "Frequency of Zygotic Seedlings from Five Polyembryonic Mango Rootstocks," *Hort. Sci.*, vol. 27, no. 2, pp. 174–176, 1992.
[10] Universiti Malaysia Perlis, "Sistem Pemantauan PSPM 2015," 2017. [Online]. Available: http://epspm.unimap.edu.my/EPSPM_V1/PSPM_ini_viewinitiative_luar.jsp?indikatorid=2&indidesc=KDNK Sektor Pertanian. [Accessed: 26-Nov-2017].
[11] H. Mohd Azhar, S. Mohd Asrul, J. Sarip, and T. A. M. Tengku Maamun, "Variation study on morphological characters among mangifera indica L. 'Chok Anan' for development of superior mango clone," *Acta Hortic.*, vol. 1012, pp. 305–314, 2013.
[12] S. Singh, A. B. Gaikwad, and J. L. Karihaloo, "Morphological and Molecular Analysis of Intracultivar Variation in Indian Mango (*Mangifera indica* L.) Cultivars," in *Proc. VIth IS on In Vitro Cult. and Hort. Breed.*, 2009, pp. 205–212.
[13] A. S. Khan, S. Ali, and I. A. Khan, "Morphological and molecular characterization and evaluation of mango germplasm: An overview," *Sci. Hortic. (Amsterdam).*, vol. 194, pp. 353–366, 2015.
[14] IPGRI, *Descriptors for Mango*. Rome, Italy: International Plant Genetic Resources Institute, 2006.
[15] M. B. Kirkham, *Principles of Soil and Plant Water Relations*. MA, USA: Elsevier Academic Press, 2014.

[16] A. Zziwa, R. K. Kambugu, S. Kizito, A. Mugisha, O. Sseremba, and A. Syofuna, "Evaluation Of Basic Strength Indicators Of Mangifera indica Timber To Ascertain Its Suitability For Furniture Construction," vol. 4, no. 1, pp. 1–9, 2016.

[17] L. Oliva Carrasco *et al.*, "Water storage dynamics in the main stem of subtropical tree species differing in wood density, growth rate and life history traits," *Tree Physiol.*, vol. 35, no. 4, pp. 354–365, Apr. 2015.

[18] S. D. Tanksley, "The Genetic, Developmental, and Molecular Bases of Fruit Size in Tomato and Shape Variation," *Plant Cell*, vol. 16, no. Supplement 2004, pp. 181–190, 2009.

[19] P. Ceccato, S. Flasse, S. Tarantola, S. Jacquemoud, and J.-M. Grégoire, "Detecting vegetation leaf water content using reflectance in the optical domain," *Remote Sens. Environ.*, vol. 77, pp. 22–33, 2001.

[20] U. C. Malavasi, A. S. Davis, M. de M. Malavasi, U. C. Malavasi, A. S. Davis, and M. de M. Malavasi, "Estimating Water in Living Woody Stems - A Review," *CERNE*, vol. 22, no. 4, pp. 415–422, Dec. 2016.

[21] R. J. Joly and J. B. Zaerr, "Alteration of Cell-Wall Water Content and Elasticity in Douglas-Fir during Periods of Water Deficit.," *Plant Physiol.*, vol. 83, no. 2, pp. 418–22, Feb. 1987.

[22] S. M. Chapotin, J. H. Razanamehararizaka, and N. M. Holbrook, "a Biomechanical Perspective on the Role of Large Stem," *Am. J. Bot.*, vol. 93, no. 9, pp. 1251–1264, 2006.

Molecular characterization of cadmium-resistant *Cupriavidus* spp. and *Ralstonia solanacearum* isolated from soil and plants in Taiwan

Ruey-Shyang Chen[1], Wen-Yu Chen[2], Jwu-Guh Tsay[3*]

[1,2]Department of Biochemical Science and Technology, National Chiayi University, Chiayi 60004, Taiwan
[*3]Department of Hospitality Management, Nan Jeon University of Science and Technology, Tainan 73746, Taiwan

Abstract*—Cadmium is a natural heavy-metal element. It is highly toxic and a vital industrial pollutant to the environment. In order to survive in heavy-metal polluted environments, some bacteria can withstand high concentrations of heavy metals in environment due to their specific mechanisms, including the transport of heavy metal ions out of the cell. Three kinds of membrane-bound-proteins are known to participate in this transport. The objective of this study was to determine the cadmium-resistant properties and possible mechanism among strains of Cupriavidus metallidurans (= Ralstonia eutropha), C. taiwanensis (=R. taiwanensis) and Ralstonia solanacearum isolated from soil and plants in Taiwan. Strains tested include six strains, rcd 6, 8, 19 (C. metallidurans) and rcd 12, 14, 21 (C. taiwanensis), from cadmium-polluted soil, seventeen strains, nod 1 to nod 5 and nod s1 to s12 (C. taiwanensis) from root nodules of two Mimosa species (M. pudica and M. diplotricha), and ten strains of R. solanacearum isolated from different diseased plants. Sequence analysis of 16S-23S rDNA ITS and nifH gene regions were used to confirm the taxonomic classification of these bacteria. However, the 624-bp PCR product of nifH gene was only amplified from strains nod 1 to nod 3 and nod s1 to s12, but not from other tested strains of Cupriavidus and Ralstonia spp. These tested strains could tolerate cadmium within the range from 0.67 to 6.70 mM. The gene, czcC, thought to encode the outer membrane factor (OMF) of czc efflux system, was found in fifteen tested strains (rcd 6, 8, 12, 14, 19, 21; nod s1 to s6, s9, s11; PSS161) by PCR. Especially, strains rcd 12, 14, and 21 which could tolerate higher cadmium concentration (6.70 mM) harbored the entire czc operon. In addition, the PCR products revealed that cadmium-tolerant strains contained at least a portion of the czc operon and efflux mechanism was confirmed by cadmium uptake test. The results here indicated that cadmium resistant capability in Cupriavidus spp. and R. solanacearum was related to the presence of czcC or czc operon.*

***Keywords*—cadmium-resistant genes (czc operon), Cupriavidus spp., efflux system, Ralstonia solanacearum.**

I. INTRODUCTION

Heavy metals most commonly found at contaminated sites are lead (Pb), chromium (Cr), arsenic (As), zinc (Zn), cadmium (Cd), copper (Cu), mercury (Hg), and nickel (Ni). Soils may become contaminated by the accumulation of heavy metals through emissions from the rapidly expanding industrial areas, disposal of high metal wastes, and sewage sludge (Khan et al., 2008). Some bacteria use heavy metals for respiration, and some have evolved mechanisms to detoxify them. Microbes have evolved several mechanisms to tolerate the presence of heavy metals (by either efflux, complexation, or reduction of metal ions) or to use them as terminal electron acceptors in anaerobic respiration. Under normal conditions, essential and non-essential metals are transported by nonspecific entry systems. However, when metal ions are in excess, specific ion efflux protein complexes may be synthesized to aid in the elimination of non-essential metals. Microbial interactions with metals may have several implications for the environment. Microbes may play a large role in the biogeochemical cycling of toxic heavy metals also in cleaning up or remediating metal-contaminated environments (Nies and Silver, 1995; Spain and Alm, 2003).

The sources of cadmium pollution include industries, such as those producing television screens, lasers, paints, cosmetics, batteries, and zinc refining. It is widely distributed in humans, cigarette smoke, welding, and contaminated food and beverages is also the major source of cadmium contamination (Bernhoft, 2013). Cadmium is highly toxic to animals, plants, microorganisms, and humans even at quite low concentrations (Belimov et al., 2005). It has been widely accepted in the model bacterium *Cupriavidus metallidurans* (=*Ralstonia eutropha*, *R. metallidurans*) that these metal resistance mechanisms appear to be cooperative, not metal specific, and are controlled by a complex regulatory network involving several clusters of genes and functions (Maynaud et al., 2013). Cadmium-resistant bacteria, *Cupriavidus metallidurans* and *C. taiwanensis* (=*R. taiwanensis*) were isolated from cadmium-polluted and tainted soil by a waterink factory in Yunlin, Taiwan. According to cadmium tolerance these bacteria were separated into two groups: lower than 400 mg/kg and ranges from 650 to 900 mg/kg,

respectively. These cadmium-resistant strains use *czc* operon, which is located on plasmid or chromosome, to release cadmium off the organism (Chen et al., 2005).

Ralstonia solanacearum is a devastating, soil-borne plant pathogen with a global distribution and an unusually wide host range. The complete genome sequence of *R. solanacearum* strain GMI1000 has been recently determined and annotated. It is organized in two replicons: a 3.7 Mb chromosome and a 2.1 Mb megaplasmid. The megaplasmid appears to encode numerous genes that might play a role in the overall fitness of the bacterium or that may provide advantages in diverse environments (for example: flagellum biosynthesis, many essential pathogenicity genes, catabolism of aromatic compounds, copper and cobalt–zinc–cadmium resistance gene clusters (Salanoubat et al., 2002). The objectives of this study were to examine the cadmium tolerance of *Cupriavidus* species (*C. metallidurans* and *C. taiwanensis*) and *Ralstonia solanacearum* collected from soil and plants in Taiwan and to determine the possible mechanism of theses bacteria resistance to cadmium.

II. MATERIAL AND METHOD

2.1 Bacterial strains

Three strains of *Cupriavidus metallidurans* (= *Ralstonia eutropha*; rcd 6, 8, and 19) and 3 strains of *C. taiwanensis* (= *R. taiwanensis*; rcd 12, 14, and 21) isolated from cadmium-polluted soil in Yunlin, Taiwan (Chen et al., 2005) and maintained in our laboratory. A total of 17 strains of *C. taiwanensis* (nod 1, 2, 3, 4, 5, s1, s2, s3, s4, s5, s6, s7, s8, s9, s10, s11, and s12) isolated from root nodules of sensitive plant (*Mimosa pudica* L.) and giant sensitive plant (*Mimosa diplotricha* C. Wright ex Sauvalle) in Chiayi, Taiwan. Ten tested *Ralstonia solanacearum* strains isolated from different host plants and locations in Taiwan, PSS36 (peanut, Taichung), PSS161 (strawberry, Taichung), PSS189 (bitter guard, Taichung), PSS225 (hot pepper, Taichung), PSS253 (potato, Kaohsiung), PS99 (eggplant, Changhua), GB03 (ginger, Nantou), Ps-Au-11 (anthunrium, Taichung), PsL-11 (loofah, Touliu), and CLWl579 (rice flat sedge, Hsinchu), were provided through the courtesy of The World Vegetable Center, Shanhua, Tainan, Taiwan.

All tested strains were grown on nutrient broth (0.3% beef extract and 0.5% peptone) for 24 hr, then 35% glycerol are added for the preservation of bacteria at -80°C. Partial 16S rDNA gene sequence analysis following polymerase chain reaction (PCR) with a primer pair, fD1/rP1, was used for preliminary identification of tested strains (Weusburg et al., 1991). Identity of 16S rDNA sequences between tested strains in this study and published data from NCBI GenBank was conducted (Table 1). Part of the *nifH* gene, encoding dinitrogenase reductase, a key enzyme in nitrogen fixation, *nifH* gene sequence analysis following polymerase chain reaction (PCR) with a primer pair, nifH3/nifH4 (Table 2), was used for preliminary identification of *C. taiwanensis* (=*R. taiwanensis*) strains (Chen et al., 2003). The representative soil samples were collected from root zone of sensitive plant and giant sensitive plant in Chiayi, Taiwan. Soil properties analysis was conducted by the Soil Survey and Testing Center, National Chung Hsing University, Taichung, Taiwan.

TABLE 1
IDENTITY OF 16S RDNA SEQUENCES BETWEEN TESTED STRAINS IN THIS STUDY AND PUBLISHED DATA FROM NCBI GENBANK

Strains	Sampling location	Bacterium (GenBank accession number)	Identity (%)
rcd 6, 8, 19[a]	Yunlin, Taiwan	*Cupriavidus* sp. KU-26 (AB266608)	98-100
rcd 12, 14, 21[b]	Yunlin, Taiwan	*Ralstonia taiwanensis* strain MS1 (AY303977) *Ralstonia taiwanensis* strain LMG 19425 (AF300325)	98-99
nod 1 to nod 5[b]	Chiayi, Taiwan	*Ralstonia taiwanensis* strain MS1 (AY303977) *Ralstonia taiwanensis* strain LMG 19425 (AF300325)	99
nod s1 to nod s12[b]	Chiayi, Taiwan	*Cupriavidus taiwanensis* strain PAS15 (AY752959) *Ralstonia taiwanensis* strain LMG 19424 (AF300324)	99
PSS36, Ps-Au-11[c]	Taichung, Taiwan	*Ralstonia solanacearum* strain LMG 17138 (EF016364)	99

[a] Cupriavidus metallidurans; [b] Cupriavidus taiwanensis; [c] Ralstonia solanacearum

2.2 Determination of cadmium tolerance and antibiotic resistance

For testing the tolerance to cadmium ion, bacterial suspension (A_{600} = 0.8-0.9) of tested bacterial strains was spread on nutrient agar plates containing 0.5 to 10 mM of cadmium chloride ($CdCl_2$), and incubated at 30°C for 3 to 6 days. In order to understand the relationship between cadmium-tolerance and drug resistance, the antibiotic resistance of the tested strains was also determined. A loop of tested bacterial colony was inoculated to nutrient broth contained ampicillin (Amp, 50 μg/ml; Sigma, USA), chloramphenicol (Chl, 20 μg/ml; Sigma, USA), kanamycin (Kmi, 30 μg/ml; Sigma, USA), nalidixic acid (Nal,

15 µg/ml; Sigma, USA), streptomycin (Str, 30 µg/ml; Sigma, USA), or tetracycline (Tet, 12 µg/ml; Sigma, USA) at 30°C for 72 hr (Ausubel et al., 2002).

TABLE 2
PRIMERS USED IN PCR ANALYSIS

Primers	Sequence (5'-3')	Position	Expected size (bp)	Reference
fD1	AGAGTTTGATCCTGGCTCAG	7-26		
rP1	ACGGTTACCTTGTTACGACTT	1505-1485	1499	Weusburg *et al.*, 1991
nifH3	ATCGGCAAGTCGACTACCTC	2-21		
nifH4	TTCTGCATGCTGGACTACGTT	625-605	624	This study
phsC1	AATACTATCTGGGGAGCGGAA	1-21		
phsC2	TCAGACGGCGGACTTATCC	762-744	762	Chen et al., 2005
Smt A1	AAGCATTCTTGGGCATGACA	1-20		
Smt A2	TTGATTCAGGTATGGTGGGTG	492-472	492	Chen et al., 2005
czcNf	CTTGCTAGGCATTCTCGGACTAGG	312-335		
czcNr	ATGGAACAGATCAAACGACTCCAC	612-589	301	This study
czcIf	GTTCTGATCTTCGTGCTGCTCATT	13-36		
czcIr	GGTCACTTCTACCCGATTCGCTAT	270-247	258	This study
CzcC1	ATGCGAAGACTATTTCTGCCG	32-52		
CzcC2	TTAACGTCCCAGAATGCGAT	1285-1266	1254	Chen et al., 2005
CzcB1	CAAACAAAAGGCTGCCATTG	15-34		
CzcB2	GTGTTCGGCGCTGGATTT	1554-1537	1540	Chen et al., 2005
CzcA1	AACCAGATCTCGCGCGAGAAC	2458-2478		
CzcA2	CGGCAACACCAGTAGGGTCAG	3090-3070	633	Burnley, 2000
CzcD1	AGCCTGGCGTTGATCTCC	118-135		
CzcD2	CCAGATGTGGAGGTCATG	717-700	600	Burnley, 2000
czcRf	GCGGGTACTTGTTGTAGAAGACGA	3-26		
czcRr	CTTGGATCGAATGGACTTGATGAC	207-184	205	This study
czcSf	AAAGTCATCGCTCATGTTCCAGTC	357-380		
czcSr	CAATGTAAAGCGTGTCTTCCCATC	1350-1327	994	This study
czcEf	GCTTCGTATGCTTTGGAAATGACC	58-81		
czcEr	AAGGTCCACACTCGTATCCCTGAC	315-292	258	This study
cadAf	AGAGAACCTCCGGCTAAAGAAGTT	399-422		
cadAr	GGTCAAGCTTTGGAGATGAGAC	1437-1416	1039	This study
zntAf	GCAAGGGCTGGATCGCAG	779-796		
zntAr	CCACGCCATCGGTTTCGG	1742-1725	964	Legatzki *et al*., 2003

2.3 Assay of Cd^{2+} uptake and efflux

Four gram dry weight of mid-exponential phase bacterial strains, rcd 6 (*C. metallidurans*), rcd 12, nod 1, nod s1, nod s7 (*C. taiwanensis*), PSS161, PSS36, PSS189, PSS225, and Ps-Au-11 (*R. solanacearum*), were resuspended in 100 ml deionized water contained 53 ppm (0.47 mM) and 390 ppm (3.48 mM) of cadmium ion and incubated at 25°C for 0, 20, 60, 120, 180, 240, 300, 360 mins, respectively. The bacterial suspension was centrifuged at 13,000 rpm for 5 min at 25°C and the residual cadmium concentrations were measured by Fast Sequential Atomic Absorption Spectrometer (AA240FS, VARIAN, USA) at different incubation time (Chen et al., 2005). Experiments were repeated three times and the mean value was calculated.

2.4 Amplification of cadmium resistance related genes

In order to determine the possible cadmium tolerance mechanism of tested strains, PCR with the specific primer was used to detect the genes. The cadmium resistance related genes include czcA and other eight components of efflux pump, *czc* operon (Burnley, 2000), *znt*A and *cad*A (P-type ATPases) (Legatzki et al., 2003), metallothionein protein gene (*smt*A), thiosulfate reductase gene (*phs*ABC) (Bang et al., 2000; Bruins et al., 2000; Stoffels et al., 2012). Primers used for PCR are listed in Table 2. PCR mixture (25 µl) contained 0.12 µM of each primer, 0.1 mM dNTPS, 1.0 U *Taq* DNA Polmerase, PCR buffer (1.5 mM $MgCl_2$) supplied with the enzyme and 1 µl (10 µg) of template DNA. The total volume of the reaction mixture was maintained with sterilized double distilled water. PCR was performed in GeneAmp PCR system 2700 (Applied Biosystems, Inc., USA) and was carried out as follows: a single denaturation step at 94°C for 5 min followed by a 30-cycle program

which included denaturation at 94°C for 1 min, annealing at 60°C for 30 s (phsC1/phsC2, cadAf/cadAr, zntAf/zntAr), 56°C for 30 s (SmtA1/SmtA2), 67°C for 15 s (czcNf/czcNr, czcIf/czcIr, czcRf/czcRr, czcSf/czcSr, czcEf/czcEr), 50°C for 30 s (CzcC1/CzcC2), 56°C for 15 s (CzcB1/CzcB2), 63.8°C for 30s (CzcA1/CzcA2), 53°C for 15s (CzcD1/CzcD2) and extension 72°C for 1 min and a final extension at 72 °C for 10 min. The amplification products were electrophoresed on a 1.5% agarose gel buffer with 0.5 X TBE at 100 V for 25 min along with standard DNA (Gen 100-3000 DNA ladder, GeneMark Biotechnology). The amplified DNA fragments were stained with ethidium bromide and photographed with a Gene Genius Bioimaging System.

2.5 Cloning and sequencing of PCR products

PCR products were extracted with a QIAquick Gel Extraction kit (Qiagen, Hilden, Germany) and the purified DNAs were then sequenced by using an automated sequencer at the Minsheng Biotechnology Co. (Taipei, Taiwan). The sequence thus obtained was analyzed using BLASTn search (http://www.ncbi.nlm.nih.gov/Blast).

2.6 Southern hybridization

In order to confirm the existence of *czcA* and *czc*C gene, total DNA from bacterial strains, rcd 6, rcd 8, rcd 12, rcd 21, nod 1, nod s1, nod s2, nod s7, PSS36, PS99, PSS161 and PSS189 were digested with 2 µl *Bam*HI (2 U/µl; New England BioLabs, Beverly, MA, USA), digoxigenin (DIG)-labelled as probes as described by the manufacturer (Boehringer, Mannheim, Germany) and used in subsequent southern hybridization procedures (Sambrook et al., 1989).

III. RESULTS AND DISCUSSION

3.1 Comparison of 16S rRNA gene sequences of tested strains

Based on the results of amplification with primer pair, fD1/rP1, a 1500-bp band was generated. Following the sequencing of theses PCR products, 16S rDNA sequence alignment among 25 tested strains in this study and published data from NCBI were made using the BLASTn program. The data revealed that the levels of sequence identity among all of the tested strains and published data from NCBI were relatively high (identity 98-100%). The results suggested that strains rcd 6, 8, and 19 were *Cupriavidus* (=*Ralstonia*) spp.; strains rcd 12, 14, and 21 were *Cupriavidus taiwanensis* (=*Ralstonia taiwanensis*)-like organisms; strains nod 1 to nod 5 and nod s1 to nod s12 and 9 were similar to *Cupriavidus taiwanensis* (=*Ralstonia taiwanensis*); the remaining strains PSS36 and Ps-Au-11 were *Ralstonia solanacearum* (Table 1). Chen et al. (2005) reported strain rcd 6 appeared to be *Ralstonia eutropha* tested by BIOLOG bacterial identification system and 16s rRNA gene sequence comparison. Their results also suggested that strain rcd 8 was similar to *Cupriavidus metallidurans* (=*Ralstonia eutropha*) and strain rcd 19 was *Cupriavidus taiwanensis* (=*Ralstonia taiwanensis*)-like organism.

Nitrogen fixation is carried out by the nitrogenase enzyme encoded by the genes *nifH*, *nifD*, and *nifK*. Of the three, *nifH* (encoding the nitrogenase reductase subunit) is the most sequenced and has become the marker gene of nitrogen-fixing microorganisms. Thus, many PCR primers have been developed for *nifH* gene with the purpose of amplifying this gene sequence (Gaby and Buckley, 2012). In order to understand whether the test strain has a nitrogen fixation ability to confirm the identity of the strain, a specific primer pair, nifH3/nifH4, was designed according to the *R. taiwanensis nifH* gene sequence. PCR amplification results showed that only strains nod 1 to 3 and nod s1 to s12 isolated from root nodules of *Mimosa* spp. with the expected size of 624 bp (Fig. 1).

These *nifH* gene sequences have more than 95% identity compared with GenBank database using the BLASTn program of NCBI. However, no products were amplified from the strains nod 4, 5 (*C. taiwanensis*), rcd 6, 8, 19 (*C. metallidurans*), rcd 12, 14, 21 (*C. taiwanensis*) and all strains of *Ralstonia solanacearum*. The specific primer pair nifH3/nifH4 has detected 15 from 20 (75%) of *R. taiwanensis*-like strains. Gaby and Buckley (2012) found that there were 15 universal *nifH* primers that targeted 90% or more of nitrogen fixers, but that there were also 23 *nifH* primers that targeted less than 50% of *nifH* sequences.

3.2 Cadmium tolerance and antibiotic resistance

Cadmium-resistant organisms were isolated from cadmium-polluted soil in Yunlin, Taiwan. The concentration of cadmium in soil was 79.6 mg/kg. Whereas the concentration of cadmium in root zone soils of *Mimosa* spp. sampled from Chiayi, Taiwan, was 0.14 to 0.23 mg/kg (Table 3).

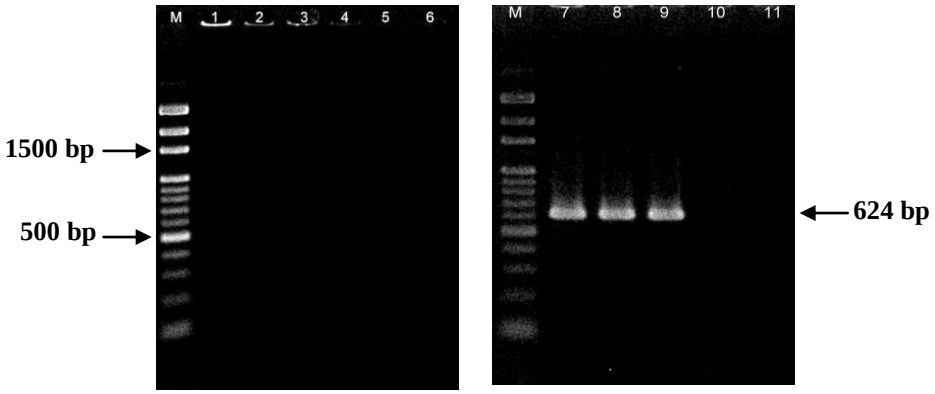


FIG. 1. PCR amplification of *nifH* gene from tested strains. M: 100 bp DNA Ladder Marker; Lanes 1-6: strains rcd 6, 8 (*Cupriavidus metallidurans*), rcd 12, 14 (*C. taiwanensis*), rcd 19 (*C. metallidurans*), rcd 21 (*C. taiwanensis*); Lanes 7-11: strains nod 1 to nod 5 (*C. taiwanensis*).

TABLE 3
CHARACTERISTICS OF TESTED SOIL

Character	Description		
	Huwei, Yunlin[1]	Holland Lake, Chiayi	Pa Cha River, Chiayi
Bacterial strains	rcd 6, 8, 12, 14, 19, 21	nod 1 to nod 5	nod s1 to s12
pH	7.5	5.3	5.3
Conductance (dS/m)	2.1	0.35	0.59
Water (%)	12	22	9.4
Texture	Loam	Silty clay loam	Sandy loam
Sand (%)	41.2	8	42
Silt (%)	44	61	43
Clay (%)	14.8	31	15
Organic matter (%)	1.58	3.3	0.4
Cadmium (mg/kg)	79.6	0.23	0.14

[1] ***Data from Chen et al. (2005).***

According to cadmium tolerance, these organism were then separated into three groups: (i) strains rcd 12, 14, 21 (*C. taiwanensis*) and PSS161 (*Ralstonia solanacearum*) higher than 5 mM; (ii) strains rcd 6, 8, and 19 (*C. taiwanensis*), nod s1-s6, s9 and s11 (*C. taiwanensis*), PS99, Ps-Au-11, CLWl579, PSS36 and PSS253 (*R. solanacearum*) ranges from 2.1 to 4.9 mM; and (iii) strains nod 1 to nod 5, nod s7, s8, s10, s12 (*C. taiwanensis*), PSS225, PSS189, GB03 and PsL-11c (*R. solanacearum*) lower than 2 mM, respectively (Table 4). Soil environment of sensitive plant and giant sensitive plant was not cadmium-contaminated and the isolated strains of *C. taiwanensis* have lower cadmium tolerance. The results were consistent with previous report that strains rcd 12, 14, 21 could be tolerant to cadmium ion at 900 mg/kg and strains rcd 6, 8, and 19 could reach 650 mg/kg (Chen et al., 2005).

TABLE 4
CADMIUM SUSCEPTIBILITY OF STRAINS OF *CUPRIAVIDUS* AND *RALSTONIA* SPP.

Strains	Cadmium concentration (mM)
rcd 12, 14, 21[b]	6.70
PSS161[c]	5.81
rcd 6, 8, 19[a]; nod s1 to s6, s9, s11[b]; PS99, Ps-Au-11, CLWl579[c]	3.13
PSS36, PSS253[c]	2.68
nod 1 to nod 5[b]; nod s7, s8, s10, s12[b]; PSS225[c]	1.34
PSS189, GB03, PsL-11[c]	0.67

[a] ***Cupriavidus metallidurans***; [b] ***Cupriavidus taiwanensis***; [c] ***Ralstonia solanacearum***

TABLE 5
ANTIBIOTIC SUSCEPTIBILITY TEST OF STRAINS OF *CUPRIAVIDUS* AND *RALSTONIA* SPP. BY BROTH METHOD

Strains	Antibiotic[1]					
	Amp	Chl	Kmi	Nal	Str	Tet
rcd 6[a]	+*	+	-	+	-	-
rcd 8[a]	+	+	-	+	-	-
rcd 19[a]	+	+	-	+	-	-
rcd 12[b]	-	+	-	-	-	-
rcd 14[b]	-	+	-	-	-	-
rcd 21[b]	-	+	-	-	-	-
nod 1[b]	+	+	-	+	-	-
nod 2[b]	+	+	-	+	-	-
nod 3[b]	+	+	-	+	-	-
nod 4[b]	+	+	-	+	-	-
nod 5[b]	+	+	-	+	-	-
nod s1[b]	+	+	-	+	+	-
nod s2[b]	+	+	-	+	+	-
nod s3[b]	+	+	-	+	+	-
nod s4[b]	+	+	-	+	+	-
nod s5[b]	+	+	-	-	+	-
nod s6[b]	+	+	-	+	+	-
nod s9[b]	+	+	-	+	+	-
nod s11[b]	+	+	-	+	+	-
nod s7[b]	+	+	-	-	+	-
nod s8[b]	+	+	-	-	+	-
nod s10[b]	+	+	-	+	+	-
nod s12[b]	+	+	-	+	+	-
PSS36[c]	-	-	-	-	-	-
PSS161[c]	+	+	-	+	-	-
PSS189[c]	+	+	+	-	+	-
PSS225[c]	-	-	-	-	-	-
PSS253[c]	-	-	-	-	-	-
PS99[c]	-	-	-	-	-	-
GB03[c]	+	+	+	-	+	-
Ps-Au-11[c]	+	+	-	-	-	-
PsL-11[c]	+	+	+	-	+	-
CLWl579[c]	-	-	-	-	-	-

****: +/-, resistance/susceptivity; [a] Cupriavidus metallidurans; [b] Cupriavidus taiwanensis; [c] Ralstonia solanacearum***
[1] Amp: ampicillin, 50 µg/ml; Chl: chloramphenicol, 20 µg/ml; Kmi: kanamycin, 30 µg/ml; Nal: nalidixic acid, 15 µg/ml; Str: streptomycin, 30 µg/ml; Tet: tetracycline, 12 µg/ml.

The strains rcd 12, 14 and 21 showed cadmium tolerance (6.70 mM) were isolated from cadmium contaminated soil. Strain PSS161 isolated from wilt strawberry stem caused by *Ralstonia solanacearum* had cadmium tolerance (5.81 mM). Strains rcd 12, 14 and 21 collected from cadmium contaminated soil were only resistant to chloramphenicol (Table 5). In addition to chloramphenicol, strains rcd 6, 8 and 19 were found to exhibit resistance to ampicillin and nalidixic acid. Whereas strains nod 1 to nod 5 had the same result as strains rcd 6, 8 and 19. Strains nod s1 to nod s12 had the same antibiotic-resistant results, in addition to strains nod s5, s7 and s8 showed sensitive to nalidixic acid. All strains of *Ralstonia solanacearum* had diverse results, strains PSS189, GB03 and PsL-11 were resistant to four antibiotics, and the remaining strains were resistant to only one of the tested antibiotics.

Among them, PSS36, PS99, PSS225, PSS253 and CLWl579 had no resistant ability to all tested antibiotics. In contrast to strains rcd 12, 14 and 21 from cadmium contaminated soil, the strains of *Ralstonia solanacearum* from fields at different

location had diverse results of antibiotics resistance. However, more informations, eg. soil characteristics and application history of bactericides, are required to confirm the relationship with these findings..

Together with other genes, *czcA* forms the *czc* determinant, which encodes a multi-protein complex associated with a high level resistance to cadmium, cobalt and zinc in bacteria (Nies, 2003). Moreover, this bacterium was resistant to antibiotics (Mowade and Bhattacharyya, 2000), which suggested the existence of multi-resistance mechanisms against drugs and/or the expression of efflux pumps (Pages et al., 2008).

Efflux pumps are transport proteins involved in the extrusion of toxic substrates (including virtually all classes of clinically relevant antibiotics) from within cells into the external environment (Andersen et al., 2001; Webber and Piddock, 2003). However, the results of the antibiotic resistance analysis showed that cadmium-resistant strains were not positively correlated to the resistance to antibiotic. It was still needed further study to investigate the relevance between cadmium efflux mechanism and antibiotic efflux system of *Ralstonia* species.

3.3 Cadmium ion uptake and efflux

In the state of 53 ppm cadmium ion, the residual cadmium ion concentration decreased at 20-min incubation by rcd 6 (*C. metallidurans*), rcd 12 and nod s1 (*C. taiwanensis*). At that time, the cadmium ion uptake by tested strains were rcd 12 (2.7 mg/kg), rcd 6 (0.54 mg/kg) and nod s1 (0.53 mg/kg). After that time, the cadmium ion efflux by bacterial cells and the residual cadmium ion concentrations were raised again. On the other hand, the strains nod 1 and nod s7 had not showed a phenomenon of cadmium ion uptake and efflux (Fig. 2).

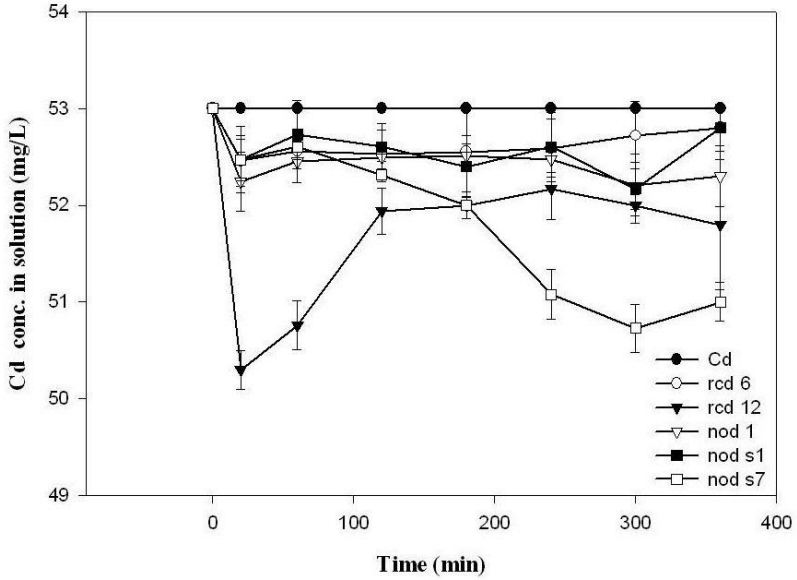


FIG. 2. Uptake of cadmium by strains of rcd 6 (*Cupriavidus metallidurans*), rcd 12, nod 1; nod s1, nod s7 (*C. taiwanensis*) at 30°C. Cd: untreated controls.

Strains PSS36 and PSS161 of *Ralstonia solanacearum* uptake cadmium ion after 20-min incubation (3.11 mg/kg, strain PSS161) and then cadmium ions were effluxed. Strain PSS225 uptaked cadmium ion after 20 to 60 minutes and then cadmium ions efflux occurred. Strain PSS189 uptake cadmium ion after 20 to 360 minutes and had no efflux trend. No cadmium ion uptake and efflux phenomenon were found for strain Ps-Au-11 (Fig. 3).

Thus, at lower cadmium ion concentration (53 ppm), it was found that the most tolerant strain (rcd12, 6.70 mM/1500 ppm) appeared to uptake and efflux cadmium ion at an earlier stage of incubation. Other than strain PSS189, whereas strains with a cadmium tolerance above 1.34 mM (300 ppm), PSS161 (5.81 mM/1300 ppm), rcd 6 and nod s1 (3.13 mM/700 ppm), Ps-Au-11 and PSS36 (2.68 mM/600 ppm), nod 1, nod s7 and PSS225 (1.34 mM/300 ppm), were also showing the phenomenon. On the other hand, when at higher cadmium ion concentration (390 ppm) state, the strains rcd 6, rcd 12 and nod s1 had the same uptake phenomenon after 20-min incubation and the bacterial cell cadmium concentrations were 30.56 mg/kg, 18.48 mg/kg and 10.67 mg/kg, respectively, and then cadmium efflux occurred (Fig. 4). The bacterial cell cadmium concentration of strain PSS161 was 33.71 mg/kg at 20 min incubation and then cadmium efflux occurred (Fig. 5).

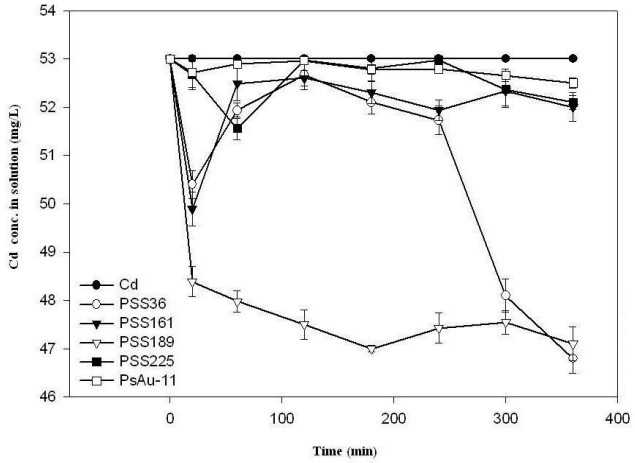


FIG. 3. Uptake of cadmium by strains PSS36, PSS161, PSS189, PSS225, Ps-Au-11 (*Ralstonia solanacearum*) at 30°C. Cd: untreated controls.

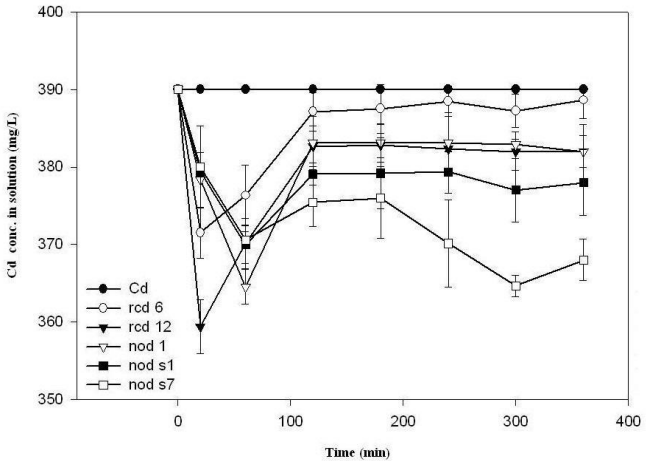


FIG. 4. Uptake of cadmium by strains of rcd 6 (*Cupriavidus metallidurans*), rcd 12, nod 1; nod s1, nod s7 (*C. taiwanensis*) at 30°C. Cd: untreated controls.

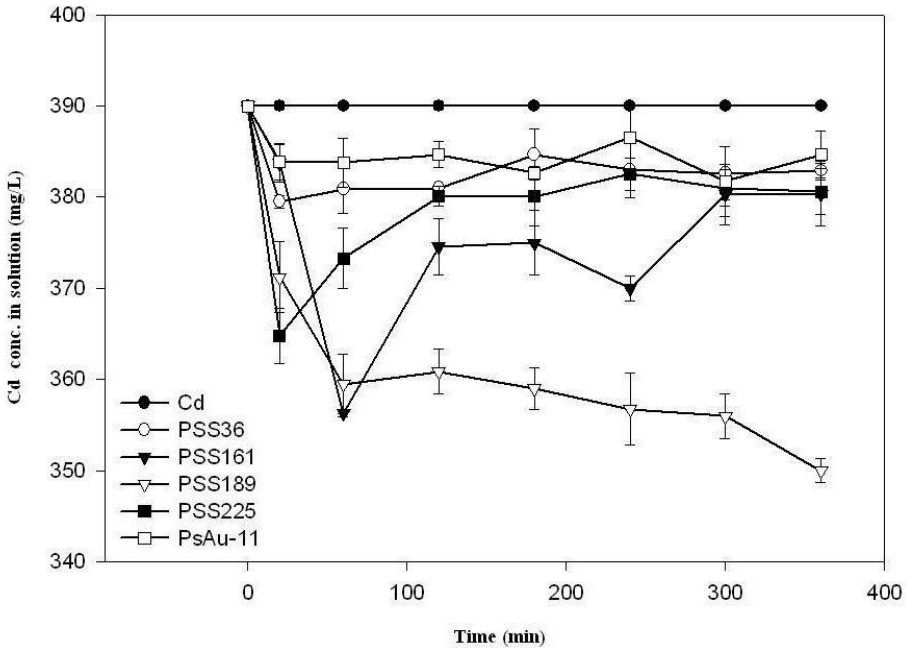


FIG. 5. Uptake of cadmium by strains PSS36, PSS161, PSS189, PSS225, Ps-Au-11 (*Ralstonia solanacearum*) at 30°C. Cd: untreated control.

3.4 Cadmium-resistant genes

It is known that bacteria are able to survive in environment with high concentrations of heavy metal due to their specific mechanisms, including the transport of heavy metal ions out of the cells. Many kinds of membrane-bound-proteins are known to participate in this transport. They are CBA efflux pumps driven by proteins of the resistance–nodulation–cell division superfamily, P-type export ATPases, CDF (cation diffusion facilitators) proteins and chromate proteins, NreB- and CnrT-like resistance factors. Heavy metal resistance is the result of multiple layers of resistance systems with overlapping substrate specificities, but unique functions (Nies, 2003). The best characterized CBA transporter is the CzcCBA complex from *Cupriavidus metallidurans* CH34. The *czc* determinant encodes resistance to Cd^{2+}, Zn^{2+} and Co^{2+} by metal-dependent efflux driven by the proton motive force (Nies, 1995; Nies et al., 1987; Nies and Silver, 1989). Chen et al. (2005) reported that cadmium-resistant *C. metallidurans* (=*Ralstonia eutropha*) and *C. taiwanensis* (=*R. taiwanensis*) strains use *czc* operon, which is located on plasmid or chromosome, to release cadmium off the organism.

TABLE 6
CHARACTERISTICS OF CD-RESISTANT MECHANISM OF TESTED STRAINS OF *CUPRIAVIDUS* AND *RALSTONIA* SPP.

Strains[1]	Cadmium (mM)	Cd-resistant genes												
		phsC	*smtA*	*cadA*	*zntA*	*czc* efflux system								
						czcN	*czcI*	*czcC*	*czcB*	*czcA*	*czcD*	*czcR*	*czcS*	*czcE*
rcd 12, 14, 21	6.70	-	-	+	-	+	+	+	+	+	+	+	+	+
PSS161	5.81	-	-	-	-	-	-	+	-	-	-	-	-	-
rcd 6, 8, 19; nod s1 to nod s6, s9, s11	3.13	-	-	-	-	-	-	+	-	-	-	-	-	-
PS99, Ps-Au-11, CLWl579	3.13	-	-	-	-	-	-	-	-	-	-	-	-	-
PSS36, PSS253	2.68	-	-	-	-	-	-	-	-	-	-	-	-	-
nod 1 to nod 5; nod s7, s8, s10, s12; PSS225	1.34	-	-	-	-	-	-	-	-	-	-	-	-	-
PSS189, GB03, PsL-11	0.67	-	-	-	-	-	-	-	-	-	-	-	-	-

[1] ***Cupriavidus metallidurans: rcd 6, 8, 19; C. taiwanensis: rcd 12, 14, 21; nod 1 to nod 5; nod s1 to nod s12; Ralstonia solanacearum: PSS36, PS99, PSS161, PSS189, PSS225, PSS253, GB03, Ps-Au-11, PsL-11, CLWl579***

The *czc* operon located on plasmid or chromosome of Cadmium-resistant *Cupriavidus metallidurans* and *C. taiwanensis* strains is resopsible for releasing cadmium out of these microorganisms (Chen et al., 2005). Cadmium and zinc are removed from cells of *Ralstonia metallidurans* by the CzcCBA efflux pump and by two soft-metal-transporting P-type ATPases, CadA and ZntA. Resistance-Nodulation-Cell division (RND)-type cation efflux systems of the Czc type comprising of an inner membrane pump CzcA associated with two membrane bound factors, CzcB and CzcC (Legatzki et al., 2003). In order to determine the genes responsible cadmium resistance, *phsC*, *smtA*, *cadA*, *zntA* and nine components of efflux pump, *czc* operon were targeted by PCR, all the tested strains consisted of this operon with some variations. Higher cadmium-resistant strains rcd 12, 14, 21 (*C. taiwanensis*) consisted of *cadA* and nine components of *czc* efflux system, whereas strain PSS161 (*Ralstonia solanacearum*) only consisted of *czcC*. Strains rcd 6, 8, 19 (*C. metallidurans*) and nod s1 to nod s6, s9, s11 (*C. taiwanensis*) could grow on moderate cadmium levels (2.1 to 4.9 mM) only consisted of *czcC* (Table 6).

However, no PCR products of *phsC*, *smtA*, *cadA*, *zntA* and nine components of efflux pump, *czc* operon were amplified from lower cadmium-resistant strains PS99, Ps-Au-11, CLWl579, PSS36, PSS253, PSS225, PSS189, GB03, PsL-11 (*R. solanacearum*) and strains nod 1 to nod 5; nod s7, s8, s10, s12 (*C. taiwanensis*). Sequence analysis of these PCR products of *C. taiwanensis* strain rcd 12 showed nucleotide and amino acid sequence differences were 1 to 3% and 0 to 2%, respectively, as compared with the published data from NCBI (Table 7).

The *czcC* nucleotide and amino acid sequences among four tested strains rcd 6 (*C. metallidurans*), rcd 12 (*C. taiwanensis*), nod s1 (*C. taiwanensis*), and PSS161 (*R. solanacearum*) showed a 1 to 25% nucleotide sequence difference and 1 to 2% amino acid sequence difference compared with the GenBank sequence database provided by the National Center for Biotechnology Information (NCBI) (Table 8).

In *Cupriavidus metallidurans* CH34, the regulatory genes of *czcCBA* are arranged in an upstream region consisting of *czcN* and *czcI*, and a downstream region consisting of *czcD*, *czcR*, *czcS* and *czcE*. CzcRS and a periplasmic copper-binding protein designated CzcE, exert metal-dependent control of *czcNICBA* expression via regulation of *czcNp* activity (Van der Lelie et al., 1997; Grosse et al., 2004; Petit-Haertlen et al., 2010). Phosphorylated CzcR activates the expression of *czcCBA* operon encoding an efflux pump specific for zinc, cadmium, and cobalt. Liu et al. (2015) reported that *czcRS*s regulated the expression of *czcCBA* and a cross-link existed between different czcRSs in the heavy metal resistance of *Pseudomonas putida* X4.

TABLE 7
IDENTITY OF CADMIUM-RESISTANT RELATED GENES NUCLEOTIDE AND AMINO ACID SEQUENCES BETWEEN STRAIN RCD 12 OF *CUPRIAVIDUS TAIWANENSIS* AND PUBLISHED DATA FROM NCBI GENBANK

Gene	Strain	NCBI GenBank (CP000354)	Nucleotide sequences identity (%)[1]	Amino acid sequences identity (%)[1]	Suppositional function
cadA	rcd 12	*R. metallidurans* CH34	98	98	Heavy metal translocating P-type ATPase
czcN	rcd 12	*R. metallidurans* CH34	98	98	Cobalt-zinc-cadmium resistance protein CzcN
czcI	rcd 12	*R. metallidurans* CH34	97	98	Cobalt-zinc-cadmium resistance protein CzcI
czcC	rcd 12	*R. metallidurans* CH34	99	99	Outer membrane efflux protein
czcB	rcd 12	*R. metallidurans* CH34	99	99	Cobalt-zinc-cadmium resistance protein CzcB, Membrane Fusion Protein cluster 2
czcA	rcd 12	*R. metallidurans* CH34	99	99	Heavy metal efflux pump CzcA, Cation efflux system protein CzcA
czcD	rcd 12	*R. metallidurans* CH34	99	100	Cobalt-zinc-cadmium resistance protein, CzcD
czcR	rcd 12	*R. metallidurans* CH34	98	98	Two component heavy metal response transcriptional regulator, winged helix family
czcS	rcd 12	*R. metallidurans* CH34	98	98	Heavy metal sensor signal transduction histidine kinase
czcE	rcd 12	*R. metallidurans* CH34	98	99	ORF131 protein

[1] Identity (%) of nucleotide acid sequences was compared using the BLASTn program and amino acid sequences were compared using the BLASTx program of NCBI.

TABLE 8
IDENTITY OF *CZCC* NUCLEOTIDE AND AMINO ACID SEQUENCES BETWEEN TESTED STRAINS OF CUPRIAVIDUS AND RALSTONIA SPP. AND PUBLISHED DATA FROM NCBI GENBANK

Strains	Species	NCBI GenBank (CP000354)	Nucleotide sequences identity (%)[1]	Amino acid sequences identity (%)[1]	Suppositional function
rcd 6	*C. metallidurans*	*C. metallidurans* CH34	75	88	Outer membrane efflux protein
rcd 12	*C. taiwanensis*	*C. metallidurans* CH34	99	99	Outer membrane efflux protein
nod s1	*C. taiwanensis*	*C. metallidurans* CH34	75	88	Outer membrane efflux protein
PSS161	*R. solanacearum*	*C. metallidurans* CH34	75	88	Outer membrane efflux protein

[1] Identity (%) of nucleotide acid sequences was compared using the BLASTn program and amino acid sequences were compared using the BLASTx program of NCBI.

CzcA is one of the primary proteins in cadmium, cobalt and zinc resistance in several microorganisms, including the tolerant bacterium *Cupriavidus metallidurans* CH34 (Nies, 2003), *Caulobacter crescentus* CB15N (Hu et al., 2005), *Pseudomonas putida* CD2 (Hu and Zhao, 2007), *Sinorhizobium meliloti* 1021 (Rossbach et al., 2008) and *Gluconacetobacter diazotrophicus* PAl 5 (Intorne et al., 2012).

When *czcA* used as probe in a Southern blot analysis, the results revealed that this operon was located on plasmid and chromosome as well (Chen et al., 2005). In order to further confirm the results of PCR amplification, the *czcA* and *czcC* probes were prepared for Southern blot hybridization. The results showed only the strain rcd 12 and rcd 21 (*Cupriavidus taiwanensis*) have the hybridization signals on the position of DNA digestion fragment (>10 kb). However, no hybridization results were found on other tested strains rcd 6, rcd 8 (*C. metallidurans*), nod 1, nod s1, nod s2, nod s7 (*C. taiwanensis*), PSS36, PS99, PSS161 and PSS189 (*R. solanacearum*) (Fig. 6 and Fig. 7).

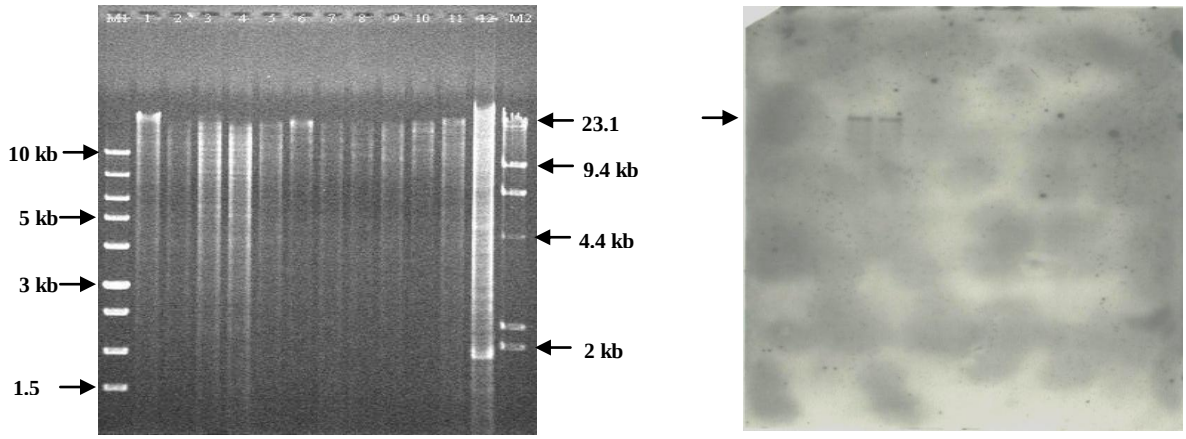


FIG. 6. Bacterial genomic DNA digested with the restriction enzyme *Bam*HI and separated by gel electrophoresis (Left). The detection of specific DNA fragment *czcC* in lanes 3 and 4 by Southern blot hybridization (Right).

M1: 1 kb DNA Ladder Marker; Lanes 1-2: strains rcd 6 and 8 (*Cupriavidus metallidurans*), Lanes 3-4: rcd 12 and 21 (*C. taiwanensis*); Lanes 5-8: strains nod 1, nod s1, s2 and s7 (*C. taiwanensis*); Lanes 9-12: strains PSS36, PS99, PSS161, and PSS189 (*Ralstonia solanacearum*); M2: Lambda DNA/*Hin*dIII Marker.

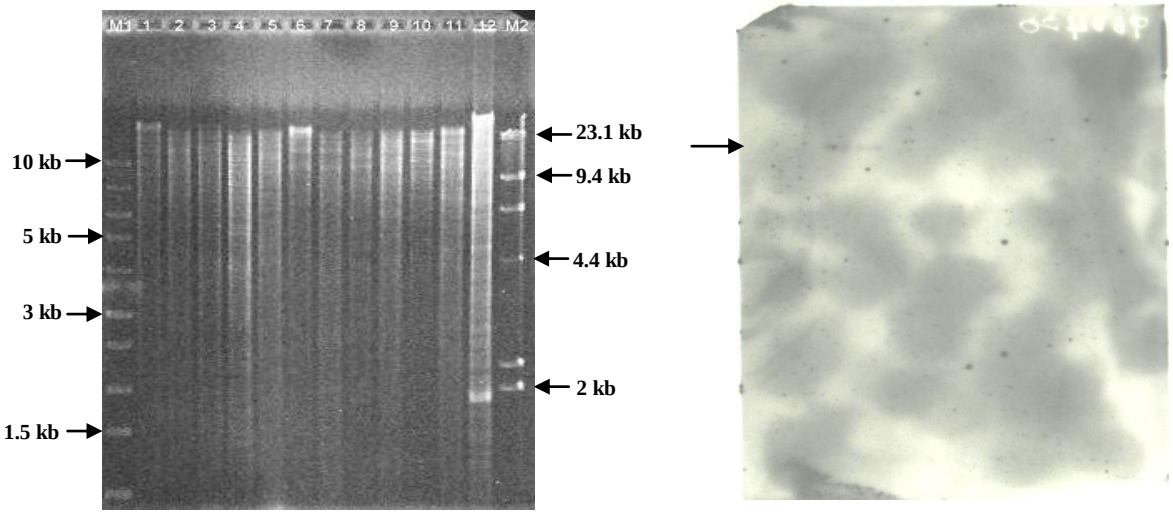


FIG. 7. Bacterial genomic DNA digested with the restriction enzyme *Bam*HI and separated by gel electrophoresis (Left). The detection of specific DNA fragment *czcA* in lanes 3 and 4 by Southern blot hybridization (Right).

M1: 1 kb DNA Ladder Marker; Lanes 1-2: strains rcd 6 and 8 (*Cupriavidus metallidurans*), Lanes 3-4: rcd 12 and 21 (*C. taiwanensis*); Lanes 5-8: strains nod 1, nod s1, s2 and s7 (*C. taiwanensis*); Lanes 9-12: strains PSS36, PS99, PSS161, and PSS189 (*Ralstonia solanacearum*); M2: Lambda DNA/*Hin*dIII Marker.

As concluding remarks, our findings have demonstrated that the higher cadmium resistant capability of strains rcd 12, 14, 21 (*Cupriavidus taiwanensis*) was related to the presence of *cadA* and *czc* efflux system (*czcN*, *czcI*, czcC, *czcB*, *czcA*, *czcD*, *czcR*, *czcS* and *czcE*). In addition to this, some strains of *Cupriavidus* species and *Ralstonia solanacearum*, rcd 6, 8, 19 (*C. metallidurans*), nod s1 to nod s6, s9, s11 (*C. taiwanensis*) and PSS161 (*R. solanacearum*), exhibit moderately elevated cadmium resistance. Our results revealed that cadmium resistant capability in these *Cupriavidus* and *Ralstonia* strains was related to the presence of *czcC*, thought to encode the outer membrane factor (OMF) of *czc* efflux system.

REFERENCES

[1] Andersen, C., Colin, H. and Vassilis, K. (2001). Protein export and drug efflux through bacterial channel-tunnels. Current Opinion in Cell Biology 13:412-416.

[2] Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (2002). Short Protocols in Molecular Biology: a Compendium of Methods from Current Protocols in Molecular Biology, Fifth Edition. John Wiley and Sons, Inc., New York.

[3] Bang, S. W., Clark, D. S. and Keasling, J. D. (2000). Engineering hydrogen sulfide production and cadmium removal by expression of the thiosulfate reductase gene (*phs*ABC) from *Salmonella enterica* Serovar Typhimurium in *Escherichia coli*. Applied and Environmental Microbiology 66:3939-3944.

[4] Belimov, A. A., Hontzeas, N., Safronova, V. I., Demchinskaya, S. V., Piluzza, G., Bullitta, S. and Glick, B. R. (2005). Cadmium-tolerant plant growth-promoting bacteria associated with the roots of Indian mustard (*Brassica juncea* L. Czern.). Soil Biology and Biochemistry 37:241-250.

[5] Bernhoft, R. A. (2013). Cadmium toxicity and treatment. The Scientific World Journal, vol. 2013, Article ID 394652, 7 pages.

[6] Bruins, M. R., Kapil, S. and Oehme, F. W. (2000). Microbial resistance to metals in the environment. Ecotoxicology and Environmental Safety 45:198-207.

[7] Burnley, L. E. (2000). Heavy metal resistance in the genus *Gluconobacter*. Master Thesis of Science in Biology, Faculty of Virginia Tech, Blacksburg, VA, USA. 81 pp.

[8] Chen, W. M., Moulin, L. Bontemps, C., Vandamme, P., Bena, G. and Boivin-Masson, C. (2003). Legume symbiotic nitrogen fixation by β-Proteobacteria is widespread in nature. Journal of Bacteriology 185:7266-7272.

[9] Chen, X. P., Chu, C., and Tsay, J. G. (2005). Efflux pump (*czc* operon)-mediated cadmium-resistance mechanism of *Cupriavidus metallidurans* (*Ralstonia eutropha*) and *C. taiwanensis* (*R. taiwanensis*). Environmental Protection 28:185-201. (in Chinese)

[10] Gaby, J. C. and Buckley, D. H. (2012). A Comprehensive evaluation of PCR Primers to amplify the *nifH* gene of nitrogenase. PLoS ONE 7(7): e42149.

[11] Grosse, C., Anton, A., Hoffmann, T., Franke, S., Schleuder, G. and Nies, D. H. (2004). Identification of a regulatory pathway that controls the heavy-metal resistance system Czc via promoter czcNp in *Ralstonia metallidurans*. Archives of Microbiology 182:109-118.

[12] Hu, P., Brodie, E. L., Suzuki, Y., McAdams, H. H. and Andersen, G. L. (2005). Whole genome transcriptional analysis of heavy metal stresses in *Caulobacter crescentus*. Journal of Bacteriology 187:8437-8449.

[13] Hu, N. and Zhao, B. (2007). Key genes involved in heavy-metal resistance in *Pseudomonas putida* CD2. FEMS Microbiology Letters 267:17-22.

[14] Intorne, A. C., de Oliveira, M. V. V., de M Pereira, L. and de Souza Filho, G. A. (2012). Essential role of the czc determinant for cadmium, cobalt and zinc resistance in *Gluconacetobacter diazotrophicus* PAl 5. International Microbiology 15:69-78.

[15] Khan, S., Cao, Q., Zheng, Y. M., Huang, Y. Z. and Zhu, Y. G. (2008). Health risks of heavy metals in contaminated soils and food crops irrigated with wastewater in Beijing, China. Environmental Pollution 152:686-692.

[16] Legatzki, A., Anton, A., Grass, G., Rensing, C. and Nies, D. H. (2003). Interplay of the Czc-system and two P-type ATPases in conferring metal resistance to *Ralstonia metallidurans*. Journal of Bacteriology 185:4354-4361.

[17] Liu, P., Chen, X., Huang, Q. and Chen, W. (2015). The role of CzcRS two-component systems in the heavy metal resistance of Pseudomonas putida X4. International Journal of Molecular Sciences 16:17005-17017.

[18] Maynaud, G., Brunel, B., Mornico, D., Durot, M., Severac, D., Dubois, E., Navarro, E., Cleyet-Marel, J.-C. and Le Quéré, A. (2013). Genome-wide transcriptional responses of two metal-tolerant symbiotic *Mesorhizobium* isolates to zinc and cadmium exposure. BMC Genomics 14:292.

[19] Mowade, S. and Bhattacharyya, P. (2000). Resistance of P-solubilizing *Acetobacter diazotrophicus* to antibiotics. Current Science 79:1591-1594.

[20] Nies, D. H. (1995). The cobalt, zinc, and cadmium efflux system CzcABC from *Alcaligenes eutrophus* functions as a cation-proton antiporter in *Escherichia coli*. Journal of Bacteriology 177:2707-2712.

[21] Nies, D. H. (2003). Efflux-mediated heavy metal resistance in prokaryotes. FEMS Microbiology Reviews 27:313-339.

[22] Nies, D., Mergeay, M., Friedrich, B. and Schlegel, H. G. (1987). Cloning of plasmid genes encoding resistance to cadmium, zinc, and cobalt in *Alcaligenes eutrophus* CH34. Journal of Bacteriology 169:4865-4868.

[23] Nies, D. H. and Silver, S. (1989). Plasmid-determined inducible efflux is responsible for resistance to cadmium, zinc, and cobalt in *Alcaligenes eutrophus*. Journal of Bacteriology 171:896-900.

[24] Nies, D. H. and Silver, S. (1995). Ion efflux systems involved in bacterial metal resistances. Journal of Industrial Microbiology 14:189-199.

[25] Pages, D., Rose, J., Conrod, S., Cuine, S., Carrier, P., Heulin, T. and Achouak, W. (2008). Heavy metal tolerance in *Stenotrophomonas maltophilia*. PLoS ONE 3:e1539.

[26] Petit-Haertlen, I., Girard, E., Sarret, G., Hazemann, J., Gourhant, P., Kahn, R. and Coves, J. (2010). Evidence for conformational changes upon copper binding to *Cupriavidus metallidurans* CzcE. Biochemistry 49, 1913-1922.

[27] Rossbach, S., Mai, D. J., Carter, E. L., Sauviac, L., Capela, D., Bruand, C. and Bruijn, F. J. (2008). Response of *Sinorhizobium meliloti* to elevated concentrations of cadmium and zinc. Applied and Environmental Microbiology 74:4218-4221.

[28] Salanoubat, M., Genin, S., Artiguenave, F., Gouzy, J., Mangenot, S., Arlat, M., Billault, A., Brottier, P., Camus, J. C., Cattolico, L., Chandler, M., Choisne, N., Claudel-Renard, C., Cunnac, S., Demange, N., Gaspin, C., Lavie, M., Moisan, A., Robert, C., Saurin, W., Schiex, T., Siguier, P., Thébault, P., Whalen, M., Wincker, P., Levy, M., Weissenbach, J. and Boucher, C. A. (2002). Genome sequence of the plant pathogen *Ralstonia solanacearum*. Nature 415:497-502.

[29] Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York.

[30] Spain, A. and Alm, E. (2003). Implications of microbial heavy metal tolerance in the environment. Reviews in Undergraduate Research 2:1-6.

[31] Stoffels, L., Krehenbrink, M., Berks, B. C. and Unden, G. (2012). Thiosulfate reduction in *Salmonella enterica* is driven by the proton motive force. Journal of Bacteriology 194:475-485.

[32] Van der Lelie, D., Schwuchow, T., Schwidetzky, U., Wuertz, S., Baeyens, W., Mergeay, M. and Nies, D. H. (1997). Two-component regulatory system involved in transcriptional control of heavy-metal homoeostasis in *Alcaligenes eutrophus*. Molecular Microbiology 23:493-503.

[33] Webber, M. A. and Piddock, L. J. V. (2003). The importance of efflux pumps in bacterial antibiotic resistance. Journal of Antimicrobial Chemotherapy 51:9-11.

[34] Weusburg, W. G., Barns, S. M., Pelletier, D. A. and Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. Journal of Bacteriology 173:697-703.

Physical and Chemical Diagnosis of Lower Sebou River for Agricultural Use (GHARB - Morocco)

HAMDAOUI Farid[1*], Saad ABOUBAKER ALZWI[2], Elmehdi ALIBRAHMI[3], Khadija EL KHARRIM[4], Driss BELGHYTI[5], Noureddine LOTFI[6*]

[1,6]Laboratory of Optoelectronic, Physicochemical, Materials and Environment. University Ibn Tofail, Kenitra.
[1,2,4,5]Laboratory of Biotechnology, Environment & Quality, Faculty of Sciences, PB: 133, 14000 Kenitra, Morocco.
[3]Laboratory of Renewable Energy & Environment. Faculty of Sciences, Kenitra, Morocco
*Corresponding author: Email: farid10hamdaoui@gmail.com

Abstract— *In the present study, it is proposed to characterize the physicochemical of the water of the Lower Sebou sub-basin in the Gharb region used mainly in irrigation. The physicochemical characterization of the raw waters of the Sebou revealed that this river is very loaded with mineral and organic matters and have a wide variation in the chemical composition:*

**the electrical conductivity (EC), varies between a minimum of 629µS/cm and a maximum of 2370 µS/cm;*
**the average pH is between 8 and 8.77. The pH is slightly basic but remains acceptable according to standard;*
**the ammonium concentration varies between 0.04 and 2.66 mg/L;*
**concentrations of nitrates NO_3- have a maximum value of 196.9 mg/L and a minimum value of 0.24 mg/L;*
**the concentration of ion Cl- has a maximum value of 385.53 mg/L and a minimum value of 145.55 mg/L;*
**for sulfate ion SO_4 --, the maximum concentrations is 359.29 mg /L and the minimum value is 37.62 mg /L;*
**the maximum and minimum bicarbonate ion concentrations are 362.34 mg / L and 75.64 mg /L;*
**calcium Ca^{2+} ion contents range from 220.4 to 97.6 mg /L;*
** for magnesium ion Mg^{2+} the maximum concentration is 124.08 mg / L and the minimum value is 17.28 mg/L;*
** Na^{+} ion concentrations in water range from 2530 mg /L to 51 mg /L;*
**K + ion concentrations in surface waters range from 17.55 mg /L to 2.54 mg /L.*

In conclusion, this study shows that the waters of the lower Sebou have a high mineral load but remain within the limits of the Moroccan irrigation standard. The waters of the Sebou are too polluted and we recommend that all domestic and industrial wastewaters should be treated appropriately to reduce the nuisance to the receiving environment and to compensate for the loss of this coveted and prized water resource.

Keywords— Sebou River, Waters, Hydrochemistry, Agricultural, Irrigation, Gharb, Morocco.

I. INTRODUCTION

Water quality is defined by physical, chemical and biological parameters, but also by its use. Thus, water unfit for human consumption can be adapted to irrigation, fish farming or to cool industrial circuits [1-3]. The rational management of water resources in the Kenitra Gharb area has become the main issue for local decision-makers to adopt a fair policy and which takes into consideration the importance of this resource and the challenge of increasing water resources. The Sebou river and its tributaries drain an area of 34000 km^2. It extends for more than 600 km starting in the Middle Atlas under the name of Guigou river. It opens in the Atlantic to Mehdia, through its estuary 35 km in length. The rise of marine waters being stopped at the level of the guard dam, immediately downstream of Sidi Allal Tazi city [4]. In addition, the Sebou river is home to many pollutant spills from a variety of sources. The Sebou watershed, an extremely important area from a socio-economic point of view, is one of the most affected areas in Morocco. The existence of two of the main agricultural plains of the country as well as the multitude and diversity of industrial units and urban wastewater effluents in the major cities of the basin (Fez, Allal Tazi, Mechraa Bel Ksiri, Dar Gueddari, Kenitra), not to mention the uncontrolled dumping of household waste, which are the main causes of the deterioration of the quality of Sebou waters.

In our present study it is proposed to examine the physicochemical surface water of the lower Sebou sub-basin. This characterization of the levels and concentrations of the organic and mineral loads of Sebou raw water consists of a

monitoring of the pH, EC electrical conductivity, sodium, chloride, sulphate, calcium, magnesium, potassium, bicarbonate, ammonium and nitrates.

II. MATERIAL AND METHOD

2.1 Study area

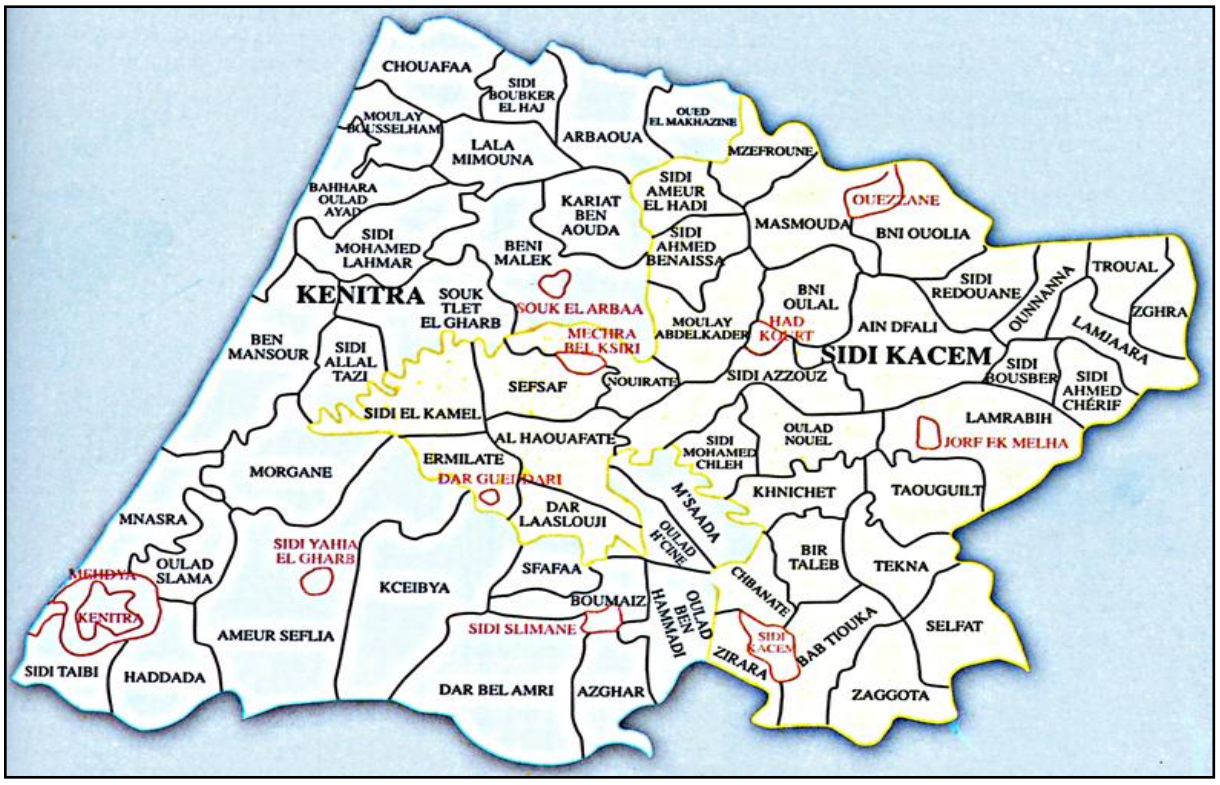
The Gharb region is bordered on the west by the Atlantic sea, bordered to the north by the pre-Rif hills and to the south by the Maâmora shelf (**Fig.1**). It is composed of a coastal zone (dune cord, flooded depressions, interior dunes), continental borders and the central alluvial plain of the lower Sebou which is the main river. The Sebou basin forms a basin between the Rif in the North, the Middle Atlas and the Meseta in the South, the Taza corridor in the East and the Atlantic sea in the West. It is the most important basin of the kingdom with approximately 38380 km^2 and currently contains a total population of 5.73 million inhabitants, of which 49% in urban and 51% in rural areas. It is characterized by an agricultural and industrial activity that contributes significantly to national economy.

The climate prevailing on the whole basin is of Mediterranean type with oceanic influence and inside the basin the climate becomes more continental. The Sebou basin has a very developed industrial activity. Large units at the basin scale are: sugar mills, paper mills, oil mills, tanneries, cement plants, the textile industry and the oil refinery. The taking of a water sample is a delicate operation to which the greatest care must be taken, it determines the analytical results and the interpretation that will be given. In general, the sample must be homogeneous and representative, and not modify the physicochemical characteristics of the water (dissolved gas, suspended matter, etc) [5]. Sampling equipment should be given special attention. The washing of the flasks will depend on the desired analyzes on the sample. The most frequently used sampling method is instant sampling. The vials are filled without shaking the water and sometimes without contact with the air [6-7].

2.2 Study method

2.2.1 Water removal

1000 ml polyethylene bottles were previously rinsed with distilled water and then with the sample water in the field. Sampling was done in areas where the water is not stagnant and in the direction of flow. It is carried out in total immersion, so that the bottles are filled flush without air bubbles, in order to minimize the contamination on the one hand, and the evolution of the samples on the other. The water samples taken for analysis were transported at low temperature (4 °C) in portable coolers to the laboratory where analyzes were carried out. In addition, from one campaign to another, the samples were taken at approximately the same time and place for the same station.



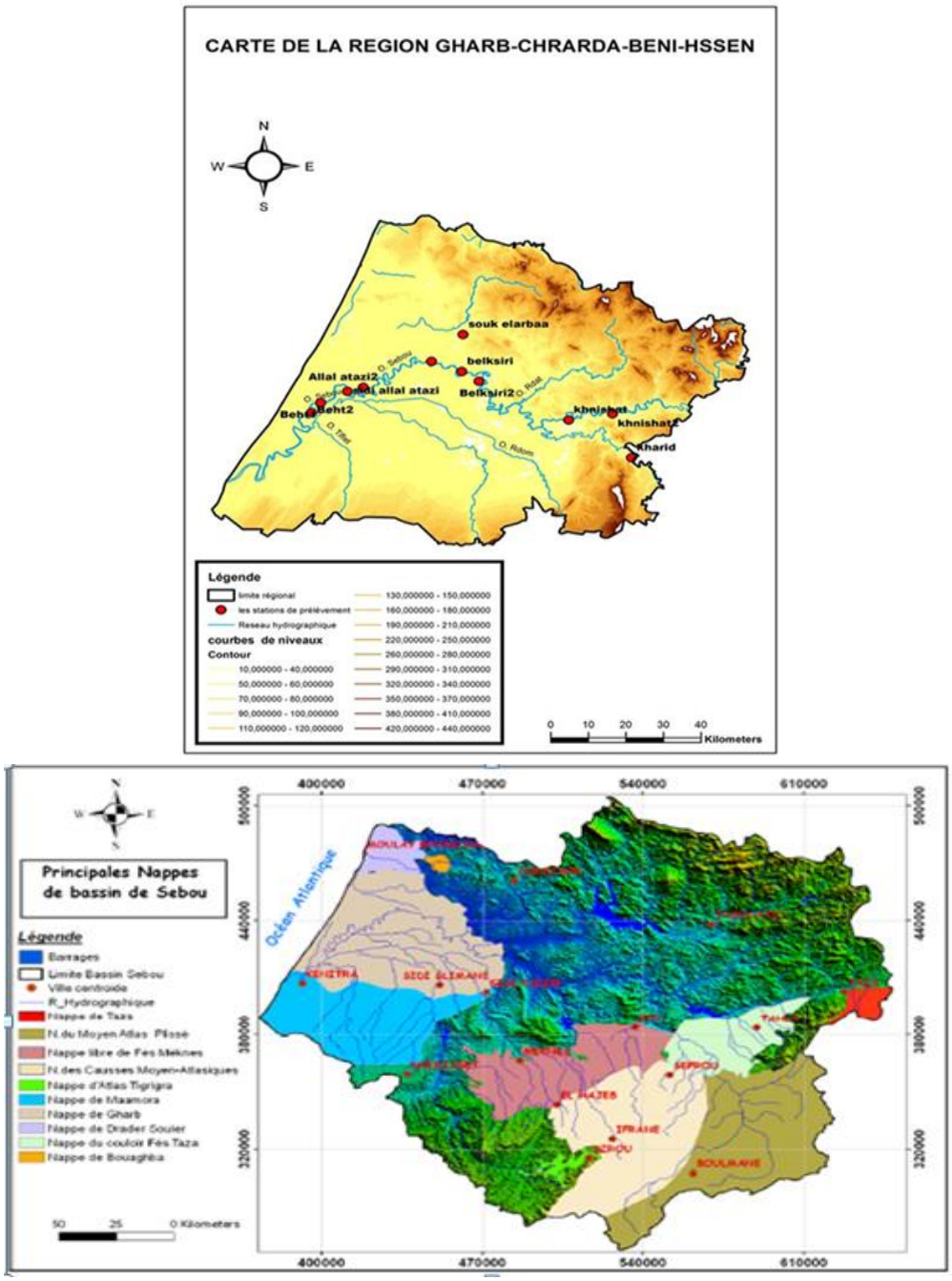


FIGURE 1: GEOGRAPHICAL LOCALIZATION OF SAMPLING AREA GHARB MOROCCO

2.2.2 Water analyzes

In the present study the parameters that were analyzed are: pH, electrical conductivity (EC), calcium (Ca^{++}), magnesium (Mg^{++}), sodium (Na^{+}), potassium (K^{+}), carbonate and bicarbonate (CO_3-- HCO_3-), chlorides (Cl-), sulphates (SO_4--), ammonium (NH_4^{+}) and nitrate (NO_3-). The devices used are Assays C831, Jenway flame photometer, NOVASPEC II pharmacy-type spectrophotometer, UV-Visible spectrophotometer (**Fig. 2, 3**) [8-11].

Calcium and magnesium are determined by complexometry with EDTA in the presence of Eriochrome black T. Determination of carbonates and bicarbonates by a solution of 0.02N sulfuric acid in the presence of phenophthalein and bromocresol green as a colored indicator. Determination of the combined chloride in the chloride state by silver nitrate, in the presence of a solution of potassium chromate. Determination of sulfates by colorimetry by precipitation of sulphate ions in the presence of barium chloride in a hydrochloric acid medium in the form of barium sulphate. Determination of nitrates and ammoniums by distillation in the presence of a catalyst respectively magnesium oxide and alloy DEVARDA. NH_4^{+} and NO_3- are collected in a boric acid solution and finally assay with H_2SO_4.

FIGURE 2: DEVICES FOR MEASURING WATER QUALITY IN THE TRAINING LABORATORY (ORMVAG-KENITRA)

FIGURE 3: METHODS OF ASSAYS AND TITRATIONS OF MINERAL ELEMENTS IN THE WATER OF THE LOWER SEBOU

III. RESULTS AND DISCUSSION

The evaluation of raw water pollution of the lower Sebou was made according to the determination of a certain number of physicochemical parameters characterizing the waters. In the light of this work which contributes to enriching the bases of the data accumulated on the Sebou basin, and to make it possible to clarify the degree of its pollution thanks to the results obtained during the period of our internship within the Regional Office of implementation agricultural value of Kenitra.

It can be deduced from **Tables 1, 2** and **Figures 4, 5** that the sub basin of the lower Sebou river is subject to different types of pollution of natural origin which are mainly mineral (by dissolution of the natural substrate, Atlantic tides) and anthropogenic (agricultural, industrial and urban).

The thermal regime of the Sebou hydrographic network follows that of the Mediterranean climate, cold in November and warmer in summer.

The pH does not show any significant variations and the waters are generally alkaline ranging between 8.0 and 8.77 (**Tab.1**) following their crossing of limestone and marl-limestone soils characterizing the basin.

Mineralization accurately follows dissolved salt, salinity, chloride, sodium and potassium levels (**Tab. 1, 2** ; **Fig.4, 5**). It results essentially from the leaching of the karstic limestone and kelp-like terrain and ocean spray. Indeed, the electrical conductivity that reflects salinity (Tab.2) varies from 629 to 22760 µS / cm and far exceeds the Moroccan irrigation standard (> 2700 µS / cm) [12-14].

TABLE 1
PHYSICOCHEMICAL DATA (ANIONS) OF THE RAW WATERS OF THE LOWER SEBOU RIVER

Stations	pH	NO_3- mg/L	CL – mg/L	SO_4- - mg/L	HCO_3- mg/L	CO_3- mg/L
S1	8,62	9,3	213	314,64	233,02	12
S2	8,65	10,42	161,88	159,18	214,72	18
S3	8,39	0,24	202,35	150,25	213,5	6
S4	8,46	17,11	154,78	141,18	275,72	0
SD5	8	63,36	243,53	181,94	246,44	0
SD6	8,12	20,58	202,35	151,62	362,34	0
SD7	8,69	86,92	248,5	183,59	241,56	21,6
SD8	8,33	188,6	385,53	258,32	323,3	13,2
SD9	8,49	827,9	230,4	106,7	75,64	0
SD10	8,24	2692	860,27	113,1	122	0
SD11	8,4	260,8	269,09	37,62	100,04	0
SD12	8,31	886,9	476,41	276,57	84,18	0
SD13	8,77	94,6	461,31	359,29	246,44	49,2
SD14	8,21	162,2	397,7	441,4	178,12	42
SD15	8,73	59,9	304,59	248,5	241,56	18
SD16	8,33	693,8	145,55	54,04	108,58	0

TABLE 2
PHYSICOCHEMICAL DATA (CATIONS) OF THE RAW WATERS OF THE LOWER SEBOU RIVER

Stations	Ca2+ mg/L	Mg2+ mg/L	K+ mg/L	Na+ mg/L	NH4+ mg/L	TH mg/L	CE µS/cm
S1	97,6	89,04	6,44	1240	0,18	6,15	1190
S2	118	46,56	6,24	1270	0,43	4,89	1120
S3	126,4	59,52	2,54	1560	0,04	5,72	1240
S4	166,4	17,76	5,27	1360	0,68	4,9	1160
SD5	150,8	44,4	4,29	1820	0,22	5,62	1430
SD6	169,2	68,64	9,56	1330	0,18	7,09	1400
SD7	148,4	51,36	4,68	1470	0,68	5,85	1490
SD8	220,4	111,6	12,48	1840	0,5	10,16	2370
SD9	217,6	17,28	3,71	51	0,68	6,16	629
SD10	914,4	631,2	5,07	140	1,76	49,16	15820
SD11	148,4	26,64	6,63	190	15,34	4,82	11960
SD12	314,8	39,36	17,55	390	0,54	9,51	22760
SD13	170,8	124,08	9,75	2530	2,66	9,44	2200
SD14	1072,8	74,88	15,99	150	1,26	57,94	16700
SD15	144,8	94,08	7,41	400	1,29	7,54	1660
SD16	174	99,36	2,73	120	1,51	8,49	880

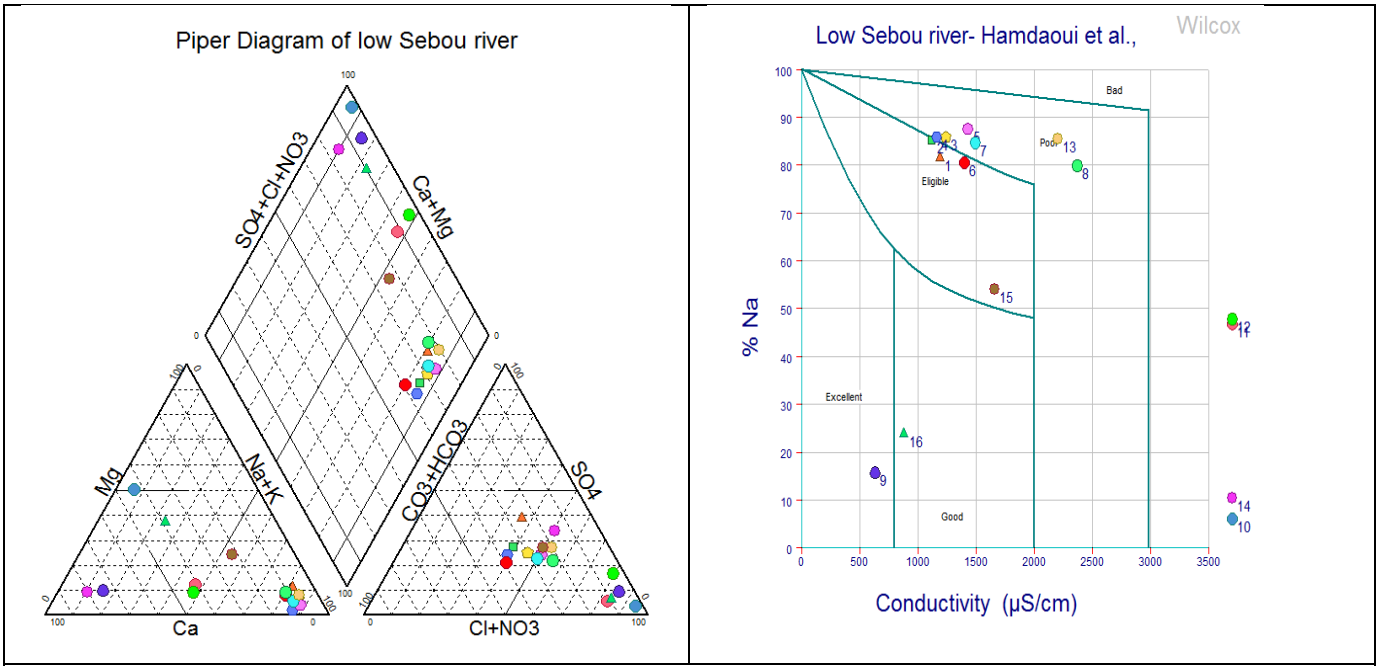


FIGURE 4: PIPER AND WILCOX DIAGRAMS OF WATER OF THE LOWER SEBOU

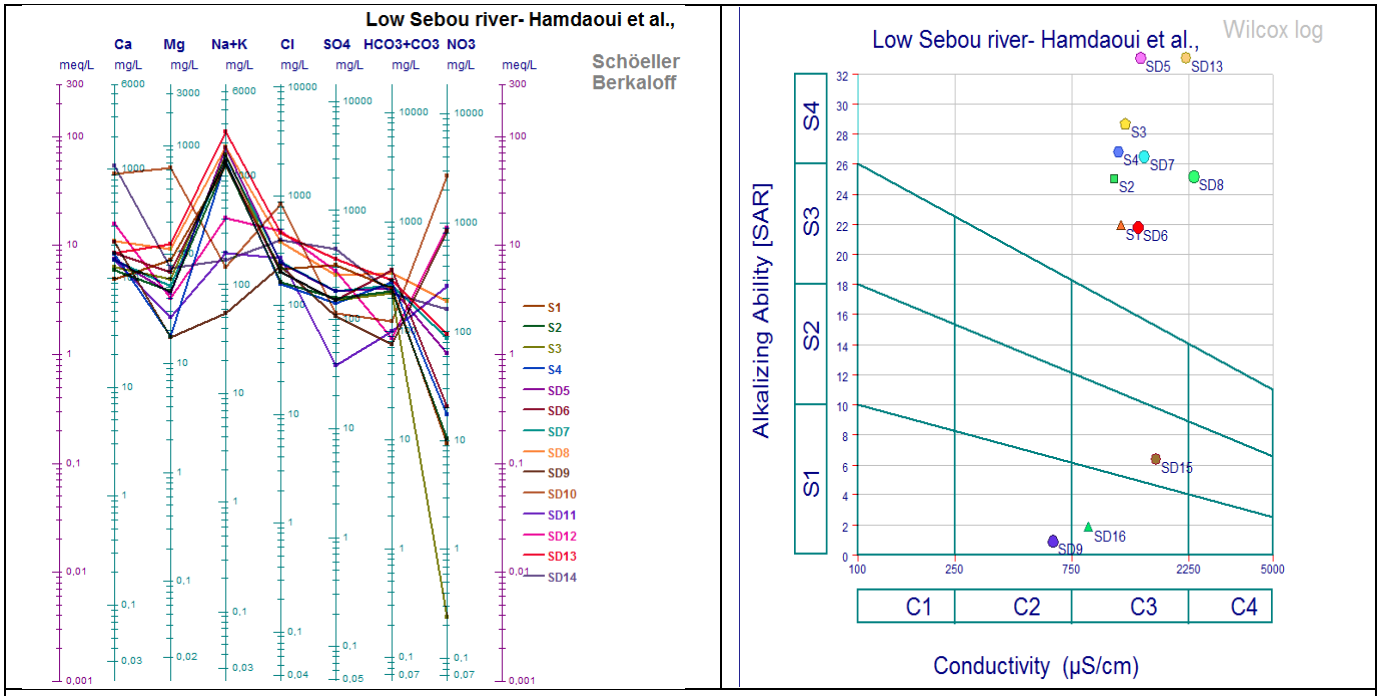


FIGURE 5: BERKALOFF AND WILCOX LOG DIAGRAMS OF WATER OF THE LOWER SEBOU

Concerning the nitrate contents (**Tab.1**), the values oscillate between 0.24 mg / L and 2692 mg / L and clearly translate the pollution of agricultural origin by the nitrogenous fertilizers, the wastewater and leachates of the wild discharges [15-21].

The Piper diagram (**Fig.4**) shows that globally the waters of the lower Sebou are hyper-chlorinated calcium, hyper-sulphated calcium, chlorinated sulphated calcium and magnesium or chlorinated sodium and potassium or sulphated sodium [22, 23].

Moreover, the projection of physicochemical data in the Wilcox diagram (**Fig.4**) and Wilcox Log diagram (**Fig.5**), shows that the quality of the waters of the lower Sebou varies between Poor and Bad and rarely excellent and especially have a degraded quality because the alkalizing power of sodium (SAR). The waters of the lower Sebou are classified in the group C3S4 and C4S4 (**Fig.5**) and are unsuitable for irrigation [24-25].

IV. CONCLUSION

Adjacent agricultural activities occur well in the waters of the Lower Sebou sub-basin by significant concentrations of nitrates and sulphates which enter the water stream by runoff and leaching of nitrogenous and phosphorus fertilizer and phytosanitary products [26-27]. The upstream-downstream distribution of physicochemical parameters, reflects deteriorated situations of water quality in salts and chlorides in relation to the rise of marine saline waters.

The present work has revealed the poor quality of the waters of the lower Sebou but remains incomplete and needs to be deepened by analyzes of trace heavy metals and pesticides to provide the scientific and technical bases for decision-makers [28-30].

ACKNOWLEDGEMENTS

The authors acknowledge all responsible from ORMVAG who have aided in accomplishing the analysis.

REFERENCES

[1] C. Boutin and N. Dias, ‘‘Impact de l’épandage des eaux usées de la ville de Marrakech sur la nappe phréatique’’, Bull. Fac. Sci. Marrakech (Sect. Sci. Vie), 3 (1987), pp. 5-25.

[2] M. Benyakhlef, S. Naji, D. Belghyti, Y. El guamri et T. Hassouni, ‘‘ Qualité de l’eau de boisson dans la région du Gharb (Kénitra, Maroc)’’ 2011.

[3] H. Abouzid et A. Outair, ‘‘ Les Nitrates dans les eaux’’, 7ème Congrès Mondial des ressources en eau, Rabat, Maroc, 13-18 Mai 1991, Volume 2.

[4] ORMVAG, ‘‘Office Régional de mise en Valeur Agricole Gharb Maroc : Etude pédologique au 1/20 000 de la Troisième Tranche d’Irrigation (TTI) sur une superficie de 100.000 ha. Zone M’nasra, Z1-Z2. Kénitra, Maroc’’, Rapport inédit, 1994, 180p.

[5] J. Rodier, ‘‘L’analyse de l’eau: eaux naturelles, eaux résiduaires, eau de mer : physico-chimie, bactériologie et biologie’’, Ed. Dunod, Paris, France, 8 (1996), 1383p.

[6] D. Belghyti, Daifi, H., Alemad, A., Elkharrim, K., Elmarkhi, M., Souidi, Y., Benelharkati, F., Joti, B., Elmoukrifi, Z., Ibeda, A., Azami-Idrissi, Y., Baroud, S., Elkhayyat, F., Elrhaouat, O., Sadeq, S., Taboz, Y., Sbai, H., Naser, R., Chigger, H., Derwich, N., ‘‘Groundwater management for sustainable production of drinking water quality in Maamora’’, 2nd International Conference on Water and Society, 4 - 6 September 2013, New Forest, UK, 2013, Vol 178, , doi:10.2495 / WS130201, 2013, pp. 242-254.

[7] O.N.E.P, ‘‘Méthodologie d’analyse de l’eau au laboratoire. Mode opératoire: Contrôle de la pollution des eaux destinées à l’alimentation en eau potable’’, 1998.

[8] L. Matini, J.M. Moutou et M.S. Kongo-Mantono, ‘‘ Evaluation hydro-chimique des eaux souterraines en milieu urbain au Sud-Ouest de Brazzaville, Congo’’, 2012.

[9] T. El Hammoumi et Belghyti D., ‘‘Caractérisation physicochimique des eaux potables Produit Par la station de traitement de Mkansa’’ (Maroc, 2012).

[10] L. Bentouati et Bouzidi A., ‘‘étude de la qualité des eaux souterraines de la wilaya de Sétif, algésaire’’, Journal Scienceslip, 2011.

[11] B. Benkabbour, ‘‘Exploration, évaluation et protection des ressources hydriques en zones côtiers Marocaines : Approche Géophysique, Hydro chimiques, modélisation et S.I.G : Cas de la Maàmora occidentale (Bassin du Rharb-Maàmora)’’, Thèse de Doctoral National, Université Ibn Tofail, 2002.

[12] Administration Hydraulique (AH), ‘‘Etat de la qualité des ressources en eaux dans le bassin du Sebou, année 1989-1990’’, Ministère des Travaux Public, de la Formation Professionnelle et de la Formation des Cadres. 1996.

[13] S. Akhiar, “Caractérisation des eaux souterraines de la ville de Mechraa Bel Ksiri”. Mémoire Master Eaux usées. Université Ibn Tofail, Kénitra, 2009.

[14] M. Hilali,. “Hydrogéologie et modélisation de l'intrusion marine dans les aquifères côtiers de Martile et de Sahel- Maroc”, Thèse de Doctorat en Sciences Appliquées. Université. Mohammed V-Agdal, Ecole Mohammedia d'ingénieurs, 2002, 158p.

[15] Secrétariat d’Etat chargé de l’Environnement, ‘‘Etude pour programme d’action visant à minimiser et à contrôler l’impact des engrais et des pesticides sur l’environnement du bassin de Sebou’’, PPES (Projet de l’environnement du Sebou), Secrétariat d’état chargé de l’environnement, (Maroc), (1999), 43p.

[16] Z. Saadi, A. Maaslouhi, M. Zeraouli et J. P Gaudet, ‘‘Analyse et modélisation des variations saisonnières des concentrations en nitrates dans les eaux souterraines de la nappe Mnasra, Maroc’’, C. R. Acad. Sci., Sér. 2, Sci. Terre Planètes, 329, 8, 1999, pp. 579-586.

[17] MPCI, ‘‘Impact environnemental de l’usage des eaux usées d’assainissement dans l’irrigation des agricultures’’, ministère de la planification et de la coopération internationale, juillet 2005.

[18] A. Alemad , Nagi M., Ibeda A., Nasser R., Alwathaf Y., Elrhaouat O., Elkharrim K., Babaqi A., Belghyti D., ‘‘The impact of sana'a solid waste on the quality of groundwater in Yemen’’, 2nd Intemational Conference on Water and Society, 4 - 6 September 2013, New Forest, UK, Paper DOI: 10,2495/WS130151.

[19] M. S. Coyne and J. M. Howell, ‘‘Agricultural Impacts on Fecal Contamination of Shallow Groundwaters in the Bluegrass Region of Kentucky’’, Soil Science News and Views, 15, 6, 1994, pp. 1-3.

[20] A., B. Krira. Chakour et H. Fouta, ‘‘Intensification de l’agriculture et son impact sur l’environnement. Cas des nitrates dans la nappe phréatique de M’nasra du Gha’’, Actes 1er Colloq. Sur le Développement agric. Rech. Agron. Au niveau de la région du Ghab, 2001.

[21] M., Zeraouli, ‘‘Pollution par les nitrates. Premiers résultats de la situation actuelle dans la nappe des Mnasra’’ (décembre 1992-janvier 1993) », Office régional de mise en valeur agricole du Gharb, Département de développement agricole, Service des études de développement agricole, Bureau Agro- Pédologique, Puplication interne ORMVAG, septembre, 1993.

[22] L. Zilliox, C.Schenc, H. Kobus et B. Huwe, ‘‘Pollution par les nitrates: Quels remèdes ? Supplément’’, La Recherche Suppl. les enjeux de l’agriculture en Europe, 227, 1990, pp. 18-21.

[23] K.. El Bouqdaoui, Aachib, M., Blaghen, M., et Kholtei, S., ‘‘Modélisation de la pollution par les nitrates de la nappe de Berrechid, au Maroc’’, 2009.

[24] L. Zouhri, “Structure et modélisation hydrodynamique de l’aquifère de la Maamora (Maroc)”. Thèse, Univ, LilleI, 2000, 218p.

[25] M. Nisbet et Verneaux J., “Composantes chimiques des eaux courantes : discussion et proposition de classes en tant que bases d’interprétation des analyses chimiques”, Annls Limnol, 6, 2, 1970.

[26] O.N.E.P, ‘‘Alimentation en eau potable, Menaces de pollution’’, 1999.

[27] Laferriere. M, J. J. Minville, J. Lavoie et P. Payment, ‘‘L’industrie porcine et les risques reliés à la santé humaine’’, Bull. Information Santé Environnem, Québec, 7, 2, 1996, pp. 1-4.

[28] Organisation Mondiale de la Santé (O.M.S.), ‘‘Charte d’Ottawa pour la promotion de la sant’’, Copenhagen, Bureau régional de l’Europe, (1986).

[29] CSE. Conseil Supérieur de l’Eau, ‘‘Aménagement optimal des eaux de l’oued Ouergha: Réalisation du barrage Mjara’’, Rabat, Maroc. 1988.

[30] A.B.H.S., ‘‘Etude d’actualisation du plan directeur d’aménagement integer des resources en eau du basin hydraulique du Sebou. Note de synthèse’’, Septembre 2011, Agence du Bassin Hydraulique du Sebou, Fès, Maroc.

Characterization of antifungal activity of endophytic *Penicillium oxalicum* T 3.3 for anthracnose biocontrol in dragon fruit (*Hylocereus* sp)

Suhaila Mamat[1], Umi Kalsom Md Shah[2], Nurul Atika Mohamad Remli[3], Khozirah Shaari[4], Rozeita Laboh[5], Nor Aini Abdul Rahman[6]

[1,2,3,6]Department of Bioprocess Technology, Faculty Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia
Email: umikalsom@upm.edu.my
[4]Faculty of Sciences, Universiti Putra Malaysia
[5]Department of Horticulture, Malaysia Agriculture Research and Development Institute, 43400 Serdang, Selangor, Malaysia

Abstract— *A group of 126 endophytic fungi was isolated from dragon fruit plants in Malaysia. Dual culture and disc diffusion test revealed that seven strains could suppress the growth of Colletotrichum gloeosporioides. Of all the potential strains, strain T3.3 exhibited the best antagonistic activity by producing an inhibition zone of 12±1.0 mm in dual culture test. Disc diffusion test using crude extract produced by this fungus could inhibit the growth of C.gloeosporioides by 33.33±2.89%. The diffused non volatile metabolite produced by this strain suppressed the growth of the pathogen by 97% after 7 days of incubation. Based on morphological characteristics and ITS region sequence, strain T3.3 was identified as Penicillium oxalicum. The carbon utilization profile in Biolog FF Microplate analysis revealed that P.oxalicum T3.3 is a versatile microorganism. P.oxalicum T3.3 was found to produce β-glucanase and chitinase with activity of 3.38 U/mL and 1.19 U/mL respectively. In addition, the ethylacetate extract of P.oxalicum T3.3 could suppress the growth of C.gloeosporioides. Scanning electron microscopy study showed that the release of extracellular antifungal metabolites from the endophytic P.oxalicum T3.3 had caused abnormal hyphal growth of C.gloeosporioides. Treatment with the crude extract from this fungus on dragon fruit could control anthracnose disease in this fruit in vivo. Thus, endophytic Penicillium oxalicum T3.3 is considered as a potential biological control agent of anthracnose disease in dragon fruit.*

Keywords— Antifungal, dragon fruit, endophytic fungi, Penicilium oxalicum.

I. INTRODUCTION

Colletotrichum.gloeosporioides is the causal agent of anthracnose disease and an important cosmopolitan fungal pathogen that infects many plant species worldwide (Cannon *et al*., 2012). This pathogen is present in various regions of the world including tropical to subtropical areas and can be destructive if left uncontrolled. In recent years, the occurrence of anthracnose disease in dragon fruit plant has affected fruit yield tremendously. (Masyahit *et al*., 2009). The disease occurs when the weather conditions, such as warm weather and frequent rains as well as inoculum were present. The common symptoms that usually observed in infected plants and fruits are sunken necrotic tissues with concentric rings where ascervuli emerge from it. The infected spot will coalesced to rot (Palmateer and Ploetz, 2006).

This disease is controlled by a combination of cultural management practices and chemical control. The infected plant parts are pruned out and destroyed by burning them. Fungicides such as Benex, Arimo 23, Maneb and Mancozeb were applied to the farm to eradicate the fungal infection. However, the usage of chemical fungicide in the dragon fruit farm gives detrimental effect to the lives of human and had polluted the environment (Crissman *et al*., 1994). Apart from that, the development of fungicide resistance pathogen had complicated the disease management (Pimenta *et al*., 2010).

Under such circumstances, there is a need to search for another alternative control method that is considered safe for human health and the environment. Currently, there is growing interest in using endophytic fungi as biological control agent (BCA). The use of endophytic fungi which reside asymptomatically inside host plant without causing any apparent disease as BCA is beneficial as they occupy the same ecological niche as the pathogens and may induce the defense responses of the hosts against the pathogens (Brum *et al*., 2012). Endophytic fungi also produce an array of secondary metabolites which may be exploited for use in agriculture and industry (Zhang *et al*., 2006).

Many species of endophytic fungi mostly those belonging to the genus *Penicillium* are identified as biocontrol agents with antifungal activities against a number of plant pathogenic fungi (Xu *et al*., 2010; Elsharkawy, 2012; Murali, 2012). *Penicillium sp*. has also been reported to produce lytic enzymes including chitinase and β-glucanase which are involved in

degrading fungal cell walls (Lee *et al*., 2009; Chen *et al*., 2012; Patil *et al*., 2012). Furthermore, *Penicillium sp*. also produce antimicrobial compounds that could control a wide spectrum of microorganisms (Komai *et al*., 2006; Wang *et al*., 2008).

In this study, we reported the screening and isolation of endophytic fungi from healthy dragon fruit plant. The antifungal activity of the potential strain against *C. gloeosporioides* was also characterized. The introduction of the paper should explain the nature of the problem, previous work, purpose, and the contribution of the paper. The contents of each section may be provided to understand easily about the paper.

II. MATERIAL AND METHOD

2.1 Sample collection

Stem and aerial root samples of healthy dragon fruit plants were collected from the dragon fruit field at three different locations in Malaysia including Telong, Kelantan; Mantin; Negeri Sembilan; and Serdang, Selangor from May 2011 to August 2011. The samples were collected using a clean knife and immediately processed for isolation of the endophytic fungi.

2.2 Fungal pathogen

The plant pathogenic fungus *C.gloeosporioides* (Accession number: MARDICG1) was obtained from the Malaysian Agriculture Research and Development Institute (MARDI), Serdang, Selangor, Malaysia. The fungus was maintained on potato dextrose agar (PDA) medium and stored at 4°C.

2.3 Isolation of endophytic fungi

The isolation of endophytic fungi followed the method described by Tan *et al*. (2006) with slight modifications. Malt extract agar (MEA), Potato Dextrose Agar (PDA) and Czapek Dox Agar (CDA) were used as isolation media. The dragon fruit plant part was cut approximately to 4 cm in length with a sterilized blade, cleaned by washing under running tap water several times and dried in a laminar airflow. Then the prepared organ was surface sterilized with 70% (v/v) ethanol for 1 min followed by 2% NaOCl for 1 min and then sequentially rinsed in sterile distilled water three times and dried on sterile filter paper. The surface sterilized organ was cut approximately to about 5 mm in length using a sterile blade and placed onto the isolation medium. All the plates were incubated at 30°C for 7 to 15 days. Individual hyphal tips developed from the stems were transferred onto fresh PDA plates to get pure culture.

2.4 Dual culture test of endophytic fungi against *C.gloeosporioides*

All the pure cultures of the endophytic fungi were tested for their antagonistic activity against *C.gloeosporioides* using dual culture test on PDA plate. Agar discs (10 mm diameter) of pure endophytic fungus and fungal pathogen from 7 day old cultures were put at the periphery of the PDA plate opposed to each other. As for the control, the pathogen was inoculated on PDA with no opposed fungi. All the plates were incubated at 30°C and observed daily for 7 days. Antagonistic activity was observed daily for seven days after incubation. Fungal strains that showed mutual inhibition were chosen and the size of the inhibition zone was measured.

2.5 Cultivation of the potential strains for production of antifungal compounds

The strains that exhibited mutual inhibiton were selected and cultivated for the production of antifungal compounds. An agar plug of the pure culture of the potential strain was inoculated into 100 ml of Richard medium (KNO_3, 1 g/l; glucose, 30 g/; KH_2PO_4, 0.5 g/l; $MgSO_4.7H_2O$, 0.25 g/l; $FeCl_3$, 0.001 g/l) pH 5.5 in a 250 ml of conical flask. The flask was incubated on rotary shaker at 30°C, 150 rpm for 10 days. Each strain was prepared in triplicates.

2.6 Extraction of metabolite

Following fermentation, the mycelia of the fungi were separated from the fermentation broth by passing through a funnel layered with cotton wool. The mycelia were placed in a beaker and an equal volume of ethylacetate was added into the beaker. This mixture was left at room temperature overnight. The solvent fraction was separated and collected from the mixture. As for the fermentation broth, the broth was transferred into a conical flask and an equal volume of ethylacetate was added into the broth. The mixture was stirred using a magnetic stirrer for an hour. After that the solvent phase was separated and collected from the mixture using a separatory funnel. An equal volume of ethylacetate was added for the second time and

the step was repeated twice. All the solvent fractions were combined and dried using a rotary evaporator at 40°C with vacuum pressure until all the solvent was removed.

2.7 Disc diffusion test using the extracted metabolites

Ten mg of the crude extract produced from each of the seven strains was weighed and dissolved in 1000 µl of ethylacetate. Ten µl of the dissolved crude extract was impregnated onto 5 mm sterile filter paper disc (Whatman filter paper No 1) and allowed to dry in a laminar airflow for 30 min. The dried filter paper disc was placed at the center of the PDA plate. An agar plug of *C.gloeosporioides* with diameter of 5 mm from 7 days old culture was placed at a distance of 10 mm opposite the filter paper disc. As for the control, the disc that was impregnated with ethylacetate was used. The test was prepared in triplicate and incubated at 30°C for 2 days. The radial growth of *C.gloeosporioides* towards the disc containing the crude extract (R_2) and that on a control plate (R_1) were measured and percentage of radial growth inhibiton (PIRG) was recorded to this formula: $(R_1-R_2)/R_1 \times 100$.

2.8 Non volatile metabolite test

The agar layer technique (Dennis and Webster, 1971) was used to detect the production of non-volatile metabolites by strain T3.3 and to evaluate their effects against the pathogen. An agar plug (10 mm in diameter) of strain T3.3 was located at the center of the PDA plate which was previously layered with sterile Visking tube and incubated at 30°C. After 7 days, the Visking tube layer containing the fungal growth was removed from the plate. An agar plug (10 mm in diameter) of pathogen was placed at the center of the prepared plate. The plate was incubated at 30°C for 7 days and observed daily. As for the control, strain T3.3 was not inoculated on the layered PDA plate. The radial growth of *C.gloeosporioides* on the plates containing the non volatile metabolite produced by strain T3.3 (R_2) and that on a control plate (R_1) were measured and the PIRG value was calculated according to the formula described previously.

2.9 Identification of potential endophytes using morphological characteristic and ITS sequence

Strain T3.3 which exhibited the highest inhibitory activity was identified macroscopically and microscopically. The morphology of the isolate such as the spore colour, growth of hyphae on PDA and the color of the bottom of the PDA was observed with the naked eyes. The microscopic characteristics of the isolate such as spore arrangement and hyphae structure were observed using a light microscope in order to identify its genus. Strain T3.3 was identified using the ITS method. Seven days old mycelia culture of this strain was used to isolate total genomic DNA. The genomic DNA of the strain was extracted using the Profound and Kestrel Laboratory (PKL) DNeasy Plant Minikit, according to the manufacturer's manual. The DNA was amplified by PCR using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCT TATTGATATGC-3') primers. The PCR was done according to the Fungal ID Kit (PKL) protocol and the conditions were as follows: predenaturation at 95 °C for 5 min, denaturation at 95 °C for 30 s, renaturation at 54.5 °C for 20 s and extension at 72 °C for 1 min per-cycle, followed by 35 cycles, and a final extension step at 72 °C for 5 min. DNA sequence homology searches were performed using the online BLAST search engine in GenBank. The phylogenetic tree for the data set was constructed using the MEGA 4.1 programme.

2.10 Biolog FF Microplate analysis

Carbon source utilization assimilation of strain T3.3 was examined by using the Biolog Filamentous Fungi Microplate according to the manufacturer's protocol. The profile was compared with the BIOLOG identification database GN4.01

2.11 Production of cell wall degrading enzymes by strain T3.3

2.11.1 Preparation of dried *C.gloeosporioides* mycelium for β-glucanase production

One cm^2 disc of actively growing mycelium of *C.gloeosporioides* was inoculated in a 250 ml conical flask containing 100 ml potato dextrose broth and incubated at 30 °C for 7 days. The mycelium was then filtered through Whatman No.1 filter paper, washed with distilled water and dried at 60°C overnight. The dried mycelium was ground with mortar and pestle until it turned into fine powder and stored at 4°C until further use.

2.11.2 Production of β-glucanase and chitinase enzyme

Strain T3.3 was grown on β-glucanase production medium (peptone, 3.0 g/l; $(NH_4)_2SO_4$, 2.0 g/l; yeast extract, 0.5 g/l; KH_2PO_4, 0.5 g/l; $CaCl_2.2H_2O$, 0.3 g/l; $MgSO_4.7H_2O$, 0.3 g/l; Tween 20, 100µl; dried cell wall of *C.gloeosporioides*, 10 g/l;

pH 5.5) and chitin medium ($NaNO_3$, 0.2 g/l; K_2HPO_4, 0.1 g/l; $MgSO_4.7H_2O$, 0.05 g/l; KCl, 0.05 g/l; $FeSO_4.7H_2O$, 0.001 g/l; pH 5.5) supplemented with 10 g/l of colloidal chitin. These media were prepared for β-glucanase and chitinase enzyme production respectively. Two discs (one cm^2) of seven days old culture were inoculated into 100 ml of each medium in 250 ml flask. All the flasks were prepared in triplicate incubated at 30°C, 150 rpm on a rotary shaker for 7 days and sampling was done daily.

2.11.3 β-glucanase and chitinase assays

β-glucanase activity was determined according to the method described by Cao et al., (2009) with some modifications. β-glucanase was assayed by incubating 0.5 ml of 1.0 % (w/v) laminarin in 50 mM acetate buffer (pH 5.5) with 0.5 ml enzyme solution at 50 °C for 30 min. Then 1.5 ml of DNS was added into the mixture and boiled for 15 min. The absorbance was read at 575 nm after the addition of 1 ml of Rochelle salt. Chitinase activity was determined using the colorimetric method described by Patil et al., (2012) with minor modifications. The reaction mixture contained 1 ml of 1% colloidal chitin 0.2 M, pH 5.5 and 1 ml of enzyme solution. The reaction mixture was incubated for 60 min at 50°C. One ml of 1% NaOH was added into the mixture and boiled for one min. The mixture was centrifuged at 7000 rpm for 10 min and 1 ml of the supernatant was collected and transferred to a new test tube. One ml of DNS was added into the supernatant and boiled for 5 min. Then the mixture was cooled at room temperature and the absorbance was read at 575 nm. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of reducing sugar per minute.

2.11.4 Dry cell weight and antifungal activity of strain T3.3

Richard medium was used as the medium to determine the dry cell weight and antifungal activity of strain T3.3. One ml spore suspension of this strain with a concentration of $1x10^6$ per ml was inoculated into 100 ml Richard medium in 250 ml conical flask. Fermentation was carried out at 30°C, 120 rpm for 10 days and sampling was done daily. The biomass produced was used to determine the dry cell weight of this fungus. The culture filtrate collected was processed according to the method described previously. The antifungal activity using the crude extract of isolate T3.3 was calculated following the method described previously.

2.12 Scanning electron microscopy

Zone of interaction from dual culture plate was used as specimen to observe the interaction between strain T3.3 and *C.gloeosporioides*. One cm^2 of the agar plug from the zone of interaction was cut from the dual culture test plate. The specimen was fixed with 4% glutaraldehyde for 12 h at 4°C. Then it was washed with 0.1 M sodium cacodylate buffer for 10 min and this step was repeated three times. The specimen was post fixed with 1% osmium tetroxide for 2 h at 4°C. After post fixation, the specimen was washed again with 0.1 M sodium cacodylate buffer for 10 min three times. The specimen later was dehydrated using a series of alcohol starting from 30%, 50%, 70%, 90% and 100% for 10 min each. The sample was further dehydrated with 100% acetone, 10 min and this step was done twice. The dehydrated specimen was dried in a critical point dryer machine for 30 min .The dried specimen was stuck onto the stub using double sided tape. Finally the specimen was coated with gold in sputter coater for 3 min and it was viewed using a scanning electron microscope LEO 1455 VP SEM attached with EDX.

2.13 Evaluation of strain T3.3 crude extract against *C.gloeosporioides* on detached dragon fruit

The inoculum of the pathogen was prepared by culturing the *C.gloeosporides* on PDA and incubated at 30°C for one week. Ten ml of sterile distilled water was added onto PDA containing the pathogen. The mycelia were harvested by scraping the surface of the plate using a sterile hockey stick. Then the suspension was filtered using sterile cheesecloth to separate the mycelia and conidia. The conidia concentration was calculated using a haemacytometer and adjusted to $1x10^6$ per ml using sterile distilled water. The conidia suspension was stored at 4°C until further use. The fresh and healthy dragon fruits were surface sterilized according to the method described previously. The fruit was artificially wounded using a sterile needle. Ten µl of crude extract with concentration of 100 mg/ml (dissolved in ethanol) was applied onto the wounded area using sterile pipette and allowed to air dry for 30 minutes. Then 10 µl ($1x10^6$ per ml) of fungal pathogen spore suspension was inoculated at the wounded site. As for positive control, the fruit was inoculated with 10 µl conidia of *C.goeosporioides*. The dragon fruit that was inoculated with ethanol was used as negative control. The experiments were prepared in triplicate for each treatment. All the fruits were kept in a moisturized cotton wool layered container at 28°C for 5 days. The disease symptoms and radial lesions were observed and measured daily.

III. RESULTS

3.1 Isolation of endophytic fungi

A group of 126 endophytic fungi were successfully isolated from healthy dragon fruit plants from three different locations in Malaysia. Of that number, 86 endophytic fungi were isolated from stem whereas 40 strains were isolated from aerial roots. Based on morphological identification of the endophtic fungi isolated, *Aspergillus sp., Phoma sp., Monilia sp., Botrytis sp., Trichoderma sp., Penicillium sp*. and *Fusarium sp.* were among the fungal species that resided in dragon fruit plants. Some endophytic fungi did not have reproductive structures and could not be identified.

3.2 Dual culture test and disc diffusion test of potential strains against *C.gloeosporioides*

Based on the interactions observed in the dual culture test plates, seven strains showed mutual inhibition interaction against *C.gloeosporioides*. Further screening of the seven potential strains was carried out by using the disc diffusion test. Referring to table 1, strain T3.3 exhibited the highest antifungal activity against the pathogen tested. The highest inhibition zone against *C.gloeosporioides* was exhibited by strain T3.3 whereas the lowest inhibition zone was shown by strain Ma6. Strain T3.3 produced inhibition zone of 12±1.00 mm (Fig 1). The 10 mg/mL of crude extract of this isolate could inhibit the growth of *C.gloeosporioides* by 33.33±2.89%.

TABLE 1
INHIBITION ZONE AND ANTIFUNGAL ACTIVITIES OF SELECTED ENDOPHYTIC FUNGI ISOLATED FROM DRAGON FRUIT PLANT AGAINST *C.GLOEOSPORIOIDES*

Strain	Origin	Inhibition zone (mm)[a]	PIRG of crude extract (%)[b]
T3.3	Telong, Kelantan	12±1.00	33.33±2.89
Mn10	Mantin, Negeri Sembilan	4±1.00	20.00±5.00
UN9	Telong, Kelantan	6±0.50	26.67±2.89
TPU22	UPM, Selangor	8±0.50	26.67±5.77
Ma6	Telong, Kelantan	3±0.50	13.33±5.77
TPU19	UPM, Selangor	4±0.86	15.00±0.00
Mn21	Mantin, Negeri Sembilan	6±1.00	23.33±5.77

[a]The size of inhibition was measured when the three colonies in the control covered the whole plate.
[b]The PIRG value was measured when the three colonies in the control reached the disc with no crude extract.

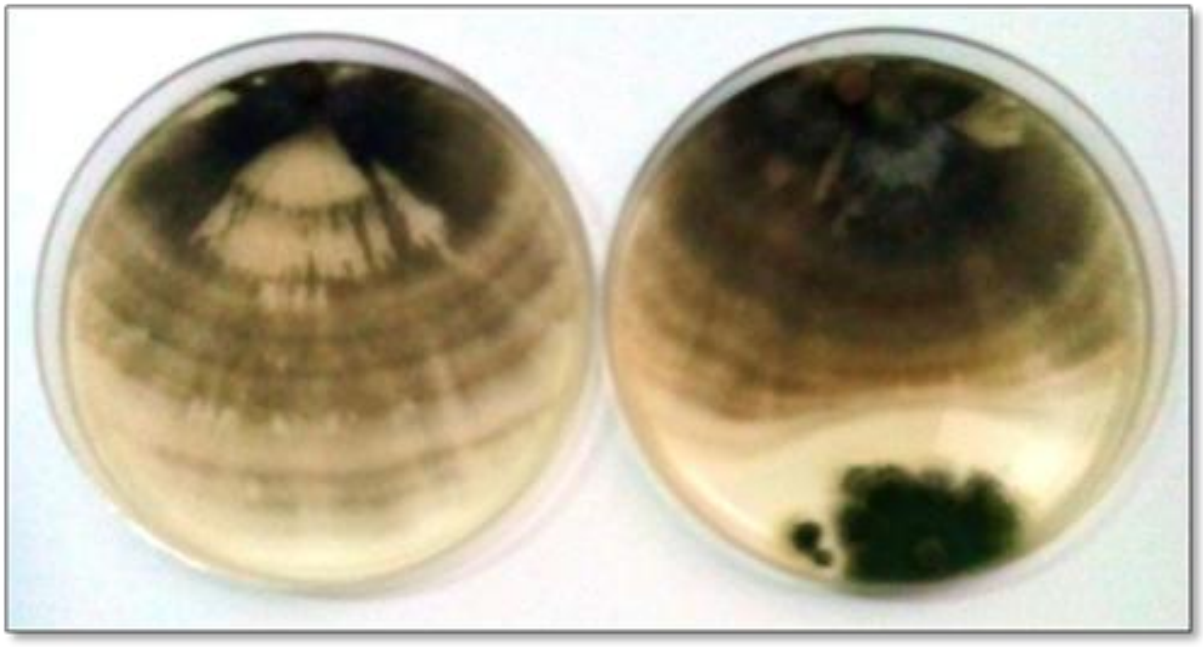


FIG. 1: Growth inhibition of *C.gloeosporioides* by endophytic strain T3.3 cultured on PDA plate at 30°C for 1 week. Left: control; right: in the presence of strain T3.3.

3.3 Non volatile metabolite test of strain T3.3

Strain T3.3 exhibited potential inhibitory activity against C.gloeosporioides. Therefore, this strain was further evaluated using the non volatile metabolite test. The growth of the pathogen on the plate containing the diffused non volatile metabolite of strain T3.3 was suppressed by 97% compared to the growth of the pathogen on the control plate. This test revealed that non volatile metabolites released by this strain contain antifungal metabolites which contribute to its antifungal property. All these characteristics, in addition to its strong inhibition, we recommend strain T3.3 for further evaluation of its biocontrol potential.

3.4 Identification of strain T3.3

Strain T3.3 showed slow and dense growth on PDA plate. The colony size was 32-36 mm in diameter, and the mycelium appeared as white radially plane or sulcate, velutinous within the first three days. After three days of incubation, the white mycelia at the center of the colony sporulated and produced heavy dark green spores. Within seven days, the entire colony appeared as dark green with narrow irregular white margin about 2 mm wide. The reverse side of the colony showed yellow colorations. Under a light microscope, conidiopohores with metulae and phialides were observed and the phialides branched asymmetrically. Conidia were budded from the phialides and were arranged in chains. The conidia were ellipsoidal in shape and the walls were smooth. Based on the phylogenetic tree constructed using ITS sequence (Fig. 2), strain T3.3 was identified as Penicillium oxalicum.

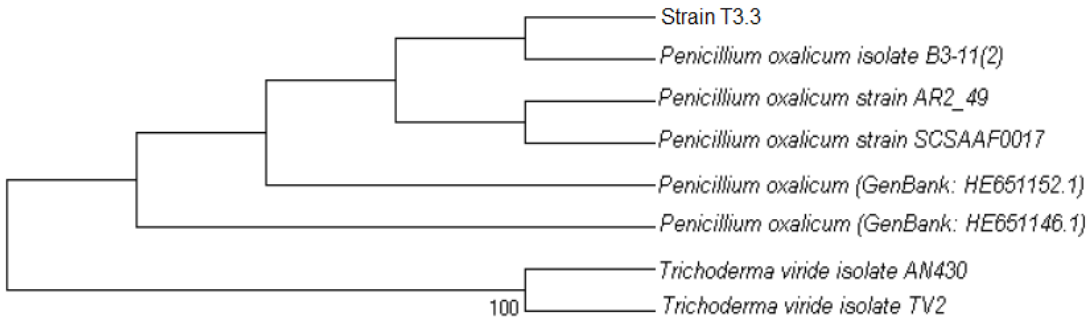


FIG. 2: Neighbour-joining phylogenetic tree showing relationships between strain T3.3 and several other strains of *Penicillium*, based on their ITS DNA sequences. *Trichoderma viride* was used as an out group for reference purposes.

3.5 Biolog FF Microplate analysis of endophytic P.oxalicum T3.3

Biolog FF Microplate was recently introduced as a new method to identify filamentous fungi instead of using morphological and DNA sequence identification. This method had also been used to characterize the metabolism of a certain microorganism (Singh, 2009; Papaspyridi *et al.*, 2011). Based on Biolog FF Microplate analysis, P.oxalicum T3.3 was identified as Penicillium oxalicum Currie and Thom. The Biolog analysis also analyzes fungal growth via turbidimetric analysis.The turbidities of this fungus were significantly high in wells containing sucrose, xylitol, maltose, maltotriose gentibiose and i-erythritol as carbon sources. These groups of carbon sources are mainly carbohydrates and sugar alcohols. The turbidities of *P.oxalicum* T3.3 in wells containing D-cellobiose, D-fructose, α- D-glucose, D-raffinose D-trehalose were considered as moderate. However, there was no growth observed in wells containing N-acetyl-D-galactosamine, N-acetyl-D-mannosamine, L-sorbose, glucuronamide, L-fucose as carbon sources.

3.6 β-glucanase and chitinase production by *P.oxalicum* T3.3

Endophytic P.oxalicum T3.3 was found to produce cell wall degrading enzymes including β-glucanase and chitinase enzymes. The highest β-glucanase and chitinase activities from this fungus were obtained on the third and sixth days of fermentation with activities of 3.38 U/ml and 1.19 U/ml respectively (Fig. 3). Both β-glucanase and chitinase enzymes were hydrolytic enzymes which degrade the cell wall of the phytopathogens and had been reported as a mechanism of suppression of fungal pathogen by some biocontrol agents (El-Katatny *et al*., 2000).

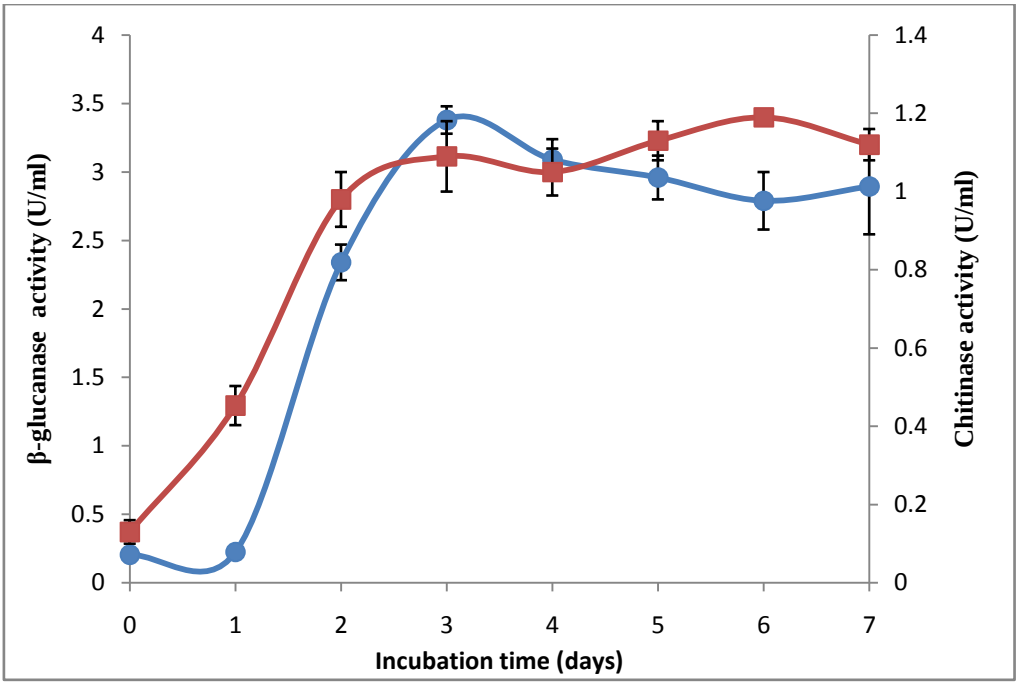


FIG. 3: Time course of β-glucanase and chitinase enzymes by *P.oxalicum* T3.3 in β-glucanase and chitinase production medium respectively at 30°C. Data represent the means from three different flasks. The highest β-glucanase activity was on the third day (3.38 U/ml) and the highest chitinase activity was on the sixth day of fermentation (1.19 U/ml). Error bars represent standard deviation from three replicates. Symbols represent: (●) β-glucanase activity; (■) chitinase activity

3.7 Antifungal activity of the extract of *P.oxalicum* T3.3

P.oxalicum T3.3 showed the presence of white smooth balls not more than one mm in diameter in Richard medium after 24 h of incubation. After two days, the ball - like masses of mycelia grew bigger ranging from two to six mm in diameter and after 10 days of fermentation, the clear medium changed to yellow colour indicating that metabolite was excreted out into the medium. Dark brown crude extract was obtained after extraction of the culture filtrate by ethylacetate. Based on Fig 4, the antifungal activities of the crude extract of *P.oxalicum* T3.3 were not detected from day 0 to day 4. On day 5, antifungal activity was detected and reached a peak on the 9th day of fermentation and decreased on the subsequent day. In this figure, it was shown that the antifungal activity was directly proportional to the biomass produced.

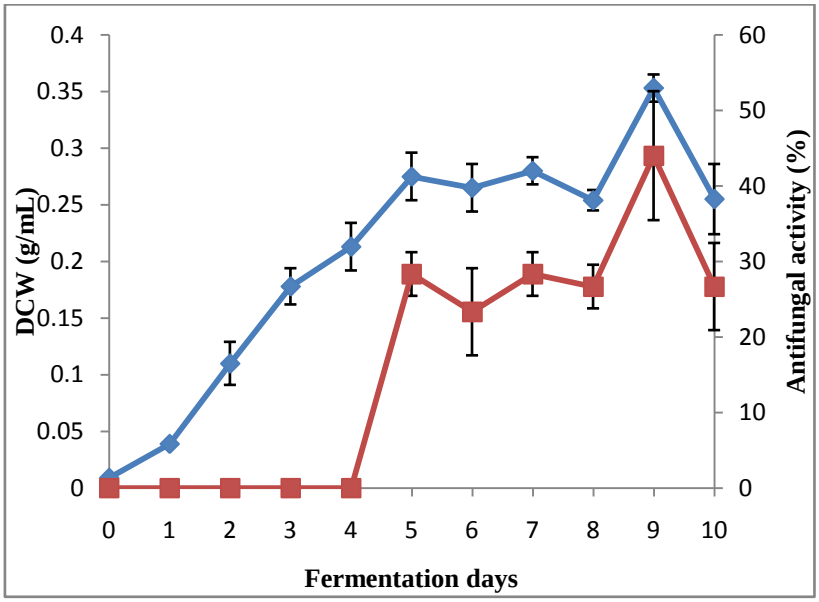


FIG. 4: Dry cell weight and antifungal activity of endophytic *P.oxalicum* T3.3 grown in Richard medium at 30°C, pH 5.5. Error bars represent standard deviation from three replicates. Symbols represent: (●) Dry cell weight; (■) antifungal activity.

3.8 Scanning electron microscopy

The SEM images revealed no direct contact between the hyphae of endophytic *P.oxalicum* T3.3 and *C.gloeosporioides*. The hyphae of *C.gloeosporioides* showed normal and rigid structure in the absence of *P.oxalicum* T3.3 (Fig. 5a). Compared to the pathogen hyphae in the control plate, there were severe morphological alterations in hyphae of this pathogen in the presence of *P.oxalicum* T3.3. The mycelial cell wall of this pathogen showed abnormal growth and became disintegrated resulting in growth inhibition of the fungal pathogen (Fig. 5b). The metabolites secreted by the endophytic *P.oxalicum* T3.3 in the medium had evoked abnormal growth of *C.gloeosporioides* where the growth of the hyphae became irregular, such as shriveling of the pathogen hyphae (Fig. 5c), necrosis of the hyphal cell wall (Fig. 5d) and leakage in the hyphae of *C.gloeosporioides* (Fig. 5e).

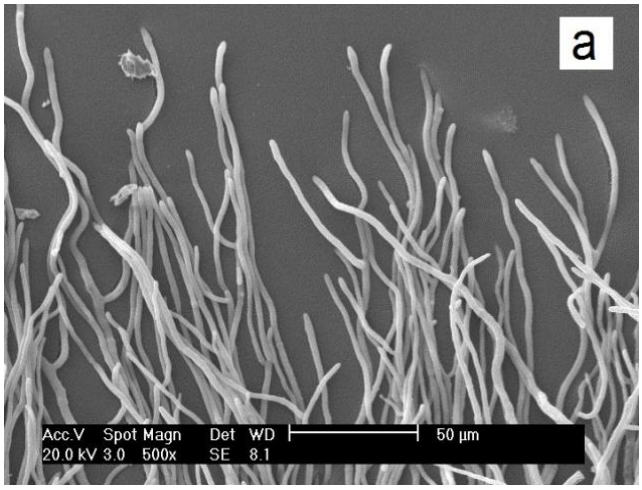


FIG. 5 (a): intact and healthy *C.gloeosporioides* hyphae from control plate

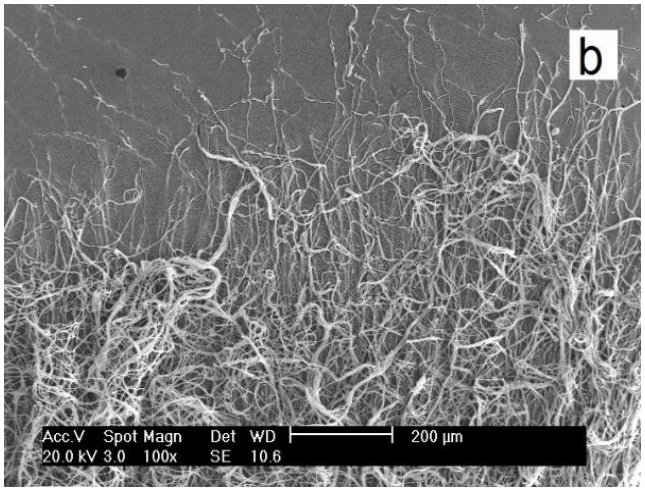


FIG. 5(b): abnormal growth of *C.gloeosporioides*

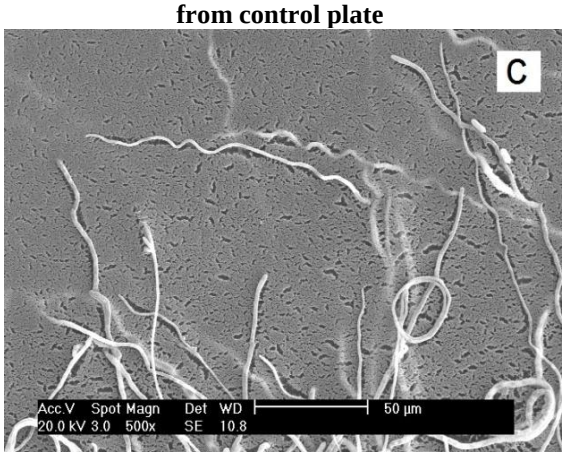


FIG. 5(C) distorted hyphal growth

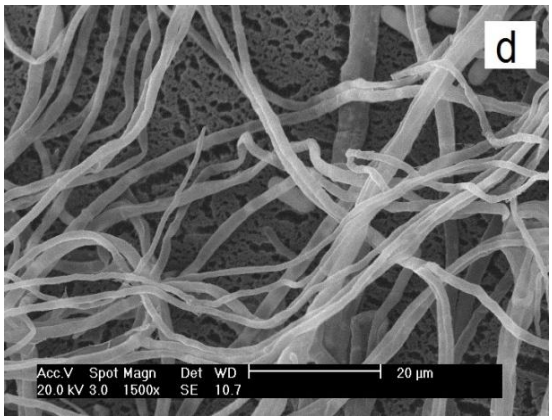


FIG. 5(d) shriveled *C.gloeosporioides* hyphae

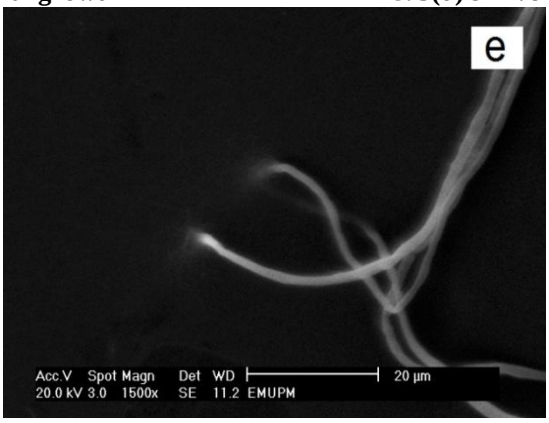


FIG. 5(e) Incomplete cell wall formation causing cytoplasm leakage from the hyphae.

3.9 Evaluation of P.oxalicum T3.3 crude extract against *C.gloeosporioides* on detached dragon fruit

The crude extract of endophytic *P.oxalicum* T3.3 was tested in vivo to evaluate its activity against anthracnose disease. Based on this work, no lesion was observed on dragon fruits from negative control (Fig. 6). Anthracnose symptoms were observed on dragon fruits from the positive control after 2 days of incubation at 28°C and it was similar to the symptoms reported by Masyahit *et al.,* (2009). Sunken necrotic lesions with radius 13.5±0.22 mm were observed on the fruit from positive control after 5 days of incubation. The dragon fruit that was treated with crude extract of *P.oxalicum* T3.3 did not exhibit serious disease symptom. Necrotic lesions with radius of 1.00±0.03 mm were observed around the inoculation site on day 5. Thus, treatment with 100 mg/mL of crude extract could reduce the lesions caused by the pathogen on the fruit.

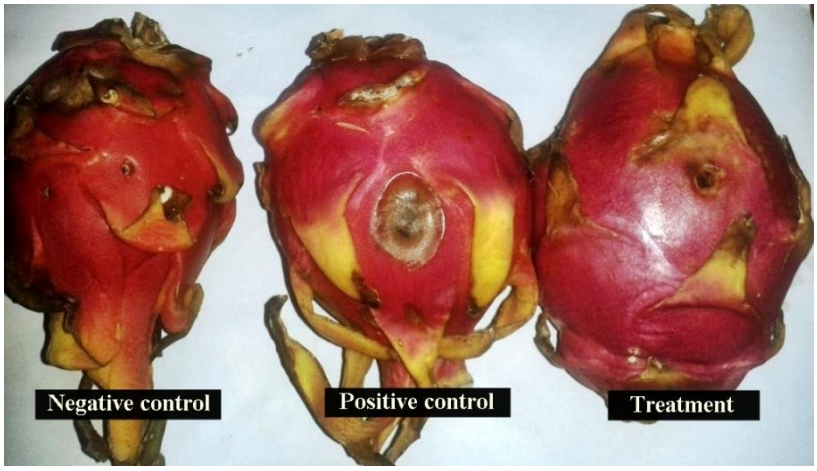


FIG. 6: Anthracnose disease suppression by 100 mg/mL of crude extract of *P.oxalicum* T3.3 on dragon fruit at 28°C on day 5. Negative control: dragon fruit treated with ethanol. Positive control: dragon fruit inoculated with *C.gloeosporioides*. Treatment: Dragon fruit inoculated with 10 µl of 100 mg/ml crude extract from *P.oxalicum* T3.3 prior to *C.gloeosporioides* inoculation

IV. DISCUSSION

Endophytic fungi are the most unexplored group of organisms and have remained uncharacterized. Out of the 1.5 million plant species worldwide, only less than 5% of endophytic microorganisms from plants have been figured out (Gilbert, 2002).To our knowledge, this is the first report regarding to the occurrence of endophytic fungi in dragon fruit plant. In this study, endophytic *Penicillium oxalicum* T3.3 has been identified as one of the endophytic fungi that are capable of living inside the dragon fruit plant. *Endophytic Penicillium* has been isolated from different host plants such as *Scoparia dulcis Linn, Hevea brasiliensis , Azadirachta indica A. Juss* (Mahesh *et al.,* 2005; Mathew *et a*l., 2010; Saithong *et al*., 2010; Gazis and Chaverri, 2010Tenguria and Khan, 2011). Different strains of *Penicillium* have also been isolated from coffee plants (Vega *et al*., 2006). *P.oxalicum* has also been frequently isolated from the soil and from various organic materials undergoing slow deterioration (Santamarina *et al*., 2002; Kurakake *et al*., 2006; Li, *et al*., 2007; Pandey *et al*., 2008).

Our findings show that endophytic *P. oxalicum* T3.3 demonstrated antagonistic activity towards *C.gloeosporioides*. The large inhibition zone produced by the endophytic *P.oxalicum* T3.3 from among the other seven potential strains indicated that this fungus contains the most potent antifungal activity against the pathogen tested. *P. oxalicum* has been reported to produce inhibition zone against a wide range of pathogenic fungi during dual culture test (Yang *et al.,* 2008, Paul *et al*., 2012). Inhibition zones were also produced by several *Penicillium* species towards *Rhizoctonia solani* (Nicoletti *et al.,* 2004). In contrast, Sempere and Santamarina (2008) reported that *P.oxalicum* overgrew *Nigrospora oryzae* and suggested that competition for space and nutrients are the mechanisms that present *P. oxalicum* as a biocontrol agent. The non volatile metabolite test revealed that this fungus also produced other antifungal metabolites that have not been extracted by ethylacetate. Hydrolytic enzymes such as β-glucanase, chitinase and protease might have contributed to the inhibitory activity of this strain as well since there has been a study which reported that endophytes directly suppress pathogens by producing either antibiotics or lytic enzymes (El-Katatny *et al.,* 2006).

The Biolog FF Microplate analysis demonstrated that *P.oxalicum* T3.3 is a versatile microorganism as it able to utilize a wide range of carbon sources including monosaccharides, disaccharides, polysaccharides and sugar alcohols. Papaspyridi *et al*., (2011) suggested that the substrate assimilation fingerprint obtained from the Biolog FF Microplate analysis is useful in selecting components for media optimization of maximum biomass production.

Biological control agents produced cell wall degrading enzymes such as chitinase, glucanase, cellulases and proteases and antifungal secondary metabolites that are involved in controlling the growth of the pathogen. In this study, endophytic *P.oxalicum* T3.3 was found to produce cell wall degrading enzymes including chitinase and β-glucanase. This is in agreement with Sempere and Santamarina (2008) who suggested that *P.oxalicum* secretes chitinases and β-glucanases to degrade and penetrate into the conidiophores and spores of *N. oryzae*. These enzymes are involved in degrading the cell wall of fungal pathogen due to fact that the cell walls of the fungi are constituted primarily by β-glucan and chitin (Cao *et al*., 2009). The highest β-glucanase and chitinase activities from endophytic P.oxalicum were obtained on the third and sixth days of fermentation respectively. El – Katatny *et al.,* (2000) reported that the maximum β-glucanase and chitinase levels from *Trichoderma harzianum* were on the fourth and seventh day respectively. As reported in another study, *P.oxalicum* produced β-glucanase with the activity of 3.2 U/mL on day five (Doughari, 2011). *P.oxalicum* also produced β-glucanase enzyme during its autolysis occuring after 28 days of fermentation (Copa-Patino *et al.,* 1990). Chitinase production in *P.oxalicum* had been previously reported in other studies (Rodriguez *et al*., 1993, Rodriguez *et al*., 1995)

Along with β-glucanase and chitinase, *P.oxalicum* T3.3 also produced antifungal compounds which contributed to its antifungal activity. This is in accordance with Yang *et al*., (2008) who reported that the ethylacetate extract of *P.oxalicum* strain PY-1 contained two active compounds which suppressed the hyphal growth of *Sclerotinia sclerotiorum*. Pandey *et al.,* (1993) suggested that *P. oxalicum* produced volatile substances which inhibited the growth of *C.gloeospoiroides* in guava. *Penicillium* genus has been reported to produce antifungal compounds. For example, endophytic *Penicillium sp.* associated with *Hopea hainanensis* produced antifungal compounds that showed antifungal activity against *Candida albicans* and *Aspergillus niger* (Wang *et al.,* 2008). *Penicillium simplicissimum* produced altenusin and dehydroaltenusin that exhibited antifungal activities against *Aspergillus fumigatus, A.niger, C. albicans* and *Candida neoformans* (Komai *et al*., 2006).

Under SEM imaging, the hyphal morphology of *C.gloeosporioides* was greatly affected by the metabolite released by *P.oxalicum* T3.3. There is no direct contact observed between the hyphae of *P.oxalicum* and *C.gloeosporioides*. This finding suggests that the release of extracellular metabolites containing both the cell wall degrading enzymes and antifungal compounds from endophytic *P.oxalicum* may be related to abnormal and distorted hyphal growth of the *C.gloeosporioides*. Sempere and Santamarina (2008) reported that *P.oxalicum* showed direct contact with *Nigrospora oryzae*. By Cryo-SEM microscopic investigations, they revealed that P. oxalicum coiled and penetrated around spores and conidiophores of N. oryzae, causing deformation, morphological changes and disintegration of the spores and conidiophores walls. P.oxalicum also mycoparasitised and penetrated into *Alternaria alternata (*Sempere and Santamarina, 2010). The disintegration of conidium of *A. alternata* by the action of *P. oxalicum* revealed by SEM images suggested that *P. oxalicum* produced antifungal components and extracellular metabolites like the cell wall degrading enzymes chitinases, β-glucanases and proteases. Thus, it was proven in this study that the abnormal growth, swelling, necrosis and hyphal leakage of *C.gloeosporioides* were mainly associated with the cell wall degrading enzymes; β-glucanase and chitinase and antifungal compounds of *P.oxalicum* T3.3.

Crude extract of *P.oxalicum* T3.3 could control the development of anthracnose disease on dragon fruit. This result clearly indicated that crude extract of this fungus could provide protection to dragon fruit from infection by *C.gloeosporioides* and could reduce disease severity. This is in agreement with Palaniyandi *et al*., (2011) who reported that the crude culture filtrate extract produced by *Streptomyces sp*. MJM5763 at 100 µg/ml suppressed anthracnose disease in detached yam leaves. The crude culture filtrate extract of *Streptomyces sp.* MJM5763 treatment was most effective to reduce anthracnose severity and incidence in yam.

The role of *P.oxalicum* in biological control had been well documented and commercial formulations had been developed (Larena *et al*., 2003; Sabuquillo *et al*., 2005). *P.oxalicum* conferred various degrees of protection on different plant species against plant pathogenic fungi. For example, *P.oxalicum* was successfully applied as a biocontrol agent against *Fusarium oxysporum f. sp. lycopersici* (De Cal and Malgarejo, 2001). The application of conidial suspension of *P.oxalicum* to tomato seedlings resulted in a significant reduction of fusarium wilt incidence. *Penicillium oxalicum* was able to suppress wilt caused by *Fusarium oxysporum f. sp. melonis* and *F. oxysporum f. sp. niveum* on melon and watermelon (De Cal *et al*., 2009). A significant reduction of powdery mildew disease also been observed in *P.oxalicum*-treated strawberry cultivars (De Cal *et al*., 2008). *P.oxalicum* also was reported as a biocontrol agent against other crop diseases as well (Larena *et al*., 2001).

V. CONCLUSION

In brief, endophytic *P.oxalicum* T3.3 has been demonstrated to be a potential BCA against anthracnose disease in dragon fruit plant by producing β-glucanase, chitinase and antifungal compounds involved in controlling the growth of the pathogen

tested. Therefore, the discovery of endophytic *Penicillium oxalicum* T3.3 in this study is significant as this fungus is a potential alternative to fungicides to control anthracnose disease in dragon fruit plant.

ACKNOWLEDGEMENTS

This research was funded by Ministry of Science, Technology and Innovation (MOSTI), Malaysia (02-01-04-SF1280). We would like to thanks to Dr Jeffrey Lim Seng Heng, from Strategic Resources Research Center, MARDI, Malaysia for providing the *C.gloeosporioides* culture.

REFERENCES

[1] Brum MCP, Araújo WL, Maki CS, & Azevedo JL (2012) Endophytic fungi from *Vitis labrusca* L.('Niagara Rosada') and its potential for the biological control of *Fusarium oxysporum*. Genet and Molecul Res 11(4):4187-4197.

[2] Cannon PF, Damm U, Johnston PR, & Weir BS (2012) *Colletotrichum*–current status and future directions. Stud in Mycol 73(1), 181-213.

[3] Cao R, Liu X, Gao K, Mendgen K, Kang Z, Gao J, Dai Y, Wang X. (2009) Mycoparasitism of endophytic fungi isolated from reed on soilborne phytopathogenic fungi and production of cell wall-degrading enzymes in vitro. Curr Microbiol

[4] Chen X, Meng K, Shi P, Bai Y, Luo H, Huang H., ... & Yao B (2012) High-level expression of a novel *Penicillium* endo-1, 3 (4)-β-d-glucanase with high specific activity in Pichia pastoris. J of Industrial Microbiol & Biotechnol 39(6): 869-876.

[5] Copa-Patino JL, Rodriguez J, Reyes F, Perez-Leblic MI (1990) Effect of β-glucanases on *Penicillium oxaficum* cell wall fractions. FEMS Microbiol Lett 70: 233-240

[6] Crissman CC, Cole DC, Carpio F (1994) Pesticide use and farm worker health in Ecuadorian potato production. Am J of Agric Economics 76(3): 593-597.

[7] De Cal A, & Melgarejo P (2001) Repeated applications of *Penicillium oxalicum* prolongs biocontrol of fusarium wilt of tomato plants. Eur J of Plant Pathol 107(8): 805-811.

[8] De Cal A, Redondo C, Sztejnberg A, Melgarejo P (2008) Biocontrol of powdery mildew by *Penicillium oxalicum* in open-field nurseries of strawberries. Biol Control 47(1): 103-107.

[9] De Cal A, Sztejnberg A, Sabuquillo P, Melgarejo P (2009) Management Fusarium wilt on melon and watermelon by *Penicillium oxalicum*. Biol Control 51(3), 480-486.

[10] Dennis C, Webster J (1971) Antagonistic properties of species group of Trichoderma I. Production of non-volatile antibiotics. Trans Brit Mycol Soc 57:25-39

[11] Doughari JH (2011). Production of β-glucanase enzyme from *Penicillium oxalicum* and *Penicillium citrinum*. African J of Biotechnol 10(47): 9657-9660.

[12] El-Katatny MH, Somitsch W, Robra KH, El-Katatny MS, & Gübitz GM (2000) Production of chitinase and β-1,3-glucanase by *Trichoderma harzianum* for control of the phytopathogenic fungus *Sclerotium rolfsii*. Food Technol and Biotechnol 38(3).

[13] El-Katatny MH, Abdelzaher HM, & Shoulkamy MA (2006) Antagonistic actions of *Pythium oligandrum* and *Trichoderma harzianum* against phytopathogenic fungi (*Fusarium oxysporum* and *Pythium ultimum* var. ultimum). Arch of Phytopathol. and Plant Protection 39(4): 289-301.

[14] Elsharkawy MM, Shimizu M, Takahashi H, & Hyakumachi M (2012) Induction of systemic resistance against Cucumber mosaic virus by *Penicillium simplicissimum* GP17-2 in Arabidopsis and tobacco. Plant Pathol 61(5): 964-976.

[15] Gazis R and Chaverri P (2010) Diversity of fungal endophytes in leaves and stems of wild rubber trees (*Hevea brasiliensis*) in Peru. Fungal Ecology 3: 240–254

[16] Gilbert GS (2002) Evolutionary ecology of plant diseases in natural ecosystems. Annu Rev of Phytopathol. 40(1): 13-43.

[17] Komai S, Hosoe T, Itabashi T, Nozawa K, Yaguchi T, Fukushima K, and Kawai KI (2006) New penicillide derivatives isolated from *Penicillium simplicissimum*. J of Nat Med 60(3), 185-190.

[18] Kurakake M, Sumida T, Masuda, D., Oonishi, S., & Komaki, T. (2006). Production of galacto-manno-oligosaccharides from guar gum by β-mannanase from *Penicillium oxalicum* SO. J of Agric and Food Chem 54(20), 7885-7889.

[19] Larena I, De Cal A, García-Lepe R, Melgarejo P, (2001) Biocontrol of tomato diseases by *Penicillium oxalicum*. In: Dehne, H.-W., Gisi, U., Kuck, K.H., Russell, P.E., Lyr, H. (Eds.), Modern Fungicides and Antifungal Compounds III. AgroConcept GmbH, Bonn, pp. 387–394.

[20] Larena I, Sabuquillo P, Melgarejo P, De Cal A (2003) Biocontrol of Fusarium and Verticillium wilt of tomato by *Penicillium oxalicum* under greenhouse and field conditions. J of Phytopathol 151(9): 507-512.

[21] Lee YG, Chung KC, Wi SG, Lee JC, Bae HJ (2009) Purification and properties of a chitinase from *Penicillium* sp. LYG 0704. Prot Expression and Purification, 65(2): 244-250.

[22] Li Y, Liu Z, Cui F, Xu Y, & Zhao H (2007) Production of xylanase from a newly isolated *Penicillium* sp. ZH-30. World J Microbiol Biotechnol, 23(6) 837-843.

[23] Mahesh B, Tejasvi MV, Nalini MS, Prakash HS, Kini KR, Subbiah V, Shetty HS (2005) Endophytic mycoflora of inner bark of *Azadirachta indica* A. Juss. Curr Sci 88(2): 218-219.

[24] Masyahit M, Sijam K, Awang Y, Satar MGM (2009) The first report of the occurrence of anthracnose disease caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. on dragon fruit (*Hylocereus* spp.) in Peninsular Malaysia. Am J of Appl Sci 6 (5): 902-912

[25] Mathew AJ, Jayachandran K, Mathew J (2010) Endophytic *Penicillium citrinum* Thom. from *Scoparia dulcis* Linn. Indian J Microbiol 50(1): 99-102.

[26] Murali M, Sudisha J, Amruthesh KN, Ito SI, Shetty HS (2013) Rhizosphere fungus *Penicillium chrysogenum* promotes growth and induces defence-related genes and downy mildew disease resistance in pearl millet. Plant Biol 15(1): 111-118.

[27] Nicoletti R, De Stefano M, De Stefano S, Trincone A, Marziano F (2004) Antagonism against *Rhizoctonia solani* and fungitoxic metabolite production by some *Penicillium* isolates. Mycopathologia 158: 465–474.

[28] Palaniyandi SA, Yang SH, Cheng JH, Meng L, Suh JW (2011) Biological control of anthracnose (*Colletotrichum gloeosporioides*) in yam by *Streptomyces* sp. MJM5763. J of Appl Microbiol 111(2): 443-455.

[29] Papaspyridi LM, Katapodis P, Gonou-Zagou Z, Kapsanaki-Gotsi E, Christakopoulos P (2011) Growth and biomass production with enhanced β-glucan and dietary fibre contents of *Ganoderma australe* ATHUM 4345 in a batch-stirred tank bioreactor. Eng in Life Sci 11(1): 65-74.

[30] Palmateer AJ and Ploetz RC (2006) Anthracnose of pitahaya: a new disease on a new crop in south florida. In Proceedings of the Florida State Horticultural Society (Vol. 119, pp. 50-51).

[31] Pandey A, Das N, Kumar B, Rinu K, Trivedi P (2008) Phosphate solubilization by *Penicillium* spp. isolated from soil samples of Indian Himalayan region. World J Microbiol Biotechnol 24:97–102

[32] Patil NS, Waghmare SR, Jadhav JP (2012) Purification and characterization of an extracellular antifungal chitinase from *Penicillium ochrochloron* MTCC 517 and its application in protoplast formation. Process Biochem

[33] Paul NC, Deng JX, Sang HK, Choi YP, Yu SH (2012) Distribution and antifungal activity of endophytic fungi in different growth stages of chili pepper (*Capsicum annuum* L.) in Korea. Plant Pathol. J, 28(1): 10-19.

[34] Pimenta RS, Silva JFM, Coelho CM, Morais PB Rosa CA (2010) Integrated control of *Penicillium digitatum* by the predacious yeast *Saccharomycopsis crataegensis* and sodium bicarbonate on oranges. Brazilian J Microbiol 41: 404–410

[35] Rodriguez J, Santos MJ, Copa-Patiño JL, Pérez-Leblic MI (1993) Chitinolytic activity produced by *Penicillium oxalicum* in different culture media. Lett in Appl Microbiol 16(2): 69-71.

[36] Rodriguez J, Copa-Patiño JL, & Pérez-Leblic MI (1995) Purification and properties of a chitinase from *Penicillium oxalicum* autolysates. Lett in Appl Microbiol 20(1): 46-49.

[37] Sabuquillo P, De Cal A, Melgarejo P (2005) Dispersal improvement of a powder formulation of *Penicillium oxalicum*, a biocontrol agent of tomato wilt. Plant Disease 89(12): 1317-1323.

[38] Sempere F, Santamarina MP (2008) Suppression of *Nigrospora oryzae* (Berk. & Broome) Petch by an aggressive mycoparasite and competitor, *Penicillium oxalicum* Currie & Thom. Int J of Food Microbiol 122: 35–43

[39] Sempere F, Santamarina MP (2010) Study of the interactions between *Penicillium oxalicum* Currie & Thom and *Alternaria alternata* (Fr.) Keissler. Brazilian J of Microbiol 41(3): 700-706.

[40] Santamarina MP, Roselló J, Llacer R, Sanchis V (2002) Antagonistic activity of *Penicillium oxalicum* Corrie and Thom, *Penicillium decumbens* Thom and *Trichoderma harzianum* Rifai isolates against fungi, bacteria and insects in vitro. Rev Iberoam Micol 19: 99-103

[41] Saithong P, Panthavee W, Stonsaovapak S, Congfa L (2010) Isolation and primary identification of endophytic fungi from *Cephalotaxus mannii* trees. Maejo Int J Sci Technol.\ 4(03): 446-453

[42] Tan HM, Cao LX, He ZF, Su GJ, Lin B, Zhou SN (2006) Isolation of endophytic actinomycetes from different cultivars of tomato and their activities against *Ralstonia solanacearum*. World J Microbiol Biotechnol 22:1275-1280.

[43] Tenguria RK and Khan FN (2011) Distribution of Endophytic Fungi in Leaves of *Azadirachta indica* A. JUSS. (Neem) of Panchmarhi Biosphere Reserve. Curr Botany 2(2): 27-29

[44] Vega FE, Posada F, Peterson SW, Gianfagna TJ, Chaves F (2006) *Penicillium* species endophytic in coffee plants and ochratoxin A production.Mycologia 98(1):31–42.

[45] Wang FW, Hou ZM, Wang CR, Li P, Shi DH (2008) Bioactive metabolites from *Penicillium* sp., an endophytic fungus residing in *Hopea hainanensis*. World J Microbiol Biotechnol 24(10): 2143-2147.

[46] Xu CL, Zeng R, Ruan CS, Wang X, Xia K, McBride G., ... & Chen S (2010) Study on the control of tobacco black shank by using dry mycelium of *Penicillium chrysogenum*. J of Life Sci 4(1): 1-6.

[47] Yang L, Xie J, Jiang D, Fu Y, Li G and Lin F (2008) Antifungal substances produced by *Penicillium oxalicum* strain PY-1—potential antibiotics against plant pathogenic fungi. World J Microbiol Biotechnol 24:909–915

[48] Zhang, HW, Song YC, Tan RX (2006) Biology and chemistry of endophytes. Nat Prod Rep 23(5): 753-771.

Assessment of potential cancer protection of cosmetic products of agro-food origin by Zeolite Scaffolds

A. Tavolaro[1], S. Catalano[2], P. Tavolaro[3]

[1]Research Institute on Membrane Technology (ITM-CNR), National Research Council of Italy c/o University of Calabria, I-87036 Arcavacata di Rende, ITALY
[2]Department of Physics, University of Calabria, I-87036 Arcavacata di Rende, ITALY
[2]Department of Pharmacy, Health and Nutritional Sciences University of Calabria, I-87036 Arcavacata di Rende, ITALY
*All authors contributed equally to this work.

***Abstract*—** *In this work, three different pure inorganic zeolite membranes and three hybrid PLA-containing Mixed Matrix Membranes (MMMs) were fabricated and compared with each other in virtue of their ability to interact with cells for in vitro test applications. Additionally, we report their performances in cell experiments of novel olive oil-containing cosmetics by using two cell lines with (MCF-10A) epithelial and (MCF-7) epithelial-like cancer characteristics. Our in vitro results revealed that all zeolite scaffolds permit to obtain higher cell densities compared to pure polymeric scaffolds. We also describe that epithelial cells preferentially adhere on pure membranes according to decreasing order: Linde type L>Fe-S-1>Fe-ZSM-5. Moreover, we evidenced an opposite behavior of cancer cells that prefer to adhere and growth on MMM scaffolds. This study shows that the anticancer characteristics of novel cosmetics of natural origin can be easily determined and emphasized using zeolite membranes.*

***Keywords*— Cell culture, Cosmetics, PZC, Scaffolds, Zeolite membranes.**

I. INTRODUCTION

Various studies reported in the literature seem to indicate that olive oil can be directly used on the skin and locally applied in the forms of creams or salves because the topical use of olive oil alone or as an ingredient in cosmetics shows therapeutic effects (anti-inflammatory, anti-neoplastic and anti-aging) [1-3]. The possible carcinogenicity of each component of a novel cosmetic product can represent an alarming characteristic, which frequently represents the biggest barrier to its commercialization. In the past, many tests on cancer induction from novel cosmetics were carried out using animals. Since 2004, in vivo tests are prohibited in the European Union and the commercialization of cosmetics containing component tested on animals was forbidden and a ban extension until March 2013 was agreed [4]. Today it is a pressing need to design novel scaffolds that have high cellular densities, durability, reproducibility and low degradation kinetic in culture media characteristics due to intrinsic properties of materials. Recently, many synthetic zeolites have been shown to be biocompatible materials [5,6] that allow the growth of different types of cells in culture with better performances than commercial polymeric supports [7].

The work we present here describes the use of inorganic and MMMs zeolite membranes as scaffolds for in vitro analyses of olive oil-containing cosmetics. The basis of this approach is that zeolite membranes with their high surface area and their crystalline porous channel system permit the free passage of culture media cations, substances, and water determining higher cell densities with respect to commercial membranes. To the best of our knowledge, this is the first work addressing a reliable and useful application of pure zeolite and mixed matrix membranes as in vitro scaffolds to test novel cosmetic products.

II. MATERIAL AND METHOD

2.1 Chemicals reagents and cell lines

Potassium fluoride (ACS reagent: minimum 99%), tetrapropylammonium bromide (TPABr, purum), fumed silica (99.8%), colloidal silica (Ludox, AS-40, 40% suspension in water), aluminum nitrate nonahydrate (ACS reagent: minimum 98%) were purchased from Sigma Aldrich (USA). Polylactic polymer granules (PLA) were obtained from Cargill-Dow Inc. (USA) with the trade name of Nature Green® 2100D. This highly crystalline type mostly consisted of the L co-monomer containing less 1.47 ± 0.2% of the D co-monomer. Phosphate buffered saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM), Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12), L-glutamine, penicillin/streptomycin was purchased from Eurobio (France), fetal bovine serum (FBS) was purchased from Life Technologies, (Life Technologies,

Paisley, UK), trypsin, sodium orthovanadate, dimethyl sulfoxide (DMSO), MTT (3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltatrazolium bromide), glutaraldehyde solution and osmium tetroxide were obtained from Sigma Aldrich (USA).

MCF-7 cells (human breast adenocarcinoma cells) and MCF-10A cells (human normal breast epithelial cells) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). MCF-7 and MCF-10A cells were authenticated, stored according to the supplier's instructions, and used within a month after frozen aliquots resuscitations. MCF-7 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM, Eurobio, France) supplemented with 10% premium fetal bovine serum (FBS, Life Technologies, Paisley, UK) and 100 U/mL penicillin−streptomycin in a 5% CO2 environment. MCF-10A cells were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12, Eurobio, France) containing 5% horse serum, 100 U/mL penicillin, 100 mg/mL streptomycin, 100 ng/mL cholera toxin, 10 ng/mL epidermal growth factor, 0.5 mg/mL hydrocortisone, 10 mg/mL insulin, and 1% L-glutamine. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2–95% air. After growing to 90% confluence, the cells were washed with Phosphate Buffer Solution (PBS, Eurobio, France) and replaced the culture medium by 1 mL PBS.

2.2 Synthesis procedure of zeolite crystals and Membrane Preparation

2.2.1 Zeolite crystals synthesis and Zeolite Membrane preparation

Fe-S-1 zeolite crystals were prepared by the following fluoride procedure. The molar composition used to obtain MFI zeolite crystals was: SiO_2: 2.4KF: $0.01Fe_2O_3$: $0.4(TPA)_2O$: $33H_2O$. The mixture was heated in stainless steel Teflon-lined autoclaves at 170 °C for 3 days. After reaction time, the crystals obtained were filtered, washed with bidistilled water, and dried overnight at 110 °C. As-synthesized crystals were thermally treated up to 550 °C in nitrogen and in the air to obtain porous crystals. Linde type L crystals synthesis was performed using the following gel composition: $10K_2O:Al_2O_3:0.1Na_2O:20SiO_2:1030H_2O$. The aged gel was transferred to a Teflon-lined stainless steel autoclave. The hydrothermal synthesis was performed at 180 °C for 3 days statically. The autoclave was then cooled down to room temperature. The resulting crystals were filtered with copious amounts of deionized water, before being left to dry at 110 °C overnight. The zeolite membranes used in this work (Table 1) as inorganic scaffolds were prepared according to the patented Tavolaro's method with a diameter equal to 13 mm [8], while MMMs preparation were reported elsewhere [7].

TABLE 1
CHARACTERISTICS OF SYNTHESIZED ZEOLITE CRYSTALS: ATOM RATIOS, PZC AND CRYSTAL SIZES

Molar atomic ratio	Fe-S-1	Fe-ZSM-5	Linde type L
Si/Al	∞	7	5
Si/Fe	50	50	∞
Si/K	0.4	0.4	1
Fe/Al	∞	0.4	∞
PZC	5.02	6.55	9.4
Length a (μm)	11.30	18.43	2.2
Length b (μm)	11.30	6.93	1.7
Length b (μm)	11.30	6.36	1.6

2.3 Cosmetic base cream preparation

The cosmetic cream used like the source is a newly formulated O/W emulsion (base), which was found stable after evaluating for pH, electrical conductivity, centrifugation, phase separation, temperature stability tests (data not shown). It was prepared according to a commercial formulation kindly suggested by SA.TE.CA. S.r.l, a local company that produces skin care products using sulfurous hyperthermal water [9].

2.4 Characterization of Pure Zeolite Membranes and MMMs

The crystalline zeolite frameworks of the materials prepared were identified by powder X-ray diffraction (XRD) patterns on a Philips Model PW 1730/10 generator equipped with a PW 1050/70 vertical goniometer (using Cu Ka radiation). The diffractograms were measured from 5 to 45° using a step size of 0.02 and a scanning speed of 2° min^{-1}. The calcination stage to eliminate the template utilized in the syntheses was performed in a Lindberg/blue STF55346C tube furnace in a static atmosphere. The zeolite morphologies and crystal sizes were determined by a FESEM, FEI – Philips Quanta 200. FTIR-ATR

spectra were directly collected from the membrane surfaces over different points of the sampling area at the same pressure with a micrometer torque (UATR crystal Diamond/ZnSe Spectrum One System by Perkin Elmer Instruments) equipped with an attenuated total reflectance accessory (ATR). This is an infrared technique particularly useful for surface analysis of membranes. For each sample, six scans were signal-averaged at a resolution of 4 cm^{-1}. The analysis was performed on the two different surfaces of each prepared membrane.

2.5 Cell adhesion and Cell Proliferation assays

MCF-10A and MCF-7 cells were cultured with a density of $1x10^5$ cell/mL in no treated 24-well plates on zeolite scaffolds. Cell adhesion was determined, after three hours from seeding, by cell count using a Burker's chamber and Polarized Light Photomicroscope and Field Emission Scanning Electron Microscope analysis. The cosmetic emulsion concentration in DMSO used in treatments was equal to 8 µg/mL. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) at 24 h post-treatment. MTT solution (5 mg/mL, Sigma Aldrich, Milan, Italy) was added to a volume of 1 mL in each well and was incubated for three hours. Then, the solution was removed, and 100 µL of DMSO was added to solubilize the crystals. The wells were then read by spectrophotometer at the wavelength of 570 nm (Olympus Instruments, Japan). The results are representative of at least three independent experiments for each cell line. The percentage of viable cells was calculated according to our previous study.

2.6 Microphotography and Imaging analysis

The morphology of MCF-10A and MCF-7 cells in different culture conditions was studied by Polarized Light Photomicroscope (PLP) and Field Emission Scanning Electron Microscope (FESEM) analyses. Samples of both cell lines were prepared for FESEM by fixation in 2.5% glutaraldehyde, pH 7.4 phosphate buffer, followed by post-fixation in 1% osmium tetroxide and by progressive dehydration in ethanol. For observations and imaging analysis, we used a FESEM, FEI-Philips, Quanta 200 equipped with detector EDX and a Polarized Photomicroscope BX 41-MLED (Olympus Instruments, Japan) equipped with camera SC30 CMOS colors 3MPIXELS and Cell A Image Plus software (Olympus Instruments, Japan). Data were expressed as the mean ± standard deviation (S.D.) of at least three independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA) with the Bonferroni's multiple comparison tests. The level of significance at * p-value < 0.05 was considered statistically significant.

III. RESULTS AND DISCUSSION

Here, we fabricated novel MMMs and pure zeolite membranes substrates for MCF-10A and MCF-7 growth to observe the possible toxic and/or inhibitory responses of epithelial and tumor cell activity after the administration of novel olive oil-containing cosmetics. Table 1 shows Si/Al, Si/Fe, Si/K, and Fe/Al ratios, PZCs and crystalline dimensions for as-made zeolite crystals prepared and used in this work. The powder X-ray diffraction (XRD) patterns of Fe-S-1, Fe-ZSM-5, and Linde type L zeolite crystals synthesized and used in this work to prepare both pure and hybrid membranes are shown in Figure 1 a, b and c, respectively. These figures exhibit the typical diffraction patterns of MFI and LTL type zeolites with high crystalline structures that matching well with the XRD powder patterns both simulated and reported in the literature, and indicate the successful syntheses [10]. FESEM microphotograph of Fe-S-1 crystals and surface pure membrane are shown in Figure 1 (d and e). EDX analyses of the zeolite crystals used to prepare all membranes showed different chemical atom ratios and compositions (Figure 1f and 1g). In particular, it is possible to observe the presence of the peak at about 0.5 KeV, which corresponds to Fe Lα1, and the peak at about 6.4 KeV, which is characteristic of Fe Kα radiations, that evidences the inclusion of iron atoms in the prepared hybrid zeolite scaffold. EDX spectrum of the crystalline as-made Linde type L sample evidences the presence of sodium and potassium atoms (Figure 1g). These images evidence the regular shape and morphology of the zeolite crystals forming the crystalline scaffold. Morphologies of Fe-containing MMM scaffolds can be observed in Figures 2 a-d that illustrate the surfaces and cross- sections of Fe-S-1 and Fe-ZSM-5 membranes. In these figures it is possible to evidence the different shape of zeolite crystals forming the scaffolds; in fact, Fe-S-1 membrane reveals the inclusion of regular spheric-like crystalline clusters, while Fe-ZSM-5 scaffold shows prismatic crystals embedded in the polymeric matrix. Both pure and hybrid zeolite membranes have been screened by Fourier transformed infrared spectroscopy (FTIR) to evidence typical absorption bands of synthetic crystalline zeolite membranes and the interactions between inorganic siloxane groups of crystals and polymeric matrix involved in prepared MMMs.

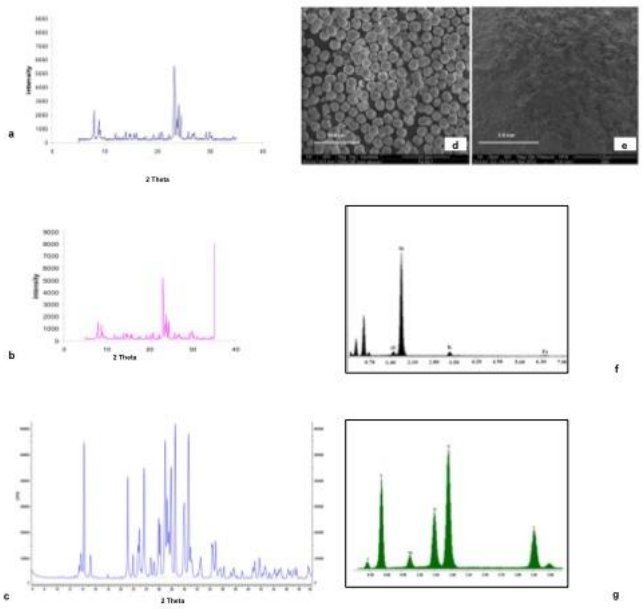


FIGURE 1. X-Ray powder diffraction spectra of zeolite crystals synthesized. a) Fe-S-1 crystals. b) Fe-ZSM-5 crystals. c) Linde type L crystals. FESEM image of Fe-S-1 materials: d) crystals and e) pure scaffold surface. EDX analyses of the zeolite crystals: f) Fe-ZSM-5; g) Linde type L.

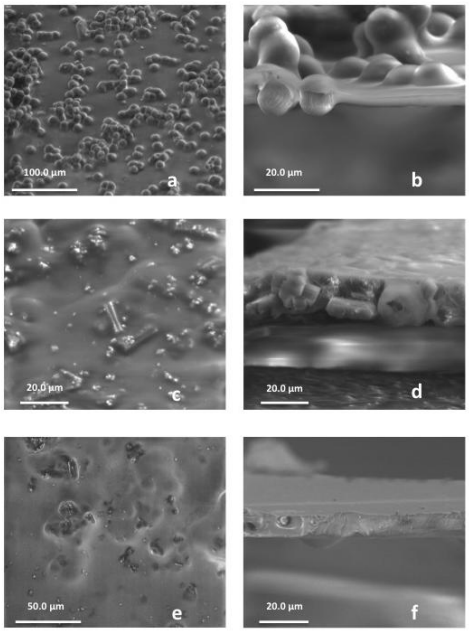


FIGURE 2. Scanning electron micrographs of MMMs fabricated: Fe-S-1 40% surface a) and cross-section b); Fe-ZSM-5 40% surface c) and cross-section d) Linde type L 40% surface e) and cross-section f).

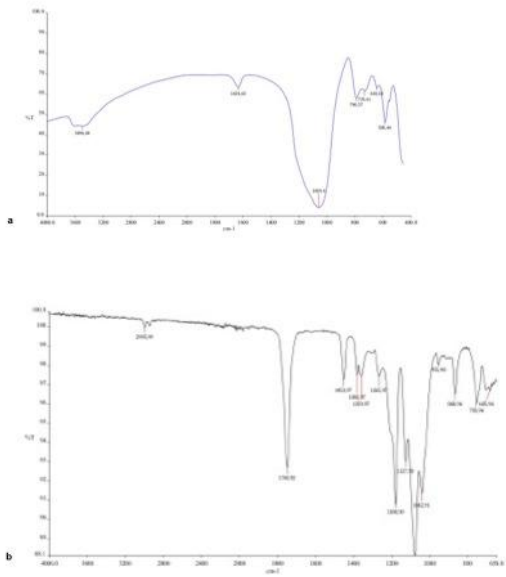


FIGURE 3. a) FTIR spectrum of Linde type L crystals synthesized in this work and used to prepare Pure and Mixed Matrix zeolite membranes. b) Infrared analysis in ATR mode collected on the neat PLA membrane

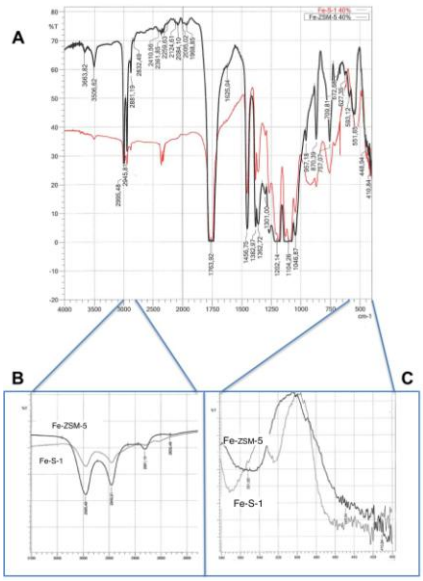


FIGURE 4. FTIR spectra of Fe-S-1 40% and Fe-ZSM-5 40% (A) MMMs; enlargement and comparison of vibrational bands of Fe-S-1 and Fe-ZSM-5 40% MMMs in the range from 3100 cm $^{-1}$ to 2800 cm^{-1} (B) and in the range from 600 cm^{-1} to 400 cm^{-1} (C)

Figures 3, and 4 reveal the vibrational spectra of Linde type L, Fe-S-1, and Fe-ZSM-5 crystals. The FTIR spectra of Fe-containing crystalline zeolite membranes show the same typical absorbance shapes as the MFI zeolite structures. In fact, in these spectra all the vibrational bands attributed to lattice modes appear with two additive shoulders centered at about 960 cm^{-1} and 1010 cm^{-1}, respectively. These two low bands were attributed in the literature to defective stretching of the silanol groups due to the polarization of the Fe••O-Si bonds caused to the presence of iron atoms in the zeolite frameworks [11]. According to the literature data, it is possible to observe that the bands, centered at about 750 cm^{-1} and 550 cm^{-1}, shift to

lower wavenumbers in the presence of the aluminum atom inclusion in the zeolite framework and the resulting decrease in the Si/Al ratio. The FTIR spectrum of Linde type L closely agrees to the literature [12]. The infrared spectrum of pure PLA membrane (Figure 3 b) in the region of 3000-1600 cm^{-1} is characterized by adsorption bands at 2996 cm^{-1}, 2947 cm^{-1} and 2877 cm^{-1} arising from the C-H stretching vibration of methyl (CH_3) groups in the side chains ($\nu_{as}CH_3$, ν_sCH_3, νCH modes) as well as a strong band related to the stretching vibration of carbonyl (C=O) groups at 1749 cm^{-1} [13]. As regards the MMMs, the infrared analysis reveals the formation of homogeneous zeolite-containing films and provides the typical bands both zeolite crystals and PLA with new modified bands, which evidence their mutual interactions. In fact, all spectra of hybrid membranes show a very similar spectroscopic behavior with the presence of both pure PLA and zeolite strong absorption bands modified. Figure 4A shows the infrared spectra of Fe-S-1 40% and Fe-ZSM-5 40% MMMs, while Figure 4B and 4C reveals the comparisons in two vibrational ranges. Infrared analyses of Fe-S-1 and Fe-ZSM-5 crystalline MMMs reveal the characteristic spectra between 1500 cm^{-1} and 400 cm^{-1} with bands of absorption centered at 1215 cm^{-1}, 1087 cm^{-1}, 800 cm^{-1} and 450 cm^{-1}, corresponding to the Si-O-Si asymmetric stretching, symmetric stretching, and symmetric bending, respectively [14]. Figure 4 reveals the enlargements of the region comprises between 3000 and 2800 cm^{-1} (B) and from 580 up to 400 cm^{-1} (C) and evidences the shift of the vibrational bands for Fe-ZSM-5 40% with respect to Fe-S-1 40% MMMs. In particular, the intense band centered at 550 cm^{-1} is characteristic of the pentasil zeolites and it is assigned to the vibration of double 5-rings in MFI lattice; no such a band appears in the spectrum of amorphous silica material then it evidences the crystallization of structures prepared [15]. All zeolite membranes fabricated and characterized were used for cell cultures. As we have already pointed out in previous works, the synthetic zeolitic scaffolds are non-cytotoxic and stable in the cell culture medium promoting a better adhesion with respect to the polymer scaffolds. Here too, our experiments have confirmed that cells react selectively with a specific zeolitic structure. In particular, MCF-10A cells preferentially adhere and grow on the pure zeolitic membrane of Linde type L (results not shown). Figure 5 shows the histograms related to the analyses of MCF-10A (A) and MCF-7 (B) cells grown on scaffolds prepared in this work in the absence and after 24 h of treatment with the cosmetic emulsion. Data obtained reveal that they favor better viability than the polystyrene (PS) control scaffold. Moreover, inorganic zeolite membranes show better viability with respect to MMMs for both cell lines. In particular, cancer cells grow better than normal cells, but after the cosmetic administration, the two cell lines show a different behavior. In fact, while MCF-10A undergoes increased cell viability, MCF-7 shows a decrease mainly on membranes. This behavior can be due to the effect of the cosmetic on the decreasing of oxidative stress in non-tumor epithelial cells and on the improving the physiological conditions, while the decrease in vitality in carcinogenic cells after administration may be caused by an increase in cosmetic-induced apoptosis.

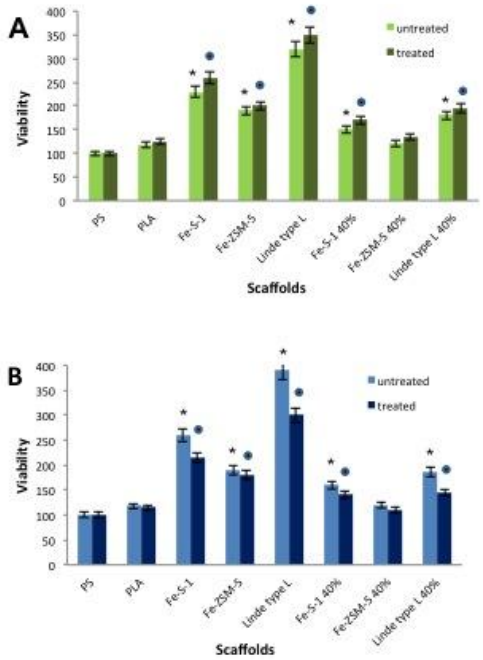


FIGURE 5. Cell viability determined by MTT test on MCF-10A (A) and MCF-7 (B) treated for 24 h with cosmetic (8 μg/mL). Columns are the mean of three independent experiments each in triplicate; bars ± S.D. (n = 4); *p < 0.05 untreated cells vs PS; ●p < 0.05 treated cells vs PS.

IV. CONCLUSION

The results reported in this article highlight the peculiarities of zeolitic scaffolds prepared as excellent cellular supports for the analysis and control of the cytotoxicological characteristics of cosmetics. A comparison between zeolite crystals-containing hybrid and pure membranes with the same framework, crystal pore and dimensions reveals that the density of cells adhered and grown on supports was always greater for inorganic scaffolds with respect to the polymeric surfaces suggesting that siloxane groups act as binding sites for the cellular membrane.

Our experimental data demonstrate that all cell adhesions are membrane-specific and, in particular, that MCF-10A cells preferentially interact with pure zeolitic membranes. Furthermore, the viability data obtained reveal that pure inorganic supports promote greater cell growth both in respect to the polystyrene control scaffold (PS) and to the MMMs membranes, for two cell lines. After administration of the solid olive oil-containing cosmetic, the normal cells show a better viability, but the tumor cells undergo an evident decrease.

In conclusion, it is evident that due to their chemical-physical peculiarities, simplicity of preparation, biocompatibility and chemical stability in both physiological and conditioned culture media, the prepared zeolitic scaffolds are excellent supports to easily determine the anticancer characteristics of novel cosmetics of natural origin.

ACKNOWLEDGEMENTS

This work was supported by "PON R&C (Programma Operativo Nazionale Ricerca e Competitività 2007-2013) project PON01_01 00293 Spread Bio-Oil". The authors would like to thank Prof. G. Martino for help with cell cultures preparation, Prof. De Cindio for its advice and Dr. S. Mazzulla for the cosmetic sample preparation.

REFERENCES

[1] T.-K. Lin, L. Zhong, J. Santiago, Int. J. Mol. Sci. 19 (2018) 70-91

[2] A. Ribeiro, M. Estanqueiro, M. Oliveira, J. Sousa Lobo, Cosmetics 2 (2015) 48-65.

[3] A. Budiyanto, N.U. Ahmed, A. Wu, T. Bito, O. Nikaido, T. Osawa, M. Ueda, M. Ichihashi, Carcinogenesis 21 (2000) 2085-2090.

[4] European Commission 2003. Directive 2003/15/EC of the European parliament and of the council. Off. J. Europ. Union 46, 26-35. http://refhub.elsevier.com/S0378-5173(17)30023-6/sbref0040.

[5] D.G. Seifu, T.T. Isimjan, K. Mequanint, Acta Biomater. 7 (2011) 3670-3678.

[6] I. Jirka, M. Vandrovcova, J. Plsek, M. Bousa, L. Bacakova, Mater. Lett. 190 (2017) 229-231.

[7] P. Tavolaro, G. Martino, S. Andò, A. Tavolaro, Mater. Sci. Eng., C 69 (2016) 894-904.

[8] A. Tavolaro, G. Martino, P. Tavolaro, Eu. Pat., PCT/EP2011/051915, 1095553 2011.

[9] F.R. Lupi, L. Gentile, D. Gabriele, S. Mazzulla, N. Baldino, B. De Cindio, J. Colloid Interface Sci. 459 (2015) 70-78.

[10] H. Robson, in Verified synthesis of zeolitic materials (second revised edition), Elsevier, Netherlands, 2001.

[11] D. Scarano, A. Zecchina, S. Bordiga, F. Geobaldo, G. Spoto, G. Petrini, G. Leofanti, M. Padovan, G. Tozzola, J. Chem. Soc., Faraday Trans. 89 (1993) 4123-4130.

[12] N.H. Phu, T.T.K. Hoa, N.V. Tan, H.V. Thang, P.L. Ha, Appl. Catal., B 34 (2001) 267-275.

[13] R. Auras, B. Harte, Macromol. Biosci. 4 (2004) 835-864.

[14] J. Qi, T. Zhao, X. Xu, F. Li, G. Sun, J. Porous Mater. 18 (2011) 509-515.

[15] M.A. Uguina, D.P. Serrano, G. Ovejero, R. van Grieken, M. Camacho, Zeolites 18 (1997) 368-378.

AD Publications
Sector-3, MP Nagar, Rajasthan, India
www.adpublications.org, www.ijoear.com, info@ijoear.com