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Preface

We would like to present, with great pleasure, the inaugural volume-7, Issue-12, December 2021, of a scholarly journal, *International Journal of Environmental & Agriculture Research*. This journal is part of the AD Publications series *in the field of Environmental & Agriculture Research Development*, and is devoted to the gamut of Environmental & Agriculture issues, from theoretical aspects to application-dependent studies and the validation of emerging technologies.

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

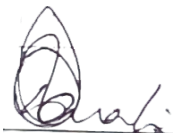
Environmental Research:

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

Agriculture Research:

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

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



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Animal Science	Agricultural Economics
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Functional and Pasting Properties of Composite Flour from Wheat, Sweet Potato and Soybean

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Abstract**—** *This work was done to ascertain the functional and pasting properties of wheat-soy-sweet potato composite flour. Wheat flour, sweet potato flour and soybean flour blends were prepared in different proportions with completely randomized design resulting to 7 samples including control which is 100% wheat flour. Functional and pasting properties of the composite flours were determined and compared with wheat flour as control. The bulk densities of the flour blends ranged from 0.717 to 0.809 g/mL, there was a significant difference in the water absorption capacity of the flour blends. Oil absorption capacity (OAC) ranged between 0.623 to 0.759 mL/g, least gelation concentration (LGC) of the flour samples ranged from 2.000% to 12.000% while the swelling capacity of different flour blends at temperature 50^{o}C ranged from 1.32% to 2.006%. Peak viscosity, sample WSO (90% wheat flour: 10% soy bean flour: 0% sweet potato flour) had the highest peak viscosity of 273.075 RVU, sample WOS (90% wheat flour: 0% soy bean flour: 10% sweet potato flour) had the highest trough viscosity (245.915 RVU) while sample WSS (80% wheat flour: 10% soy bean flour: 10% sweet potato flour) had the highest breakdown viscosity value (94.245 RVU) compared to the control sample WOO which had 100% wheat flour. This investigation shows that set back value was highest for sample WSS (80% wheat flour +10% soy bean flour + 10 % sweet potato flour) at 220.325 RVU. Sample SSO (0% Wheat flour: 50% Soybean: 50% Sweet potato flour) had highest pasting temperature of 93.450^{o}C compared to control flour samples.*

Keywords**— functional properties, pasting properties, composite flour, wheat flour, soy flour, sweet potato flour.**

I. INTRODUCTION

Wheat (*Triticum* spp.) is the most popular cereal grain that is consumed worldwide. It is the most important stable food crop for more than one third of the world population and contributes more calories and proteins to the world diet than any other cereal crops (Shewry *et al.*, 2009). Wheat is the leading crop due to the elastic property of gluten which is essentially used for the production of numerous baked products such as bread, biscuits, cookies, doughnuts and cakes, of which bread is the most common among them (Dewettinck *et al*., 2008). Many people like wheat-based products because of the taste, and particularly the texture. Wheat is unique among cereals because its flour possesses the ability to form a visco-elastic dough when mixed with water

Sweet potato (*Ipomea batatas*), is a root crop that is grown in developing countries especially the tropics and subtropics. It is high yielding, but bulky and perishable because of its moisture content. However, because of its high dry matter yield, it could be an attractive source of flour in many places if efficient, economical methods of drying could be found. Sweet potato is high in carbohydrates, vitamins (A and C), calories, minerals and precursor of vitamin A (Antonio *et al.*, 2011). According to Akoroda (2009), it provides food security and farmers in Africa produce about 7 million tons of sweetpotato each year of which the majority are lost due to improper postharvest handling.

Soybean is a leguminous vegetable of the pea family that grows in tropical, subtropical, and temperate climates. It was domesticated in the 11th century BC around northeast of China. It is believed to have been introduced to Africa in the 19th century by Chinese traders along the east coast of Africa (IITA, 2015). The eastern half of North China in the eleventh century B.C. has been traced to the first domestication of soybean. Islam *et al.* (2007) reported that soybean is one of the most important oil and protein crops of the world of which Serrem *et al.* (2011) also stated that it contains 30 to 45% protein with a good source of all indispensable amino acids. The protein content of soybean is about 2 times of other pulses, 4 times

of wheat, 6 times of rice grain, 4 times of egg and 12 times of milk. Soybean has 3% lecithin, which is helpful for brain development. It is also rich in calcium, phosphorous and Vitamins A, B, C and D. It has been referred to as "the protein hope of the future" (Islam *et al.*, 2007).

The growing concern about a healthier life style and healthy foods has necessitated the food industries to utilize indigenous food to create new products. In essence, various processing technologies have helped in transforming food ingredients into healthier products with maximum nutritional value to ensure nutrient security of the population in developing countries (kumar, 2010). Hasmadi *et al.* (2014) stated that composite flour is considered advantageous in developing countries as it reduces the importation of wheat flour and encourages the use of locally grown crops as flour.

Functional properties are the fundamental physico-chemical properties that reflect the complex interaction between the composition, structure, molecular conformation and physico-chemical properties of food components together with the nature of environment in which these are associated and measured (Siddiq *et al.*, 2009). Functional characteristics are required to evaluate and possibly help to predict how new proteins, fat, fibre and carbohydrates may behave in specific systems as well as demonstrate whether or not such protein can be used to stimulate or replace conventional protein (Siddiq *et al.*, 2009).

The pasting property is one of the important starch physicochemical properties and is affected by multiple factors (Bemiller, 2011). According to Srichwong *et al.* (2005) starch pasting properties are highly influenced by the composition proportion and structure of starch (e.g. total starch as well as amylose and amylopectin content, the ratio of amylose and amylopectin, the proportion of starch granules with distinct size, distribution of chain length).

This work is aimed at studying the functional and pasting properties of composite flours of wheat, sweet potato and soybean.

II. MATERIALS AND METHODS

The sweet potato tubers, soybean grains and wheat flour that are used for this work were bought from Eke-Awka Market in Anambra state, Nigeria. Equipment that were used include flour milling machine, mechanical sieve, oven, desiccators, centrifuge, weighing balance, Rapid Visco Analyzer (RVA) , stirrer, knife, water, pipettes crucibles, bowls and napkin.

2.1 Production of Flours

2.1.1 Production of Sweet Potato Flour

The method described by Adeleke and Odedeji (2010) was used. Sweet potato tubers were thoroughly sorted to remove bad ones, washed to remove adhering soil, dirts and extraneous materials and thereafter peeled and sliced to 2 mm thickness. The sliced tubers were blanched in water at temperature of 60^{o}C for 2 min to inactivate enzymes that may catalyze browning reaction, and drained followed by drying. Following drying, the sliced tubers were milled, sieved with a mesh of 250 µm into fine flour and packaged for use.

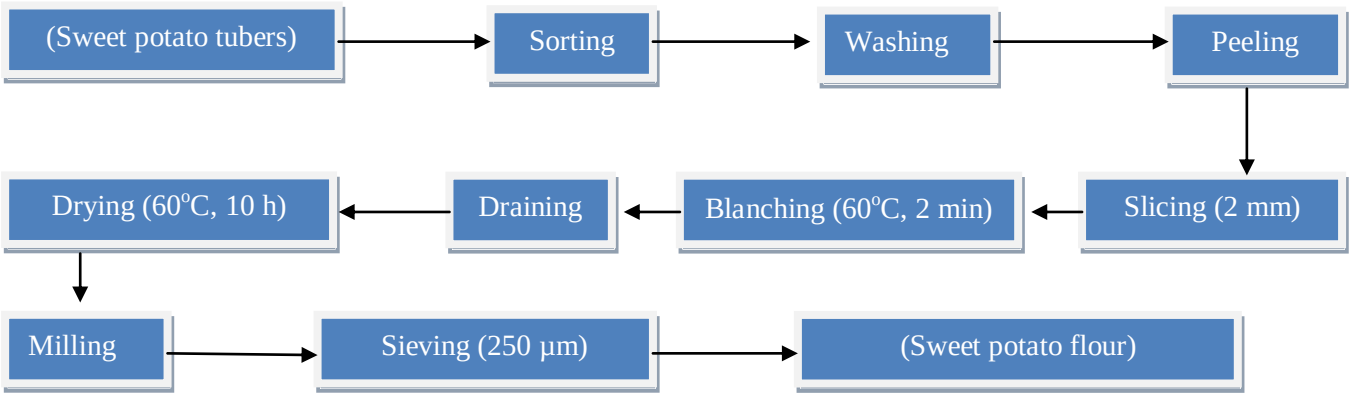

FIGURE 1: flow chart for the production of sweet potato flour

2.1.2 Production of Soybean Flour

The flour was prepared using the method of Bonsi *et al.* (2014) shown in Figure 2. Stones, damaged seeds and other foreign particles were removed from the seeds. The seeds were soaked in portable water for 1 h and the water decanted. The seeds were then boiled in water for 20 min and the water decanted. The seeds were allowed to cool for 10 min and manual

decortications by hand. The hulls were separated from the seed by floatation in water. The dehulled seeds were dried in an oven at 60°C to less than 10 % moisture content, milled and sieved with a 250 µm.

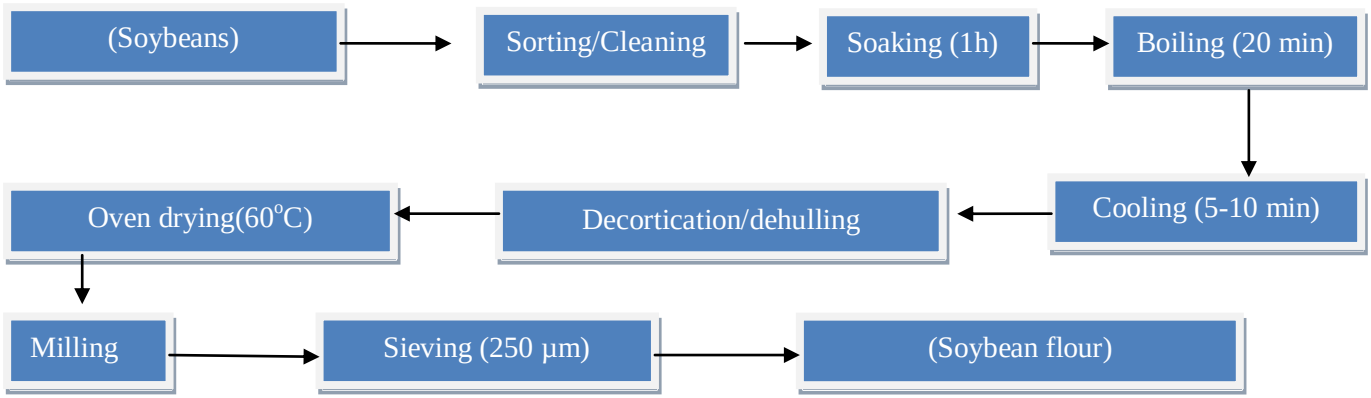

FIGURE 2: Flow chart for the production of soybean flour

TABLE 1
THE FORMULATIONS OF THE FLOUR BLENDS

Code	Wheat (g)	Soybean (g)	Sweet Potato (g)
WOO	100	-	-
WSO	90	10	-
WOS	90	-	10
WSS	80	10	10
$WSS_1$70	10	10	20
$WSS_2$70	20	20	10
SSO	-	50	50

III. METHODS OF ANALYSIS

3.1 Functional Properties

3.1.1 Water Absorption Capacity

The method described by Majzoobi and Abedi (2014) was employed in the determination of the water absorption capacity of the flour samples. One gram of the flour was mixed with 10 ml of water in a centrifuge tube and allowed to stand at room temperature (30 ± 2°C) for 1 h. It was then centrifuged at 5000 rpm for 30 min. The volume of free water was read from the calibrated centrifuge tube. Water absorption capacity was calculated as ml of water absorbed per gram of flour (i.e. the difference in volume of the initial amount of water added to that decanted after centrifugation).

3.1.2 Oil Absorption Capacity

This was determined by the method of Nwosu *et al.* (2010). Exactly 1 g of the sample was measured and 10 mL refined corn oil was measured into a dry, clean centrifuge tube and both weight was noted. Then 10 mL of refined corn oil was poured into the tube and properly mixed with the flour. The suspension was then centrifuged at a speed of 3500 rpm for 15 min. The supernatant thereafter was discarded and the tube content re-weighed. The gain in mass was recorded as the oil absorption capacity of the sample.

3.1.3 Least Gelation Concentration

Least gelation concentration was determined by the method described by Adeleke and Odedeji (2010). Test tubes containing suspensions of 2%, 4%, 6%, 8%,10%, 12%, 14%, 16%, 18%, 20% (w/v) of flours in 5 mL distilled water was heated for 1 h in boiling water bath. This was followed by rapid cooling under cold running tap water. The least gelation concentration (LGC) was taken as that concentration at which the sample in the inverted test tube did not fall down or slip.

3.1.4 Bulk Density

The method described by Heny *et al*. (2015) was used in the determination of bulk density. A 50 g flour sample was put into a 100 mL measuring cylinder. The cylinder was tapped continuously until a constant volume is obtained. The bulk density (g/cm^3) was calculated as weight of flour (g) divided by flour volume (cm^3).

3.1.5 Swelling Power

The swelling powers of the samples was determined with the method described by Heny *et al.* (2015) with slight modification. 1 g of flour sample was mixed with 10 mL of distilled water in a centrifuge tube and heated at 80°C for 30 min in a water bath with continuous shaking during the heating period. After heating, the suspension was centrifuged at 1000 × g for 15 min. The supernatant was decanted and the weight of the paste taken. This was also done at temperatures 50°C, 60°C, 70°C and 90°C.

$$SP = \frac{\text{Weight of paste}}{\text{Weight of dry flour}}$$

3.2 Determination of Pasting Properties

3.2.1 Determination of Pasting Properties

This was determined according to the method described by Efuribe *et al.* (2018) with a Rapid Visco Analyzer (Tecmaster Perten N103802 Australia). Exactly 3.5 g of the samples was weighed into the test canister. Then 25 mL of distilled water was dispensed into the canister containing the sample. The solution was thoroughly mixed and the canister well fitted into the RVA as recommended. The slurry was heated from 50 to 95°C with a holding time of 2 min followed by cooling to 50°C with 2 min holding time. The rate of heating and cooling was at constant rate of 11.25°C/min. Peak, trough, breakdown, final, and setback viscosities, peak time and pasting temperature were read from the pasting profile with the aid of Thermocline for Windows Software connected to a computer.

3.3 Statistical Analysis

The data were subjected to analysis of variance (ANOVA) using Statistical Packaging for Social Science(SPSS) version 20.0 software 2011 to test the level of significance ($p<0.05$). Duncan New Multiple Range Test was used to separate the means where different.

IV. RESULTS AND DISCUSSION

4.1 Functional properties of the flour blends

The functional properties of wheat, soybean and sweet potato composite flours are presented in Table 1. The bulk densities of the flour blends ranged from 0.717 to 0.809 g/mL. From the table, bulk densities of composite flour increased with increase in the level of substitution of wheat flour with sweet potato flour. Sample SSO (0% Wheat flour: 50% Soybean flour: 50% Sweet potato flour) has the lowest bulk density. Formation of an open-bed structure supported by inter-particle forces might have resulted in decreased bulk density of the flours with increased moisture (Fitzpatrick *et al.*, 2004). This decrease occurred mainly because of the increased volume of flours rather than an increase in mass. However, the observed low bulk density of sample SSO (0% Wheat flour: 50%Soybean flour: 50% Sweet potato flour) suggests it's suitability in the formulation of food for babies where high nutrient density to low bulk is desired though such products will not offer packaging advantage compared to wheat flour.

Water absorption capacities (WAC) of the blends increased progressively as the level of sweet potato flour and soy bean increased. However, there was a significant difference ($p\leq0.05$). The increase in WAC of the flour blends may be due to increase in the amylose leaching and solubility and loss of starch crystalline structure (Suresh *et al.*, 2015). Similarly, protein has both hydrophilic and hydrophobic groups and therefore they can interact with water in foods. Thus, the observed variation in different flour blends may be due to different protein concentration, their degree of interaction with water and conformational characteristics (Butt and Batool, 2010).

Water absorption capacity is important with regards to the consistency of product as well as in baking applications. High WAC of composite flours suggests that the flours can be used in formulation of some foods such as sausage, dough and bakery products.

Oil absorption capacity (OAC) ranged between 0.623 to 0.759 g/g among all the flours. The flour sample WOS (90% Wheat flour: 0% Soybean flour: 10% Sweet potato flour) had higher OAC values as compared to sample WOO (100% wheat flour).

Similarly, oil absorption capacity values increased with increased level of sweet potato flour in the mixture. The possible reason for increase in the OAC of composite flours could be variations in the presence of non-polar amino acid side chains of protein which might bind the hydrocarbon side chain of the oil among the flours as reported by Jitngarmkusol *et al*. (2008). This is an indication that the blends would be useful in structural interaction in food especially in flavor retention, improvement of palatability and extension of shelf life particularly in bakery or meat products where oil absorption property is of prime importance. The presence of high-fat content in flours might have affected the oil absorption capacity (OAC) of the composite flours adversely (Chandra *et al*., 2014).

Least gelation concentration (LGC) of the flour samples ranged from 2.000% to 12.000%. The least gelation is defined as the lowest protein concentration at which gel remained in inverted tube. There were no significant difference between the samples . Sample WSO (90% wheat flour: 10% Soybean flour: 0% Sweet potato flour) which has 10% incorporation of soy bean flour has the least level of LGC. Sample WOO (100% wheat flour) and sample WOS (90% wheat flour: 0% Soybean flour: 10% sweet potato flour) formed gel at a significantly low concentration (4.000%) while samples WSS, WSS_1 and WSS_2 formed gel at very high concentration (6.000, 6.000 and 8.000%) respectively. However, sample SSO (50% soybean flour: 50% sweet potato flour) required a significantly higher concentration (12.000%) for gel formation. This is an indication that gelling capacity of wheat flour reduces as the level of other flours increases. The variation in the gelling properties may be ascribed to ratios of the different constituents such as protein, carbohydrates and lipids in different flours, suggesting that interaction between such components may also have a significant role in functional properties (Aremu *et al*., 2007). The lower the LGC, the better the gelling ability of protein ingredient in food formulations. Meanwhile, the observed low gelation concentration of sample WSO (90% wheat flour: 10% Soybean flour: 0% sweet potato flour) as composite flour may be an asset for the formation of curd or as an additive to other gel forming materials in food products.

The swelling capacity of different flour blends at temperature 50^{o}c ranged from 1.32% to 2.006%. It is worthy to note that swelling capacity is an evidence of non-covalent bonding between molecules within starch granules and also a factor of the ratio of α-amylose and amylopectin ratios. However, the swelling capacity of flours depends on size of particles, types of variety and types of processing methods or unit operations (Suresh *et al*., 2015).

At 60% the values ranged from 2.213 to 3.617, there was decrease in swelling capacity as the level of incoporation increase. At 70% the values ranged from 3.698 to 5.488 levels ranged from 4.327 to 5.537, there was much increase in samples WSS (80% Wheat flour: 10 % Soybean flour: 10% Sweet potato flour), WSS_1 (70% Wheat flour: 10% Soybean flour: 20% Sweet potato flour) , WSS_2 (70% Wheat flour: 20% Soybean flour: 10% Sweet potato flour) and SSO (0% Wheat flour:50% soybean flour: 50% sweet potato flour) as compared to the control sample WOO (100% Wheat Flour: 0% Soybean flour: 0% Sweet potato flour).At 90% ,the values ranged from 4.916 to 5.637 with sample WSO (90% wheat flour: 10% Soybean flour: 0% sweet potato flour) being the highest.

TABLE 2
FUNCTIONAL PROPERTIES OF THE FLOUR FORMULATIONS

Samples	Bulk Density (g/cm^3)	Water Absorption Capacity (mL/g)	Oil Absorption Capacity (g/g)	LGC (%)	Swelling Power @ 50^{o}C (g/g)	Swelling Power @ 60^{o}C (g/g)	Swelling Power @ 70^{o}C (g/g)	Swelling Power @ 80^{o}C (g/g)	Swelling Power @ 90^{o}C (g/g)
WOO	0.763^{ab} ± 0.005	0.594^{a} ± 0.515	0.694^{bc} ± 0.039	4.000^{a} ± 0.000	1.851^{f} ± 0.014	3.617^{f} ± 0.084	4.861^{a} ± 0.046	4.869^{c} ± 0.077	5.146^{b} ± 0.111
WSO	0.772^{ab}± 0.007	0.935^{a} ± 0.005	0.623^{b} ± 0.032	2.000^{a} ± 0.000	1.667^{d} ± 0.027	3.470^{e} ± 0.010	4.493^{a} ± 0.110	4.327^{a} ± 0.281	5.637^{c} ± 0.031
WOS	0.809^{c} ± 0.002	0.960^{a} ± 0.040	0.759^{c} ± 0.013	4.000^{a}± 0.000	1.621^{c} ± 0.013	2.867^{c} ± 0.062	3.903^{a} ± 0.042	4.617^{b} ± 0.016	5.204^{b} ± 0.084
WSS	0.782^{b} ± 0.021	0.9533^{a} ± 0.040	0.710^{c} ± 0.050	6.000^{a} ± 0.000	1.320^{a} ± 0.016	3.251^{d} ± 0.028	3.713^{a} ± 0.060	5.404^{e} ± 0.160	5.470^{c} ± 0.044
WSS_1	0.783^{b} ± 0.017	0.950^{a} ± 0.020	0.747^{c} ± 0.012	6.000^{a} ± 0.000	1.741^{e} ± 0.037	3.230^{d} ± 0.046	3.698^{a} ± 0.003	5.308^{de} ± 0.017	5.469 ± 0.017^{c}
WSS_2	0.752^{a} ± 0.023	0.920^{a} ± 0.020	0.640^{ab} ± 0.035	8.000^{a} ± 0.000	1.435^{b} ± 0.013	2.716^{b} ± 0.073	4.488^{a} ± 0.029	5.142^{d} ± 0.098	4.916^{a} ± 0.070
SSO	0.71^{7d} ± 0.011	1.648^{b} ± 0.143	0.726^{c} ± 0.049	12.000^{a}± 3.138	2.006^{g} ± 0.021	2.213^{a} ± 0.037	5.488^{a} ± 2.884	5.537^{e} ± 0.023	5.447^{c} ± 0.237

Values are Mean ± Standard. Means with the same superscripts (alphabets) along each column are not significantly different whereas different superscript are significantly different (P≤0.05).

WOO = 100% Wheat Flour: 0% Soybean flour: 0% Sweet potato flour

WSO = 90% Wheat flour: 10% Soybean flour: 0% Sweet potato flour

WOS = 90% Wheat flour: 0% Soybean flour: 10% Sweet potato flour

WSS = 80% Wheat flour: 10% Soybean flour: 10% Sweet potato flour

WSS_1 = 70% Wheat flour: 10% Soybean flour: 20% Sweet potato flour

WSS_2 = 70% Wheat flour: 20% Soybean flour: 10% Sweet potato flour

SSO = 0% Wheat flour: 50% Soybean flour: 50% Sweet potato flour

4.2 The pasting properties of the flour blends

The pasting properties of the flour blends is shown in Table 2. The peak viscosity ranged from 49.255 to 273.8075 RVU which had sample SSO (0% Wheat flour: 50% Soybean flour: 50% Sweet potato flour) with the lowest value while sample WSO (90% wheat flour: 10% soy bean flour: 0% sweet potato flour) had the highest peak viscosity. High peak viscosity is an index of high starch content. This explains why sample WOO (100% wheat flour) and sample WSO (90% wheat flour: 10% soy bean flour: 0% sweet potato flour) had the highest value indicating high starch content as compared to the other blends. However, incorporation of soybean bean flour and sweet potato flours at different percentages significantly from samples WOS (90% Wheat flour: 0% Soybean flour: 10% Sweet potato flour) to SSO (0% Wheat flour: 50% Soybean flour: 50% Sweet potato flour) decreased the peak viscosity. The relatively low peak is the ability of starch to swell freely before physical breakdown (Sanni *et al*., 2004). The decrease in viscosity could be due to the high fat content. This result is in agreement with Eke *et al*. (2018) on functional and pasting properties of Acha, deffated soybean and groundnut flour blends.

The table revealed that the trough viscosity ranged from sample 5.415 to 245.915 RVU where sample SSO (0% Wheat flour: 50% Soybean flour: 50% Sweet potato flour) had the lowest while sample WOS (90% Wheat flour: 0% Soybean flour: 10% Sweet potato flour) had the highest. Trough viscosity also known as hold period is the point at which viscosity reaches its minimum during either heating or cooling process. The result indicated a decrease in value with an increase in substitution of soybean and sweet potato flour. The values obtained were higher than values (39.60-59.19 RVU) reported for wheat and walnut by ofia-olua (2014) but they are in agreement with Kiin-kabari (2015) on functional and pasting properties of wheat and plantain flours enriched with bambara groundnut protein concentrate.

Breakdown viscosity ranged from 8.245 to 94.245 RVU of which sample WOS (90% Wheat flour: 0% Soybean flour: 10% Sweet potato flour) had the lowest while sample WSS (80% Wheat flour: 10% Soybean flour: 10% Sweet potato flour) had the highest value. The higher the breakdown viscosity, the lower the ability of starch in the flour samples to withstand heating and shear stress while lower breakdown value indicates that the starch in question possess cross-linking properties (Chinma *et al*., 2010). The table showed that there was significant difference (p≥0.05) in the flour blends and this result is in agreement with (Eke *et al*., 2018)

Final viscosity ranged from 118.415 to 377.165 RVU where sample SSO (0% Wheat flour: 50% Soybean flour: 50% Sweet potato flour) had the lowest and WOS (90% wheat flour: 0% Soybean flour: 10% sweet potato flour) had the highest. There were no stability in decrease and increase of values as substitution of soybean and sweetpotato flours increased. There were significant difference (p≥ 0.05) among the samples. The final viscosity is the most commonly used parameters to determine a particular starch-based sample quality. It gives an idea of the ability of a material to gel after cooking. Final viscosities are important in determining the ability of flour to form gel during processing (Liang and King, 2003). The values obtained were above the range reported by Ofia-olua (2014) for wheat and walnut blends (95.51-252) RVU.

Set back viscosity ranged from 69.165 to 220.325 RVU having sample SSO (0% Wheat flour: 50% soybean flour: 50% sweet potato flour) obtaining the least value while WSS (80% wheat flour: 10% soy bean flour: 10 % sweet potato flour) obtained the highest value. Adebowale *et al*. (2005) reported that high set back value is an indication of the propensity of starch molecules to disperse in hot paste and re-associate readily during cooling. Setback viscosity values are reported to correlate with ability of starches to gel into semi solid pastes. This result is slightly higher than that reported by Eke *et al*. (2018) this could be due to the individual flours used.

Peak time ranged from 4.865 to 6.55 min where sample WSS (80% Wheat flour: 10% Soybean flour: 10% Sweet potato flour) obtained the highest value while sample WOS (90% Wheat flour: 0% Soybean flour: 10% Sweet potato flour) obtained the lowest value. The pasting temperature of the flour blends ranged from 80.125 to 93.450 ^{o}C. Sample WOS (90% Wheat flour: 0% Soybean flour: 10% Sweet potato flour) obtained the lowest value while sample SSO (0% Wheat flour: 50% Soybean flour: 50% Sweet potato flour) obtained the highest. This result indicates an increase in peak time and pasting temperature as substitution of soybean and sweet potato flour increased except for sample WOS which obviously decreased. Peak time is a measure of the cooking time while pasting temperature is the temperature at which viscosity starts to raise (Eke *et al*., 2018). Since pasting temperature is a measure of the minimum temperature required to cook a given food sample, flour blends with higher pasting temperature may not be recommended for certain product due to high cost of energy. The values obtained from this study are in agreement with (Eke *et al*., 2018).

TABLE 3
PASTING PROPERTIES AND FALLING NUMBER OF THE FLOUR FORMULATIONS

Samples	Peak 1	Trough 1	Breakdown	Final Visc	Setback	Peak Time	Pasting Temp
WOO	$268.165^{a} \pm 0.007$	$205.825^{a} \pm 0.007$	$62.325^{a} \pm 0.007$	$338.915^{a} \pm 0.007$	$133.075^{a} \pm 0.007$	$5.065^{a} \pm 0.007$	$81.415^{a} \pm 0.007$
WSO	$273.075^{b} \pm 0.007$	$228.245^{b} \pm 0.007$	$44.825^{b} \pm 0.007$	$341.495^{b} \pm 0.007$	$113.245^{b} \pm 0.007$	$5.205^{b} \pm 0.007$	$80.655^{b} \pm 0.007$
WOS	$254.165^{c} \pm 0.007$	$245.915^{c} \pm 0.007$	$8.245^{c} \pm 0.007$	$377.165^{c} \pm 0.007$	$131.245^{c} \pm 0.007$	$4.865^{c} \pm 0.007$	$80.125^{c} \pm 0.007$
WSS	$244.925^{d} \pm 0.007$	$150.665^{d} \pm 0.007$	$94.245^{d} \pm 0.007$	$371.005^{d} \pm 0.007$	$220.325^{d} \pm 0.007$	$6.555^{d} \pm 0.007$	$91.845^{d} \pm 0.007$
WSS_1	$165.295^{e} \pm 0.007$	$108.075^{e} \pm 0.007$	$57.215^{e} \pm 0.007$	$236.415^{e} \pm 0.007$	$71.115^{e} \pm 0.007$	$6.045^{e} \pm 0.007$	$91.445^{e} \pm 0.007$
WSS_2	$161.925^{f} \pm 0.007$	$100.565^{f} \pm 0.007$	$61.345^{f} \pm 0.007$	$232.915^{f} \pm 0.007$	$71.005^{f} \pm 0.007$	$6.365^{f} \pm 0.007$	$90.655^{f} \pm 0.007$
SSO	$49.255^{g} \pm 0.007$	$5.415^{g} \pm 0.007$	$43.825^{g} \pm 0.007$	$118.415^{g} \pm 0.007$	$69.165^{g} \pm 0.007$	$6.365^{f} \pm 0.007$	$93.450^{g} \pm 0.014$

Values are Mean ± Standard. Means with the same superscripts (alphabets) along each column are not significantly different whereas different superscript are significantly different ($P \leq 0.05$).

WOO = 100% Wheat Flour: 0% Soybean flour: 0% Sweet potato flour

WSO = 90% Wheat flour: 10% Soybean flour: 0% Sweet potato flour

WOS = 90% Wheat flour: 0% Soybean flour: 10% Sweet potato flour

WSS = 80% Wheat flour: 10% Soybean flour: 10% Sweet potato flour

WSS_1 = 70% Wheat flour: 10% Soybean flour: 20% Sweet potato flour

WSS_2 = 70% Wheat flour: 20% Soybean flour: 10% Sweet potato flour

SSO = 0% Wheat flour: 50% Soybean flour: 50% Sweet potato flour

V. CONCLUSION

The study revealed that composite flour with good nutritional value could be produced with wheat flour, sweet potato flour and soybean flour. The functional and pasting properties of the composite flours were determined by the proportion of each constituent flour. There exist variations in the functional and pasting properties of the composite flours which are desirable characteristics for the manufacture of various food products. Sweet potato flour and soybean flour have great potential as a functional ingredient in partial substitution of wheat flour in the diets, particularly in the developing countries. This work

recommends the promotion and utilization of wheat-sweet potato-soybean flour. However further research work should be focused on how to improve the sensory quality and hence acceptability.

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The Effect of Gaz Fire on Economic Development of Rwanda "Case Study of Kitchen Fires for Nyarugenge and Gitega Sectors of Nyarugenge District in Kigali City"

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Abstract— *The whole world, Rwanda inclusive is striving to improve access to affordable and reliable modern forms of energy services which may result in poverty and environmental related effects alleviation and sustaining the global economy (Leach, 1992; UNDP, 2005; Modi et al., 2005; WHO, 2006a; UNDP and WHO, 2009; UNIDO, 2009; AGECC, 2010; World Bank, 2011a; Barnes et al., 2011; Ekouevi and Tuntivate, 2012). However, currently 1.26 billion people do not have access to electricity and 2.64 billion people rely on traditional biomass (firewood, charcoal, dung and agricultural residues) for cooking mainly in rural areas of developing countries (IEA, 2013).*

In the recent years Cooking gases were introduced in developed, developing and underdeveloped countries as best approach of cleaner energy which takes into account the protection from prevention from the emission of greenhouse gases which led to the global warming effect and air pollution (UNDP and WHO, UNIDO, 2009).

Several researchers have conducted many studies on the approach of introducing the use of gases in cooking as previously charcoal and fire woods were used in daily life as available approach of cooking while they increase the rate of environmental degradation due to deforestation during the production of charcoal and fire woods (World Bank, 2011a).

Research study was conducted on effect of gaz fire on economic development of Rwanda, case of Nyarugenge and Gitega Secctors in Nyarugenge District of Kigali City where different perception on the cause of gas fire or Incendies were elaborated, among 96 interviewed and consulted people selected from 2123people of Nyarugenge and Gitega sectors 14 agreed on poor installation, 10 agreed on Shortage of area to hold cooking gases, 18 agreed on Carelessness during using gases,30 agreed on the Lack of fire extinguishers while 24 remaining consulted populations agreed on Lack of enough skills and training.

The Corelation of progress in gas use and fire accidents in Gitega and Nyaugenge sectors were also analyzed where the research findings showed that there is a high increment in gases use in the years of 2016 ,2017,2018,2018 and 2020 as the following percentage 0.4;0.8;11;30,63 respectively, however, the increments in accidents were related to the stated years are 0;3;4;6'11 respectively.

Keywords— Effect of gas fire on economic development, clean energy, Kitchen Fires for Nyarugenge, Kitchen Fires for Gitega, KIGALI CITY, energy services, Cooking gases, greenhouse gases, charcoal and fire woods.

I. INTRODUCTION

The whole world, Rwanda inclusive is striving to improve access to affordable and reliable modern forms of energy services which may result in poverty and environmental related effects alleviation and sustaining the global economy (Leach, 1992; UNDP, 2005; Modi et al., 2005; WHO, 2006a; UNDP and WHO, 2009; UNIDO, 2009; AGECC, 2010; World Bank, 2011a; Barnes *et al.*, 2011; Ekouevi and Tuntivate, 2012). However, currently 1.26 billion people do not have access to electricity and 2.64 billion people rely on traditional biomass (firewood, charcoal, dung and agricultural residues) for cooking mainly in rural areas of developing countries (IEA, 2013).

Household cooking consumes more energy than any other end-use services in low-income developing countries (IEA, 2006; Daioglou *et al.,* 2012). The widespread cooking practice with solid fuels, such as traditional biomass and coal, can have severe implications for human health, forest/land degradation and climate change. Bruce et al. (2000) and WHO (2006a), findings on air pollutants emitted from solid fuels often burned indoors on inefficient cook stoves, is one of biggest challenges to human health in developing countries (Lim *et al.* (2012), for example, estimated that in 2010, about 3.5 million premature deaths were caused by household air pollution (HAP) resulting primarily from cooking with solid fuels.

Hence, the World Bank has recently launched a number of regional clean cooking initiatives, such as the Africa Clean Cooking Energy Solutions to promote enterprise based, large scale dissemination and adoption of clean cooking solutions and the East Asia and Pacific Region's Clean Stove Initiative (CSI) to scale up access to advanced cooking stoves for rural poor households through country-specific technical assistance and a regional knowledge sharing and cooperation forum. Besides these global initiatives, there are several initiatives to promote clean cooking.

Rwanda has also adopted such clean cooking initiatives through use of low-fuel energy cooking stoves known as Rondereza in rural areas and gas cookers in Kigali, secondary cities and other civilized people in rural areas to reduce poverty and related environmental effects by promoting sustainable economic development. However, gas related fire accidents have increased up to killing more than 6 people and damaging 50 houses since 2016 in Kigali (Kwizera, 2019).

Due to accidents related to gas kitchen fires and the high price of buying gas, Umurerwa (2018) asserted that the gas users in Rwanda may continue to diminish. Recent studies, have shown that at least 5

minor accidents of kitchen fires related gas take place in Kigali per day because of lack of expertise in using gases and lack of portable fire extinguishers and advanced prevention technology. Though, this case does not get the level of involving fire brigade (Musonera, 2018; and Kaneza, 2018). It is in this regard; the researcher wants to conduct a study to demonstrate the cause, effect and the level of gas kitchen fires on socio-economic development of Kigali especially in Nyarugenge in the sector of Gitega and Nyarugenge.

II. MATERIALS AND METHODS

2.1 Population of the study

The target population of the study was composed by households using gases in the period ranging from 3 months to 3 years in both Gitega and Nyarugenge Sectors in Nyarugenge District, all categories of population were taken into account by considering marital status, education background and level, sexes, ages, 46 respondents and 50 respondents to make 96 respondents from different households were selected in Gitega and Nyarugenge Sectors respectively. Among 150 households, each household has represented by one respondent, to fill the questionnaire. Before filling the questionnaire, the questionnaires were previously revised using chief of villages in the sectors of Gitega and Nyarugenge, Nyarugenge district is part of Kigali City where people emigrate from different areas for different reasons including socio-economic needs, like formal and informal jobs, business and life style Nyarugenge and Gitega sectors comprised by 3,326 households where 2,123 use gases in their daily life. In this study, a researcher focused on households using gases in their cooking activities.

2.2 Sample size

William (2004) noted that sampling is a device or a way that is used in selecting of the members is able to question, or who are a fair presentation of all the members in a union. Sampling techniques may be defined as the method used to select sample elements in the population. At this time the researcher will use purposive sampling technique in order to come up with the relevant information to the study. This technique of purposive sampling will be applied to 96 respondents from kitchen fires in Nyarugenge and Gitega Sectors.

However, the formula of Taro Yamane was used to calculate the sample size

Therefore, the fact that it is not possible to meet the whole population to achieve the research objectives, for the purpose of collecting detailed information that can lead to paramount decision making, a sub-set of the total population (sample size) would be selected. From the population of 2123 respondents of Kigali kitchen fires, the sample size of 96 respondents will selected. As such, Sloven's formula for determining sample size was applied as illustrated below:

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

Source: Yamane, 1967 Where N= Total population, n=Sample size, and e= Error margin, e^2 is 0.01 level of significance.

Applying the above formula, the sample employed for this study was calculated from the total 2123 households using gases in both Gitega and Nyarugenge sectors of Nyarugenge District. The sample was calculated as follows:

$$n = \frac{2123}{1+(2123)e^2} = \frac{2123}{1+(2123)\,(0.1)^2} = \frac{2123}{1+(2123)\,0.01} = \frac{2123}{1+21.23} = \frac{2123}{22.23} \quad (2)$$

N = 95.5 which makes approximately 96 participants; whereby, n is the sample size, N stands for population and e² is 0.01 level of significance.

III. RESULTS AND DISCUSSIONS

3.1 Respondents' perception on the cause of fire accidents

Research study was conducted on effect of gaz fire on economic development of Rwanda, case of Nyarugenge and Gitega Sectors in Nyarugenge District of Kigali City where different perception on the cause of gas fire or Incendies were elaborated, among 96 interviewed and consulted people selected from 2123 people of Nyarugenge and Gitega sectors 14 agreed on poor installation, 10 agreed on Shortage of area to hold cooking gases, 18 agreed on Carelessness during using gases, 30 agreed on the Lack of fire extinguishers while 24 remaining consulted populations agreed on Lack of enough skills and training.

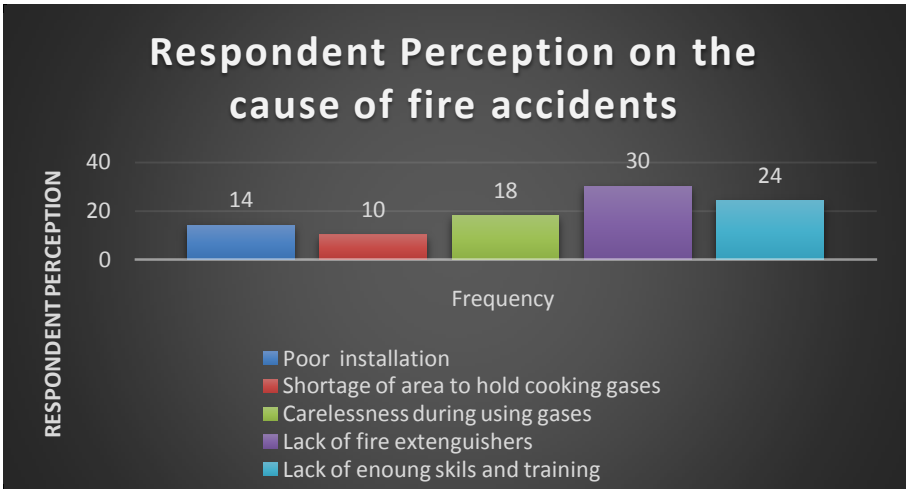

FIGURE 1: Respondents' perception on the cause of fire accidents

3.2 Correlation of progress in gas use and fire accidents in Gitega and Nyarugenge sectors

The Correlation of progress in gas use and fire accidents in Gitega and Nyaugenge sectors were also analyzed where the research findings showed that there is a high increment in gases use in the years of 2016, 2017, 2018, 2018 and 2020 as the following percentage 0.4;0.8;11;30,63 respectively, however, the increments in accidents were related to the stated years are 0;3;4;6'11 respectively.

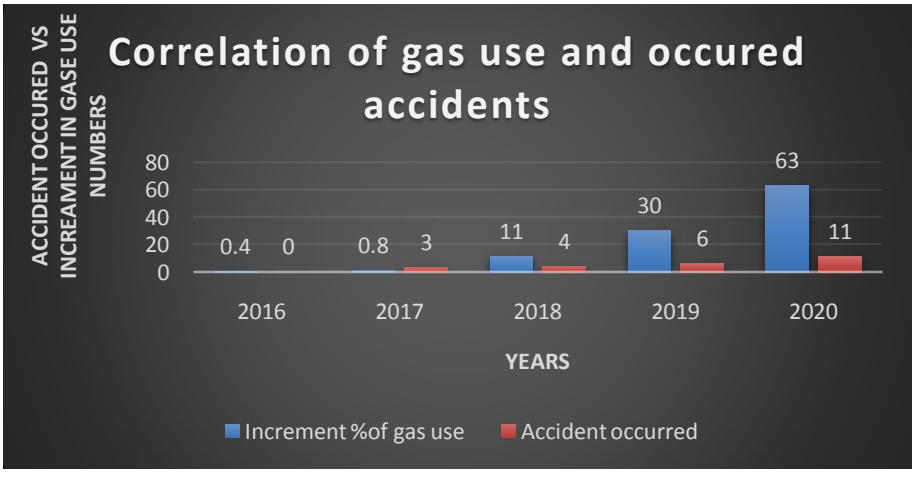

FIGURE 2: Correlation of progress in gas use and fire accidents in Gitega and Nyarugenge sectors

IV. CONCLUSION AND RECOMMENDATIONS

4.1 Conclusion

The increments of gases use in the recent years were highly proven by the data of market analysis for Gases consumable perception and the increase of LPG prices day to day. The need of gas on the market is highly motivated by the will of different country's will which enhance the use of cleaner energy, but without immediate action on the regulation of cost analysis of gases, the cost of cleaner energy or gases should be matched with the capacity of the consumer. Therefore, political will is highly needed.

4.2 Recommendations

Referring on research findings, the following recommendations were developed:

1. Several trainings on gases firefighting are highly needed in all sectors by starting where gases and LPG are being sold.
2. Regulation on the cost of LPG should designed by considering the purchasing power of the consumer
3. Campaigns and political will are highly needed in enhancing the use of cleaner energy which prevents the pollution of environments.
4. Future researchers are highly recommended to conduct further studies to support the research finding of the study.

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Socio-Economic Factors Affecting Fish Farmers in Abia State, Nigeria

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Abstract— *Study of socio-economic factors affecting fish farmers in Aba state, Nigeria was carried out between January, 2017 to January, 2018 with the aim of identifying socio-economic factors affecting fish farming in the area and proffer solutions for potential government support towards aquaculture development in Abia State as well as to recommend ways fish farming can be improved and promoted. Abia State was visited through the assistant of the staffs of their various ministries of agriculture and natural resources as well as those from agricultural development programmes. Identified farmers were subsequently counted according to their fish farm circles, blocks, and agricultural zones in their respective states. The population sample comprised of sixty four (64) fish farmers that were randomly selected within the farm circles, blocks and agricultural zones of the states through Taro Yamani formula. Questionnaires, interviews, field observations, visits to some fish farms were photographs were taken where all used during data collection. Questionnaires used were divided into five sections that covered the objectives of the study. Data collected were then analyzed using descriptive statistics (frequency, percentage, mean, etc.), inferential statistics and SPSS version 2020 were all used. The study revealed that majority of the fish farmers were males(76.9%),who were married (64.1%) with an average age that ranged between 41—50 years (37.9%), with a low level of education mainly primary education (39.1%) and a household size of 4—6 persons (50.9%). They used mainly family labour (60.0%) with a relative low level of production 4—5 ponds (46.6%) and an average annual income that ranges between ₦100, 000---- ₦500, 000 (60.0%) among them. Marketing constraints was noticed to be among the most ranked major constraints faced by these fish farmers, it was also noticed that there were no government support towards fish farming activities in the state. Consequently, the study recommended that fish farmers in the States be fortified to form cooperative societies in order to gain access to credit facilities*.

Keywords— socio-economic factors, fish farmers, credit facilities.

I. INTRODUCTION

Aquaculture which is also known as fish farming is the husbandry of aquatic food organisms. The need arose from the decrease in supply from fresh water and marine fisheries as a result of over-fishing, habitat destruction and pollution. One of the ways to bridge the gap between the reduced fish supply and increased world food fish demand is through aquaculture. Unlike Asia, Africa has little aquaculture tradition and has been affected by a number of external problems that have prevented proper management and development of the sector despite investment (Edward, 2000; Palmguist and Danelsam, 1991).

Aquaculture has been demonstrated as a cheap source of animal protein (FAO, 2016). FAO (2016) reported that an estimated 690 million people lack adequate access to food; and about 15% of these are in sub-Saharan Africa. Tunde *et al.* (2015) asserted that as the population grows and puts more pressure on natural resources, more people will probably become food insecure, lacking access to sufficient amount of safe and nutritious food for normal growth, development, and an active and healthy life. A number of countries in sub-Saharan Africa are characterized by low agricultural production, widespread economic stagnation, persistent political instability, increasing environmental damage, and severe poverty. Given this situation, it is therefore pertinent to provide the poor and hungry with a low cost and readily available strategy to increase food production using less land per output, and less water without further damage to the environment (Tunde *et al.*, 2015). In Nigeria, aquaculture development has been driven by social and economic objectives, such as nutrition improvement in rural

areas, generation of supplementary income, diversification of income activities, and the creation of employment. This is especially true in rural communities, where opportunities for economic activities are limited. Only in recent years has aquaculture been viewed as an activity likely to meet national shortfalls in fish supplies, thereby reducing fish imports. According to Ekunwe and Emokaro (2016), statistics indicate that Nigeria is the largest African aquaculture producer, with production output of over 2.7 million metric tonnes (mmt) per annum; this is closely followed by Egypt with output of about 2.1 million tonnes. Five other countries produce: Zambia (over 70 000 tons of fish annually), Madagascar (over 12,700 tonnes per annum), Togo (28,000 tons per annum), Kenya (24,000 metric tonnes of fish annually) and Sudan (2,000 tons per annum). Ekunwe and Emokaro (2016) further showed that Nigeria imports about 560,000 tonnes of fish estimated at about $400 million annually while annual domestic fish supply in Nigeria stands at about 400,000 tonnes. The fisheries sector accounts for about 2% of national G.D.P, 40% of the animal protein intake and a substantial proportion of employment, especially in the rural areas; the sector is a principal source of livelihood for over three million people in Nigeria.

The government research institutions and the universities have made effort in developing improved strategies and technologies so as to increase production to meet the demand of the country and even export. These technologies are new improved ideas, methods, practices, innovations and inputs which supersede the ones previously in use. It also provides the means of achieving a sustainable increase in fish farm productivity and consequently leading to an improved living standard of the people as stated by Ifejika and Ayanda (2014). But according to Bolorundu (2016) the level of adoption of these technologies by the fish farmers is very low. This is due to the combination of various constraints among which are faulty aquaculture policies, institutional framework and unfavourable socio-economic environment. Hence, this study aimed at identifying socio-economic factors affecting fish farming in the area and proffer solutions for potential government support

II. MATERIALS AND METHODS

2.1 Study Area

Abia State, which occupies about 6,320 square kilometers, the state which is approximately within latitude 4^{o} 41' and 6^{o} 14' North of the Equator and Longitude $7^{o,}$ 10' and 8^{o} East of Greenwich meridian, is bounded on the North and North East by the states of Anambra, Enugu, and Ebonyi. To the West of Abia is Imo state, to the East and South East are cross river state and Akwa Ibom State respectively and to the South is Rivers State. Abia State is made of three agricultural zones that were made of;

1) Aba agricultural zone is made up of seven local government Areas; namely; Aba North , Aba South , Osisioma Ngwa, Obioma Ngwa North, Ukwa West and Ugwunagbo Local Government Areas. The zone is located between latitudes 5^{o} and 39^{o} N and longitude 2^{o} and 0^{o}E, has a total land mass of 810,160ha and with a population of 1,167,698 persons (NPC 2006).
2) Ohafia zone is made up of five Local Government Areas namely; Isiukwuato, Ohafia , Bende , Arochukwu and Umunneochi.
3) Umuahia agricultural zones covers five (5) Local Government areas of namely; Umuahia North and Umuahia South, Ikwuano, Isala Ngwa North and Isiala Ngwa South.

2.2 Sampling Size and Technique

The population sample comprise of three hundred and twenty(320) fish farmers that were randomly selected within the following circles, blocks and the agricultural zones in the state as summarized in Table 1.

TABLE 1
LISTS OF CIRCLES, BLOCKS AND AGRICULTURAL ZONES IN ABIA STATE

Sr. No.	State	Agricultural zone	Fish block	Fish circle
1	Abia	Aba	15	30
		Ohafia	9	20
		Umuahia	10	21
		Total	**34**	**71**

Source: Field survey (2019)

Then Taro Yamani formula, Yamani Taro (1967) the following number of respondents were identified.

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

Where n = Sample size

N = population size

e = 0.05 based on research condition

III. METHOD OF DATA COLLECTION

In this study, questionnaires, interviews, field observation, visits to fish farms where photographs were taken which was used during data collection. However, questionnaire was the major tool used for gathering necessary data from fish farmers (respondents). The questionnaire was structured in such a way that it provided answers to the research questions.

1. What is the effect of socio-economic characteristics of fish farmers on aquaculture productivity within the state?
2. In what ways can aquaculture be improved and promoted within the States?
3. What are those major constraints faced by fish farmers in improving aquaculture productivity within the States?
4. What are the potential of governmental support towards aquaculture improvement and productivity within the State?

3.1 Viability of the instrument

Copies of the questionnaires were given to experts in Agricultural Economics, Fisheries Economics and Statistics and Computer Science in Nnamdi Azikiwe University for Validation.

3.2 Data Analysis

Analytical tools that was adopted in this study were descriptive statistics (frequency, percentage, mean), inferential statistics and SPSS version 2020 was used to analyse research questions, research hypothesis and objectives of the study. Multiple Regression and Z statistics was adopted to test the hypothesis of the study. The computation was done using SPSS 22 package).

IV. RESULT AND DISCUSSION

Gender respondents of fish farmers in Abia state showed that men were (73.8%) while female were (24.6%) as shown in table 2 (A). Those between the age ranges of 41-50 years had the percentage value of (52.5%) while those from 20-30 years had the least percentage (6.6%). Single Farmers hade (55.7%) married farmers had percentage (32.8%) while the least were widowers (1.5%). Fish farmers that had only primary education had (34.4%), those with tertiary education were (16.4%) respectively, while household house range of 4-6 persons had (67.2%) and the least were those above 9 persons (4.9%) as shown in tables 2 (B-E).

Farming experience showed that those who had 4 to 6years experience had (39.3%) as shown in table 2 (F), while those above 10 years had (9.0%). Farmers who had 1-5 ponds had the highest percentage (57.4%) and those with below 16 ponds (1.6%) were the least. Those who used family labour had (68.9%) while those who hired labour had the least (11.5%). In Abia state those who earned around N100,000 – N500,000 naira had the highest percentage (60.7%) while those who earned between 1,000,000 – 5,000,000 had the least percentage (6.5%) as shown in tables 2 (F-I).

TABLE 2
SOCIO-ECONOMIC FACTORS OF FISH FARMERS IN ABIA STATE.

A. Gender of respondents in Abia State					
		Frequency	**Percent**	**Valid Percent**	**Cumulative Percent**
	Male	45	73.8	75	75
	Female	15	24.6	25	100
	Total	60	98.4	100	
Missing	System	1	1.6		
Total		61	100		

B. Age of respondents in Abia State					
		Frequency	Percent	Valid Percent	Cumulative Percent
	21-30 Years	4	6.6	6.7	6.7
	31-40 Years	16	26.6	26.7	33.3
	41-50 Years	32	52.5	53.3	86.7
	Above 50 years	8	13.1	13.3	100
	Total	60	98.4	100	
Missing	System	1	1.6		
Total		61	100		
C. Marital Status of Respondents in Abia State					
		Frequency	Percent	Valid Percent	Cumulative Percent
	Single	34	55.7	56.7	56.7
	Married	20	32.8	33.3	90
	Divorced	3	4.9	5	95
	Widowed	2	3.3	3.3	98.3
	Widower	1	1.6	1.7	100
	Total	60	98.4	100	
Missing	System	1	1.6		
Total		61	100		
D. Educational Qualification in Abia State					
		Frequency	Percent	Valid Percent	Cumulative Percent
	Non Formal Education	10	16.4	16.7	16.7
	Primary	21	34.4	35	51.7
	Secondary	19	31.1	31.7	83.3
	Teritary	10	16.4	16.7	100
	Total	60	98.4	100	
Missing	System	1	1.6		
Total		61	100		
E. Household size in Abia State					
		Frequency	Percent	Valid Percent	Cumulative Percent
	1--3 persons	9	14.8	15	15
	4--6 persons	41	67.2	68.3	83.3
	7--8 persons	7	11.5	11.7	95
	9 persons and above	3	4.9	5	100
	Total	60	98.4	100	
Missing	System	1	1.6		
Total		61	100		
F. Farming Experiences in Abia State					
		Frequency	Percent	Valid Percent	Cumulative Percent
	1--3 yrs	20	32.8	33.3	33.3
	4--6 yrs	24	39.3	40	73.3
	7--9 yrs	10	16.4	16.7	90
	Above 10 yrs	6	9.8	10	100
	Total	60	98.4	100	
Missing	System	1	1.6		
Total		61	100		

G. Number of ponds used by fish farmers in Abia State					
		Frequency	**Percent**	**Valid Percent**	**Cumulative Percent**
	1--5 ponds	35	57.4	58.3	58.3
	6--10 ponds	19	31.1	31.7	90
	10--15 ponds	5	8.2	8.3	98.3
	Above 16 ponds	1	1.6	1.7	100
	Total	60	98.4	100	
Missing	System	1	1.6		
Total		61	100		
H. Source of labour of respondents in Abia State					
		Frequency	**Percent**	**Valid Percent**	**Cumulative Percent**
	Hired	7	11.5	11.7	11.7
	Family	42	68.9	70	81.7
	Hired and family	11	18	18.3	100
	Total	60	98.4	100	
Missing	System	1	1.6		
Total		61	100		
I. Annual income of respondents in Abia State					
		Frequency	**Percent**	**Valid Percent**	**Cumulative Percent**
	100000--500000 naira	37	60.7	61.7	61.7
	600000--10000000 naira	19	31.1	31.7	93.3
	1000000—5000000	4	6.6	6.7	100
	Total	60	98.4	100	
Missing	System	1	1.6		
Total		61	100		

Result obtained from socio-economic data showed that there were 76.9% male fish farmers in Abia state and 23.0% females. Similar results were obtained by Osondu and Ijeoma (2014), Oguntade, (2007), and Wayep and Rapede (2018) in their various works. These results were different from results obtained by Igwe and Mgbaja (2014) on their work on the evaluation of pond fish production in Umuahia South Local Government Area of Abia State were male respondents were 37.5 percent while 62.5 percent of the respondents were female. The result of this study shows that more male fish farmers were involved in fisheries activities in Abia State. Age brackets of 41 – 50 years had the highest percentage of 52.5 percent. This gives insight into the prospects that fish farming business is viable as its operation is predominately in the hands of farmers in their productive age and no doubt of youthful vigour. This goes to emphasis that fish farming is not done by the aged in Abia state. Thus it has prospects of being an enterprise that if well-developed could become a tool in dealing with youth unemployment. Age is an important factor in determining the productive and adoption of an innovation by farmers (Kebede, 2001). At the youthful age, decision making for improved production and ability to take risk for expansion of production frontier by the farmers would not be too difficult for these fish farmers to adjust. This agrees with the findings of Eze (2002) which reported that achieve age of farmers is a positive factor for decision making, Nwaru (2004) also had opined that the ability of the farmers to bear risk, do manual work and be innovative decreases with increase in age.

Furthermore people who are single constitute greater number of the respondents, followed by the married people and the least being widowers and divorcees. This indicates that most fish farmers in Abia were singles and married. This evinced that the expected support from the spouse(s) and children of these fish farmers with a view of improving and increasing fish production and improved management of fish farming. Family members would most likely see the farm business as one that directly or indirectly contributes to the economy of the home and so would not work against the enterprise. The farm fish farmers being married are themselves assumed to be responsible.

Most respondents in South East had only primary education while the least was tertiary education.

The main education level for most fish farmers were primary school and this implies that most attend secondary school which meant that most critical decisions concerning their farming enterprise could not be taken by them as a result of lack of education. This result disagrees with the work of Nnamdi *et al*. (2017). Education of farmers is therefore necessary for farmers' adoption of improved farm land management practices and reaction to policy issues (Ebii, 2000).

Household size of fish farmers as recorded on this study was between 4 – 6 household members which is 50.9 percent. Invariably, household size of 4 – 6 persons is within the desirable range and of great importance to rural household as they rely more on their family members than hired labourers in their family activities thereby reducing production cost. This finding is consistent with the report of Palmguist (1989).

Forty percent of the farmers had been in the business of fish farming between 4 – 6 years while the least in the state where those with over 10 years of fish farming experience. The mean fish farming experience are 5 years which implies that most fish farmers are new in the fish farming business. According to Ekanem *et al*. (2015) the years of farming experience of a farmer enables him to acquire practical and relevant farming knowledge which drive his ability to efficiently utilize available resources with discretion which is lacking among most fish farmers.

The overall result from these member of ponds implies that most fish farmers in Abia state operated a small farm size which have an inverse relationship on fish productivity or output of the fish farms. This further shows that most of the farmers were operating on subsistence level. Studies have shown that most rural farmers in Nigeria operated on small scale basis (Godwin et al, 2003).

Respondents from this study who used family labour were very high while fish farmers who hired labourer were very low in the state. With relatively availability family cheap labour in Abia, extensive use of human labour for fish farming especially in south east has been shown to fish famring profitable (Enete, and Okon 2008).

Result from respondents showed that most fish farmers in Abia State earned an average annual income of ₦100, 000 – ₦500, 000 are 60.7 percent, while the least were those who earned above ₦5, 000,000 who were 1.6 percent. This result showed that an average fish farmer earns between ₦5, 000 – ₦50, 000 per month. This is an indication that fish farming activity is not only affording these farmers a source of income but also provides a source of protein to them and agreed with (Sanusi *et al*, 2015).

V. CONCLUSION

This study noticed that majority of fish farmers in Abia state were males, who were married with a low level of education. They maintained a large family size whom they use as source of cheap labourer. Output from this fish farmers are relatively small as majority of them practice subsistence fish farming. The use of new technologies like intensive system of culture, use of modified drum ovum and other new technologies assisted most of the fish farmers in their farming activities.

This study also noticed that there are lots of ways aquaculture can be improved and promoted in the zone through increased government support. Major constraints were also identified during the cause of this study. Marketing constraints were noticed to be among the major constraints. Finally, Government of the entire south east were not putting serious effort towards aquaculture developments in the region as majority of the fish farmers complained of lack of government support or assistance.

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Vestibular Syndrome Associated to Ranavirus in Farmed American Bullfrogs

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Abstract**—** *Frog farming is a consolidated aquaculture activity in Brazil. American bullfrogs (Lithobates catesbeianus) are raised in high densities and fed artificial diets, conditions that favors the emergence of diseases. A common disease affecting farmed frogs is a nervous syndrome characterized by postural and locomotive alterations resembling a vestibular disease in other species. Ninety-one sick frogs were analyzed from two different farms located at Goiás State in Central-Western Brazil. Frogs were first clinically assessed and further necropsied to identify lesions. Samples from the whole head, liver, kidney, spleen, stomach, intestine, lungs, gonads, skin and blood were obtained for histopathology, immunohistochemistry (IHQ), bacteriology, transmission electron microscopy (TEM) and real-time PCR. Macroscopic lesions affecting the inner ear region were collected for histopathology. Inflammatory lesions with necrosis and lymphocytic infiltrate affecting the labyrinthine endorgans, with necrotic foci, abundant macrophages and cellular debris associated with inflammatory infiltrates and acidophilic inclusion bodies were observed. No bacteria were isolated or identified in these lesions. IHQ showed positive staining for Iridoviridae and TEM confirmed viral particles. qPCR also detected a virus from Ranavirus genus. These findings indicate that Ranavirus cause frog vestibular syndrome. As far as we know this is the first report of Ranavirus producing nervous lesions.*

Keywords— Ranavirus, Vestibular syndrome, American bullfrogs, Iridoviridae.

I. INTRODUCTION

Frog farming is a consolidated aquaculture activity in Brazil. American bullfrogs (*Rana catesbeiana*, Shaw 1802/*Lithobates catesbeianus*) are raised intensively with high population densities and fed with artificial diets. Those culture conditions trigger disease occurrence favored by close animal contact, increased pathogen densities and stress-induced immune depletion (Bondad-Reantaso et al., 2005).

A common chronic syndrome, with high prevalence in frogs' farms, is characterized by diverse degree of postural and locomotive dysfunctions. Despite morbidity will be high in some conditions, low mortality was reported and farmers did not show great concern with this problem. The most common measures are isolating those frogs into a "hospital pen" with low densities and strong water recirculation. According to farmers' experience, some recoveries were reported after this management. From a clinical point of view, signs observed in sick frogs resemble a vestibular disease in another species. To our knowledge, no description of this syndrome is available in farmed American bullfrogs and etiology, pathogenesis and affected tissues are completely unknown.

The bullfrog ear, as in most amphibian consists of a middle ear and an inner ear, but no external ear. The inner ear consists of a system of fluid-filled tubes and sacs called the labyrinth. Vestibular system is highly preserved a cross vertebrate

phylogeny and frogs have been used as models for several studies concerning this organ and its functions [2,3]. Early experimental work with frogs (Goltz, 1870 and Edwald 1892, cited by Straka et al. 2016) showed semicircular canals were distinct organs responsible for posture and equilibrium in three spatial orientations, and the semicircular canals sense head rotations [4].

For a more detailed description of labyrinth and vestibular system [5], gives useful information. In summary, the brain integrates balance signals sent through the vestibular nerve from the right and left ear. Vestibular sensory system has functional significance for gaze and posture stabilization, and has the capability to ensure accurate spatial orientation perception and navigation [4].

Vestibular neuritis and labyrinthitis result from an infection that inflames the inner ear or the nerves connecting the inner ear to the brain damaging normal connections between them and, consequently, faulty signals are sent. The brain thus receives mismatched information and infected individuals may show a complex syndrome of static (in the absence of body motion) and dynamic (during body motion) ocular, motor, postural and cognitive deficits that may include vertigo, dizziness, and difficulties with balance, vision or hearing [2,5,6]. To assess possible etiologies involved in farmed frogs' vestibular syndrome, a bibliography research was performed. Results showed that many cases of inner ear infections in humans are usually viral [5] and may be consequence of a systemic viral illness, such as infectious mononucleosis or measles, herpes viruses (such as the ones that cause cold sores or chicken pox and shingles), influenza, rubella, mumps, polio, hepatitis, respiratory syncytial virus and Epstein-Barr [6], usually affecting only one ear. In domestic animals, some viruses that have been associated with vestibular neuritis or labyrinthitis include canine distemper in dogs, and feline infectious peritonitis in cats. These syndromes have been described as a paradoxical vestibular syndrome and labyrinthitis [7,8,9,10].

Surveillance on Ranavirus presence in Brazilian farms showed high prevalence, with few disease signs or mortality episodes in frogs, but several disease outbreaks with high mortality were reported in young tadpoles [11]. This high viral prevalence, associated to the information reported from other species induced the authors to analyze possible involvement of Ranavirus in vestibular syndrome, despite infections have been described as sudden death with a variety of clinical signs depending on the affected species and development stage but few nervous symptoms were related to these pathogens [12,13].

Ranaviruses are double stranded DNA viruses which have worldwide distribution being identified as responsible for an emerging systemic disease with global occurrence affecting ectothermic vertebrates, both in the wild and in captivity [14,15,16]. Due to their severity and potential threat to wild populations Ranaviruses became a notifiable disease of amphibians according to the World Organization for Animal Health [17].

These agents have been already detected in Brazilian frog farms affecting young tadpoles [18,19], but they were not yet confirmed as pathogens for adult American bullfrogs, as this species seems to be quite resistant to these viruses and may become healthy carriers [12,20].

The clinical pattern resembling labyrinthitis and vestibular syndrome induced the authors to thoroughly study those frogs, focusing on possible lesions in central nervous system (CNS) and ear region. Several necropsies were performed and gross lesions affecting posterior CNS regions with unilateral extension to the ear were observed (see results) confirming the clinical suspect about the vestibular origin of the syndrome.

Considering these issues, the objective of this work was to study the vestibular syndrome in farmed adult American bullfrogs to identify affected organs, type of lesions and possible etiology focused on Ranavirus.

II. MATERIAL AND METHODS

2.1 Samples

Samplings were performed at a slaughtering plant with official inspection service of the Brazilian Ministry of Agriculture. Frogs (*Lithobates catesbeianus*) were delivered from several Brazilian farms located at Goiás State in Central-Western region and Sao Paulo in the South-East first. A total of 250 frogs were sampled, 91 of them evidencing diverse degree of postural and locomotive dysfunctions.

Frogs were first clinically assessed for describing postural and locomotive dysfunctions and further euthanized by using tricaine methanesulfonate (MS 222) 0.5 g/L followed by a sharp cervical cut [21]. Samples from the whole head were formalin fixed for histological analysis. For ranavirus detection by real-time PCR, brain, liver, kidney and spleen were obtained and preserved in 95% ethanol.

2.2 qPCR

High Pure PCR Template Preparation Kit ® (Roche Diagnostics) method for tissue extraction was applied in 50 mg samples. Genomic DNA concentration and quality of each sample were assessed using NanoPhotometer® (IMPLEN). The DNA samples were diluted with TE buffer (Tris-HCl 10mM, EDTA 1mM) to achieve maximum concentration of 50ng/µL.

Primer and probe sets for TaqMan real-time PCR were designed (FVF 5 'GAGCGTCACCCTCTCATTC 3', FVR 5' GCGTCCAGGTATGCCGTG and FVP 5' FAM-CGACATCAGCGCCCAGTC-MGB 3) using Primer express (Applied Biosystems, Foster City, CA, USA) targeting the MCP gene encoding the major capsid protein (GenBank accession number DQ897669.1) annealing between positions 345 and 407. Each reaction in the TaqMan assay contained TaqMan® Universal Master Mix 1x (Applied Biosystems), 900 mM forward and reverse primers, 250 nm TaqMan probe, 100 ng of template DNA and water to a final volume of 20 µL. Real-time PCR reactions were carried out in the following conditions: 60 ºC for 1 min, then 95 ºC for 10 min, 95 ºC for 15 s and 60 ºC for 1 min for 40 cycles, followed by a post-PCR reaction at 60 ºC for 1 min.

2.3 Necropsy and Histopatology

Fresh tissue samples were fixed in neutral buffered 10% formalin and thereafter, the fragments were decalcified with Ethylene Diamine Tetra-Acetic acid prior to processing. To assess macroscopic lesions in central nervous system (CNS) and/or associated labyrinth endorgans, frogs' heads were transected in three planes to detect the location and extension of lesions. A midsagittal plane cut through the midline structures was first performed separating left from right side, followed by a series of parallel cuts slicing the head laterally. A second series were coronal or frontal cuts from dorsal to ventral head surfaces. Finally, a series of horizontal plane cuts from rostral to caudal regions were performed. All cuts delivered slices of approximately 2 mm wide.

Samples were embedded in paraffin wax, sectioned for histological examination in 5µ cuts and stained with hematoxylin and eosin following routine histological methods [22]. Frontal, transverse, and sagittal sections of healthy and posture changing frogs were analyzed. Fresh fragments were fixed in 10% buffered formalin for 24 hours. For bacterial visualization associated to areas where lesions were observed, sections were stained with MacCallum-Goodpasture method [22].

2.4 Immunohistochemial Analyses

Immunohistochemial analysis (IHQ) was performed on tissues where suspicious lesions characterized by necrosis, inflammatory infiltration and cytoplasmic inclusions were detected during histopathological analysis. For detection of viral antigen, primary polyclonal antibody against the Iridovirus Major Capsid Protein (Abcam ®) was diluted 1:200. Antibody-antigen interaction was visualized by streptavidin-biotin-peroxidase [23].

2.5 Transmission Electron Microscopy

Material for transmission electron microscopy (TEM) was selected from macroscopically identified lesions located at the vestibular region from frogs showing nervous syndrome. Tissues were cut into approximately 1 mm cubes and fixed in 2,5% glutaraldehyde in phosphate buffer 0.1M, pH 7.0 for two hours, washed in phosphate buffer and finally post-fixed in 1% osmium tetroxide for 1 h. Tissues were dehydrated in a graded series of acetone (50 to 100%) and later embedded in Spurr's resin. Ultrathin sections were double-stained with 2% uranyl acetate and lead-citrate. Samples were examined with a Philips EM 208 electron microscope.

2.6 Statistical analysis

The average results (means) obtained by qPCR from apparently healthy frogs and diseased frogs were compared by Tukey test ($p < 0.05$).

2.7 Ethical Statement

All methods, including necropsy and sampling, were carried out in accordance with internal guidelines and regulations of the Ministry of Agriculture – Federal Inspection Service (SIF).

III. RESULTS

3.1 Clinical signs

Frogs with nervous syndrome showed static and dynamic behavioral deficits with asymmetric posture of head, body, and limbs. These abnormal postures included head tilt roll either to the left or right side, asymmetric limb and body positions with lordosis and slight upward tilt of the head or various degrees of scoliosis. Some frogs evidenced combinations of asymmetric limb and head positions with head roll tilt towards the same side of the lesion and ipsilesional flexed and contralesional extended fore and hind limbs respectively.

Dynamic deficits included lack of coordination with locomotive difficulties with continuous rolling and circling movements either intending to swim or walk. In many cases affected frogs showed loss of righting reflex remaining upside-down buoying in water or laying on the floor. (Fig. 1 and Supplementary video V1 online). Other external or internal signs were not observed.

(A) **(B)**

FIGURE 1. (A) Adult frog showing head roll tilt towards the same side of the lesion and ipsilesional flexed and contralesional extended fore and hind limbs. (B) Frogs with different posture abnormalities with loss of righting reflex buoying in water.

Despite these symptoms, some frogs could continue feeding, but most frogs showed mild to severe loss of body weight, depending on the time after onset of the symptoms. Although some frogs died due to emaciation and infected skin lesions consequence of strong uncoordinated movements, the disease is considered a chronic syndrome as frogs remain sick for long periods. According to farmers' experiences, some frogs may recover after a certain period, mostly if they were isolated and kept in a new pond with lower density.

3.2 Necropsy, histopathology and TEM

The necropsy of bullfrogs with vestibular syndrome focused on the location and extension of lesions when heads were transected in the three planes described in methods. When a midsagittal plane cut through the midline structures was performed separating left from right side, no lesions were identified in many frogs (Figure 2). In these specimens, the first macroscopic lesions were observed approximately 2 mm from the midline. Frontal and transverse cuts showed the lesions spreading along the labyrinth affecting the inner and middle ear regions (Figure 2). Dark colored hemorrhagic lesions immediately contiguous to the central nervous system, comprising labyrinthine-vestibular region were observed (Fig. 2). Histopathological analysis of these tissues revealed hemorrhages and inflammatory infiltrate in meninges and ventricular space. Lesions were observed affecting the posterior region of the cerebellum, choroid plexus and labyrinthine endorgans. Main features were inflammatory infiltrate, mostly involving macrophages, lymphocytes and plasmocytes, with hemorrhages, edema, fibrinous exudate, and cell necrosis with abundant cellular debris. The exudate was observed filling the semi-circular channels. Lesions were plenty of inflammatory cells with their cytoplasm showing rounded acidophilic particles consistent with viral inclusion bodies. There were no bacteria associated with lesions ruling out the involvement of these agents in the pathogenesis of the syndrome. Tissue slides obtained from identified labyrinthine lesions showed positive staining with specific anti-iridovirus antibody highlighting abundance of viral inclusion bodies (Fig. 3). Images obtained from affected labyrinth region through transmission electron microscopy showed viral inclusion bodies with several viral particles been assembled and virus particles with similar characteristics to the Ranavirus with icosahedral symmetry and size ranging from 120 to 150 nm (Fig. 4).

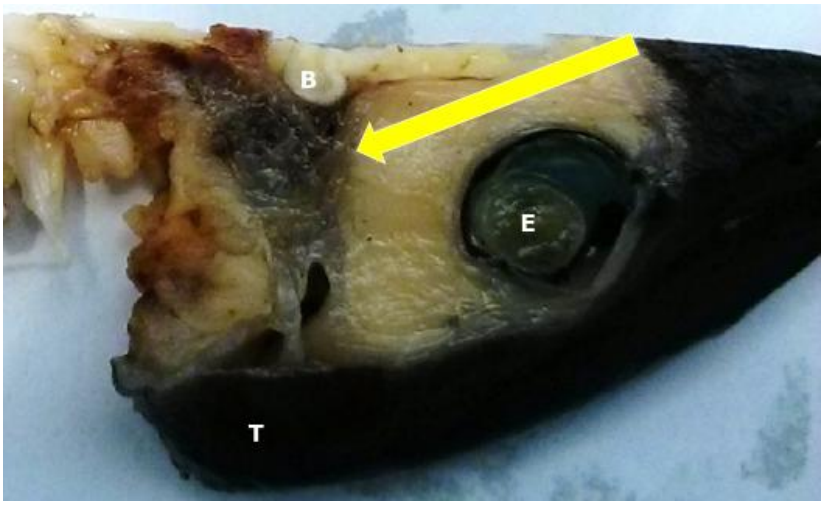
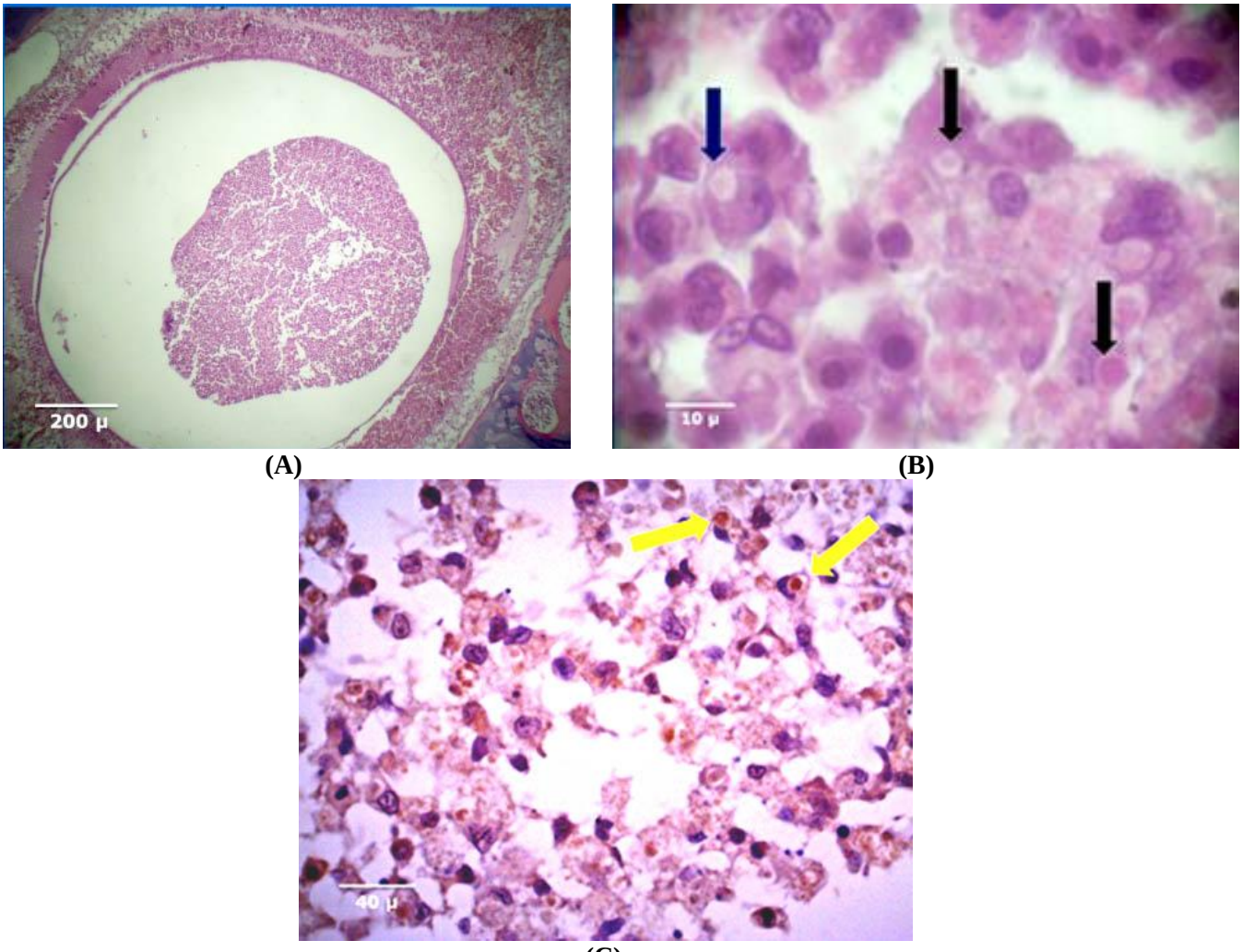


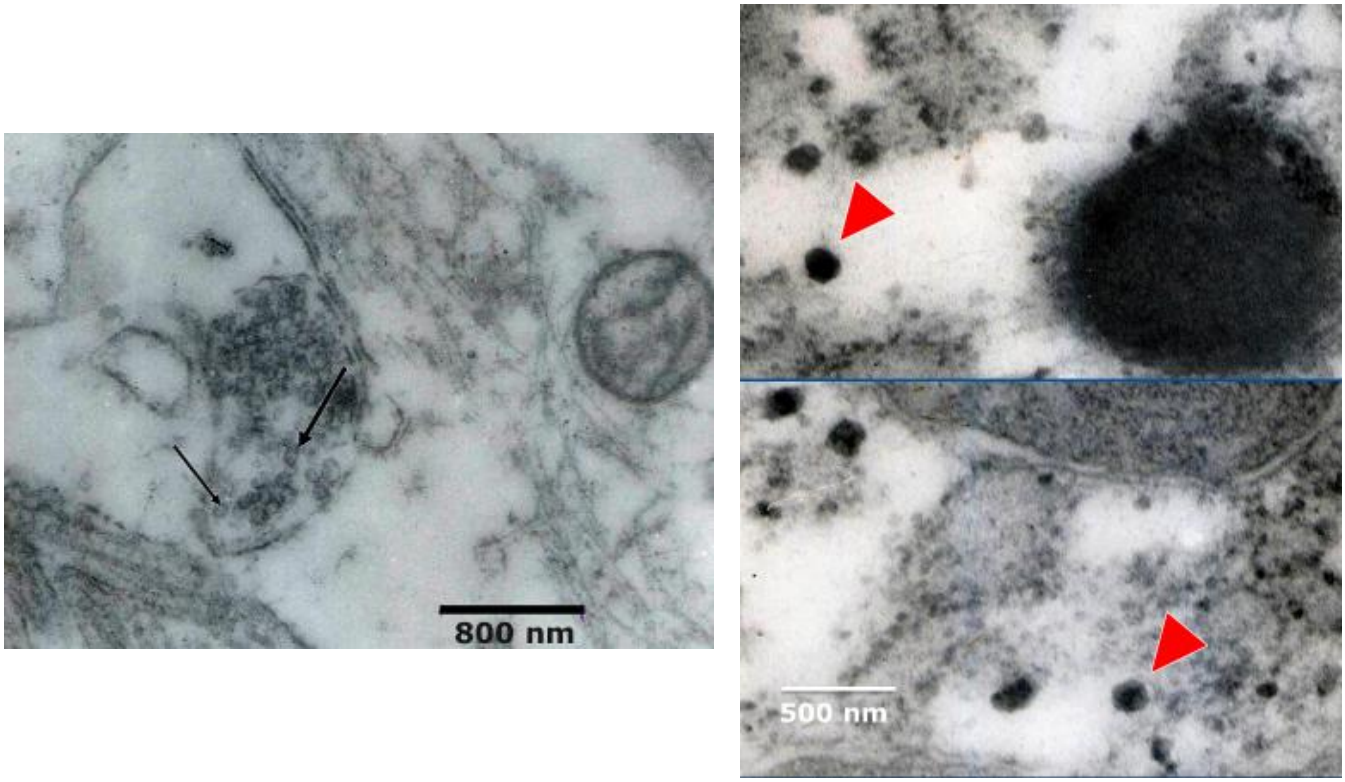
FIGURE 2: Frog with head tilted to the right. Head sectioned first through the midsagittal plane and after through the horizontal plane. Hemorrhagic lesion in right vestibular region (arrow). B: Brain; E: right eye sectioned; T: right tympanic membrane.



(A) **(B)**

(C)

FIGURE 3: (A) Histology slide from the macroscopic lesion in Figure 2 showing inflammatory exudate filling one semi-circular channel. (B) Magnification of the exudate showing necrosis and rounded acidophilic particles consistent with viral inclusion bodies (arrows). Slide stained with H&E. (C) Same histology block used for IHQ slide colored with specific anti-iridovirus antibody showing positive staining with abundance of viral cytoplasmic inclusion bodies into macrophages (arrows)



(A) **(B)**

FIGURE 4: Electron micrograph images showing labyrinth region. (A) Viral inclusion body and viral particles been assembled (arrows).(B) Vestibular region demonstrating viral particles with typical icosahedral shape (arrowheads).

3.3 Ranavirus detection by Real-time PCR (qPCR)

Results in Table 1 showed that 34 (83%) were positive to Ranavirus and 7 (17%) were negative when sick frogs were analyzed. When 50 samples from frogs without clinical signs were analyzed 29 (58%) were positive and 21 (42%) negative. The results showed a higher prevalence of Ranavirus in frogs showing vestibular syndrome ($p<0.05$).

TABLE 1

PERCENTAGE OF *RANAVIRUS* PRESENCE DETECTED BY qPCR IN FROGS WITH AND WITHOUT NERVOUS SYNDROME. DIFFERENT LETTERS IN THE SAME COLUMN REPRESENT STATISTICAL DIFFERENCES ($p<0.05$).

Samples	Results q-PCR	
	Positive	Negative
Frogs without clinical signs	29[a] (58%)	21[a] (42%)
Frogs with clinical signs	34[b] (83%)	7[b] (17%)
Total	63 (69.2%)	28 (30.8%)

Results from qPCR also showed 100% presence of ranavirus in brain of frogs showing vestibular syndrome, as well as high detection in hematopoyetic organs (Table 2).

TABLE 2

PERCENTAGE OF RANAVIRUS PRESENCE DETECTED BY qPCR IN SAMPLED ORGANS OF FROGS WITH NERVOUS SYNDROME.

Sampled Organ	Results %	
	Positive	Negative
Kidney	72,55	28,5
Liver	85,7	14,3
Spleen	85,7	14,3
Brain	100	0

IV. DISCUSSION

To our knowledge, this is the first case of vestibular syndrome involving Ranavirus as the etiological agent.

Reports involving Ranavirus infections and nervous syndromes have been rarely documented and were mentioned only as part of a set of symptom associated with the disease. Spotted salamanders suffering Ranavirus infection swam in circles, had buoyancy problems, and were unable to remain upright [24] and the LMBV-artificially-infected largemouth bass showed spiral swimming after three days of infections [25]. However, neurological abnormalities as the predominant outcome are consistent with tropism described for many viruses responsible for inner ear infections associated to systemic illness in humans and animals, where some strains are more likely to cause acute otitis than others [5,6,7,8,9,10].

Typical vestibular syndrome is characterized by some static and dynamic deficits [2,26,27] that exactly matched the clinical findings observed in the American bullfrogs during our study. Necropsy and histopathology results also confirmed the presence of lesions located in the labyrinthine region supporting the vestibular origin of the observed symptoms. Furthermore, IHQ and TEM confirmed the presence of Ranavirus particles in lesions affecting vestibular endorgans. These findings suggested a preference of Ranavirus for the labyrinthine region and central nervous system in farmed American bullfrogs, already not mentioned.

Previous studies showed that Ranavirus isolated from American bullfrogs in Brazil, was a member of the family *Iridoviridae* evidencing high homologies with FV-3, the type species of genus *Ranavirus* [18,19]. FV3-like Ranavirus, have been associated with mortality events in farmed American bullfrog (*Rana catesbeiana*/*Lithobates catesbeianus*) tadpoles in Brazil [19] and a high prevalence was detected in adults without clinical signs or internal lesions characteristic of Ranavirus infections [11]. Our surveillance of virus presence by qPCR, using specific primers and probe, indicated higher prevalence in frogs showing nervous symptoms (83%) than in non-symptomatic frogs (58%) suggesting an association between the FV3-like Ranavirus and vestibular syndrome in farmed American bullfrogs ($p<0.05$).

There is limited information on the mechanisms that affect host–ranavirus interactions and factors that lead to disease and/or mortality events [13,28]. Phylogeny, the amount of genetic variability, life history characteristics, and habitat associations of amphibians have the potential to impact susceptibility to ranaviruses [29,30]. Ranavirus disease in mature frogs has been linked to immune suppression [31,32] and may be associated to some environmental insults, e.g. crowding among farmed animals [33]. Thus, high densities, management practices and dependence on artificial feeding may affect immune response increasing susceptibility to Ranavirus infections and vestibular syndrome in American bullfrogs. There may also be a genetic predisposition to labyrinth infections in farmed bullfrogs as inbreeding among farmed populations is not properly controlled.

The pathogenesis of these viral infections is not fully understood. Despite the existence of the protection offered by the blood-brain barrier (BBB), the choroid plexus has been identified as an especially attractive target for viruses and constitutes a point of preference for the invasion of the CNS [34,35].

Our results showed lesions involving the choroid plexus and adjacent labyrinth, which supports this hypothesis. Additionally, recent finding showed that FV3 infection alters the BBB integrity leading to viral dissemination –mediated by macrophages- into the central nervous system in *Xenopus laevis* tadpoles but not in adults [36] suggesting that FV3 persistent infections in farmed frogs may allow virus penetration into the CNS during larval development and subsequent production of disease in adults.

Although recovery episodes were not studied during this work, it is well known among Brazilian farmers that moving sick frogs into new ponds with high water circulation and low densities leads to partial recoveries or significant improvements in frogs' conditions. This recovery might be explained by vestibular compensation, which is the regenerative capacity and reorganizational neural flexibility that occurs following a vestibular lesion as frogs were among the first animal models to show these capacity after a vestibular lesion, attributed to the plasticity within the central nervous system [2,26,37].

Frogs have been one of the most common medical models for studies applied to animal and human diseases, and their use for understanding labyrinth lesions was not an exception. Thus, this syndrome will provide an excellent opportunity for future veterinary and medical research on viral-induced vestibular disease pathogenesis and virulence genes involved. As an endemic disease, vestibular syndrome will be easily found at frog farms, which will offer abundant research material for a better disease understanding.

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AUTHOR CONTRIBUTIONS

R.M. and A.Q.M. designed and performed the experiments, completed analysis of data and wrote the main manuscript text and L.F.F.F, M.H, A.M.C.M. and M.C.N. performed histopathology, IHQ and TEM and prepared Figures 3–4. All authors reviewed the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

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Isolation, Selection and Identification of Nitrogen Fixation Rhizospheric and Endophytic Bacteria from Maize (*Zea Mays* L.) Grown on the Soil

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Abstract— *N-free medium (Nfb, LGI and Burks) were used to isolate bacteria having nitrogen fixation characteristics. Quantitative measurement by colorimetric methods helped to select the best isolates of nitrogen-fixing and IAA biosynthesis. The result of isolation was a total of 30 rhizopheric and 150 endophytic isolates having the both abilities. Sixty isolates having good biofertilizer activity were chosen to study. Isolates strains have both nitrogen fixation produced indole-3-acetic acid (IAA) in vitro. Six selected isolates had 16S rDNA sequences similarities with bacterial strains in data of GenBank with the values ranging between 94% and 99% of similarity in which includes strains of Enterobacter ludwigii DNL14, Enterobacter kobei DNT5, Bacillus pumilus DBT4, Klebsiella pneumoniae DNR5, Lactobacillus plantarum DLR6, Pseudomonas nitroreducens DND5. Especially, strains of endophytic isolates root maize origin as Klebsiella pneumoniae DNR5 biofertilizer activities synthesized average highest as NH4 (5.64 mg/L) and IAA (5.29mg/L) which can be exploited for enhancing soil fertility and plant growth.*

Keywords— Bacillus sp, Endophytic, Klebsiella sp, Maize, Nitrogen fixing bacteria, Rhizosphere.

I. INTRODUCTION

Maize (*Zea may* L) is an important food crop in the world economy and Vietnam. Maize kernels are used as human food, animal feed and raw materials for industry. Maize needs to absorb a large amount of fertilizers to grow and develop [1]. However, chemical fertilizer applied to maize too much will cause environmental pollution, harmful effects on human and animal health. High chemical nitrogen fertilization increases investment costs, causes imbalances in natural ecosystems such as soil erosion, increases in nitrate concentrations in surface water, groundwater and discharges nitrous oxide during denitrification [2]. In today's agricultural production, improving soil fertility, reducing the amount of chemical fertilizers, increasing biological fertilizers and including N-fixing bacteria are necessary to develop sustainable agriculture. Nitrogen-fixing bacteria convert the free nitrogen of the biosphere to NH_3 by ATP energy and catalysis enzyme nitrogenase under normal conditions [3]. Plants absorb nitrogen to synthesize protein for growth and development.

Most microorganisms present in the rhizosphere play important roles in the growth and in the ecological fitness of their plant host [4]. Many studies showed that biofertilizer containing nitrogen fixing bacteria promoted plant growth and high yield could be achieved [5]. Several Plant Growth Promoting Rhizobia (PGPR) were isolated from maize grown in field soils such as *Azospirillum lipoferum*, *Bacillus polymixa*, *Pseudomonas putida* [6], [7]. Inoculated maize with *Azospirillum* sp. could support growth parameters including plant height. Nitrogen fixing bacteria such as Pseudomonas, Klebsiella, Enterrobacter, could use as growth promoters of maize plants [8]. Bacterial strains isolated from soil samples and maize roots include dominant bacterial genera identified were Klebsiella and Burksholderia potential as plant growth-promoting on maize plants [9]. Only inoculation of Azotobacter was done just a few hours before seed sowing. It helps on growth and yield of maize increasing 15 to 35% grain yield over non-inoculated treatments [10]. The results showed that strain AC had the highest nitrogen and phosphatase activity and it helped increase plant biomass up to 39%. In Vietnam, the application of PGPR for maize grown in the field was limited [11].

Dong Thap is a province in the Mekong River Delta, within the limit of 10°07'-10°58' North latitude and 105°12'-105°56' East. The area has alluvial soil with a pH range of 5.76-7.02 and freshwater where crops grow well. This province has a large maize growing area of 4,800 hectares, identified as the second-largest maize area in the Mekong Delta. However, Farmers often provide maize with a big amount of chemical fertilizers (180 kg N+135 kg P2O5+90 kg K2O/1ha) to get high grain yield (6-8 tons/ha). The fields must be irrigated, sprayed with herbicides, pesticides and especially urea with high prices, so investment costs are high and incomes are low.

The application of native, adapted microorganisms might improve yields by direct plant growth promotion and increasing grain yield, decreasing cost in maize cultivation in order to enhance income for the farmers. The aims of this study were (i) isolation and characterization of rhizospheric and endophytic bacteria (ii) studying characteristic such as nitrogen fixation and IAA production, (iii) the genetic diversity of isolated strains from maize plant and soil was evaluated in order to identify an efficient growth promotion strains that can be also improve the growth of maize plant as biofertilizer.

II. MATERIALS AND METHODS

2.1 Materials

Maize rhizosphere soil samples and plant samples of maize were collected from in 8 sites villages Tan Thuan Tay, Tinh Thoi, Tan Binh Thuong, Tan Long, Bình Thanh, Binh Hoa, Long Khanh, Tan Thuan) of four as Lap Vo, Cao Lanh, Thanh Binh, Hong Ngu districts, Dong Thap province, Vietnam (Figure 1) from 10°34'12" to 10° 80'83" East and 105°28'74'' to 105°67'48" North. The maize plants were sampled at the stage plant growth stage from 30 to 45 days from the fields.

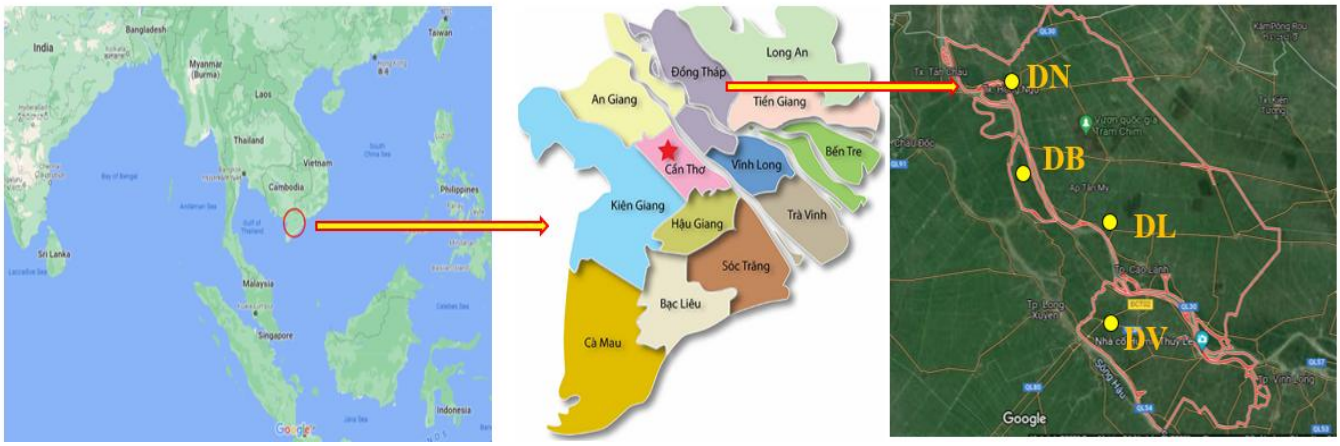

FIGURE 1: The geographic map and location of maize samples and soil were collected at the these sites four districts, Dong thap Province

**** Note* district of s*ymbol DL (Cao Lanh), DB (Thanh Binh), DV (Lap Vo) and DN (Hong Ngu)***

2.2 Methods

2.2.1 Sample collect operation

Samples were taken whole plant with leaf, stem, root (10-20 cm depth) together with soil which around maize plants roots. Collected only samples that were free from pests and diseases. Each site selected 5 maize (4 trees at the base and 1 tree in the middle of the field). Samples were obtained whole plant after that soil rhizosphere was separated for further experiments. Lightly separating muddy soil around the maize roots into plastic bags, labeled (approximately 500 g/sample). Samples were kept in 18°C plastic box before tranferred to laboratory in Can Tho University. Rhizosphere soil around maize plants and leaf, stem, root were of maize plant will be used in an experiment, kept in the refrigerator (5°C), and brought for isolation.

2.2.2 Isolation and culture

Weighed 10 g of soil samples, added 90 mL of sterile distilled water, put into sterile flasks, samples were stirred by magnetic stirrer for 2 hours, let stand for 1 hour, then diluted into decimal range 10^0, 10^{-1}, 10^{-2}, 10^{-3}… Pipetted 50 µl of samples (in each concentration) using a micropipette, dripped on agar plates containing medium without nitrogen mineral (each concentration 3 plates). There are three different types of isolation media: Nfb [12], Burks N-free [13] and LGI [14]. Using a

sterile glass rod spread the sample drops on the surface of medium, covered the plate and standed for a few minutes then turning the disk down, incubated cultural plates at 30^{o}C in incubator.

Samples were obtained whole plant after that soil rhizosphere and separated for further experiments. Maize roots were washed with tap water to remove attached clay; maize leaf, stem and root were cut separately. Subsequently, the stems and roots were immersed in 70% ethanol in 3 min, washed with fresh sodium hypochlorite solution (2.5% available CT) for 5 min, rinsed with 70% ethanol for 30 s and finally washed five times with sterile distilled water. To confirm that the sterilization process was successful, the aliquots of the sterile distilled water used in the final rinse were set on tryptone-yeast extract-glucose agar medium plates. Bacterial growth were examined after incubation at 28^{o}C for 3 days. Maize leafs, stems and roots samples that were not contaminated as detected by culture-dependent sterility test were used for further analysis. Samples (leafs or stems or roots) were cut to 1-2 cm pieces and macerated with a sterile mortar and pestle; tissue extracts were then serially (tenfold dilution) in sterile water, 200 µl-aliquot samples were used to inoculate in (in triplicate) Nitrogen-free semisolid Nfb, LGI, Burks in 5 mL tubes. After 48-72 h of incubation, bacteria growing in tubes as a white or yellow pellicle at a depth of 1 to 4 mm were streaked on Nfb, LGI, Burks agar plates, cultures were streaked on media to obtain single colonies.

Bacterial colonies were differentiated on the basis of colony morphology and pigmentation. This isolation process carries out in shifts of the agar-based culture medium to the agar-based subculture medium until monocultures were obtained. Monocultures were cultured on the agar-based culture medium slant in the test-tube (12 mL) and incubated at 30^{o}C for 4 days following by stored 10^{o}C in refrigerator.

2.2.3 Colony Characteristic and Microscopic Examination

The characteristics of colony such as size, color, shape etc. were presented in each group, cell morphologies of the isolates were observed using an optical microscope.

2.2.4 Screening for Biofertilizer Activities

Nitrogen-fixing bacteria can thrive on cultural medium without nitrogen mineral due to their ability to synthesize ammonium from atmosphere nitrogen. The bacterial strains with capability of growing well were selected and subcultured in liquid Nfb or LGI or Burks medium to measure levels of ammonium in cultural medium by Indophenol Blue method [15] after 2,4 and 6 day inoculation (DAI).

The qualitative detection of indole-3-acetic acid (IAA) production was carried out based on the colorimetric method [16]. Precultures were grown in Nfb or LGI or Burks's N free (100 mL) with 100 mg/L tryptophan in 250mL-flask at 30^{o}C on a rotary shaker at 100 rpm and samples were taken from at 2, 4 and 6 DAI, cell free supernatants were mixed 2:1 with Salkowki reagent (0.01 M FeCl3 in 35% perchloric acid) and incubated in the dark for 20 min at RT. IAA-containing solutions were indicated by reddish color with an absorption peak at 530 nm on Genesys 10uv Thermo Scientific spectrophotometer.

2.2.5 16S rDNA Gene Amplification and Sequencing

Bacterial DNA was isolated following published protocols [17]**.** Amplification of 16S rDNA of rhizosphere soil bacteria by PCR was carried out using the universal primers with primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') [18] and 1492R (5'- TACGGTTACCTTGTTACGACTT-3'). [19] Cyclying condition were as follows: initial denaturation at 95°C for 5 min, 30 cycles of 95°C for 1 min, 53°C for 1 min, and 72°C for 1.5 min, and a final extension of 5 min for 72°C.

Amplification of 16S rDNA of endophytic bacteria by PCR was carried out using primers:

p515FPL (5'-GTGCCAGCAGCCGCGGTAA- 3') [20], p13B (5'-AGGCCCGGGAACGTATTCAC-3',

PCR-1 5' AGTTTGATCCTGGCTCAGGA-3') [21]. The thermocycling profide was carried out with an initial denaturation at 94^{o}C (3 min) fol owed by 30 cycles of denaturation at 94^{o}C (1 min), annealing at 57^{o}C (1 min), extension at 72^{o}C (2 min) and a final polymerization step 72^{o}C (4 min) in C1000 Thermal Cycler (Bio-Rad). Aliquots (10 µl) of PCR products were electrophoresed and visualized in 1% agarose gels using standard electrophoresis procedures.

Partial 16S rRNA genes of selected isolates in each site were sequenced by MACROGEN, Republic of Korea (dna.macrogen.com). Finally, 16S rRNA sequence of the isolate was compared with that of other microorganisms by way

BLAST. Among the best isolates (high ability of nitrogen fixation, phosphate solubilization and IAA synthesis) of 8 sites, 6 isolates were chosen to sequence and were compared to results with sequences of GenBank based on partial 16S rRNA sequences to show relationships between endophytic and rhizosphere strains and phylogenetic tree were constructed by the neighbor-joining method using the MEGA X software version [22] based on 1000 boot straps.

III. RESULTS AND DISCUSSION

3.1 Bacterial Isolation and Colony Characteristic

From 40 maize plant samples and 8 rhizosphere soil samples which were collected from 4 districts in Dong Thap provinces on 3 kinds of medium (Nfb, LGI and Burks). A total of 180 bacterial strains were isolated (Table 1). Seventy bacterial strains were isolated from Nfb medium, 70 ones on LGI and 40 ones on Burks medium. Thirty three strains were isolated from roots (18.33%), 51 isolates from stems (28.33%), 66 isolates from leaf (36.67%) and 30 isolates from soil (16.67%)

TABLE 1
SUMMARY OF BACTERIAL STRAINS RHIZOSPHERE SOIL AND ENDOPHYTE ISOLATED FROM MAIZ

No	Site (districts)	Samples		Number of strains	Symbol of the sample group
		Maize	Soil		
1	Cao Lanh	10	2	40	DL
2	Thanh Binh	10	2	49	DB
3	Lap Vo	10	2	40	BV
4	Hong Ngu	10	2	51	DN
Total		**40**	**8**	**180**	

On semi-solid, isolates of endophytic bacteria all grew and developed under microaerobic conditions and formed an opaque white pellicle far from the medium surface 1- 4 mm after 48 hours).They developed very well on these media from 24 h at 30^{o}C. Colonies had various colors on 3 kinds of medium. Colors of colony as dark white or light white 147/180 colonies, light yellow, pink. Their colonies had round-shape 167/180 colonies, entire 145/180 colonies, emerge 122/180 colonies and glossy surfaced 155/180 colonies, raised on medium (Figure 2). Diameter size of these colonies varied from 0.3 to 4.0 mm after 48 hours (Figure 3). The results of the study demonstrated that endogenous bacteria on maize plants all form opaque white pellicle rings in the culture medium. Nfb medium, after culturing the bacteria for 4 DAI, the growth of endophytic bacteria causes the medium to completely turn blue, light or dark depending on the strain [23].

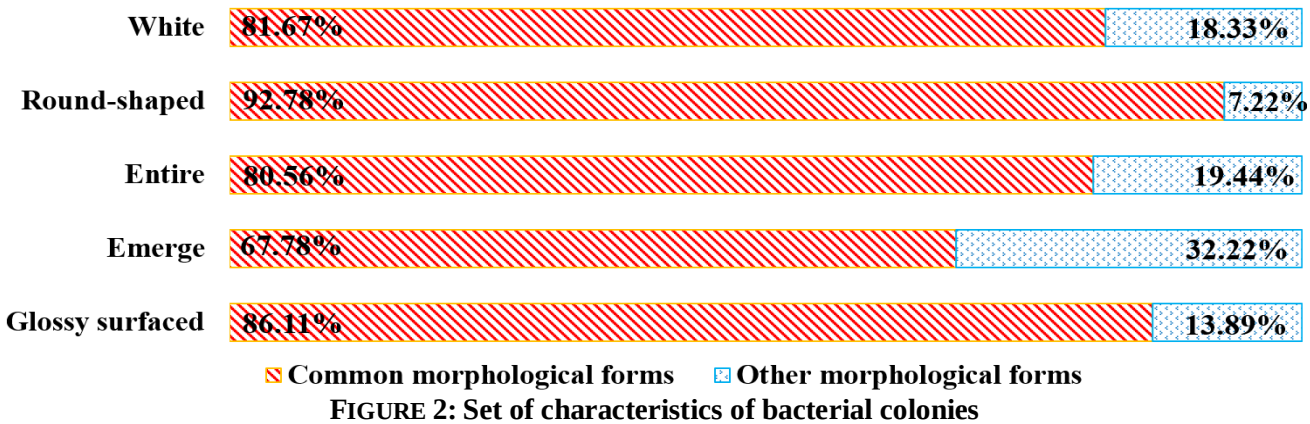

FIGURE 2: Set of characteristics of bacterial colonies

The cells were observed on 3 kinds of medium had short rod, few of long rod and round (Table 2), most of them have motility and Gram- negative by Gram stain.

TABLE 2
SET OF CHARACTERISTICS OF BACTERIAL CELLS

No	Characteristics of cell bacterial	Maize sample	Rate (%)
1	Cell shape	Rod-shaped	76.11
		Round	23.89
2	Motile	Fast	42.22
		Slow	32.78
		Not motile	25.00
3	Gram reaction	Positive	37.22
		Negative	62.78

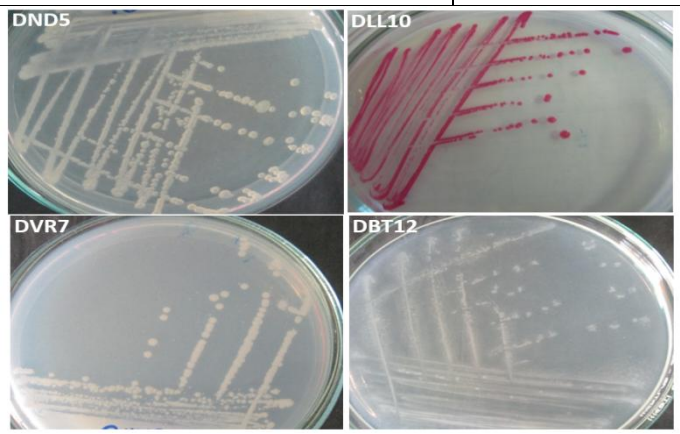

FIGURE 3: The colonies of several rhizosphere soil and endophyte isolate from maize

The results of previous studies reported that rhizosphere soil and endophyte include genus *Azospirillum*, *Pseudomonas*, *Burksholderia*, *Herbaspirillum*, *Gluconacetobacter*, *Enterobacter*, *Klebsiella*,... of Gram-negative, Bacillus of Gram-positive. The cells were observed had short rod, few of long rod *(Bacillus)* and round, have flagella, most of them are motile. These bacteria have also been investigated for their ability to fix nitrogen, dissolve insoluble phosphorus, decompose potassium and some other benefits to plants [24].

3.2 Screening for Biofertilizer Activities

3.2.1 Testing the NH_4^+ synthetic ability of isolated bacterial strains

Among 180 isolated strains, 60 isolates grew strongly on Nfb, LGI, Burks's N-free agar after 24, 48 and 72 hours at 30°C. Include, 20 isolated bacterial strains from Nfb medium, 20 isolated bacterial strains from LGI medium, 20 isolated bacterial strains from Burks medium. The result of the study on NH_4^+ synthesis ability of bacterial strains from different area ecosystem habitats soil and sample maize on 3 type Nfb, LGI, Burks was presented in Table 2. All 60 isolates have produced NH_4^+ and all of them can be amount of NH_4^+ produced from different habitats varied significantly over the time period and was sigNfbicantly different when compared with each other. The NH_4^+ producing capacity of bacterial strains was synthesized very early even after one day of incubation.

Based on the survey results on the ability to synthesize NH_4^+ of 20 bacterial strains isolated on Nfb medium, indicating that all 20 bacterial strains on Nfb medium were capable of synthesizing NH_4^+ (Table 3). By the 2nd day, the NH_4^+ content of all bacterial strains ranged from 0.96-3.61 mg/L. Only 3 DNR5, DLD1, DBT5 bacterial strains had the synthesized NH_4^+ content higher than 3 mg/L, respectively 3.61 mg/L: 3.41 mg/L: 3.32 mg/L, and had a statistically significantly difference from other strains. Bacterial strains produced highly NH_4^+ content at the 4th day and reached the highest level of DNR5 (7.52 mg/L). By the 6th day, the NH_4^+ content of all bacterial strains ranged from 0.88 to 5.78 mg/L. The average results of NH_4^+ concentration synthesized over days 2, 4 and 6 of 20 bacterial strains in Nfb environment ranged from 0.88 to 5.64 mg/L. Two strains, DNR5 (5.64 mg/L) and DBT4 (4.90 mg/L), which had high amount of NH_4^+ were selected for gene sequencing.

TABLE 3
TOTAL NH_4^+ CONCENTRATION IN NFB LIQUID MEDIUM OF 20 ISOLATES WITHIN SIX DAYS OF INCUBATION (n=3 AND STANDARD DEVIATION)

No	Sample	Bacterial isolates	Day 2 (mg/L)	Day 4 (mg/L)	Day 6 (mg/L)	Average value (mg/L)	Site
1	Leaf	DLL1	2.02 e	1.99 h	2.18 d	2.06	Cao Lanh
2		DBL7	1.53 h	1.73 i	0.93 i	1.40	Thanh Binh
3		DVL2	1.31 i	3.53 d	1.72 g	2.19	Lap Vo
4		DNL3	1.36 i	1.58 i	1.02 i	1.32	Hong Ngu
5		DNL5	0.96 j	0.60 k	1.07 hi	0.88	Hong Ngu
6		DNL6	1.59 h	1.98 h	1.26 h	1.61	Hong Ngu
7	Stem	DLT1	1.65 h	2.62 fg	2.08 de	2.12	Cao Lanh
8		DBT4	2.24 d	7.43 a	5.05 b	4.90	Thanh Binh
9		DBT5	3.32 b	3.84 c	1.90 efg	3.02	Thanh Binh
10		DVT1	1.89 efg	2.01 h	2.14 d	2.02	Lap Vo
11		DNT3	1.52 h	1.69 i	1.26 h	1.49	Hong Ngu
12	Root	DLR1	0.98 j	1.15 j	0.88 i	1.00	Cao Lanh
13		DBR2	1.61 h	2.64 fg	2.20 d	2.15	Thanh Binh
14		DVR2	2.62 c	1.69 i	2.03 def	2.11	Lap Vo
15		DNR5	3.61 a	7.52 a	5.78 a	5.64	Hong Ngu
16		DNR6	1.99 ef	2.55 g	2.18 d	2.24	Hong Ngu
17		DNR7	1.86 fg	2.78 ef	1.82 g	2.15	Hong Ngu
18	Soil	DLD1	3.41 b	4.61 b	2.79 c	3.60	Cao Lanh
19		DVD1	1.82 f	2.87 e	1.82 g	2.17	Lap Vo
20		DND1	2.02 g	2.44 g	1.84 fg	2.10	Hong Ngu
CV (%)			**3.29**	**3.36**	**4.42**		

Means within a column followed by the same letter/s are not significantly different at p < 0.01

Twenty bacterial strains isolated on LGI medium were able to synthesize $NH4^+$ but lower than bacterial strains isolated on Nfb medium (Table 4). On the 2nd day, the NH_4^+ content of all bacterial strains ranged from 0.54 to 3.78 mg/L. On the 4th day, the NH_4^+ content of all bacterial strains ranged from 0.46 to 6.00 mg/L. In those strains, 2 strains DNL14 (6.00 mg/L) and DNT5 (5.07 mg/L), had a high NH_4^+ content and a statistically significant difference from the rest of the strains. Similarly, DNL14 and DNT5 with a high NH_4^+ content had a statistically significant difference from the rest of bacterial strains at the 6 day. The average results of $NH4^+$ content synthesized over days 2, 4, and 6 of 20 bacterial strains in LGI medium ranged from 0.78 to 4.47 mg/L. Selecting 2 strains, DNL14 (4.47 mg/L) and DNT5 (4.23 mg/L) with a high amount of NH_4^+, were selected for gene sequencing.

TABLE 4
TOTAL NH_4^+ CONCENTRATION IN LGI LIQUID MEDIUM OF 20 ISOLATES WITHIN SIX DAYS OF INCUBATION (n =3 AND STANDARD DEVIATION)

No	Sample	Bacterial isolates	Day 2 (mg/L)	Day 4 (mg/L)	Day 6 (mg/L)	Average value (mg/L)	Site
1	Leaf	DLL10	1.10 ghi	4.87 c	2.21 f	2.73	Cao Lanh
2		DBL15	1.26 fg	1.26 m	0.73 k	1.08	Thanh Binh
3		DBL18	0.99 hi	3.31 e	1.72 g	2.01	Thanh Binh
4		DVL9	2.13 d	1.98 ij	2.23 ef	2.11	Lap Vo
5		DVL11	0.96 hi	2.64 g	2.43 de	2.01	Lap Vo
6		DNL12	0.72 jk	3.05 f	2.70 c	2.16	Hong Ngu
7		DNL14	2.46 b	6.00 a	4.93 a	4.47	Hong Ngu
8		DNL17	2.19 cd	4.31 d	3.11 b	3.21	Hong Ngu
9	Stem	DLT8	0.67 k	1.57 l	0.72 k	0.99	Cao Lanh
10		DBT11	0.54 k	0.75 n	1.20 ij	0.83	Thanh Binh
11		DVT4	3.78 a	3.47 e	2.24 ef	3.16	Lap Vo
12		DNT5	2.34 bc	5.27 b	5.07 a	4.23	Hong Ngu
13		DNT9	0.74 jk	0.46 o	1.13 j	0.78	Hong Ngu
14	Root	DBR4	2.43 b	4.23 d	2.56 cd	3.07	Thanh Binh
15		DBR6	1.05 hi	1.83 jk	1.48 h	1.45	Thanh Binh
16		DVR5	1.35 f	1.68 kl	1.46 h	1.49	Lap Vo
17		DNR8	1.81 e	2.43 h	1.32 hij	1.85	Hong Ngu
18		DNR10	1.67 e	2.01 ij	1.35 hi	1.68	Hong Ngu
19	Soil	DVD4	0.92 ij	2.06 i	1.25 ij	1.41	Lap Vo
20		DND7	1.16 fgh	1.91 ij	1.20 ij	1.42	Hong Ngu
CV (%)			**5.98**	**3.05**	**4.41**		

Means within a column followed by the same letter/s are not significantly different at p < 0.01.

Twenty bacterial strains isolated in Burks environment were able to synthesize NH_4^+ with different amounts. On the 2nd day, the 2nd day, the NH_4^+ content of all bacterial strains ranged from 0.40 to 4.25 mg/L (Table 5). On the 2nd day, the NH_4^+content of all bacterial strains ranged from 1.03 to 5.76 mg/L. In those, two lines DLR6 (5.76 mg/L) and DND5 (4.87 mg/L) had a high NH_4^+ content and had a statistically significant difference from the rest of the strains. Similarly, DLR6 (5.24 mg/L) and DND5 (3.57 mg/L) with a high NH_4^+ content had a statistically significant difference from the rest of the strains at the 6th day. The average results of the amount of NH_4^+ concentration synthesized over days 2, 4, and 6 of 20 bacterial strains in Burks medium ranged from 0.71 to 4.98 mg/L. Selecting two strains, DLR6 (4.98 mg/L) and DND5 (4.02 mg/L) with produced high amount of NH_4^+, were selected for gene sequencing.

TABLE 5
TOTAL NH_4^+ CONCENTRATION IN BURKS LIQUID MEDIUM OF 20 ISOLATES WITHIN SIX DAYS OF INCUBATION (n =3 AND STANDARD DEVIATION)

No	Sample	Bacterial isolates	Day 2 (mg/L)	Day 4 (mg/L)	Day 6 (mg/L)	Average value (mg/L)	Site
1	Leaf	DLL15	2.46 f	3.93 c	3.16 cd	3.19	Cao Lanh
2		DBL20	0.77 k	1.30 k	0.88 l	0.98	Thanh Binh
3		DNL18	0.99 j	1.09 l	0.76 lm	0.95	Hong Ngu
4		DNL20	0.75 k	1.18 kl	0.83 lm	0.92	Hong Ngu
5	Stem	DBT14	3.82 c	3.25 f	2.99 e	3.35	Thanh Binh
6		DBT15	0.40 m	1.03 l	0.69 m	0.71	Hong Ngu
7		DVT9	4.25 a	3.29 ef	2.14 h	3.23	Hong Ngu
8		DVT10	0.62 l	1.90 i	1.36 j	1.29	Lap Vo
9		DNT10	3.65 d	3.67 d	2.21 gh	3.18	Hong Ngu
10	Root	DLR6	3.95 b	5.76 a	5.24 a	4.98	Cao Lanh
11		DBR8	0.60 l	1.59 j	1.24 jk	1.14	Thanh Binh
12		DVR7	3.24 e	3.43 e	3.25 c	3.31	Lap Vo
13		DNR1	0.97 j	1.28 k	0.80 lm	1.02	Hong Ngu
14		DNR2	3.72 cd	2.99 g	2.31 gh	3.01	Hong Ngu
15		DNR3	3.65 d	2.85 gh	2.71 f	3.07	Hong Ngu
16	Soil	DBD3	1.15 i	1.49 j	1.10 k	1.25	Thanh Binh
17		DBD7	1.83 g	3.85 c	3.04 de	2.91	Thanh Binh
18		DBD8	1.18 i	2.80 h	1.74 i	1.91	Thanh Binh
19		DND4	1.61 h	2.23 i	2.33 g	2.06	Hong Ngu
20		DND5	3.61 d	4.87 b	3.57 b	4.02	Hong Ngu
CV (%)			**2.51**	**2,62**	**3,64**		

Means within a column followed by the same letter/s are not significantly different at p < 0.01.

The results of surveying 60 endogenous bacterial strains of the roots, stems, leaves of maize, and the soil of the maize rhizosphere that synthesized the amounts of NH_4^+ was similar to the results of previous studies in the world [25][26]. Endogenous bacteria at roots had a higher fixing activity than endogenous bacteria in stems and leaves, and the nitrogen fixation capacity of endophytic bacteria strains in the different plant species was different. Isolated of high nitrogen fixation bacteria strains from alkaline soil (PH=7.45-8.22), amounts of the synthesized organic substances ranged from 0.225-1.1%. *Pseudomonas* sp VS2 was 12.02 ppm/mL after 12 days of incubation, Paenibacillus sp VS3 was 10,635 ppm/mL after 9 days of incubation [27]. Bacteria from the maize soil with the highest ability to synthesize NH_4^+ on the 3rd day was 9.30 mg/L, and gradually decreased to 5.70 mg/L on the 5th day [28].

The general trend of bacteria isolated on all 3 medium Nfb, LGI, Burks was that the amount of NH_4^+ synthesized gradually increased to the 4^{th} day and gradually decreased to the 6^{th} day. Because of bacteria growing and increasing biomass over time, the amount of NH_4^+ synthesized gradually increased. On the 4th day, the amount of NH_4^+ synthesized went high. When the amount of NH_4^+ in the medium exceeded the limitation, it inhibited reversedly the bacteria, then the bacteria would use the available NH_4^+ in the medium and do not synthesize any more NH_4^+, so the amount of NH_4^+ surveyed on the 6^{th} day decreased [29].

The second reason is that the nutrient content in the medium was gradually depleted over time, which inhibited the growth of bacterial strains and inhibited the synthesis of NH_4^+. Isolated nitrogen-fixing bacteria from rhizosphere soil and soybean plant parts in Daknong province [24]. Bacteria strains that were grown on nitrogen-free Burks medium and NBRIP liquid media including strains of *Bacillus subtilis*, *Acinetobacter lwoffii*, *Agrobacterium tumefaciens* synthesized NH_4^+ at the highest 3.86 mg/L on the 2nd day after inoculated. Bacterial strains isolated from the rice rhizosphere in Tra Vinh grown on Burks medium synthesized NH_4^+ at the highest 4.65 mg/L on the 4th day after inoculation [30]. Similarly, isolated nitrogen-fixing bacteria strains from oil-contaminated soil at Tuticorin harbor. *Azotobacter chroococcum* strains with nitrogen-fixing capacity reached 4.2 mg/L on the 4th day after inoculation [31]. The research result of nitrogen-fixing bacterial strains were isolated from rice rhizosphere soils in the Mekong Delta on nitrogen-free Burks medium. The bacteria strains synthesized the

average NH_4^+ over days 2, 4, and 6 including *Stenotrophomonas maltophilia* was 1.76 mg/L, *Serratia marcescens* was 1.87 mg/L, *Bacillus megaterium* was 2.21mg/L, *Ideonella* sp. was 3.52 mg/L. [32].

3.2.2 Test of IAA-biosynthesis of bacteria

All 60 isolates have produced IAA from different habitats varied. The IAA producing capacity of bacterial strains was synthesized very early even after one day of incubation. The highest amount of IAA was observed after 6 DAI of incubation. The synthesized IAA content of the bacterial strains varied largely from 0.18 mg/L to 32.64 mg/L mg/L (Table 6). 16/60 isolates had the high IAA biosynthesis than 19 mg/L as DND4 isolate (32.64 mg/L) from Burks medium, DND7 (28.20 mg/L) from LGI medium, DLL1 (27.33 mg/L) from Nfb medium.

TABLE 6
CONCENTRATION OF SYNTHESIZED IAA PRODUCTION OF 60 ISOLATE IN LIQUID MEDIUM WITHIN 6 DAYS OF INCUBATION (n =3, STANDARD DEVIATION)

No	Isolate number were isolated from Nfb			Isolate number were isolated from LGI medium			Isolate number were isolated from Burks		
	Sample	Isolate	IAA (mg/L)	Sample	Isolate	IAA (mg/L)	Sample	Isolate	IAA (mg/L)
1	Leaf	DLL1	27.33 a	Leaf	DLL10	10.70 h	Leaf	DLL15	6.13 i
2		DBL7	21.02 c		DBL15	0.18 n		DBL20	21.18 c
3		DVL2	3.67 j		DBL18	7.43 i		DNL18	13.35 e
4		DNL3	19.43 d		DVL9	5.52 jk		DNL20	1.44 l
5		DNL5	21.28 c		DVL11	6.39 ij	Stem	DBT14	16.13 d
6		DNL6	2.57 k		DNL12	4.32 kl		DBT15	11.31 f
7	Stem	DLT1	1.41 l		DNL14	20.76 d		DVT9	1.70 l
8		DBT4	19.89 d		DNL17	4.42 kl		DVT10	1.18 l
9		DBT5	4.81 i	Stem	DLT8	3.80 l		DNT10	9.60 g
10		DVT1	2.35 k		DBT11	12.51 g	Root	DLR6	21.18 c
11		DNT3	2.06 kl		DVT4	5.84 j		DBR8	4.45 j
12	Root	DLR1	5.71 h		DNT5	22.73 c		DVR7	6.42 i
13		DBR2	18.62 e		DNT9	26.59 b		DNR1	7.46 h
14		DVR2	18.14 e	Root	DBR4	19.17 e		DNR2	2.70 k
15		DNR5	25.29 b		DBR6	27.01 ab		DNR3	3.74 j
16		DNR6	5.36 hi		DVR5	5.78 j	Soil	DBD3	9.34 g
17		DNR7	6.91 g		DNR8	0.92 mn		DBD7	7.56 h
18	Soil	DLD1	8.56 f		DNR10	1.63 m		DBD8	2.02 kl
19		DVD1	7.43 g	Soil	DVD4	17.65 f		DND4	32.64 a
20		DND1	8.43 f		DND7	28.20 a		DND5	25.32 b
CV (%)			**2.62**			**5.03**			**4.24**

Means within a column followed by the same letter/s are not significantly different at p < 0.01

Auxins play a cardinal role in coordination of many growth and behavioral processes in plant life cycles and are essential for plant body development [33]. In addition to endogenous IAA, plant growth is affected by a low amount of auxin outside of the plant from a IAA synthesis of microorganism. Moreover, several recent reports indicate that IAA can also be a signaling molecule in bacteria and therefore can have a direct effect on bacterial physiology [34]. These indigenous colonizers reside in almost all internal tissues/cells of plant ranging from tissues of the roots to stem, leaf, flower, fruit and seed [35]. The fact that bacteria use this phytohormone IAA to interact with plants as part of their colonization strategy, including phytostimulation and circumvention of basal plant defense mechanisms [36]. Different bacterial isolates biosynthesize IAA at differently quantities [37]. There are many studies on the ability to synthesize IAA of endophytic and rhizobacteria to help plant growth. The presence of endophytic and rhizosphere bacterial strains isolated from IAA-synthetic rice increased the

size and weight of rice roots [38]. IAA promote plant growth through the improved cycling of nutrients and minerals such as nitrogen, phosphate and other nutrients [39]. Over 73% of the bacteria isolated from the rhizosphere are capable to synthesize IAA [40].

All strains of endophytic bacteria of root rice having IAA product. In which, there are 2 strains of *Pseudomonas* sp. RE1 and RE17 produced high amount of IAA. Using ELISA based studies in the presence of maize root exudates in growth chamber study, it was revealed that strain RE1 and RE17 inoculated into germinated maize kernels resulted in an increase in the amount of IAA produced from the roots by 2.8 pmol/mL and 3.4 pmol/mL respectively compared with the uninoculated control by 0.2 pmol/mL [41]. Strains Bacillus sp. (br1, br3, wr2) và Lactobacillus sp. (br2, mr2) were successfully isolated from wheat and maize rhizosphere. The concentration of IAA increased in medium with or without tryptophan Bacillus sp. to 60µg/mL after 120 min of inoculation [42]. The IAA synthesis capacity reached 24.8 mg/L at day 4 after inoculation. Four bacterial strains Ha21, Ha22, Ha23 and Ha30 synthesized high auxin including IAA and IAM, in which Ha22 was the highest at 66.3 mg/kg at normal conditions, resulting in maximum growth and yield of wheat [40]. Bacteria group of innate microbial consortium that inhabits the soil and the surfaces of all living things had the highest ability to synthesize IAA on the 3rd day was 48.45 mg/L, gradually decreased to the 6th day at 23.81 mg/L [28]. The results of the experiment proved that 60 strains of endophytic and rhizosphere bacteria of maize are capable of synthesizing IAA similar to previous studies. In which, 16/60 potential bacterial strains synthesize high amount of IAA.

3.3 Identification and construction of the phylogenetic tree bacteria having high ability of NH4+ and IAA biosynthesis.

Six good bacterial isolates: DNL14, DNT5, DBT4, DNR5, DLR6, DND5 were selected to PCR and sequencing. The result was presented in (Table 7).

TABLE 7
PHYLOGENETIC AFFILIATION OF ISOLATES ON THE BASIS OF 16S rRNA GENES SEQUENCES BY USING BLAST PROGRAMME IN THE GENBANK DATABASE BASED ON SEQUENCE SIMILARITY

Sample	Symbol of isolate	Closest species relative	Gene length (nu)	Similarity (%)	NCBI Number
Leaf	DNL14	*Enterobacter ludwigii strain AA1*	2235	97	MT613360.1
Stem	DNT5	*Enterobacter kobei strain BLR45*	2150	97	MW624688.1
	DBT4	*Bacillus pumilus strain NSB-10*	2257	97	KR010180.1
Root	DNR5	*Klebsiella pneumoniae strain BB-301*	2230	99	MN844878.1
	DLR6	*Lactobacillus plantarum*	1428	94	KJ690749.1
Soil	DND5	*Pseudomonas nitroreducens*	2239	99	KY292456.1

The result of identifying the nitrogen-fixing bacteria presented in table showed a total of 6 isolated bacteria strains, in which 5 strains were isolated from leaves, stem, maize root, and a strain isolated from maize root-soil. This shows that the ability to isolate many endogenous high nitrogen-fixing bacteria in maize will be higher than bacteria living in the maize root soil (Table 7). On the other hand, Six nitrogen-fixing bacteria strain isolated belong to 2 classes, including Gammaproteobacteria (4 species): *Enterobacter* (2 strains), *Pseudomonas, Klebsiella* and Bacilli (2 species) including *Bacillus* and *Lactobacillus.* They were classified to Bacilli (20%) and Gammaproteobacteria (80%) (Figure 4). This result demonstrates that there are a very high diversity of bacteria isolated from the maize, from the different positions in the root-soil and other parts of maize.

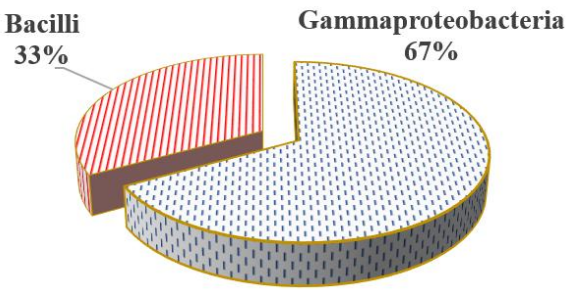

FIGURE 4: The ratio of 2 classes distributed of nitrogen-fixing bacteria strains

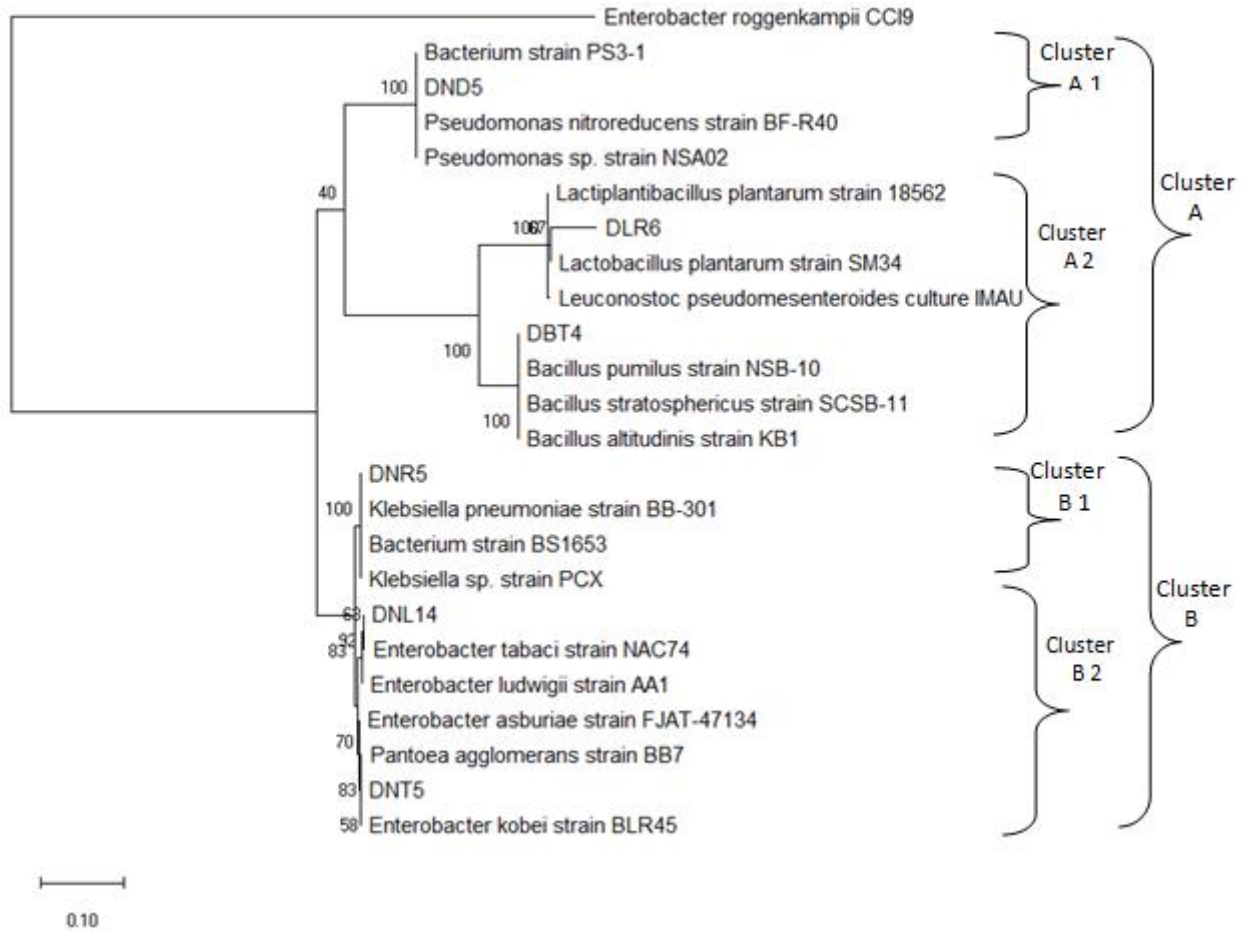

FIGURE 5: Phylogenetic tree showing the relative position of bacteria by the Maximum Likehood method of complete 16S rRNA sequences

The determination of nearest phylogenetic neighbor sequences for 16S rRNA gene sequence of the 6 isolates by the BLAST search program showed that they grouped into two clusters (Figure 5). Cluster A composed of two clusters: A1 cluster consisted of the strains DND5 closely related to *Pseudomonas* bacteria. Cluster A2 with strain DLR6 is closely related to *lactobacillus* and DBT4 closely related to *Bacillus* which originated from soil, stem and leaf. This showed that 3 strains had relationship closely eventhrough they were isolated from 3 regions districts far from 90 km. Cluster B is composed of two clusters: B1 cluster consisted of the strains DNR5 closely related to *Klebsiella*. While cluster B2 has consisted of two strains (DNL14, DNT5) closely related to Enterobacter which is endophytic bacteria.

The sequences 16S rRNA of bacterial strains in different plant species are also different for example in rice [26] and maize [43]. In addition, in different parts of the same plant species, the endogenous bacterial species and nitrogen fixation capacity are also different. These bacterial classes showed a fairly good ability of nitrogen fixation, according to previously published studies [44], [45], [46]. *Pseudomonas niethanitrificans* bacteria use the energy source of methane to synthesize NH_4^+, averaging to reach 70 mg/L after 2 months of inoculation [47]. *Enterobacter* sp. FD17 fixed nitrogen synthesis NH_4^+, synthesizing IAA reaches 12.30 mg/L. The results promote the weight of shoots, roots, amount of leaves, leaf size, and the increase in maize yield by 42% as compared to the non-inoculated control [48]. Bacterial strains isolated from the maize root-soil of transgenic maize and non-transgenic maize grown in the field in South Africa including *Bacillus, Pseudomonas, Aeromonas, Sphingomonas, Burksholderia, Stenotrophomonas, Achromobacter, Ewingella*, both synthesizing ammonium and producing IAA [49]. Similarly, bacterial strains *Klebsiella pneumoniae* 2028 and *Klebsiella pneumoniae* 342 endogenously living in the roots of maize or the maize root-soil do synthesize NH_4^+ to supply maize plants [50]. The results of study are similar to the study of endogenous bacteria and rhizosphere soil in the world.

IV. CONCLUSION

From 40 cultivated maize samples and 8 soil-root samples of districts in Dong Thap Province, Vietnam, 180 isolates were isolated and identified as nitrogen-fixing rhizospheric and endophytic bacteria and 60 isolates having good plant growth

promotion. Six bacteria strains as DNL14, DNT5, DNR5, DND5, DLR6, DBT4, were chosen to analyze their relationship. These strains should be tested in pot and in the field experiments in order to confirm their capacity to improve maize yields and soil fertility of this region.

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Rapid Micropropagation of Sapium Sebiferum using Segments with Axillary Bud for Forest Bioenergy

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Abstract— *Sapium sebiferum is one of the four woody oil tree species in China for bioenergy forest. Its seeds have high oil content and are widely used. It is an excellent tree species that can be cultivated as a renewable energy source. An experiment was conducted to study culture in vitro and rapid propagation of S. sebiferum using segments with axillary bud. The results showed that the survival rate could be reached to 50.0% when the explants were disinfected with 70% ethanol for 30 seconds and with 0.1% $HgCl_2$ for 10 minutes. In induction culture, the lateral buds began to sprout about 5 days later, and after 20 days, the buds gradually differentiated. Among the three basic mediums, WPM medium has the highest effective bud induction rate, the effect of 6-benzyladenine (6-BA) mass concentration on axillary bud induction rate is extremely significant. The optimal medium WPM +6-BA 0.5mg/L+ NAA 0.05mg/L, conducting bud induction, and the induction rate could reach 62.9% after 30 days. Multiplication culture was carried out in the medium MS + 6-BA 1.0 mg/L +NAA 0.5 mg/L+ KT 0.5 mg/L, the proliferation effect was the best, with the multiplication rate of 81.2% and proliferation multiple of 3.2. The results of variance analysis showed that Naphthalene acetic acid (NAA) is the main factor affecting bud proliferation, followed by 6-BA. The rooting rate was up to 93.3% after induction in the medium 1/2 MS+ IBA 0.5mg/L. The establishment of rapid propagation technology system through tissue culture is an important way to solve the shortage of high-quality seedlings and provide technical support for the establishment of large scale S. sebiferum energy forest.*

Keywords— Sapium sebiferum; Bioenergy; Stem section; Culture in vitro; Rapid propagation.

I. INTRODUCTION

Sapium sebiferum (Roxb.) is a deciduous tree of Sapium in Euphorbiaceae , also known as wood wax tree. S. sebiferum is a drought- tolerant, short-term water-tolerant tree, There is a cultivation history of more than 1400 years in China (Editorial committee of Chinese flora 1980). It is one of the four major woody oil-bearing plants with strong cultivation adaptability, high oil content and wide use. It is an important economic bioenergy tree in China (Li et al. 2011). S. sebiferum is an excellent afforestation tree for timber forest and shelter forest in China with fast growth and long life. Especially as a scenic forest, it has a tall crown and different colors in four seasons, which is of great ornamental value. The root bark, bark and leaves of *Sapium sebiferum* are important raw materials of traditional Chinese medicine for the treatment of cirrhosis ascites, eczema and dermatitis. The leaf phenol extract has many effects, such as antioxidant, anti-inflammatory and anti ACD (Fu et al. 2015) (allergic contact dermatitis). The seed is covered with a layer of wax skin, which can be used to extract sapium fat (also known as Chinese (vegetable) tallow); the kernel can be extracted sapium oil (Li and Liu 2011). 100 kg seeds can be extracted 23 to 25 kg of sapium fat and 16 to 18kg of sapium oil. The well-operated sapium forest, the oil yield of seeds is as high as 40%, higher than that of Camellia oleifera , and it is also higher than Elaeis guineensis Jacq known as "oil king". The oil extracted from the seeds can be used not only as an edible oil source or an industrial oil source, but also as a power oil source, and is an inexhaustible source of renewable oil(Li et al. 2011; Shi and Guo 2001; Tan 2013; Li and Xu 2006; Peng 2013). With the deepening of biodiesel research and the accelerated development of biomass energy forestry industrialization, As a kind of high oil content substance, *S. sebiferu*m seeds have a quite considerable prospect to be converted into biodiesel. Biodiesel is a typical “green energy” with the advantages of extremely low sulfur content, low aromatic hydrocarbon content, high oxygen content, high cetane content, and low exhaust gas emission(Wu et al. 2011; Helwani et al. 2009; Chen 2006; Campanelli et al. 2010; Hayyan and Mjalli 2010). The importance of wood biomass energy has been raised many times (Venketeswaran et al. 1983; Bajaj 1986).

Therefore, it is necessary to carry out the research on the breeding, the rapid propagation, cytochemical composition, secondary metabolites, and the comprehensive utilization technology of seed production biodiesel, which is of great significance to alleviate the energy shortage, improve the ecological environment, and promote the adjustment of rural industrial structure. The establishment of *S. sebiferum* energy forest requires a large number of high-quality seedlings. Compared with traditional propagation methods (such as seedling planting or grafting), this method can produce plant materials for breeding or other purposes with less labor and lower cost in a shorter time (Bommineni et al. 2001). The establishment of rapid propagation technology system through tissue culture is an important way to solve the shortage of high quality seedlings. With regard to tissue culture, previous researcher used callus to regenerate the plants in vitro (Venketeswaran and Gandhi 1982), and performed in vitro propagation by means of bud proliferation from nodal explants (Siril and Dhar 1997). In this study, stem segments of S. sebiferum were cultured in vitro, and its high-quality seedlings rapid propagation technology system was studied to provide technical support for the establishment of large-scale S. sebiferum energy forest.

II. MATERIALS AND METHODS

The test materials were collected from *S. sebiferum* plantation of Huazhong Agricultural University. The semi lignified branches on the healthy, vigorous and disease-free plants with a diameter of 0.2-0.4cm were selected.

Primary culture: The inoculated explants were stem segments with buds. Remove the leaves from the branches indoors and wash them with tap water several times. After soaking in 1% washing powder water for 10 minutes, the surface of the explants was cleaned with a brush, and then washed with water for 2 hours.

In aseptic condition, 70% alcohol was used to disinfect for 30 s, then 0.1% $HgCl_2$ was used to disinfect at different minutes (5, 8, 10, 12). After 4 rinses with sterile distilled water, the stem segment with axillary bud was cut into 1-2 cm, each stem segment has one or two buds, which was used to select the optimal conditions for disinfection. Each treatment was repeated 3 times, with 16 explants.

Selection of optimal inducement conditions: The basic medium was MS, WPM and B5, and the growth regulators were 6-BA (0.5, 1.0, 2.0) and NAA (0.05, 0.1, 0), 6-BA and NAA are mass concentration (mg/L). The orthogonal experiment was carried out with 3 factors and 3 levels of basic medium. There are 9 treatments in total; each treatment was repeated 3 times, with 30 explants.

Subculture: After 30 days of primary culture, the induced buds were cut and inoculated on subculture medium, observe their proliferation and make statistical analysis. In the subculture, MS was used as the basic medium, and the orthogonal experiment was carried out at the level of 6-BA (0.5, 1.0, 2.0) mass concentration, KT (0.1, 0.5, 1.0) mass concentration and NAA (0.1, 0.5, 0) mass concentration. Each treatment was inoculated with 30 explants, and each treatment was repeated 3 times. There are 9 treatments in total.

Induction rate = (number of induced explants / total number of inoculated explants) × 100%.

Rooting culture: Inoculate the healthy shoots obtained from Subculture on rooting medium for root induction. The rooting was counted after 30 days. 1 / 2MS were used as the basic medium for rooting induction, and 3 treatments were carried out with different mass concentrations of IBA (0.1, 0.5, 1.0). Each treatment was inoculated with 15 explants, and each treatment was repeated 3 times.

The culture conditions, unless otherwise specified, are (25 ± 2) ℃(degree centigrade), 14h / d, 1500-2000lx. For each medium, 0.7% agar and 3% sucrose (1.5% for rooting medium) were added. The pH of the medium is 5.8-6.0, the growth regulators units are mg/L.

Data analysis: SAS 9.4 and EXCEL2003 are used for data statistics, analysis of variance and multiple comparisons.

Data availability: The manuscript includes all data that support the findings of the present study.

III. RESULTS AND ANALYSIS

3.1 Effect of different treatment time of 0.1% $HgCl_2$ on pollution rate of explants.

In aseptic condition, *S. sebiferum* explant was sterilized with 70% alcohol for 30s, and then sterilized with 0.1% $HgCl_2$ for 5, 8, 10 and 12 minutes, than the pollution rate and survival rate was counted after 2 weeks (Table 1). With the increase of 0.1% mercuric chloride treatment time, the pollution rate decreased, but the germination rate also decreased. This show that the

treatment time of 0.1% $HgCl_2$ is increased, which is beneficial to reduce pollution; when the treatment time exceeds a certain limit, it will cause serious damage to plant tissue cells, so it should be considered comprehensively according to the survival rate and pollution rate. In terms of *S. sebiferum* explants, 0.1% $HgCl_2$ treatment for 10 minutes is the best sterilization method, the pollution rate can be reduced to 26.5%, and the survival rate can reach 50.0%.

TABLE 1
EFFECT OF DIFFERENT TREATMENT TIME OF 0.1% HgCl ON POLLUTION RATE OF *S. sebiferum* EXPLANTS

Treatment time/min	Pollution rate%	Browning mortality%	Survival rate%
5	46.8	15.6	37.6
8	28.6	21.4	50.0
10	26.5	23.5	50.0
12	25.8	25.8	48.4

3.2 Effects of different hormone combinations on axillary bud induction of *S. sebiferum*.

The stem segment of *S. sebiferum* was inoculated in the medium, and the lateral buds began to sprout about 5 days later (Fig.1-A). At the same time, a small number of stem base began to expand, and buds appeared. After 15 days, the lateral buds opened, and after 20 days, the buds gradually differentiated (Fig.1-B). The statistical results of the effects of different hormone combinations on the axillary bud induction of *S. sebiferum* are shown in Table 2. The effective bud induction rate of stem segment with axillary bud in medium 4 was significantly higher than that of other treatments, and the induction rate was 62. 9%

The axillary buds can be activated as early as 3 days. Medium 1 and medium 5 were also effective, the induction rates were 44.4% and 45.7% respectively. Treatment 9 was significantly lower than other treatments, only 28.6%, and could be activated in 5-7 days.

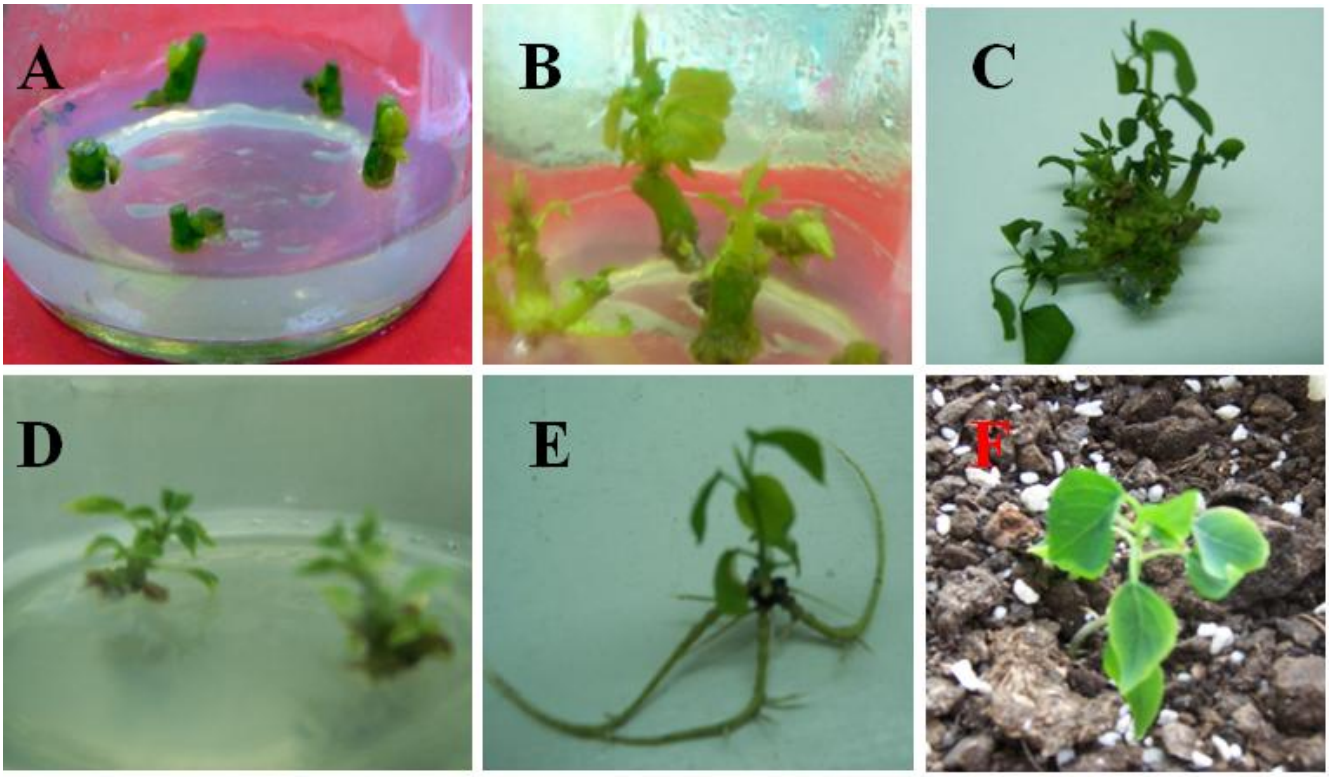

FIGURE 1: Growth of *S. sebiferum*in different stages of tissue culture. A: the buds are beginning to sprout after 5 days. B: The axillary buds grew well after 20 days in suitable medium. C: The multiple shoots induced from the buds in after 15 days, the optimal formula of culture medium of *S. sebiferum* bud proliferation is: MS + 6-BA1.0mg/L+KT 0.5mg/L + NAA0.5mg/L. D: Root culture of single seedling. E: The average root length was 2.5cm in the medium 1/2MS+0.5mg/L IBA F:Transplanted seedlings survived after 25 days.

TABLE 2
EFFECT OF DIFFERENT MEDIUM ON THE EFFECTIVE BUD INDUCTION RATE OF STEM SEGMENT WITH AXILLARY BUD

Treatment number	medium	Hormone mg/L		Induction rate%	Activated days/d
		6-BA	NAA		
1	MS	0.5	0	44.4	4-6
2	MS	1.0	0.05	40.7	4-6
3	MS	2.0	0.10	33.3	4-6
4	WPM	0.5	0.05	62.9	3-5
5	WPM	1.0	0.10	45.7	3-5
6	WPM	2.0	0	42.9	4-6
7	B5	0.5	0.10	35.5	4-7
8	B5	1.0	0	32.1	5-7
9	B5	2.0	0.05	28.6	5-7

Analysis of variance showed that among the basic media, $P < 0.0001$, its F value was 16.89, far greater than $F_{0.01}$ (2, 20) = 5.85; among the Ba mass concentration, $P < 0.0027$, its F value was 8.08, far greater than $F_{0.01}$ (2, 20) = 5.85; among the NAA mass concentration, $P < 0.1877$, its F value was 1.82, less than $F_{0.05}$ (2, 20) = 3.49. It can be seen that the effect of NAA concentration on axillary bud induction rate is not significant. The effect of basic medium and 6-BA concentration on axillary bud induction rate is extremely significant. Among the three basic media, WPM medium has the highest effective bud induction rate, which reflects that WPM medium is conducive to the induction and growth of axillary buds of woody plants.

There is significant difference in axillary bud induction rate among MS, WPM and B5 medium levels (Table 3). The average induction rate was 51.50% at WPM medium. When the mass concentration (MC) of 6-BA was 0.5mg/L, the average induction rate was 47.60%, which was significantly higher than the other two levels. and there was no significant difference between the concentrations of 1.0 and 2.0mg/L. So, 0.5mg/L 6-BA has the best induction effect on the stem segment of *S. sebiferum*. There was no significant difference in NAA concentrations. However, when the mass concentration of NAA is 0.05mg/L, the average induction rate is 44.07%, which is better than the other two levels. Therefore, the most favorable medium combination for effective bud induction is WPM + 6-BA0.5mg/L + NAA0.05mg/L.

TABLE 3
MULTIPLE COMPARISON OF EFFECTIVE BUD INDUCTION OF STEM SEGMENT WITH AXILLARY BUD

Medium	Average rate of induction	6-BA		NAA	
		Mc mg/L	Average%	Mc mg/L	Average%
MS	39.47[b]	0.5	47.60a	0	39.80a
WPM	51.50[a]	1.0	39.50b	0.05	44.07a
B5	32.07[c]	2.0	34,93b	0.10	38.17a

3.3 The effect of different hormone combinations on the axillary bud proliferation of *S. sebiferum.*

In the subsequent generation proliferation test, the cut shoots were cultured for proliferation (Fig.1-C), the growth of the shoots was observed and proliferation was counted after 25 days, as shown in Table 4. the proliferation rate of medium 3 and 5 is good, 78.5% and 81.2% respectively, and their multiplication times 2.7 and 3.2 are also higher than other medium,

The worst medium 9, only 51.3% of buds proliferated, and the proliferation coefficient was only 2.1. The results of variance analysis showed that 6-BA 、KT (Kinten) and NAA concentration had a significant effect on bud proliferation rate, F value was 11.83, 9.16 and 38.32 respectively, P value was less than 0.0004, 0.0015 and 0.0001 respectively. It can be seen from the F values of 6-BA, KT and NAA that NAA is the main factor affecting bud proliferation, followed by 6-BA.

TABLE 4
RELATIONSHIP BETWEEN AXILLARY BUD PROLIFERATION RATE, PROLIFERATION COEFFICIENT AND HORMONE RATIO IN SUBCULTURE

Treatment	Hormone ratio mg/ L			Proliferation rate %	Proliferation coefficient%
	6-BA	KT	NAA		
1	0.5	0.1	0	52.3	2.1
2	0.5	0.5	0.1	51.4	2.0
3	0.5	1.0	0.5	78.5	2.7
4	1.0	0.1	0.1	54.9	2.2
5	1.0	0.5	0.5	81.2	3.2
6	1.0	1.0	0	72.4	2.5
7	2.0	0.1	0.5	63.5	2.4
8	2.0	0.5	0	58.7	2.2
9	2.0	1.0	0.1	51.3	2.1

Table 5 showed the multiple comparison results of the effects of 6-BA, KT and NAA concentration on the proliferation rate of bud proliferation culture. When the concentration of 6-BA was 1.0mg/L, the average proliferation rate was 69.50%, which was significantly higher than the other two levels. When the concentration of KT is 0.5 and 1.0mg/L, it is also significantly higher than 0.1mg/L, but not between 0.5 and 1.0mg/L. Considering the high price of KT, 0.5mg/L is the best. The average proliferation rate was 74.33% when the concentration of NAA was 0.5mg/L.

TABLE 5
MULTIPLE COMPARISON OF THE EFFECTS OF 6-BA, KT AND NAA ON THE PROLIFERATION RATE OF BUD PROLIFERATION CULTURE

6-BA		KT		NAA	
MC mg/L	Average%	MC mg/L	Average%	MC mg/L	Average%
0.5	60.73b	0.1	56.83b	0	61.13b
1.0	69.50a	0.5	63.77a	0.1	52.53c
2.0	57.77b	1.0	67.40a	0.5	74.33a

The analysis of variance of multiplication coefficient of shoot proliferation culture showed that there was no significant difference between the three factors of 6-BA, KT and NAA concentration on the multiplication coefficient of *S. sebiferum* shoot (table omitted), which indicated that the three levels of 6-BA, KT and naa3 had little effect on the multiplication coefficient. Therefore, considering the proliferation rate, the most favorable medium combination was MS + 6-BA 1.0mg/L+KT 0.5mg/L + NAA 0.5mg/L.

3.4 The effect of IBA concentration on the rooting of *S. sebiferum*.

This experiment used 1/2MS medium with a large number of elements halved, selected IBA as exogenous hormone, inoculated about 1.5 cm stems in rooting medium, cultured under light. Generally, about 10 days, the base of the stem was swollen, and white root buds can be observed. After 30 days, the rooting rate, rooting multiple and rooting length were counted (Fig.1-D). As shown in table 6, it was found that the mass concentration of IBA had a great influence on the rooting. The rooting rate and rooting number of the 3 treatments were significantly different. Among them, the mass concentration of IBA was 0.5mg/L, the highest rooting rate was 93.3%; the average rooting multiple was 3.2, and a few bases produced callus. When the IBA mass concentration was 1.0 mg/L, the base was prone to produce a large number of callus, only straight roots, and thick, and some pseudo roots (invalid roots). Treatment 3, the rooting rate was relatively low. It can be seen that in the tissue rooting process of *S. sebiferum*, the IBA mass concentration is better with 0.5mg/L (Fig.1-E).

TABLE 6
EFFECT OF IBA ON ROOTING OF *S. sebiferum*

NO.	MC mg/L	Rooting rate %	Rooting multiple	root length
1	0.1	84.4Bb	2.7b	1.7b
2	0.5	93.3Aa	3.2a	2.5a
3	1.0	71.8Bc	1.8c	2.0b

3.5 Transplanting of rooting seedlings for sterile seedlings with strong rooting growth.

The sealing film on the bottle will be unraveled first, and the sealing film will not be removed. After 1 day, the sealing film will be completely removed and opened for 1-2 d; then, the seedlings were taken out and the medium on the roots was washed. The roots were further immersed in 0.2% carbendazim solution for 3 minutes and then transplanted into a seedling substrate of m (river sand): m (nutrient soil) = 1:1. In the environment where the temperature was about 25 ℃ and the humidity was maintained at 90% or more, the survival rate was 83.3% at 30 days (Fig.1-F).

IV. CONCLUSIONS AND DISCUSSION

The modernization of plant biotechnology began at the beginning of the 20th century, Plant tissue cell culture and regeneration, and cloning of new plants. After that, through molecular breeding to modify their genetic characteristics, including maker-assisted selection, genetic transformation and gene editing, there are likely to be more new programs in the future (Altman 2019). However, as a basic technology, tissue culture has made a great contribution. Among them, micropropagation technology has the advantages of rapid propagation, maintaining in vitro activity and protecting germplasm resources (Amirchakhmaghi et al. 2019). Just to solve the problem that some woody plants are difficult to be sowed or cut propagation and the vitality is easy to degenerate. Difficulty in sowing or cutting propagation.

During the cultivation of *S. sebiferum* stem as explant, the pollution is more serious. After 30 s of disinfection with 70% alcohol and 10 minutes of sterilization with 0.1% mercuric chloride, the pollution rate is still high, which may be related to endophytic bacteria. In many woody plant species, especially in the wild, bacterial contamination is particularly difficult to treat and detect, because bacterial infection is large and often cannot be accurately identified and disposed in plant tissues (Viss et al.1991; Buckley et al. 1995). Antibiotics can be added to the culture medium to prevent the pollution of stem culture, which may needs further study. Ascorbic Acid (VC), Polyviny Pyrrolidone (PVP) or Sodium thiosulfate pentahydrate (SH) can be added to the medium optionally to control browning.

The success of tree tissue culture system largely depends on the selection of medium (Liu et al. 2017). WPM medium is conducive to the induction and growth of axillary buds of woody plants. It has been reported that the possibility of variation in vitro is much lower through axillary bud and adventitious bud as explants (Sharma et al. 2007; Shenoy and Vasil 1992). In this experiment, stem segment with buds of Sapium sebiferum was used as explant, and buds were induced in medium of WPM + 6-BA0.5mg/L + NAA 0.05mg/L. The induction rate was 62.9% after 30 days. In the 3 levels of 6-BA concentration , when the concentration was 0.5mg/L, the average induction rate was 47.60%, significantly higher than the other two levels, it is also confirmed that 6-BA, at high concentration can stimulate shoot multiplication and inhibit their elongation (Lee and Wetzstein 1990; Jaime and Teixeira 2003). The variety and combination of growth regulators are more comprehensive, which is beneficial to screening of culture conditions conductively. Multiple analysis also be more scientific selection.

In the process of proliferation, the medium of MS + 6-BA1.0 mg/L + KT 0.5mg/L + NAA0.5mg/L had the best proliferation effect, and the multiplication multiple was up to 3.2. IBA is the most commonly used for rooting induction (Keresa et al. 2012; Xu et al. 2008). Single bud was inoculated on 1/2MS + IBA 0.5mg/L medium for rooting induction, with the best effect and the rooting rate of 93.3%. These results showed that the concentration of hormones used in the medium significantly affected the regeneration mode and differentiation type of the culture. In our study, axillary bud stem segment was used as explants, there is recent scientific study (Hou 2020) on the establishment of in vitro plant regeneration systems directly derived from leaves and petioles of *S. sebiferum*, and adventitious bud induction rate is very high. This study has completed the study of aseptic culture of tissues, and the next step is the study of cell suspension culture and secondary metabolites, which is devoted to the identification of chemical components and pharmacological analysis of cell products, and in the hope of contributing to human health and forest bioenergy.

LIST OF ABBREVIATIONS

Abbreviation	Full name
6-BA	6-benzyladenine
NAA	Naphthalene acetic acid
KT	Kinten
$HgCl_2$	Mercuric Chloride

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