Journal of Food and Agriculture
DOI: http://dx.doi.org/xxxxxxxxxxx

Detection of Viruses Infecting Lettuce (*Lactuca sativa* L.) in the Derived Savanna of Nigeria and Influence on Proximate Composition

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ABSTRACT

A survey was accomplished in the derived savanna of Oyo State, Nigeria during 2019/2020 cropping season to evaluate the incidence and severity of viruses infecting Lettuce. Laboratory experiments were performed serologically to identify the infecting viruses and to verify possible variations in the proximate composition. A total of 100 symptomatic and asymptomatic lettuce leaves were collected from different fields across the study area. Fifty of the leaves which were representative of the locations were then subjected to Antigen Coat Plate (ACP) and Double Antigen Sandwich Enzyme Linked Immunosorbent Assay (DAS ELISA) using specific polyclonal antisera against Cucumber mosaic virus (CMV), broad spectrum potyvirus, Lettuce mosaic virus (LMV) and Lettuce virus X (LeVX). The 50 other samples were subjected to proximate analysis. The incidence of virus infection varied across survey locations from 7.14% to 39.22% with a severity range from 10.33% to 41.32%. ELISA indicated single or multiple virus infection of CMV, LMV and/or Potyvirus while LeVX was not detected in the study. Proximate analysis indicated that the chlorophyll content of infected plants (0.6) was significantly lower than healthy plants (1.4). The total carbohydrate content was also significantly higher in healthy plants (13.8) compared to infected plants (7.7). This study is the first report confirming the infection of Cucumber mosaic virus, Lettuce mosaic virus and Potyvirus in lettuce in the selected agroecological zone. To enhance growth and quality of nutrients derived from lettuce, plant-virus management strategies should be adopted coupled with periodic virus surveys to detect newly emerging virus strains.

Keywords: ELISA, Polyclonal antisera, Nutrient content, Vegetables, Virus strains.

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INTRODUCTION

Leafy green vegetables are an important component of a balanced diet, and their consumption has increased worldwide in recent years (Machado-Moreira *et al*., 2019). Lettuce (*Lactuca sativa* L.) belongs to the family

Asteraceae, alternatively Compositae, and is the most popular vegetable in terms of its highest consumption rate and economic importance throughout the world (M'hamdi *et al*., 2014). It contains several minerals that are important for human health such as iron (Fe), zinc (Zn), calcium (Ca), phosphorus (P), magnesium (Mg), manganese (Mn), and potassium (K), in addition to other health-promoting bioactive compounds (Mulabagal *et al*., 2010; Kim *et al*., 2016). Epidemiological studies have reported a correlation between fresh vegetable consumption and reduced risk of chronic diseases (Rodriguez-Casado, 2016). Lettuce has been confirmed as a replacer for some vegetables like waterleaf, bitter leaf and pumpkins for common dishes in the Southern part of Nigeria (Ogunbo, 2015). In Asian countries, lettuce and asparagus lettuce are traditionally cooked rather than consumed raw (Mou, 2008).

Generally, the production of vegetables such as lettuce is an important component of the farming systems in Nigeria. Its cultivation has grown into either as backyard farming or commercial farming. It is now a very lucrative economic activity due to the availability of markets in the vicinity of the production areas and in the southern states where there is high demand for them (Ibrahim and Omotesho, 2011). A large number of Lettuce varieties can be successfully cultivated all year round with a high production potential (Schneider and Goss, 2011; Yang *et al*., 2017). The most favorable temperatures for optimum growth and development are daily temperature means between 15°C and 18°C, with monthly means between 7°C and 24°C (Shah *et al*., 2011). Day temperatures ranging from about 17°C to 27°C, and night temperatures between 2°C and 12°C, are most suitable (Foteinis and Chatzisymeon, 2016).

Plant diseases can cause considerable damage to quality and yield of lettuce. The extent of losses caused varies considerably according to the disease and severity, method of crop production, lettuce variety, season and other factors (Barak *et al*., 2002). Plant virus infections are economically important and cause great yield reduction in lettuce cultivation (Verbeek *et al*., 2014). LMV and CMV are major viruses infecting lettuce worldwide (Soleimani *et al*., 2011). LMV is transmitted by seeds and aphid vectors (Moreno and Fereres, 2012). It

causes severe systemic mosaic infection on lettuce plants. CMV causes similar symptoms on lettuce leaves. It is also a seed and aphid-transmitted virus infection (King *et al.,* 2012). Virus surveys of lettuce crops over the past seasons have however confirmed that a number of other virus diseases can threaten production (Fletcher *et al.,* 2005).

In Nigeria, the bulk of the lettuce consumed comes from the Northern part of the country. The long-distance of transportation between the North and South and poor storage facilities make the crops costly (Ogbodo *et al.,* 2010). There is need therefore to explore the challenges of lettuce production associated with virus disease infection in the derived savanna agroecology of Nigeria with a view of increasing the productive capacity. The objectives of the study were to survey for incidence and severity of viruses infecting lettuce; serologically identify the predominant viruses infecting the crop and verify possible variations happened on proximate composition resulting from virus infection.

MATERIALS AND METHODS

Survey Site and Sample Collection

Virus survey was undertaken from January to November, 2020 during the wet and dry cropping seasons. The primary focus was on areas where production is greatest (Table 1). A total of 100 farmers' fields (20 from each of the 5 locations) were surveyed and crops were surveyed along rows in a 'W' pattern. The percentage incidence was calculated based on the number of diseased plants relative to the total number of plants sampled as the following equation.

% incidence =

$$\frac{Number\ of\ diseased\ plants}{Total\ number\ of\ plants\ sampled}\ X\ 100$$

The percentage severity was calculated based on number of diseased leaves relative to the total number of leaves of plant as the following equation.

% severity =

$$\frac{Number\ of\ diseased\ leaves}{Total\ number\ of\ leaves\ of\ plant}\ X\ 100$$

Table 1. Survey location

S/no	Location	Altitude (m)	Longitude	Latitude
1	Moniya	204	003⁰53.630	07⁰30.785
2	Saki	473	003⁰24.525	08⁰38.253
3	Yejide	166	003⁰53.570	07⁰21.503
4	Beere	180	003⁰54.109	07⁰22.222
5	Olukitipi	193	003⁰56.180	07⁰33.659

Enzyme Linked Immunosorbent Assay (ELISA)

A total of fifty samples were subjected to Antigen Coated Plate (ACP) and Double Antibody Sandwich (DAS) Enzyme Linked Immunosorbent Assay using specific polyclonal antisera against CMV, Broad spectrum potyvirus, LMV and LeVX. The antibodies were obtained from Leibniz-Institute DSMZ-Deutsche Sammlung Von, Germany. Goat anti-rabbit-IgG alkaline phosphatase conjugate was used as a secondary antibody at a 1:7.500 dilution (catalogue number AP132A). Absorbance was measured using an ELISA reader (Anthos-2.020 microplate reader) at 405 nm. Samples were considered positive if their absorbance was equal to or greater than twice as that of healthy control mean values. Each sample was tested in duplicate (Aliyu *et al.,* 2021).

Proximate Analysis

Estimation of Chlorophyll Content

For chlorophyll estimation, the method of Kamble *et al.*, (2015) was adopted. One gram of freshly cut and well mixed representative samples of leaves was taken. It was ground to a fine pulp with addition of 20 ml of 80% chilled acetone. It was centrifuged at 5000 rpm for five minutes at 4°C and the supernatant was transferred into a 100 ml volumetric flask. The residue was again ground in 20 ml of 80% chilled acetone, centrifuged as earlier and the supernatant was transferred to the same volumetric flask. This procedure was repeated until the residue became colourless. The mortar and pestle were washed thoroughly with 80% acetone and the clear washings were collected in the volumetric flask. The volume was made up to 100 ml using 80% acetone. The absorbance values were read at 645

and 663 nm in a spectrophotometer against the blank, 80% acetone. Chlorophyll a, chlorophyll b and total chlorophyll contents were calculated using the following formulae in mg chlorophyll per gram tissue,

Chlorophyll a =

$$[12.7 (A\ 663) - 2.69 (A\ 645)] \times \frac{v}{1000w},$$

Chlorophyll b =

$$[22.9 (A\ 645) - 4.68 (A\ 663)] \times \frac{v}{1000w}$$

and

Total chlorophyll =

$$[20.2(A\ 645) + 8.02 (A\ 663)] \times \frac{v}{1000w}$$

where, A = absorbance at specific wave length, v = final volume of the chlorophyll extract and w = fresh weight of the tissue extracted.

Carbohydrates Determination

Carbohydrates content (C) of the plant samples was evaluated by the method described by Unuofin *et al.*, (2017). The carbohydrate content was calculated by the difference of the total dry matter and the addition of the percentage ash, crude fat, crude protein and Neutral detergent fiber (NDF) using the formula,

% C = 100 - (% Ash + % Crude fat + % Crude protein + % NDF).

Crude Fiber Determination

The crude fiber determination was done according to the method adopted by Miteu and Ezeh (2022). Three (3) grams of each of the samples were defatted with light petroleum ether (60 ^{0}C BP) for 1 hour. Two gram of the samples (W1) were weighed into 250 ml round bottom quick fit flask and 100 ml of crude fiber reagent (mixture of 500ml glacial acetate acid, 20g trichloroacetic acid, 50 ml Nitric acid, 450 ml distilled water) added and refluxed with occasional shaking for 50 minutes on a heating mantle. The mixtures were cooled and filtered with a Buchner funnel. The residues were rinsed with methylated spirit and hot water. These were then carefully transferred into silica crucible and dried in an oven at 105 ^{0}C overnight. After drying, the samples were cooled in a desiccator and weights were taken (W2). Finally, the samples were kept into a muffle furnace at a temperature of 600°C for 6 hours, cooled and weighed (W3).

$$\% \text{ Crude Fiber (CF)} = \frac{W2 - W3}{W1} \times 100$$

Determination of Crude Protein

The pulverized plant sample 2g was weighed in a 300 mL Kjeldahl flask and digested by a volume of 20mL concentrated sulfuric acid (H_2SO_4). The process was aided with a catalyst until a clear solution was obtained. The digest was allowed to cool, diluted with 250 mL distilled water and transferred into a 500 mL Kjeldahl flask, containing anti-bumping chips and 40 mL of 40% NaOH. The distillate from the solution was transferred in a collecting liquid (250 mL of 2% Boric acid and a few drops of mixed indicator) by immersing the end of the condenser inside the liquid to trap the gaseous ammonia being liberated. The resultant liquid was then back-titrated against 0.01 M hydrochloric acid until the endpoint violet color was reached and the percentage nitrogen content was calculated according to the method of Gandji *et al.,* (2019) as follows.

$$\% \text{ Wn2} = \frac{14 \text{ x M x V1 x V100}}{Weight\ of\ sample\ (mg) x\ Va} x\ 100$$

Percentage crude protein was expressed as; % Wp = % Wn2 x 6.25,

where, M is actual molarity of acid (HCl), V100 is the titre value (cm^3) of HCl used, V1 is the total volume of the diluted digest, Va is the aliquot volume distilled, Wp is the crude protein content, Wn2 is the Nitrogen content.

Determination of Ash Content

The AOAC (2016) method was used for the ash content assay. A heat-resistant porcelain crucible was dried in an oven for 10 min at 105 ^{0}C, cooled in a desiccator and dry weight (W1) was measured. Thereafter 2g of the pulverized plant sample was measured in the porcelain crucible and reweighed (W2). The crucible with the sample was incinerated in a furnace, first at 250 ^{0}C for 1 h and 550 ^{0}C for 7 hours to ensure proper ashing. The crucible was removed, allowed to cool in a desiccator and weighed (W3). The percentage of ash content was thus evaluated as,

$$\% \text{ Ash content} = \frac{W3-W1}{W2-W1} x\ 100$$

Determination of Crude Fat

The crude lipid content of the sample was determined by soxhlet extraction method as described by AOAC (2016). A pulverized sample of 5g was measured in a 500mL round bottom flask, containing a few grams of anti-bumping granules and was weighed

(W1) all together. The fat content of the pulverized plant samples was extracted in 100mL of diethyl ether at 40–60 ^{0}C for 6 hours in the flask attached to the Soxhlet extractor, at reflux. The filtrate was concentrated, diethyl ether was recovered and the oil in the round bottom flask was dried in an oven. The oil and the round bottom flask were thereafter weighed (W2). The percentage of crude fat content was calculated as,

$$\% \text{ Crude fat} = \frac{W2 - W1}{Weight\ of\ sample} x\ 100$$

Data Analysis

The Statistical Package for Social Sciences (SPSS), alongside with Microsoft Excel (2010) package was used for statistical analysis to showcase the variabilities among the locations and samples. Statistical tests were carried out at the 0.05 significance probability level.

RESULTS AND DISCUSSION

Symptoms Observed on Lettuce Plants

The commonest varieties of lettuce grown in the study area are Crip head lettuce, Romaine lettuce, Leaf lettuce, Iceberg lettuce and Argula lettuce. The most notable symptoms (Figure 1) observed during the survey on lettuce were: yellow-green mottling, mosaic patterns on leaves, leaf puckering, accentuated serration of the leaf margins, leaf distortion, marginal necrosis of leaves and plant dwarfism. The observed symptoms are consistent with those reported by Zhang *et al.,* (2020), to be occurring on virus-infected lettuce plants. Tordo *et al.,* (2005) attributed the phenomenon to mixed infection of both cucumber mosaic virus and lettuce mosaic virus.

Incidence and Severity of Virus Infection on Lettuce Plants

The results of the survey on virus incidence and severity across five farm locations (Saki, Moniya, Yejide, Beere and Olukitipi) located within the derived savanna agroecological zone of Oyo state-Nigeria are presented in Table 2. The results indicated the prevalence of viruses in all of the locations. However, the percentage incidence of lettuce plants showing symptomatic expressions in the different locations varied from 7.14% to 39.22%. The top two highest incidences were in Saki (39.22%) and Yejide (28.42%), while Beere and Olokitipi had lower values of 14.87% and 7.14%

(a) **(b)**

Figure 1. Some virus disease symptoms observed on lettuce plants during the survey. (a) Yellow-green mottling, (b) Mosaic patterns on leaves.

respectively. Over all, symptomatic expressions were most severe in Saki (32.12%) and least severe in Moniya (10.27%). Table 2 indicated that the mean disease incidence for the locations was 23.26 % with a range of 25% to 45% and the mean severity was 19.99% with a range of 10.33% to 41.32%.

Viruses have a great potential for high genetic variability due to their rapid replication and generation of large populations (Gago *et al.,* 2009). Viral populations in individual plants can be even more complex, since mixed infections with different virus species (Juárez *et al.,* 2013) or divergent variants of the same virus species (Gómez *et al.,* 2009) are frequent as a consequence of successive inoculations by vectors. The most important factor causing virus epidemics in lettuce plantations according to Moreno *et al.,* (2004) and Li *et al.,* (2022) is the presence and infection of the virus in seed materials that cause the primary spread of the virus. The present work revealed that farmers in the study area use lettuce seeds of different varieties from untreated stock. The disparity observed in infectivity levels is therefore a consequence of varietal inherencies. This view is supported by Sreenivasaprasad (2004), who reported that the cultivation of different crop varieties resulted in fluctuating virus occurrence within the surveyed area.

Enzyme-Linked Immunosorbent Assay Results

The Enzyme-Linked Immunosorbent assay results on lettuce samples collected from the study area are presented in Tables 3. The results indicated that CMV was confirmed in all 10 samples from Yejide (100% CMV infection), 5 samples from Bere (50% CMV infection) and 4 samples from Shaki (40% CMV infection). LMV was detected infecting 4 samples each from Yejide and Saki (40% LMV infection). Potyvirus infection was detected only on 2 samples from Bere (20% Potyvirus infection). LeVX was however not positive in all the 50 samples tested. The results indicated multiple virus infection of CMV, LMV and/or Potyvirus in Yejide and Bere, while single virus infection of only CMV or Potyvirus in Saki and Bere respectively. The optical range values (nm) for all the samples from Moniya and Olukitipi were below the range and confirmed negative to all viruses tested. It can be deduced that 50% of the samples tested positive for at least one virus, of which 38% tested positive for CMV, 8% for LMV and 4% for broad spectrum potyvirus. However, for mixed infections of CMV and LMV, 8% of the samples were positive. LeVX was not detected in any of the samples.

Viral disease management is dependent upon an accurate detection procedure that is convenient, reproducible and scalable for a wide range of samples (Viswanathan *et al*., 2013). CMV was the most widespread virus disease of lettuce over the survey area with proportional to infected crops from 40% up to 100% in some locations. This finding justifies the claim of CMV being one of the most widespread plant viruses causing significant economic losses for numerous vegetable and horticultural crops (Roossinck, 1999). The combination of CMV and LMV and sparsely Potyvirus were found infecting lettuce during the survey. The combination of these viruses was similar to those reported by Soleimani *et al.,* (2011). These viruses directly affected the quality of leaves and preventing sale of affected plants (Pavan *et al.,* 2008). The non-detection of LeVX in the present samples is an indication that the lettuce varieties planted by farmers in the study are not susceptible to the virus and could therefore be explored for plant breeding programmes for resistant development. Innate immunity is an evolutionary mechanism that protects

Table 3. ELISA results of lettuce samples from study area.

Location	Sample Code	Optical Density (nm/100μ)			
		CMV	LMV	Potyvirus	LeVX
Yejide	Y001	1.297 ×	0.733	0.150	0.56
	Y002	0.991 ×	0.713	0.169	0.521
	Y003	0.912 ×	0.766	0.174	0.587
	Y004	0.928 ×	0.722	0.202	0.55
	Y005	1.039 ×	0.839	0.168	0.561
	Y006	1.110 ×	1.147 ×	0.162	0.557
	Y007	1.192 ×	0.881 ×	0.145	0.571
	Y008	1.327 ×	0.828	0.158	0.467
	Y009	1.243 ×	1.158 ×	0.195	0.573
	Y010	1.429 ×	1.134 ×	0.197	0.576
Bere	B001	1.391 ×	0.652	0.306	0.491
	B002	1.586 ×	0.502	0.266	0.621
	B003	0.905 ×	0.679	0.288	0.487
	B004	0.696	0.571	0.394 ×	0.423
	B005	0.570	0.605	0.362 ×	0.421
	B006	0.657	0.593	0.346	0.386
	B007	0.686	0.585	0.121	0.463
	B008	0.717	0.711	0.114	0.459
	B009	0.893 ×	0.588	0.109	0.447
	B010	0.796 ×	0.650	0.111	0.43
Saki	S001	0.836 ×	0.765	0.174	0.539
	S002	0.990 ×	0.809	0.147	0.532
	S003	0.930 ×	0.297	0.167	0.396
	S004	1.008 ×	0.048	0.152	0.606
	S005	0.517	0.625	0.172	0.484
	S006	0.327	0.566	0.197	0.449
	S007	0.306	0.622	0.301	0.694
	S008	0.291	0.739	0.262	0.411
	S009	0.310	0.592	0.299	0.545
	S010	0.291	0.608	0.310	0.511
Moniya	M001	0.392	0.549	0.307	0.506
	M002	0.369	0.504	0.345	0.310
	M003	0.349	0.514	0.150	0.510

	M004	0.428	0.505	0.120	0.543
	M005	0.404	0.432	0.133	0.541
	M006	0.606	0.237	0.131	0.544
	M007	0.437	0.521	0.167	0.704
	M008	0.243	0.509	0.141	0.496
	M009	0.228	0.612	0.150	0.572
	M010	0.198	0.500	0.147	0.522
Olukitipi	O001	0.137	0.134	0.181	0.232
	O002	0.173	0.154	0.137	0.296
	O003	0.216	0.189	0.177	0.106
	O004	0.193	0.177	0.162	0.174
	O005	0.180	0.171	0.184	0.219
	O006	0.191	0.167	0.171	0.294
	O007	0.174	0.141	0.201	0.161
	O008	0.179	0.212	0.178	0.175
	O009	0.180	0.219	0.192	0.211
	O010	0.183	0.197	0.205	0.138
	B	0.394	0.290	0.146	0.272
	NC	0.329	0.316	0.208	0.313
	PC	0.578	0.381	0.229	0.365

Key: Sample (×) is considered virus positive when the optical density (OD) reading at 450nm is twice greater than the absorbance from healthy control. B = Buffer; NC = Negative control; PC = Positive control.

plants from a wide range of pathogens. According to the opinion of Glazebrook (2005) the resistance to different pathogen types is regulated by salicylic acid and ethylene/jasmonic acid-dependent defense mechanisms.

Effect of Virus Infection on Proximate Content of Lettuce Leaves

The result of the proximate composition of the diseased and healthy lettuce leaves is represented in Figure 2. The chlorophyll content of the infected plants (0.6) was significantly ($P< 0.05$) lower than the healthy plants (1.4). Chlorophyll degradation is a common characteristic phenomenon in viral

infections and the present findings are in line with that of Ananthu and Umamaheswaran (2019). The total carbohydrate content was found significantly ($P < 0.05$) higher in healthy plants (13.8) than that of infected plants (7.7). The findings related to carbohydrate are in concurrence with that reported by Handford and Carr (2007). Due to virus infection, there is an increase demand for abnormal protein production resulting in the decreased production of carbohydrate level of plant leaves. The crude fiber level of both healthy and diseased samples was 2.7% and therefore not significantly ($P \geq 0.05$) different. This result however, contrasts with those of Mofunnaya *et al.,* (2015), who reported increased crude fiber level in *Amaranthus hybridus* infected with Telfairia Mosaic Virus (TeMV).

The protein level was significantly ($P > 0.05$) lower in healthy plants (7.4 %) compared with virus infected plants (13.6 %). This could be due to alterations in plant metabolism and increased levels of viral proteins in the infected plants. Previous reports of virus infection on protein contents seem dependent on host-virus combination. Mofunanya *et al.,* (2008) have also reported a decrease in the protein

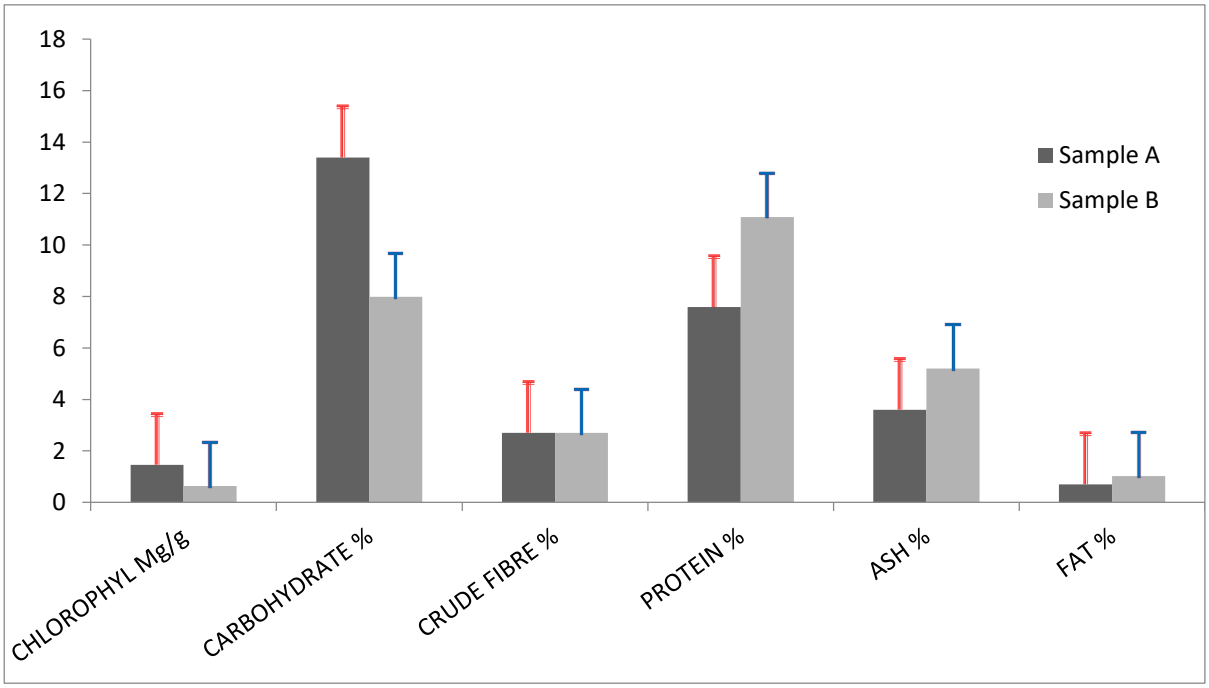

Figure 2. Results of proximate analysis. Samples A: Healthy plants, B: virus infected plants.

content to *Telfairia occidentalis* inoculated with TeMV. Owolabi *et al.*, (2010) also observed a decrease in The results of this study however, contrasts with those of Hemida (2005), which documented increases in the protein content in potato-PVY^{NTN} and *Vicia faba*- BYMV combination.

The ash (5.2%) and Fat (1.0%) content of virus infected samples were found significantly (P > 0.05) higher than those of healthy plants which had values of 3.6% and 0.7% respectively, which is different from Yardımcı *et al.*, (2007). Many researchers have reported physiological and biochemical disorders due to pathologic changes in plants as a result of virus infection. Sing *et al.* (1977) had earlier reported that leaves of plants infected by viruses cause high enzymatic activities. This could be one of the reasons why higher ash and fat contents were recorded in this present study.

CONCLUSION

The infections of cucumber mosaic virus, Lettuce mosaic virus and Potyvirus have been identified for the first time in Lettuce in the derived savannah agroecology of Oyo State, Nigeria. The viruses were detected protein content in Ivy gourd infected by a Nigeria strain of Moroccan Watermelon Mosaic Virus (MWMV). infecting lettuce either singly or in mixed infections with characteristic symptoms. The prevalence of virus infection was diverse across locations. Proximate analysis indicated reduced chlorophyll and carbohydrate contents in infected lettuce plants. It is recommended to conduct virus survey on lettuce to detect newly emerging virus strains to be regularly supported in effective virus management strategies. This will enhance crop growth and improve the quality of nutrients derived from the plant.

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