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Full Length Research Paper

Molecular and physiological characterization of bread wheat (*Triticum aestivum* L.) breeding lines for fungal diseases tolerance

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Fungal diseases are one of the major causes of losses on wheat yield in the world. Recent studies on plant defense mechanisms have highlighted the role of amino acids and total polyphenols in disease tolerance. Thus, with the objective of identifying on the basis of morphological and physiological variables the high-performance wheat accessions in high and low altitude, we characterized sixteen wheat accessions via quantification of polyphenols (TPP) and amino acids (AA) and identified fungal diseases affecting wheat in Centre Cameroon. Using the set of the three hexaploid wheat cultivars where the 11 microsatellite markers data was available, a total of 29 alleles were detected among cultivars and the number of alleles per locus ranged from 2 to 3 with an average of 2.64. Gene diversity ranged from 0.44 to 0.67 with an average of 0.59, increasing with the number of alleles. Microsatellites markers used had an average Polymorphic Information Content (PIC) value of 0.50, indicating that these markers are useful and will make a contribution to the studies in hexaploid wheat. The assessment of wheat plant to disease tolerance permitted to identify Septoria, Fusarium wilt; tanned spot and powdery mildew in high and low altitude. Analyzes of TPP and AA have made it possible to discriminate accessions that are tolerant to diseases. Hence, accession Atilla 1 was highly tolerant and had high levels of TPP (3.5 ± 0.36 µg/mg) and AA (40.1 ± 1.21 µg/mg) while the accession Sup 152 highly susceptible to disease had low levels (2.3 ± 0.1 and 2.1 ± 0.36 µg/mg, respectively). This study will contribute to the extension of wheat in areas where conditions similar to the study sites will be localized.

Key words: Amino acids, bread wheat, high and low altitude, fungal diseases, polyphenols.

INTRODUCTION

Wheat (*Triticum aestivum* L.) is one of the most valuable crops with a rate of more than 45% in world trade (Mohsen et al., 2014). Cereal of the Poaceae family, its global production increased from 655 million tons in 2014

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to 734.9 million tons in 2017 with 26 million tons coming from Africa. In Cameroon wheat is still the most consumed cereal. A highly strategic commodity, with a view to food security and sovereignty, wheat cultivation in Cameroon is limited to small areas despite the country's potential. (Boutraid (1982). With its 66 tons, Cameroon, then unable to meet the needs of its population which amounts to nearly 598,000 tons allocates about 144 billion CFA francs each year for the import of wheat estimated at nearly 750,000 ton. Cameroon is thus very dependent on wheat import and therefore, in order to be able to withstand the financial consequences of price fluctuations, it must imperatively reduce this dependence and work to reach a minimum threshold of self-sufficiency for this speculation. Before the closure of the company Development of Wheat Cultivation in Cameroon (Sodéblé) in 1982, Kouébo and Monté (1979) had worked on fungal diseases attacking wheat and had identified Solanika considered today as a highly productive local accession and resistant to fungal diseases. However, for intensive and extensive cultivation, it is urgent to find new accessions that can guarantee competitive yields. It then appears necessary to introduce and popularize accessions with satisfactory ecological and economic solutions. However, the extension of wheat cultivation depends on the resolution of the main constraint related to its production, which is on one hand the problem of altitudes, which seems to be one of the main factors limiting production to nearly 75% (Jabal et al., 2017). Known as Africa in miniature, Cameroon has a high level of topographic variation. However, the majority of cultivable areas are located in low-lying areas (<800 m), since bread wheat usually grows at high altitude, hence it will be important to find accessions adapted to local situations in order to increase the area under cultivation. On the other hand, the problems of fungal infestations such as rust, septoria and fusariosis account for nearly 20 to 100% of the losses on production (Tesfay and Araya, 2015). Several authors have shown that the narrowness of crop genetic diversity could lead to increased susceptibility to diseases and pests, as well as the inability of plants to respond to different environmental constraints (Bendif, 1994; Gorji and Zolnoori, 2011). So, evaluating the genetic diversity is a prerequisite for studying the adaptation of populations to new environmental conditions and hence for the selection of new varieties. In this context, the use of Simple Sequence Repeats (SSRs) markers combines many desirable marker properties such as abundance, high levels of polymorphism (unlike RFLP), very good reproducibility (compared to RAPD), and co-dominance (contrary to the AFLP for which codominance is not exploitable), but also an even coverage of the genome and the specificity of amplification (Tekeu et al., 2017).

On the other hand, phytosanitary constraints being a function of environmental conditions (Siou, 2013), knowledge of the potential of accessions through agronomic and biochemical descriptors remains to be done to choose and make an informed decision on the best accession to introduce (Nda et al., 2014). Several methods are then available for such work, namely the identification of local situations that meet the biological requirements of each accession, and the detection of the elements involved in the defense mechanism against diseases. Indeed, Bushnell (1984); Lattanzio et al., (2006) and Patil et al. (2011) have shown that wheat genotypes in response to pathogens synthesize substances such as polyphenols and total amino acids that, in addition to their roles in growth metabolism, also protect the plant. Our study has been investigated to identify high performance wheat accessions in high and low altitude zone in Cameroon.

MATERIALS AND METHODS

Plant material and morphological characterization

A total of 16 winter and spring wheat varieties were obtained from the International Maize and Wheat Improvement Center (CIMMYT) and the International Center for Agricultural Research in the Dry Areas (ICARDA) (Table 1). Field trials were conducted in two areas of different altitudes of the bimodal humid forest zone of Cameroon (Mount Mbankolo: high-altitude area of 1057 m above the sea level; and Nkolbisson: low altitude area of 650 m above the sea level). At each trial site, an incomplete alpha-lattice design with two replications was used. Each accession was planted in a single 3-m row with 25-cm spacing between the rows.

For each wheat accession, plant height (measured using a graduated ruler) and systemic identification of pathogens were assessed, based on observation of symptoms on infected plants and appearance of colonies on the plants via culture medium. Thus, seeding was done under a laminar flow host near a Bunsen burner flame. After visual examination of organs affected by pathogens, isolations were performed according to the protocol of Mahfoud and Lasbuhani (2015). From organ fragments with characteristic symptoms of diseases, including leaves, neck, roots or seeds, small portions were cut (leaves and stems) and disinfected with 2% sodium hypochlorite (1 minute). These fragments were then rinsed three times with sterile distilled water (1 min each time). Using a scalpel previously flamed and immersed in an alcoholic solution, the dried fragments on blotting paper were seeded in Petri dishes containing Potato Dextrose Agar (PDA) on which was indicated the name of the sample, as well as the date of seeding. Then, the seeded Petri dishes were incubated for 7 days until a good fungal development appeared. After good development, colonies were sub-cultured into new Petri dishes containing PDA. The goal was to purify the mushrooms by minimizing the risk of contamination. Finally, these boxes were also incubated for 3 to 4 days. The microscopic identification consisted in observing after putting on the slide a few drops of methyl blue, the appearance of the mycelium and spores if sporulation existed thanks to a photonic microscope with a magnification of 400 x after which the different species observed were determined by criteria established by (Leslie and Summerell (2006).

Genomic DNA extraction

An adjusted Doyle and Doyle (1990) protocol was used to extract genomic DNA (gDNA) from seedlings at the two to three leaf stage. Approximately, 100 mg of plant tissue were cut up and placed into a micro centrifuge tube. 500 µl of 2% (m/v) CTAB extraction buffer [1.4 M NaCl, 20 mM Na2EDTA (pH 8), 100 mM Tris-HCl (pH 8)]

Table 1. Different accessions used.

S/N	Name	Origin	S/N	Name	Origin
1	Attila 1	Icarda	9	Seri1 b1	Icarda
2	Whear 1	Cymmit	10	Schwink1	Cymmit
3	Kakaba	Ethiopia	11	Vee	Icarda
4	Egypte 3	Cymmit	12	Kachu 4	Cymmit
5	Kiskadee	Cymmit	13	Kenya 11	Cymmit
6	Watan-7- sekhra	Icarda	14	Atlass66	South Africa
7	Sup-152-2	Cymmit	15	Crows	Icarda
8	Pauraq	Cymmit	16	Attila 5	Icarda

Source: Author

and tow sterilized steel bearings were added to each sample. A Qiagen®TissueLyser (Qiagen (Pty) Ltd; local distributer: Southern Cross Biotechnology, Claremont, RSA) was used to grind the samples 2 times for 2 min (min) at 30 Hz. This mixture was then incubated in a water bath for 20 min at 60°C. 200 µl and 50 µl of chloroform: isoamyl-alcohol (C:I::24:1, v/v) were added and the solution was centrifuged for 5 min at 12000 rpm. The supernatant was transferred to a clean centrifuge tube and another 250 µl of chloroform: isoamyl-alcohol was added, followed by centrifugation for 5 min at 12000 rpm. The supernatant was once again transferred to a clean centrifuge tube. 50 µl of 3 M Sodium acetate (pH 5.0) was added, followed by 500 µl of ice cold 100% ethanol. The tubes were carefully inverted and the gDNA was precipitated. After incubation, the pellet underwent two wash steps with 500 µl of 70% ethanol. Then, the tubes were centrifuged for 5 min at 12000 rpm. The supernatant was discarded and the pellet was left to air dry. The pellet was finally re-suspended in 30 µl of DNase/RNase-free water and incubated at 60°C for 2 min. The extracted gDNA was quantified using a Nanodrop® ND-1000 spectrophotometer. The DNA was diluted with DNase/RNase-free water to a concentration of 100 ng/µl and stored at the fridge (-20ºC).

Microsatellite markers and PCR amplification

Eleven wheat microsatellite markers for 11 loci located in the chromosomes 1A, 2A, 2D, 3A, 3B, 4D, 5D, 6B and 7D, were used for genetic diversity analysis. Xgwm and Xwmc markers were obtained respectively from Röder et al. (1998) and Somers and Isaac (2004).

PCR reactions were carried out in 14 µl reaction mixture of KAPA2GTM Fast Multiplex PCR Mix, 6.25 µM of each forward and reverse primer (Stellenbosch University, Stellenbosch, South Africa), 1 µl gDNA and dH2O. The PCR cycling conditions was set at 94ºC for 3 min of denaturation, followed by 45 cycles of 1 min at 94°C, 1 min at the annealing temperature (Ta), 2 min at 72°C and then 72°C for 10 min for extension. The PCR products were electrophoresed on 6% denaturing polyacrylamide gels containing 1xTBE (Tris Borate EDTA). The amplified band sizes for each SSR locus were determined on the basis of their migration relative to the 50 bp marker.

Assessment of the incidence and prevalence of fungal diseases

The incidence was calculated using the formula of Dajoz, (1985): I = Nm / NT × 100, where Nm: number of diseased plants per accession, NT: total number of plants per accession, and I: incidence (%). The prevalence was expressed through the formula of Zahri et al. (2014): P = n / N × 100, where n: number of sick accessions for each disease, N: total accession number, and P: prevalence (%). To estimate the ability of plant to fight against the pathogen, the severity was expressed as a percentage of sick leaf area on a log scale (Table 3) described by Notteghem et al. (1980), and is calculated using the formula preconized by Chumakov (1990)., where Σ (a x b): sum of sick plants (a) having the degree of infection (b); N: Total number of diseased plants; S: Severity (% of SFM).

Biochemical analyzes

Extraction and dosage of total polyphenols

The extraction of the total polypheanols was carried out following the modified method of Boizot (2006). Briefly, 1 g of leaves was mixed with 2 mL of 80% methanol and incubated at room temperature. The mixture was centrifuged (Labofuge 400R) at 4500 rpm for 15 min. However, the method used to assay polyphenols is that of Marigo (1973). 50 µL of each extract was added 3 mL of distilled water and 0.5 mL of Folin-ciocalteu reagent. The mixture was incubated for 2 min in dark and room temperature. Then, 2 mL of Na2CO3 (75 g/L) were added and the whole was stirred on a vortex and then incubated for 15 min at 50°C in a water bath in the dark at 760 nm and was immediately measured (Jenway 6305, spectrophotometer) against a blank in which the extract is replaced by 80% methanol. Results were expressed in µg/mg gallic acid equivalents (GAE).

Extraction and determination of total amino acids

To extract the total amino acids from wheat leaf, 1 g of plant material was crushed and then diluted in 2 ml of citrate buffer (0.2 M, pH 4.5). The mixture obtained was homogenized using a vortex and centrifuged at 4500 rpm for 15 min at room temperature (25 ± 2°C). The supernatants were recovered and placed in the 2 ml tubes. Further extractions were repeated with 1 ml of solution and the two supernatants were mixed into a single extract according to the method of Singh et al. (1999). The total amino acids were then determined according to the ninhydrin modified method of Yemm and Cocking (1955). Briefly in tubes, 5.1 ml of the reaction medium, 0.5 ml of 80% ethanol, 25 µl of alcohol extract, 0.5 ml of citrate buffer (0.2 M, pH 5) and 2 ml of reagent Potassium Cyanide-Ninhydrin acetone (1 % ninhydrin and 0.06 % KCN in acetone). The mixture was heated in a boiling water bath for 15 min. A blue-purple complex was formed whose intensity of the coloration was

Table 2. Description of SSR markers.

Symptom	Note	Foliar surfaces affected (%)
No diseases	1	0
Very few spots on lower leaves	2	2.5
Few spots on the first two leaves	3	12.5
Few spots on 1/3 basal plants	4	25
Necrosis scattered on the 1/2 of the height of the plant	5	50
Necrosis not exceeding half of the plant	6	62.5
Attack pronounced at 1/3 of the base of the plant and the middle leaves	7	75
Pronounced attack on the middle leaves and at the top of the plant	8	87.5
Very pronounced attack on all leaves even the ear can be reached	9	100

Source: Author

proportional to the concentration of the amino acid in the solution. After cooling at room temperature, 8 ml of distilled water was added to the mixture and finally the absorbance was read at 570 nm with the spectrophotometer. For each extract, three readings were made and the amino acid content was evaluated by reference to the standard curve made with pure glycine.

Data analysis

The molecular diversity within all accessions was estimated for each SSR locus, using the PowerMarker 3.25 software (Liu and Muse, 2005). To measure the informative character of the SSR markers, the Polymorphism Information Contents (PIC) for each marker was calculated using the formula of Nei (1973): PIC = $1-\Sigma_{i=1}^{k} P^2$, where k is the total number of alleles detected per locus and Pi the frequency of the allele i in all 17 accessions.

Genetic similarity (GS; Dice, 1945) was calculated as: GS = $2N_{ij}/(N_i + N_j)$ where, N_{ij} is the number of fragment common to individual i and j, and (Ni + Nj) is the total number of fragment in both individuals.

Genetic distance (GD) among group pairs was calculated following Nei and Li (1979); $(GD_{XY}) = 1-(2N_{XY}/N_X + N_Y)$. The dendrogram was constructed using the method based on the genetic distance (SAHN method, UPGMA algorithm) of the 17 accessions using the software Statistica 12. To calculate allele frequency (Axy) from one of variation to another in each locus, the formula of Khlestkina et al. (2004) was used: Axy = ΣIPxi – PyiINxy, where Pxi and Pyi are the frequencies of the ith allele in regions X and Y, respectively, and Nxy is the total number of alleles for the two groups X and Y. The allelic frequency variation was calculated separately for each of the 11 loci and then for all of them as an average. All fragments were used to generate a GS matrix for Principal Component Analysis (Sneath and Sokal, 1973).

The statistical analyzes as well as the classification of the accessions in the dendrogram were carried out using the SPSS 20.0 software. The data of different variables were subjected to an analysis of variances (ANOVA), the averages compared to the Tukey test at the threshold of 0.05 and the correlations carried out using the Pearson test.

RESULTS

Diversity of wheat microsatellite markers

All pairs of primers specific for SSR locus used gave amplifications with allelic variations in size on all DNA wheat accessions. A total of 29 microsatellite alleles were detected. The number of alleles per locus varied from 2 to 3, with an average of 2.64 alleles per locus. The polymorphism information Content (PIC) varied from 0.35 to 0.59, with an average of 0.50 (Table 2). The results indicated a significant correlation over 11 loci (R2=1, $P < 0.01$) between gene diversity and number of alleles across wheat accessions (Table 3).

Phenotypic characterization of accessions

A great variability in the height of the wheat plants was observed according to the accessions but also according to the sites. Within accessions, Attila 1 (60.16 ± 0.85 cm) and Attila 5 (64.16 ± 2 cm) appeared best in Mbankolo while Kakaba (52.00 ± 2 cm) and Kiskadee (49.13 ± 1.61 cm) are the best in Nkolbisson. However, the Watan 7 accession behaves both in Mbankolo and Nkolbisson (48.10 ± 2.02 and 46.83 ± 10.53 cm respectively). Significant differences between the two sites have been observed and it appears that all accessions in general develop better in Mbankolo than in Nkolbisson. For Mount Mbankolo (high altitude), values between accessions range from 37 cm (Sup 152-2) to 64.16 cm (Attila 5) versus 22.03 cm (sup 152-2) to 52 cm (Kakaba) for the Nkolbisson site (low altitude) (Table 4).

Assessment of the status of diseases

Observations of cultures isolates from fragments of infected plants on the macroscopic and microscopic level allowed the realization that isolates obtained were characteristic of the pathogens responsible for the main diseases of wheat such as: Tan spot (*Pyrenophara tritici*-repentis), Fusarium wilt (*Fusarium* sp.), Septoria leaf blotch (*Septoria* sp.), and powdery mildew (*Erysiphe graminis*). On PDA media, showed from colonies obtained from *Pyrenophora-tritici*-repentis, a streak and low green mycelium that turned gray on aging. This was observed in Attila 1 (3.5 ± 0.36 µg EAG/mg), whereas accession Sup-152-2 had lower accumulation (2.3 ± 0.1

Table 3. Rating scale of severities.

Locus	Chr position	Primers sequences	Repeat	Bases expected	Ann. temp.	Alleles frequency	Number	Gene diversity	PIC
Xwmc 11	1A, 3A	5' TTGTGATCCTGGTTGTGTTGTGA 3' 5' CACCCAGCCGTTATATATGTTGA 3'	CT	177	61	0.67	2	0.44	0.35
Xwmc 59	1A. 6A	5' TCATTCGTTGCAGATACACCAC 3' 5' TCAATGCCCTTGTTTCTGACCT 3'	(CA)19	197	58	0.33	3	0.67	0.59
Xwmc 177	2A	5' AGGGCTCTCTTTAATTCTTGCT 3' 5' GGTCTATCGTAATCCACCTGTA 3'	(CA)21	184	52	0.33	3	0.67	0.59
Xgwm 190	5D	5' GTGCTTGCTGAGCTATGAGTC 3' 5' GTGCCACGTGGTACCTTTG 3'	(CT)22	201-253	55	0.33	3	0.67	0.59
Xgwm 437	7D	5' GATCAAGACTTTTGTATCTCTC 3' 5' GATGTCCAACAGTTAGCTTA 3'	(CT)24	109-111	47	0.33	3	0.67	0.59
Xgwm 539	2D	5' CTGCTCTAAGATTCATGCAACC 3' 5' GAGGCTTGTGCCCTCTGTAG 3'	(GA)27	143-157	60	0.33	3	0.67	0.59
Xdgm 125	4D	5' GCAGGCGTGTTACTCCAAGT 3' 5' CCGAGGTGGATAGGAGGAAA 3'			60	0.33	3	0.67	0.59
Xwmc 331	4D	5' CCTGTTGCATACTTGACCTTTTT 3' 5' GGAGTTCAATCTTTCATCACCAT 3'		128	61	0.67	3	0.50	0.39
Barc 133	3B	5' AGCGCTCGAAAAGTCAG 3' 5' GGCAGGTCCAACTCCAG 3'	(CT)24			0.67	2	0.44	0.35
Xgwm 133	6B	5' ATCTAAACAAGACGGCGGTG 3' 5' ATCTGTGACAACCGGTGAGA 3'	(CT)39			0.33	3	0.67	0.59
Xgwm 644	6B	5' GTGGGTCAAGGCCAAGG 3' 5' AGGAGTAGCGTGAGGGGC 3'	(GA)20			0.67	2	0.44	0.35
Mean						**0.45**	**2.64**	**0.59**	**0.50**

Source: Author

µg EAG/mg) in condition of sickness. The sample from healthy accessions had accumulated lower total polyphenol content than the sample from sick accessions with values that ranged between 0.8 and 2.2 µg EAG/mg (Figure 9A). Accumulation of Amino acid was very important in Attila 1 accession with 40.1 ± 1.21 µg conidia observed were cylindrical and slightly tapered (Figure 1). The isolation on PDA medium from *Fusarium* sp. showed colonies with whitish to pinkish mycelium. Microscopic observation showed spores in short and thinned form at both ends with 2 to 4 septa (Figure 2). Colonies obtained on PDA *Septoria* sp. were whitish in the form of cream. The pycnidiospores observed under the microscope, after 10 days of incubation, were hyaline, narrow, curved at the end and filiform divided by 3 to 7 septa (Figure 3).

Concerning powdery mildew, microscopic observation showed ovoid, hyaline and unicellular conidia (Figure 4), with a thin, slightly transparent wall. The conidiophore was composed of a graduated series of conidia with progressive maturity.

Distribution of different diseases at both sites

A high variability was observed in the distribution of diseases. Regarding the tanned spot, it was regularly distributed on both sites. Indeed, its prevalences were more or less similar and varied very little between 2 and 9% in Mbankolo

Table 4. Size of plants in high (HA) and low altitude (LA).

S/N	Accession	Mbankolo (HA)	Nkolbisson (BA)
1	Kakaba	48.00±2.00^{ef}*	52.00±2.00^{h}*
2	Attila5	64.16±1.04^{j}**	39.26±0.38^{cdef} *
3	Attila1	60.16±0.85^{ij}**	31.01±1.00^{abc}*
4	Seri1b-1	38.00±1.00^{ab}*	24.17±0.66^{ab}*
5	Vee	58.00±1.50^{hi}*	42.00±1.00^{defg}*
6	Watan -7-sehkra	48.10±2.02^{f}*	42.27±1.10^{efg}*
7	Crows	46.00±1.00^{def}**	24.00±1.00^{ab}*
8	Egypte 3	43.17±1.33^{cd}*	33.11±3.00^{bcde}*
9	Kenya 11	42.00±1.00^{bcd}*	40.21±2.52^{cdefg}*
10	Kiskadee	56.00±0.40^{gh}*	49.13±1.61^{gh}*
11	Pauraq	53.16±2.71^{g}**	39.26±0.38^{cdef} *
12	Schwink 1	44.00±0.10^{cde}*	46.83±10.53^{fgh}*
13	Whear 1	57.00±1.00^{ghi}**	33.05±0.21^{bcd}*
14	Kachu 4	44.00±1.00^{cde}*	32.66±0.80^{bc}*
15	Sup 152-2	37.00±1.30^{a}*	22.03±1.00^{a}*
16	Atlass 66	41.70±0.75^{bc}*	26.00±2.00^{ab}*

Tukey test at P< 0.05; values followed by the same letter in a column are not significantly different; the values followed by an asterisk (*) between the same accessions of two different column are not significantly different; HA, High altitude; LA, Low altitude.
Source: Author

Figure 1. Healthy wheat plant and different aspects of the Tanned Blot. (A) Disease free wheat leaf (B) Symptom in field of leaf disease. (C) Colony of Pyrenophora-tritici-repentis on PDA. (D) Conidia of *Pyrenophora-tritici-repentis* observed at magnification (400 X).
Source: Author

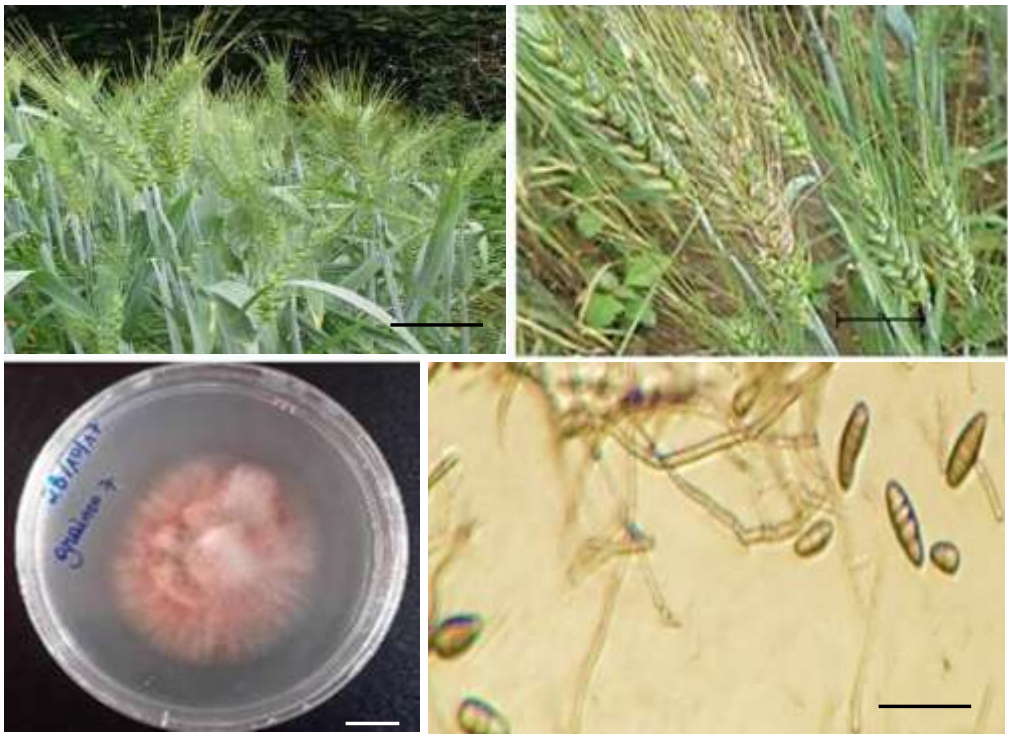

Figure 2. Healthy wheat plant and different aspects of Fusarium wilt. (A) Ears of healthy wheat. (B) Symptom in field of the disease on ears. (C) Colony of Fusarium sp. on PDA. (D) Conidia of *Fusarium* sp. observed at magnification (400 X).
Source: Author

Figure 3. Ears and leaves of healthy wheat and different aspects of septoria. (A) Ears and leaves of healthy wheat. (B and C) Symptoms in field of disease on ears and on leaf. (D) Colony of *Septoria* sp. on PDA. (E) Mycelium of *Septoria* sp. observed at magnification (400 X).
Source: Author

and Nkolbisson respectively (Figure 5). All the same, the incidences were slightly higher in Mbankolo (23%) than in Nkolbisson (19.86%) and the severity was slightly higher in Mbankolo (19.7%) compared to Nkolbisson

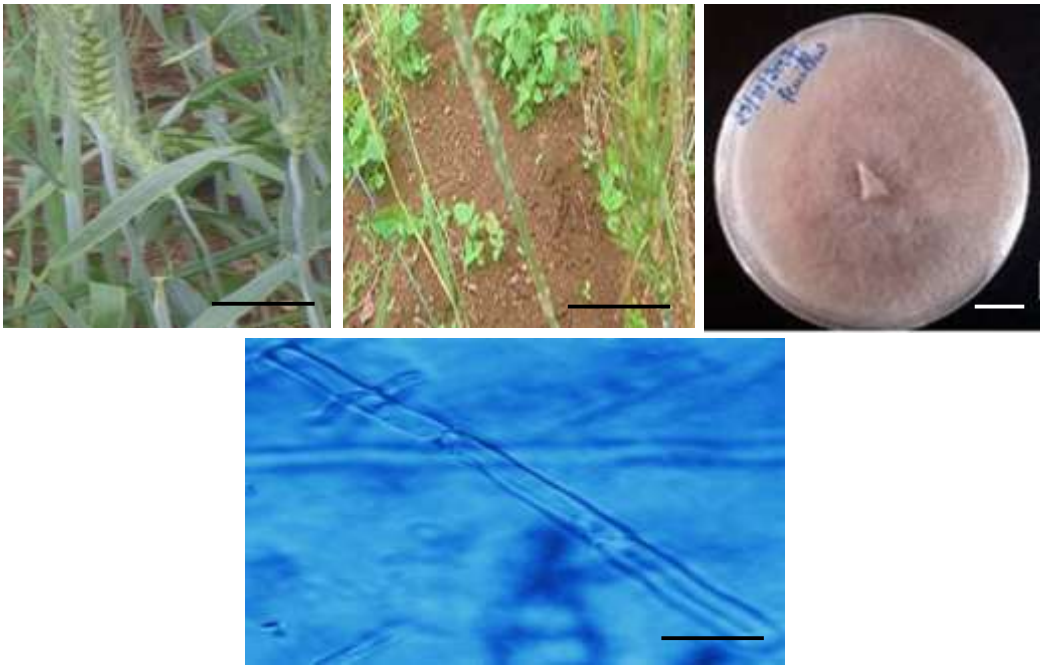

Figure 4. Healthy wheat plants and different aspects of powdery mildew. (A) Stalks of healthy wheat. (B) Symptoms in field of the disease on the stem. (C) Colony of Erysiphe graminis on PDA. (D) Conidiophore of Erysiphe graminis observed at magnification (400 X).
Source: Author

(16.55%). Fusarium wilt was prevalent at both sites but with a high prevalence in Mbankolo (31%) compared to Nkolbisson (23.3%). The disease was more severe in Mbankolo with average attack severity of 27.5% compared with 19 % in Nkolbisson. The incidences were also higher in Mbankolo (30 to 37%) contrary to Nkolbisson (23 to 28%). Like Fusarium wilt and blotch, septoria was also present at both sites with a marked predominance at Mbankolo. Indeed, it was present at 37% in Mbankolo against 21.12% in Nkolbisson. Its incidence is also higher in Mbankolo fluctuating between 40 and 44% against 27.5 to 33% in Nkolbisson although at the level of some plots, they were 100%. As regards severity, it was higher in Mbankolo than in Nkolbisson (31.15 and 22.86%, respectively) (Figure 5). In the case of powdery mildew, it was not observed at all sites. Indeed, during the surveys, it appeared only in Mbankolo with a low prevalence (2%), a low severity (1.5%), but also a low average incidence compared to other diseases (Figure 5).

Behavior of accessions to fungal diseases

On common wheat accessions, a classification analysis was carried out on the basis of the observation of the epidemiological characteristics (infection rates) presented by each accession on different sites (Figure 6). The result obtained showed that common wheat accessions on both sites were principally affected by four diseases, identified as: Tanned spot, fusarium, septoria, and powdery mildew. Indeed, we noted that all accessions of common wheat were sensitive to fungal diseases but to varying degrees. Thus, more sensitive accessions (Atlass 66, seri1b1, sup 152-2) recorded high infection rates (57.6, 60.66 and 62.5%, respectively) in Mbankolo and Nkolbisson sites. This sensitivity varied very strongly between accessions on a given site, but also on an intra-varietal scale at both sites. However, accessions Kabaka [Nkolbisson (10%) and Mbankolo (30%)] and Attila 5 [Nkolbisson (43%) and Mbankolo (11%)] showed depending on the site concerning infection (fungal disease). On the order hand, Watan-7-sehkra presented behavior substantial with infection rates in high (30%) and low (33%) altitude (Figure 6) in relation to others accessions.

Hierarchical cluster of accessions to tolerant level of disease

The analysis of the percentage of infection of accessions at high and low altitude revealed the existence of a great variability within these accessions (Figure 7). The dendrogram obtained made it possible to group these accessions according to their ability to be less or more

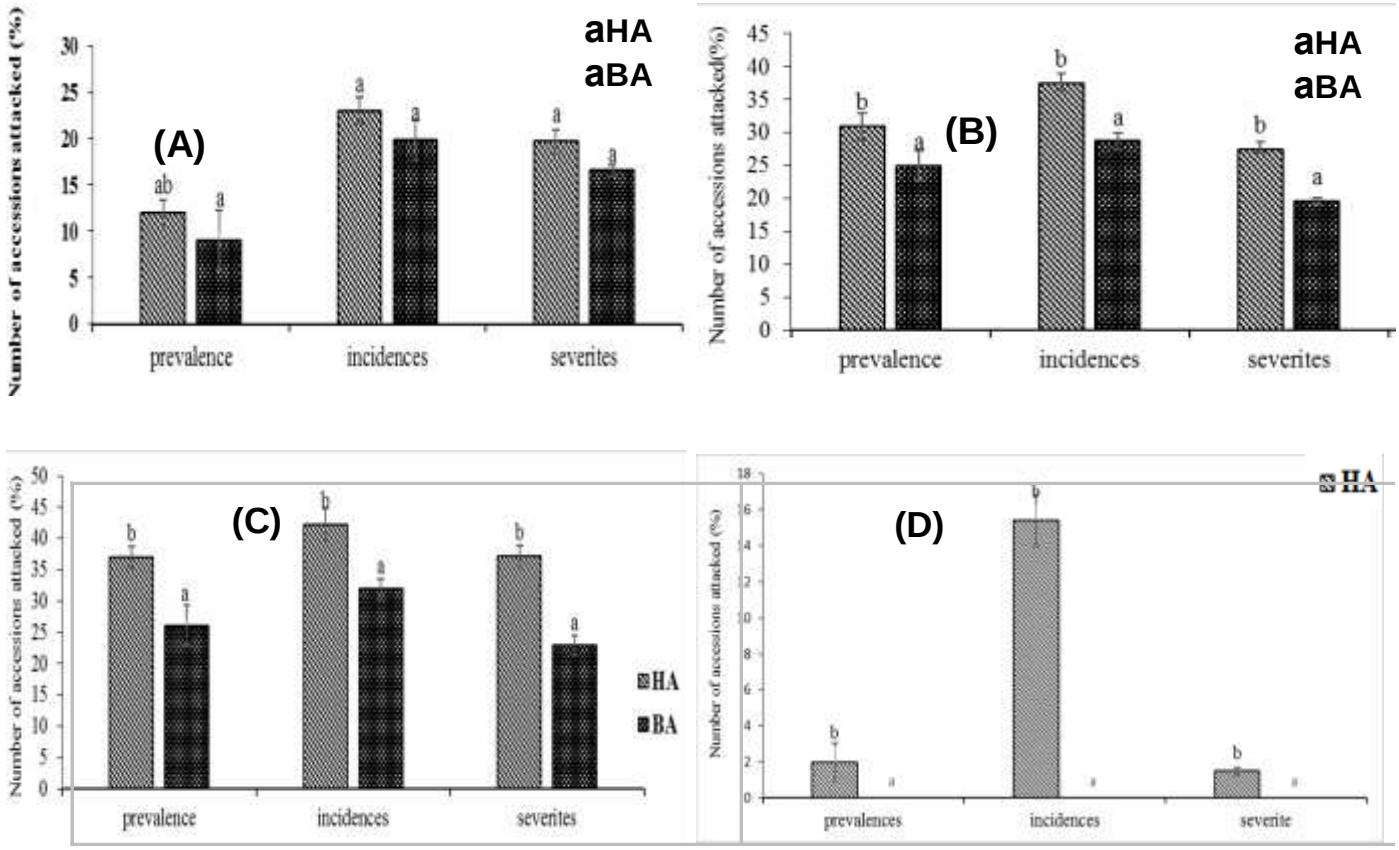

Figure 5: Distribution of fungal diseases at high and low altitude. (A) Tan spot; (B) Fusarium wilt; (C) Septoria; (D) Powdery mildew. The average histograms between two sites (for each variable) bearing the same letters are not significantly different at the 5% threshold. HA: high altitude; BA: low altitude.
Source: Author

attacked by pathogens. At 10 % similarity, analysis revealed that the 16 wheat accessions evaluated form four distinct groups (Figure 8). Indeed, each of these groups showed clear specificities for which the performances differ from those of the others.

Group 1 contained seven accessions (Kiskadee, Kakaba, Schwink1, Vee, Pauraq, Watan 7 and Kenya 11), characterized mainly by an average infection percentage of 31%. Group 2 was constituted of Attila 1 and Attila 5 accessions, which presented relatively low percentages of infections (23.17 % on average). Group 3 included the susceptible accessions (Seri1b-1, Sup-152-2 and Atlass 66) that presented significant infection percentage of 67.5%; however, it was less important (high infection percentage of 54%) in the fourth group embodied: Egypt 3, Kachu 4, Crows and Whear1 accessions (Figure 8).

Variation of biochemical compounds

This study revealed that biochemical activities in the leaves of soft wheat accessions attacked with fungal diseases displayed significant variations among the sick and healthy accessions for total polyphenols and amino acid content between tolerant and susceptible accessions (Figures 9A and 9B). The highest total polyphenol content GE/mg, while Sup-152-2 accession recorded 2.1 ± 0.36 µg GE/mg (Figure 9B).

DISCUSSION

In this study, 11 microsatellite markers revealing 29 alleles allowed to discriminate three cultivars of hexaploid wheat introduced in Cameroon. The number of alleles per locus ranged from 2 to 3 with an average of 2.64. Röder et al. (2002) detected an average of 10.5 alleles per locus from 502 recent European wheat varieties, using 19 microsatellite markers. Khaled et al. (2015) used 17 SSR markers to assess genetic diversity of 33 genotypes of hexaploid wheat from Egypt and detected an average of 5.59 alleles per locus. In addition, the microsatellite markers used had an average PIC value of 0.59, which

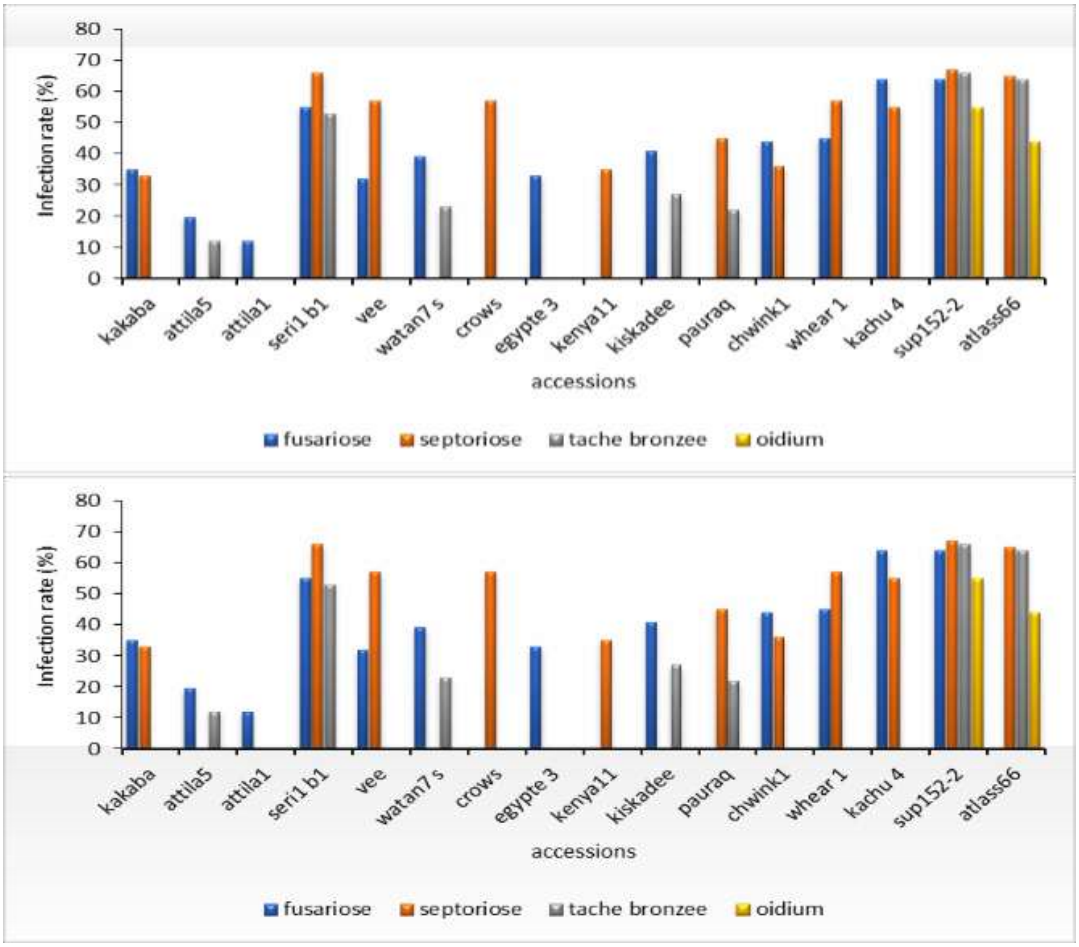

Figure 6. Behavior of wheat accessions in the face of diseases. (A) High altitude accessions behavior. (B) Behavior of accessions at low altitude.
Source: Author

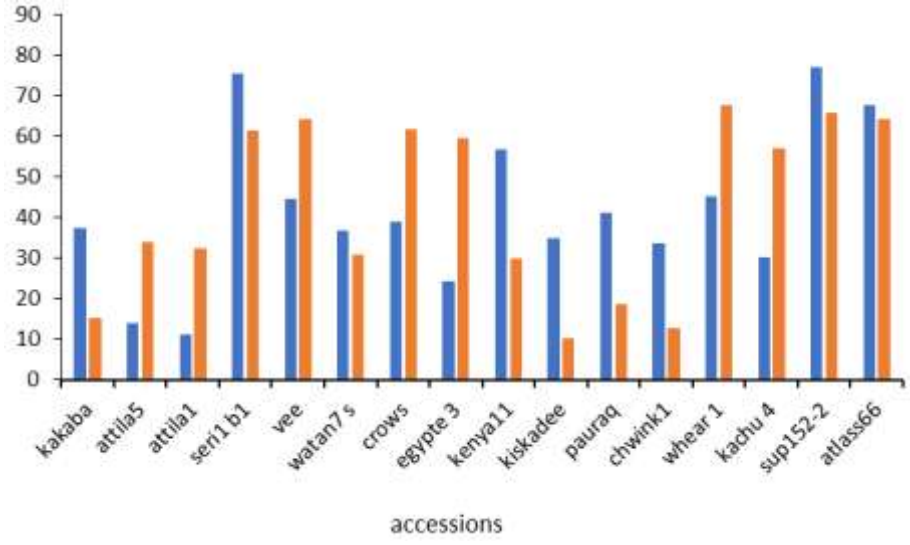

Figure 7. Diagram of percentages of average infections accumulated by accessions at high (HA) and low altitudes (BA).
Source: Author

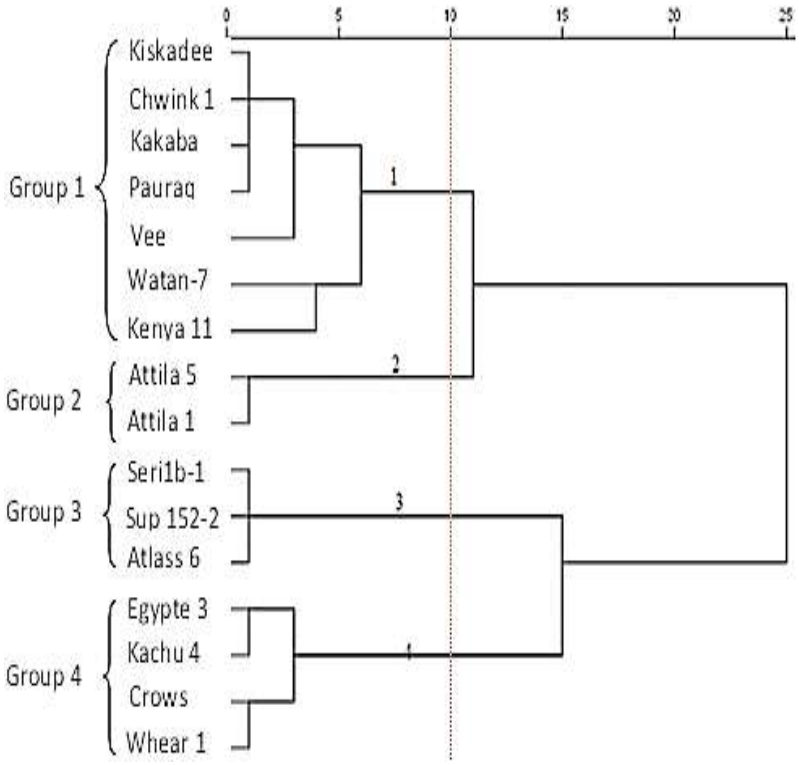

Figure 8. Dendrogram based on the percentage of infection accumulated by the different accessions at the two study sites.
Source: Author

means that these markers were highly informative in this study, since, Botstein et al. (1980) reported that a PIC value > 0.5 is considered to be a sign of a very high informative marker, while 0.5> PIC> 0.25 corresponding to a low informative marker. In previous studies, Röder et al. (2002) found an average PIC value of 0.67 in 500 genotypes. The choice of these SSR loci is therefore relevant for future study in wheat. On the other hand, a high variability in plant height was observed between different accessions in both study sites. These results can be explained by the genetic capacity of different accessions. Indeed, Hsissou (1994) underlined that the growth, development and production of wheat genotypes is the result of numerous morphological changes resulting from the biochemical and molecular expression of the plant. At the inter-site level, the high adaptation capacity observed in Mbankolo can be explained by the climatic conditions present on the site, which are closer to the conditions required for the cultivation of soft wheat, which is in line with results obtained by Ngo Ngom (2017), which states that Mount Mbankolo with its good humidity is ideal for growing wheat in Cameroon. These results also in conformity with the study made by Othmani (2016) and Tsimi (2018) who worked respectively on the performance of selected wheat lines in two ecosystems and on the potential of adaptation of soft wheat varieties in high and low altitudes conditions of the wet zone with bimodal rainfall. Indeed, these authors have also noted that the intense heat at low altitude negatively affects the genetic expression of varieties at the level of growth and development which is materialized by the reduction of the dimensions of the organs of the plant and the reduction of yield (Salawa et al., 2014).

The mycological analysis showed the presence of four diseases attacking wheat (septoria, fusariosis, tanned spot and powdery mildew). These diseases are the same as those reported by Siou (2013), Ayad et al. (2014) and Azoui (2015) who identified wheat pathogens according to standards previously defined by some authors such as Zillinsky (1983), Eyal et al. (1987) and Ezzahiri (2001). This fungal polymorphism may be due to the presence on the culture sites of inocula from the previous crops such as wheat in Mbankolo and corn in Nkolbisson. These works are in agreement with those of Pereyra et al. (2004), who observed that a disease-sensitive crop precedent during its cycle is a potential source of inoculum for the next crop through its residues.

For each disease, except the tanned spot, significant differences were observed between the two sites for prevalences, and incidences with marked dominance at Mbankolo. This suggests that in high altitude conditions, accessions of soft wheat are more prone to fungal fact

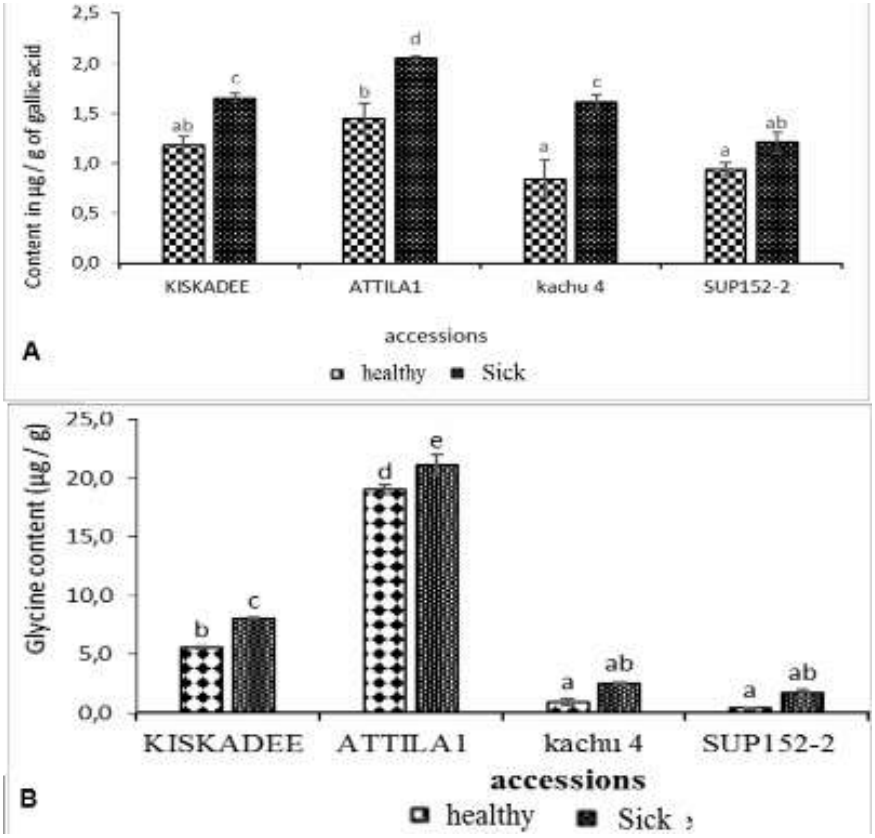

Figure 9. Variations in biochemical compounds of accessions in healthy and sick conditions. (A) Total polyphenol contents. (B) Total amino acids. The averages of healthy and sick histograms (for each variable) bearing the same letters are not significantly different at the 5% threshold.
Source: Author

invasions. Although not statistically significant, for some diseases it has also been shown that all the diseases observed were generally more severe in Mbankolo than in Nkolbisson. These results can be explained by the that Mbankolo's hot and humid climate favors the development and intensification of diseases. In fact, climatic factors (humidity and temperature) play a decisive role in the transmission and evolution of the disease. These results are consistent with those of Nguyen (2007), Moreau (2011) and Zahri et al. (2014) who state directly that the environment of the pathogen has an influence on its development and that the majority of fungal diseases prefer humidity and high temperatures. According to Mathieu et al. (2012), temperature and humidity are responsible for variations in disease. Indeed, a great variability in the distribution of diseases on both sites was observed. Powdery mildew was observed during surveys only at the Mbankolo site. Knowing that each pathogen has specific climatic requirements for its development, this contrast can be justified by the climatic conditions that prevailed at both sites. Mbankolo is characterized by a high hygrometry (15.53% higher than Nkolbisson) and lower temperatures (lower than 6.5°C, compared to Nkolbisson), thus appears very favorable to the development of pathogens.

Based on the results of the biochemical markers of tolerance, it is noted that at the intra-varietal level, the polyphenol and total amino acid contents were appreciably higher in sick leaves for all the accessions analyzed. This result can be explained by the fact that in response to the fungal infection, plants synthesize specific substances that are phenolic compounds and amino acids to defend themselves. These results are similar to the work of Lattanzio et al. (2006), and Patil et al. (2011) who reported that during fungal invasion, one of the most important aspects of the defense strategy is the stimulation of phenolic compounds as well as the increase of the amount of amino acid from 20 to 25% in infected wheat tissues.

At the varietal scale, the results obtained indicate that the highest levels of total polyphenols and amino acids were observed in Attila 1 (highly tolerant accessions to diseases), and the lowest in Sup-152-2 which is characterized by a high sensitivity to diseases. This

difference can be explained by several factors including the intrinsic factors of accession including the genotype. Indeed, the quantitative and qualitative variability of these metabolites is the result of the expression of the genes of the different accessions, which can be linked to the growth metabolism. In addition to their defense functions, these metabolites are involved in the growth of the plant. Several studies (Benbrook, 2005, Beta et al., 2005, Zouaoui, 2012) have reported that the concentration of polyphenols and amino acids is generally higher in wheat-resistant genotypes than in disease-susceptible genotypes fungal. These results are consistent with those of Manga et al. (2016) and Effa et al. (2017) which emphasize that these compounds act as barriers against pathogen invasion and hence constitute part of host resistance mechanisms of cocoa (*Theobroma cacao* L.).

Nevertheless, a significant negative correlation found between total polyphenol, amino acid and level of tolerance (percentage of infection) has been observed. These results are consistent with those of Bhuiyan et al. (2009) who also reported a correlation between the levels of these compounds and resistance to fungal infection, state that a high polyphenol and amino acid content prevents the development of pathogens and therefore, reduces the percentage of infection. Moreover, these findings agree with that of Effa et al. (2017) and Manga et al. (2018) observations who reported that tolerant genotype accumulates high amount of biochemical substances. These can be used as markers for selection of tolerant genotypes. Tolerant plants, when subjected to biotic stress, showed elevated levels of free phenolics and amino acid (Djocgoue et al., 2011).

Conclusion

The results obtained in this study provided new information about characterization of wheat cultivars for fungal disease tolerance in Cameroon. The set of the used microsatellite markers showed a high level of polymorphism and sufficient information to discriminate the cultivars of hexaploid wheat introduced in Cameroon. In this study, Mbankolo represents the most suitable site for wheat cultivation. However, specificity for the Nkolbisson site has been noted in accessions such as Kakaba and Kiskadee suggesting a possible adaptation of wheat at low altitude. Four diseases have been identified in wheat, namely Septoria, Fusarium, Tanned Spot and Powdery Mildew. In addition, the influence of the site on the evolution and intensity of the disease was determined. With a hygrometry higher than 15.53%, the site of Mbankolo is more conducive to the development of diseases. The percentage of infection, the total polyphenol content and the amino acid content were discriminating factors that could serve as selection criteria for the accessions to be introduced according to the sites. Of these criteria, 2 accessions to Mbankolo (Attila 1 and Attila 5), 2 to Nkolbisson (Kakaba and Kiskadee) and 1 common to both sites (Watan-7-sehkra) were among the 16 as high-performance and can then be recommended for growing wheat in Cameroon. For future investigation, a thorough mycological study could be carried out to identify the strains of the various pathogens responsible for the diseases observed and make a molecular characterization of the different pathogens.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Some physicochemical properties of tyrosinase from sweet potato (*Ipomea batatas*)

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Tyrosinase catalyzes formation of browning in plants, foods and vegetables. Sweet potato (*Ipomea batatas*) undergoes browning after harvesting or during post-harvest operations leading to spoilage and loss of economic value. The physico-chemical properties of purified tyrosinase from *I. batatas* are here described with a view to providing information on the suitability or otherwise of the enzyme for several industrial and biotechnological processes. The enzyme was purified using new approach resulting into final yield and purification fold of 76% and 7.1, respectively. The molecular weight (native) was 48.3 ± 2.5 kDa as estimated on Sephadex G-100. Highest tyrosinase activity was obtained at pH 6.5 while that of temperature was 50°C. Kinetic parameters studies resulted to 2.5 ± 1.2 mM and 451 ± 23.7 units/mg for Michaelis constant (K_m) and maximum velocity (V_{max}), respectively. This led to catalytic efficiency, k_{cat}/K_m of 1.45 × 10^5 s^{-1} M^{-1}. It was concluded that, tyrosinase from *I. batatas* possesses remarkable properties that could be exploited for biotechnological processes.

Key words: Polyphenols, Solanaceae, biotechnology, biocatalysis.

INTRODUCTION

Tyrosinase (TYR) is a metalloenzyme with a highly conserved copper binding region and exists in fruits, fungi, vegetables, mammals, cuticle sclerosis and wound healing in insects (Song et al., 2022). They have been reported to be responsible for melanogenesis in humans and browining reactions in plants, fruits and vegetables (Halaouli et al., 2006). Tyrosinase has the ability to react with polyphenols; it has found various applications in biotechnology (Jus et al., 2009). Such functions include synthesis of important drugs (Ates et al., 2007), removal of phenols in wastewater reported by Martorell et al. (2012), grafting of silk proteins onto chitosan through tyrosinase reactions (Anghileri et al., 2007) and cross-linking abilities used in food processing (Ilesanmi et al., 2021). Tyrosinase has been reported in several organisms, but the most characterized is from micro-organism. Recently, higher plants have been exploited for the isolation tyrosinase.

Sweet potato (*Ipomea batatas*) is a dicotyledonous plant that belongs to the order Solanales and family Convolulaceae. It's large, starchy, sweet-tasting, and tuberous roots are used as a root vegetable. Sweet potato tubers undergo browning reactions when harvested and/or during processing. These could be associated with the presence of tyrosinase converting the released polyphenols into polymeric products. Tyrosinase

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has been enzyme of interest for biotechnologists, biochemists and industries because of its increasing use in several applications (Sabarre and Yagonia-Lobarbio, 2021). Hence, the need to exploit several sources for the enzyme to replace traditional source. The presence of tyrosinase in different yam species has been reported (Ilesanmi et al., 2014). In that work, the levels of tyrosinase in seven yam species were reported and four species (*Dioscorea praehensilis*, *Dioscorea rotundata*, *Dioscorea alata* and *Colocasia esculenta*) with highest activities were well characterized. Tyrosinase from *I. batatas* was not characterized. Whether the tyrosinase from *I. batatas* possesses even interesting and remarkable properties, in terms of stability and usability, is not known. The aim of this study, therefore was to investigate and document the physicochemical properties of tyrosinase from the sweet potato for it's suitability in biotechnological applications and industrial processes.

MATERIALS AND METHODS

Fresh tuber of sweet potato was obtained from local farmers in Owo, Ondo State, Southwestern Nigeria.

Chemicals

Anhydrous sodium phosphate monobasic ($NaHPO_4$), bovine serum albumin (BSA), L-dihydroxyphenyl-3,4-alanine (L-DOPA), blue dextran, tris base, trizma acid, sodium phosphate dibasic (Na_2HPO_4), glutathione, and Coomassie brilliant blue R-250 were obtained from Sigma, USA. Ammonium sulphate, *t*-butanol was obtained from Carl Roth, Karlsruhe, Germany. Sephadex G-100 was purchased from GE Healthcare Bio-sciences, Sweden. All other reagents were of analytical grade.

Preparation of sweet potato homogenate

Thirty grams of the fresh sweet potato tuber were rinsed with distilled water, peeled and homogenized using mortar and pestle in 70 mL of Na-phosphate buffer (0.05 M, pH 6.5) at 4°C to obtain 57 mL homogenate (30%). The homogenate prepared was subjected to centrifugation at 12,000 rpm for 30 min in a cold centrifuge (4°C) to obtain crude supernatants. The crude supernatant was assayed for tyrosinase activity using L-DOPA as substrate. The supernatants were separated and stored at -20°C.

Tyrosinase activity assay

The activity of tyrosinase from *I. batatas* was determined according to Ilesanmi et al. (2014). It involved addition of 50 µL of enzyme, 750 µL homogenizing buffer and 200 µL (1 mM final concentration) of L-DOPA to initiate the reaction. The activity was measured kinetically in spectrophotometer.

Determination of protein concentration

Protein concentration was determined according to the method by Bradford (1976) using bovine serum albumin as standard.

Purification using three phase partitioning and gel filtration chromatography

Three phase partitioning (TPP) of the crude enzyme was carried out according to the method of Akardere et al. (2010). Briefly, ammonium sulfate was added to the crude tyrosinase to achieve 70% saturation. The mixture was stirred using vortex machine at 4°C. After, *t*-butanol was added at different ratios (1:0.5, 1:1 and 1:1.5 v/v). The mixture was allowed to stand for 1 h at room temperature and centrifuged at 4000 rpm for 10 min. The mixture phase was separated into three layers. The upper *t*-butanol layer was removed carefully. The interfacial precipitate (middle phase) and the lower aqueous layer (bottom phase) were collected separately. The middle phase precipitate was dissolved in 1 mL of sodium 0.2 M acetate buffer, pH 5.0. Thereafter, all three phases were dialyzed to remove ammonium sulfate and then analyzed for tyrosinase activity. The middle phase of all TPP systems gave the highest tyrosinase activity recovery.

The post TPP bottom rich was then further purified by layering on column packed with Sephadex G-100 previously washed and equilibrated with 0.05 M Na-phosphate buffer (pH 6.5). Fractions obtained were assayed for tyrosinase activity and protein profile measured at 280 nm. The active fractions were pooled together and concentrated using lyophilization. About 10 g of the lyophilisate was redissolved in phosphate buffer (50 mM, pH 6.5) for further use.

Estimation of molecular weight for native tyrosinase

The molecular weight of the enzyme was determined on calibrated Sephadex G-100 column. The calibration curve for the estimation was obtained as plot of partition coefficient (k_{av}) of the standard proteins versus their respective molecular weights. Interpolation of the k_{av} values on the standard curve was used to obtain the molecular weight of tyrosinase from sweet potato.

Estimation of kinetic parameters

The kinetic parameters (K_m, V_{max}, k_{cat} and k_{cat}/K_m) were determined from the plot of varied concentrations of L-DOPA against tyrosinase activities at these concentrations. The K_m and V_{max} were analysed using Graph pad prism 5 and the data obtained were used for estimation of k_{cat} and k_{cat}/K_m.

Effect of temperature on tyrosinase activity

This involved incubation of the substrate solution (1 mM L-DOPA in final mixture), 0.05 M phosphate buffer at pH 6.5 at the different temperature ranging between 10 and 80°C for 5 min. Enzyme was introduced immediately and read at 475 nm in the spectrophotometer. The activity was plotted against their respective temperature.

Thermal stability of *I. batatas* tyrosinase

The tyrosinase from *I. batatas* was incubated differently at 20, 30, 40, 50, 60 and 70°C for 1 h. At 10 min interval, aliquot was taken for residual activity determination. The percentage residual activity obtained by comparing the activity at zero time was plotted against the time of incubation.

Effect of pH on *I. batatas* tyrosinase

The determination of pH influence on tyrosinase activity was carried

Table 1. Purification summary of tyrosinase from *I. batata*.

Sample	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)	% Yield	Purification fold
Crude	20	11000	30.0	367	100	1.0
TTP	12	13970	9.0	1541	127	4.2
Sephadex G-100	30	8360	3.2	2606	76	7.1

TPP: Three phase partitioning.
Source: Author

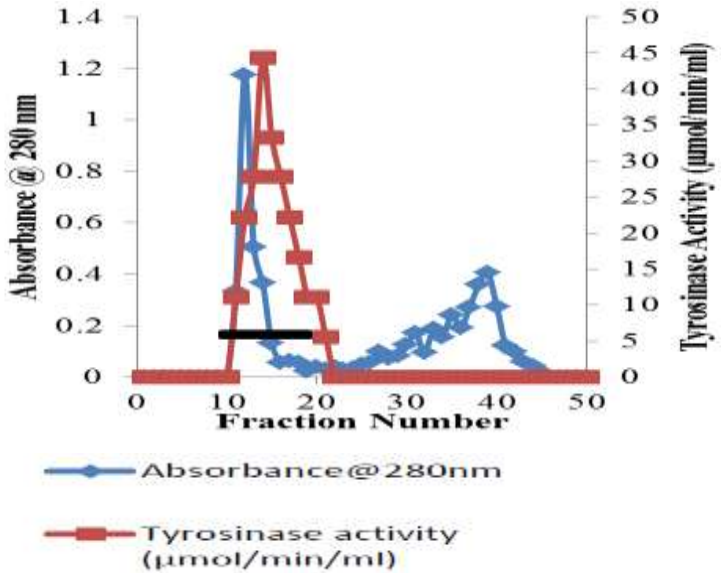

Figure 1. Elution profile of partially purified tyrosinase from *I. batata* on Sephadex G-100. The ATPP pool was then layered on Sephadex G-100 column (1.0 cm × 40.0 cm). The proteins were eluted with the equilibration buffer at a flow rate of 12 ml/h. Fractions of 1 ml each were collected and active fractions were pooled and concentrated.
Source: Author

out using pH range of 3.0 to 11.0. The buffer systems used were 0.05 M citrate buffer (pH 3.0 to 5.0), 0.05 M acetate buffer (pH 5.5 to 6.0), 0.05 M phosphate buffer (pH 6.5 to 8.5) and 0.05 M glycine-NaOH buffer (pH 9.0 to 11.0).

RESULTS AND DISCUSSION

Enzyme purification

Several traditional purification processes have been employed for tyrosinase from different sources. Most of these purification schemes are expensive and cumbersome and involved a number of steps (Ilesanmi and Adewale, 2020). In this work, a simple, more efficient and economical method we have been developed for separation and purification of target proteins. The three-phase partitioning (TPP) gave percentage yield and purification fold of 127% and 4.2, respectively (Table 1). After further purification using gel filtration chromatography on Sephadex G-100, a single peak of activity was obtained (Figure 1). The percentage recovery and purification fold of 76% and 7.1 were achieved, respectively. The summary of purification is shown in Table 1. ATPS purification method has continued to find application because of the advantage over the chromatographic techniques. The method combines both purification and concentration of the resulting enzyme. High yield obtained may be due to preferential partitioning of the enzyme from other unwanted materials.

Molecular weight determination

The native enzyme had molecular weights of 48.3 ± 2.5 kDa as estimated on Sephadex G-100 column. This was estimated on calibrated column of Sephadex G-100. Shuster and Fishman (2009) reported molecular weight

Table 2. Kinetic Parameters of tyrosinase from *I. batata*.

Species	K_m (mM)	V_{max} (units/mg protein)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
I. batata	2.5 ± 1.2	451 ± 23.6	363.1	1.45×10^5

The data are the mean ± standard deviation (SD) of three independent determinations.
Source: Author

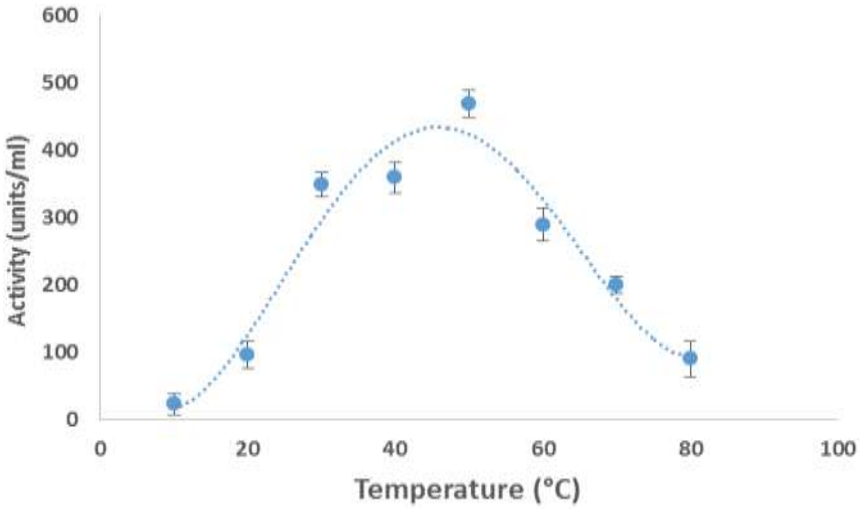

Figure 2. Effect of temperature on the activity of tyrosinase from *I. batata*. Activity at each temperature was determined under the standard reaction conditions. The activity was plotted against temperature. From the plot, the optimum was estimated to be 50°C for the tyrosinase.
Source: Author

of approximately 31 kDa for native *Bacillus megaterium* tyrosinase. The native molecular weight of tyrosinase from different sources is usually in the range between 35 and 55 kDa (Ilesanmi et al., 2014; Zekiri et al., 2014). The *Thermomicrobium roseum* tyrosinase was different from the enzymes of *Agaricus bisporous* (Strothkamp et al., 1976) which is composed of two H subunits (43 kDa) and two L subunits (13 kDa), and of *Neurospora crassa* (Lerch, 1983) and *Streptomyces glaucescens* (Huber and Lerch, 1985) which are monomers of 46 and 31 kDa, respectively.

Kinetic parameters of Tyrosinase from *I. batatas*

The Michaelis constant, K_m of the tyrosinase from *I. batatas* for L-DOPA was 2.5 ± 1.2 mM while that of the maximum velocity, V_{max} was 451 ± 23.6 units/mg proteins (Table 2). This led to catalytic efficiency, k_{cat}/K_m value of 1.45×10^5 s^{-1} M^{-1}. The kinetic properties obtained in this study are similar to that obtained for tyrosinase from yam species (Ilesanmi et al., 2014). Dolashki et al. (2009) reported a K_m of 7.8 mM for L-DOPA for *Streptomyces albus* tyrosinase. The low *K*m value obtained for the tyrosinase from *I*. *batatas* revealed that the enzyme has good affinity for L-DOPA as substrate. However, it was not tightly bound to the active site of the enzyme leading to rapid release of the product. The catalytic efficiency of the enzyme could be an advantage in its application for biotechnological processes.

Effect of temperature on tyrosinase from *I. batatas*

Figure 2 shows the influence of temperature on tyrosinase from *I. batatas*. The optimal temperature obtained was 50°C. The enzyme was not fully stable at temperatures below 50°C. The enzyme was fully stable at temperatures of ≥50°C, but rapidly lost its activity above 70°C. Thus, the enzyme is stable at higher temperatures when compared with tyrosinase from other plant. The data obtained in this work is also comparable with tyrosinase from *Rhizobium etli* and *B. megaterium* displaying optimum activity at 50°C (Cabrera-Valladares et al., 2006; Shuster and Fishman, 2009), but higher than those obtained from other microorganism sources such as *Pseudomonas putida* F6 and *Trichoderma reesei* (30°C) (McMahon et al., 2007). In thermal stability,

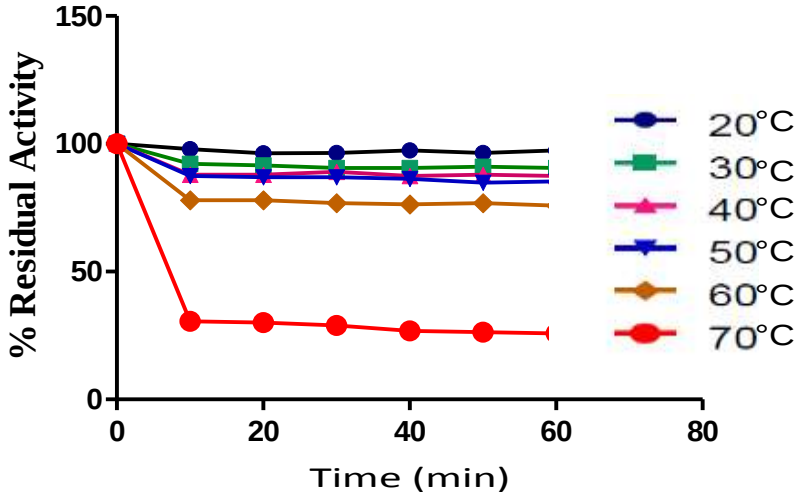

Figure 3. Thermal Stability of Tyrosinase from *I. batata.* The enzyme was incubated at different temperatures (20 – 70°C) for 1 h. An aliquot was taken at 10 min interval and assayed for tyrosinase activity and the residual activity was determined under the standard reaction conditions. The activity at zero time was taken as 100%. The residual activity was plotted against the time of incubation.
Source: Author

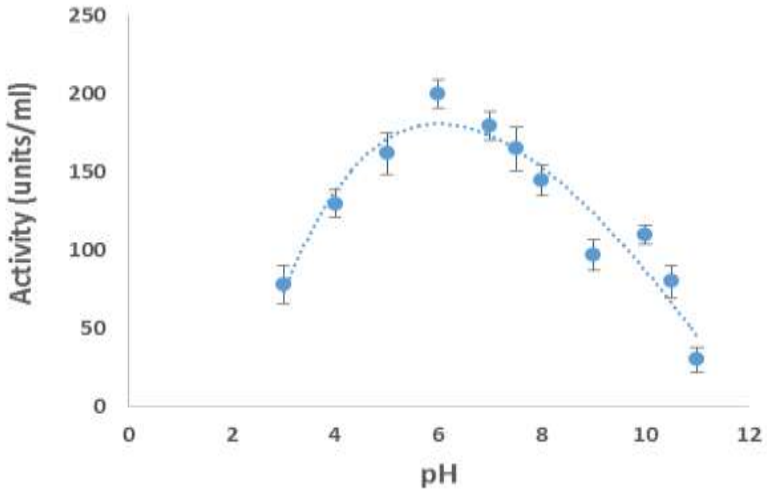

Figure 4. Effect of pH on the Activity of Tyrosinase from *I. batata.* The enzyme was assayed for activity at the indicated pH values. The highest activity was obtained at pH of 6.5.
Source: Author

residual activity was plotted against the time of incubation (Figure 3). About 70% activity was retained at 60°C for the enzyme after 1 h of incubation. About 35% activity was retained even at up to 70°C. The activity and stability of the enzyme was retained at temperatures between 30 and 60°C.

Thermostable enzymes are advantageous as several industrial processes are usually carried out at high temperature. The heat stability of *I. batatas* tyrosinase would be an advantage for its application in these processes especially in wastewater treatment.

Effect of pH on tyrosinase from *I. batatas*

When the activities of enzyme were plotted against the pH, (Figure 4), maximum activity was obtained at pH 6.5. The value is in agreement with that obtained for

tyrosinase from *Dioscorea bulbifera*, *Pseudomonas putida* F6 (pH 7.0), *Streptomyces* species (pH 6.8) (McMahon et al., 2007); *Vibrio tyrosinaticus* (pH 6.6-7.8) (Pomerantz and Murthy, 1974), portabella mushrooms with optimum pH of 7.0 (Fan and Flurkey, 2004); hamster (pH 6.8) (Hearing, 1987); *B. megaterium* of optimum pH 7.0 (Shuster and Fishman, 2009); and fungal tyrosinase from *Pycnoporus* strains (Halaouli et al., 2005). Most results confirm that tyrosinase is not significantly active under basic conditions. The pH dependence of tyrosinase activity showed that the enzyme was more active in acidic buffers than basic buffers.

Conclusion

This study has been able to establish fast purification protocol for tyrosinase from *I. batatas*. It was further established that the enzyme possess several interesting properties that could be exploited in several technical and biotechnological processes.

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

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Full Length Research Paper

Microbial analysis and detection of Aflatoxin from *Irvingia gabonensis* kernels sold in Oyo Town, Oyo State, Nigeria

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This research work aimed at screening for different microorganisms associated with *Irvingia gabonensis* var. *gabonesis* Kernels, its nutritional value and detection of aflatoxins from some of the infested *I. gabonensis* Kernels sold in Oyo town. A total of 30 different *I. gabonensis* var. *gabonesis* Kernels were randomly purchased from six different points in the five major markets in Oyo town and isolation was done on Nutrient agar (NA), McConkey agar, Eosine Methylene Blue Agar (EMB) and Potato Dextrose Agar (PDA) using pour plate method. The isolates were culturally, morphologically and biochemically characterized. The mineral, proximate and aflatoxin detection of the *I. gabonensis* Kernels with high microbial load and growth of *Aspergillus flavus* was carried out using standard methods. A total of 25 bacteria and 18 fungal were isolated which include *Bacillus* spp*., Staphylococcus* spp., *Aspergillus* spp*., Penicillium* spp., and yeast. *Irvingia gabonensis* seeds with growth of *A. flavus* (OOW1) had the least mineral composition with 5.4% sodium, 20.0 mg/kg vitamin C, 29.4 mg/kg calcium, 0.9 mg/100g iron, 34.4 mg/100 g magnesium and 0.02 mg/100 g zinc. The *I. gabonensis* Kernels with the growth of *A. flavus* (OOW1) had the least mineral composition with 5.5% moisture content, 4.2% crude protein, 45.7% crude fat, 9.1% crude fibre and 1.5% total ash. The *I. gabonensis* Kernels with the growth of *A. flavus* (OOW4, OOJ6 and OOW1) had aflatoxin level of 3.47, 3.69 and 5.10 ppb, respectively. *Irvingia gabonensis* seed with high microbial load and growth of *A. flavus* had low nutritional value making them unsafe for consumption.

Key words: Nutritional value, kernels, mineral composition, microbial load, proximate analysis.

INTRODUCTION

Food is a vital part of the cultural identity of people all over the world. In African countries such as Nigeria, some foods are consumed during religious or cultural festivals, while condiments and soup thickeners such as melon and *Irvingia gabonensis* Kernels (Ogbono) are consumed as a normal culinary practice (Chibundu et al.,

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2016). *I. gabonensis* commonly known as Dikanut, Africa mango, bush mango or wild mango is an essential product that serve as a source of valuable income to both rural and urban settlers in Africa (Arowosoge, 2017). It is known in South Western part of Nigeria (Yoruba land) as "Apon" and South Eastern part of Nigeria (Igbo land) as "Ogbono".

The tree of *I. gabonensis* grows well in tropical rainforests of Africa. The freshly harvested seeds are sun-dried, grinded and used as recipe for "ogbono" soup (Bamidele et al., 2015). The two known species of *I. gabonensis* Kernels that grow freely are the *I. Gabonensis* var. *Gabonesis* and *I. gabonensis* var. *Excelsa*. The pulp of the *I. gabonesis* var. *Excelsa* is classified as oil seed due to its high fatty matter (54 to 67%) (Bamidele et al., 2015; Akusuand and Kiin-Kabari, 2016). The kernels of *I. gabonensis* contain oil which is sometimes extracted and often used in the production of margarine and drugs. The residue from the extracted kernel is then used as thickening agent in soup (Arowosege, 2017). The pulp of the *I. gabonensis* var. *gabonensis* is sweet, smooth in the mouth and has brittle pulp but its kernel draws less than that of *I. gabonensis* var. *Excelsa* (Akusuand and Kiin-Kabari, 2016). *I. gabonensis* var. *gabonensis* is highly demanded due to its nutritional, economic and medicinal worth. Due to the high moisture content of the African bush mango, the best method of preserving the *I. gabonensis* is by sun-drying the seeds which help to extend the shelf life of the Kernels (Vihotogbé et al., 2019).

However, these Kernels of *I. gabonensis* have been reported to be prone to fungi attack causing the food to become tasteless, loose its thickness, nutritional value and produce mycotoxins (Sanyaolu et al., 2014; Chuku and Aggrey, 2017). Fungi are plant pathogens and major spoilage agents of foods and foodstuffs. During favorable environmental conditions, some fungal strains may release metabolites such as mycotoxins into food hence making it poisonous and unfit for human consumption (Jonathan et al., 2016). Mycotoxins are mainly produced by certain filamentous fungi belonging to *Aspergillus*, *Penicillium* and *Fusarium* genera. The major agro-economic important mycotoxins produce includes aflatoxins, ochratoxins, trichothecenes, zearelenone, fumonisins and tremorgenic. Aflatoxins have been observed as the most toxic because of their highly carcinogenic and hepatotoxic effect, especially *Aspergillus flavus* and *Aspergillus parasiticus* (Ubwa, 2014; Ozer et al., 2012; Menza et al*.,* 2015).

The growth of molds on the seeds is majorly as a result of poor post-harvest handling, especially during the process of cracking, drying, storage and transportation (Chuku and Aggrey, 2017). An investigation into the aflatoxin content of African bush mango seeds in Nigeria revealed a 35% non-compliance with the European Union standard (Adebayo-Tayo et al., 2006). Due to the infestation of microorganisms in *I. gabonensis* Kernels which has resulted in loss of income and nutritional value of the seeds (Azuonwu et al., 2019), there is need to investigate the different microorganisms associated with *I. gabonensis* Kernels as well as the mineral, proximate and nutritional value of some of the *I. gabonensis* Kernels sold in Oyo town.

MATERIALS AND METHODS

The study was conducted between the months of January to June, 2021. Thirty *I. gabonensis* var. *gabonesis* Kernels were randomly collected from display retailers at six different shops in the five major markets in Atiba Local Government Area of Oyo town, Oyo State. All samples were aseptically packaged and transported to the laboratory for analyses.

Enumeration, isolation and identification of microorganisms

One gram of each samples was weighed and mashed in a stomacher bag containing 9 mL of distilled water using a stomacher machine (Seward STOMACHER® 80 Lab System). One mL aliquot from the stomacher bag was pipetted and transferred into a sterile test tube containing 9 mL of 0.1% peptone water. This process was repeated for each of five sets of test tubes until a dilution of 10^{-6}. 1 mL from the dilution 10^{-5} were plated in duplicate into 15 ml of sterilized and cooled Nutrient Agar (NA), Mac Conkey agar, Eosine Methylene Blue (EMB) agar and Potato Dextrose Agar (PDA). The inverted plates were incubated at 37°C for 24 hours for the bacteria isolates while the PDA plates were inverted and incubated for 5 days at 30°C for the fungal isolates. Distinct colonies were sub-cultured to obtain a pure culture. The inverted plates were incubated for 24 hours in NA, EMB and Mac Conkey agar while the un-inverted PDA plate was incubated for 5 days at 30°C after which the colonies were counted (Kidd et al., 2016). The pure colonies of the bacterial isolates were subjected to gram staining, spore staining, oxidase, catalase and starch hydrolysis. While the pure colonies of the fungal isolates were examined under the microscope after staining with lactophenol cotton blue. The isolates were identified using their morphological characteristics and microscopic structures (Tersoo-Abiem et al., 2020).

Mineral analysis

The mineral analysis of the *I. gabonensis* Kernels with high microbial load and growth of *Aspergillus flavus* were done according to the methods of AOAC (2005). A gram of each sample was digested with 10% HNO_3 after ashing. The sample was filtered after digestion and the filtrate made up to 100 mL with distilled deionized water. Atomic Absorption Spectrometer (Buck Scientific East Norwalk, USA) was used to determine the concentration of iron, magnesium, zinc and calcium while Flame Photometry (Jenway Ltd, Dunmow Essex UK) was used for the determination of Na.

Proximate analysis

Determination of the proximate composition which include moisture, fats, ash, carbohydrate and protein contents of the *I. gabonensis* Kernels with high microbial load and growth of *A. flavus* were done according to the methods of AOAC (2016).

Determination of Moisture content

Moisture content of *I. gabonensis* Kernels samples with high

microbial load and growth of *A. flavus* were analysed using the gravimetric method reported by AOAC, (2016). A 5 g was measured into a previously measured moisture sampler. The sample in the can was allowed to dry by air over a steam bath and then dried in the 105°C for three hours in the oven. It was cooled in a desiccator and weighed. It was then returned to the oven for proper drying. The sample was further dried, cooled and weighed until a regular weight was achieved. Weight of lost moisture content was obtained by difference and calculated as percentage of the weight of sample analyzed.

$$\% \text{ Moisture (\%MC)} = \text{M2-M3/M2-M1} - 100/1$$

where M1 = Measurement of empty moisture can, M2 = Measurement of can + sample before drying and M3 =Measurement of can + sample after drying

Determination of Ash content

Content if the ash in *I. gabonensis* Kernels sample was analyzed by the furnace incineration gravimetric process (AOAC, 2005). A 5 g sample was weighed into previously weighed crucible. It was evaporated to dryness over a steam bath and then burnt in a muffle furnace at 550°C until it becomes grey ash. The ashes in the crucible were carefully removed and chill in a desiccator and weighed again. As the measurement increased, the weight of ash was obtained and expressed as percentage of the sample analyzed and calculated using the formula as shown below.

$$\% \text{ Ash} = \text{W2} - \text{W1} \times 100$$

where, W1 = weight of empty crucible and W2 = weight of crucible + ash.

Determination of Protein content

Protein content was done by Greenfield and Southgate (2003) in which the total nitrogen was obtained and multiplied with the factor 6.38. 10 mL of concentrated H_2SO_4 and 0.5 g of the *I. gabonensis* var. *gabonesis* Kernels was boiled with selenium as a catalyst. Digestion was done in a fume cupboard until a clear mixture was achieved. This breakdown was transferred quantitatively to a standard container and diluted with 100 mL distilled water. 10 mL of the breakdown was mixed with the same volume of 45% NaOH solution and distilled in semi micro- Kjeldahl apparatus. The distillate was transferred into 10% boric acid solution and 3 drops of mixed methyl red and bromocresol green indicator. A total of 50 mL distillate was collected and titrated against 0.02 N H_2SO_4. Titration was done from green colouration to a deep red end- point. Blank was also treated just as described. The Normality (N2) content and protein was calculated as shown below.

$$\text{N2} = \frac{100}{\text{W}} \times 14 \times \frac{\text{N}}{1000} \times \frac{\text{VF}}{\text{Va}} \times \text{T} - \text{Blank}$$

W =weight of sample, N =Normality of titrant, Vf = Total digest volume, Va = Volume of digest analyzed, T = Sample titre, BLK = Reagent Blank titre.

Determination of carbohydrate content of the produced *I. Gabonensis* kernels

Carbohydrate was calculated as nitrogen free extractives using the formula described by Greenfield and Southgate (2003).

$$\% \text{ CHO} = 100 - \% \text{ (Protein + ash + fat + moisture content)}$$

Determination of fat content of the produced ogbono samples

Fat content of *I. gabonensis* Kernels was carried out by measuring 0.5 g of the sample into a conical flask. 0.88 mL ammonia solution and 10 mL of 95% ethanol was added to it and mixed properly. 25 mL of diethyl ether was added and mixed properly for 1 minute. 25 mL of petroleum ether was properly mixed with it. Mixture separated into phases and after standing for 1 hour. The fat extract (ether phase) was collected and the sample was re-extracted with the same solvent and the extracts was pooled together. The extract was then transferred to a pre-weighed flask and the solvent recovered. The fat in the container was oven dried at 100°C for 30 minutes. The dried samples were cooled in a dessicator and measured. Dried sample was weighed and fat was assayed. The amount was written as a percentage of the sample analysed. It was calculated as shown below (Greenfield and Southgate, 2003).

$$\% \text{ fat} = \text{W2} - \text{W3} \times 100$$

where W1 =weight of flask alone and W2 =weight of flask and extract

Aflatoxin detection and quantification

Detection of aflatoxin levels from *I. gabonensis* Kernels with high microbial load and growth of *A. flavus* was carried out by Enzyme-Linked Immunosorbent Assay (ELISA) method. 10 g of each sample was extracted with 20 mL methanol: water (70: 30). The residue was dissolved in 1 mL of methanol: water (3:1, v/v) and 200 ml of diluted extract was applied to the enzyme immuno-sorbent assay (ELISA) plate in order to determine the total aflatoxin content. Each one of the samples and standards were applied in duplicates. Testing for total aflatoxin content was carried out on each sample after the extraction process, using AgraQuant assay kit (Romer Labs) according to the manufacturer's instructions in the ELISA kit. The total aflatoxin concentration was read at 450 to 630 nm. The optical densities (ODs) were compared to those of the standards. Total aflatoxin concentration in each sample was expressed in parts per billion (ppb) (Tersoo-Abiem et al., 2020).

RESULTS

Microorganisms were enumerated from *I. gabonensis* Kernels samples obtained from five different markets in Oyo town is shown in Table 1. Regardless of the high dilution factor (10^5) used for the six samples of *I. gabonensis* Kernels randomly purchased from each market, the highest bacterial count was observed in OOJ1 with 2.80 x 10^6 cfu/g on Nutrient agar, 4.8 x 10^6 cfu/g on McMackey agar and 4.4 x 10^4 cfu/g on Eosine. Methylene Blue agar while OOW1 had high fungal count (7.0 x 10^3) on Potato Dextrose Agar. Tables 2 and 3 shows the macroscopic characteristics of isolates on Nutrient agar, Eosine Methylene Blue (EMB) and MacConkey Agar. A total of 25 bacteria were isolated. The bacterial isolates showed various colonial appearances on Nutrient agar ranging from smooth

Table 1. Microbial count of *Irvingia gabonensis* Kernels at 10^5 dilution factor

Sample	NA		Mc Conkey agar		EMB agar		Potato dextrose agar (PDA)	
	No. of colonies	Viable count (cfu/g)	No. of colonies	Viable count (cfu/g)	No. of colonies	Viable count (cfu/g)	No. of colonies	Viable count (cfu/g)
OOW1	245	2.45×10^4	093	6.3×10^4	051	5.1×10^4	07	7.0×10^3
OOW2	034	3.4×10^6	014	1.4×10^6	014	1.4×10^6	02	2.0×10^5
OOW3	016	1.6×10^6	046	2.6×10^6	021	2.1×10^6	02	2.0×10^5
OOW4	230	2.30×10^4	078	7.8×10^4	056	5.6×10^4	06	6.0×10^3
OOW5	140	1.40×10^4	083	1.3×10^4	035	3.5×10^4	02	2.0×10^3
OOW6	109	1.09×10^4	019	1.9×10^4	042	4.2×10^4	02	2.0×10^3
OAJ1	214	2.14×10^4	51	5.1×10^4	011	1.1×10^4	05	5.0×10^3
OAJ2	267	2.67×10^6	042	9.2×10^6	025	7.5×10^6	04	4.0×10^5
OAJ3	183	1.83×10^6	014	1.4×10^6	062	6.2×10^6	-	-
OAJ4	283	2.83×10^6	048	4.8×10^6	018	1.8×10^6	06	6.0×10^3
OAJ5	190	1.90×10^4	012	1.2×10^4	011	1.1×10^4	01	1.0×10^3
OAJ6	083	8.3×10^6	023	2.3×10^6	062	3.1×10^6	-	-
OAK1	120	1.20×10^6	072	5.2×10^6	033	3.3×10^4	-	-
OAK2	279	2.79×10^6	057	8.7×10^6	027	6.7×10^4	06	6.0×10^3
OAK3	187	1.87×10^6	076	2.6×10^4	021	2.1×10^4	01	1.0×10^5
OAK4	129	1.29×10^6	072	4.2×10^6	035	3.5×10^6	04	4.0×10^5
OAK5	192	1.92×10^6	029	2.9×10^6	034	3.4×10^6	-	-
OAK6	192	1.92×10^6	029	2.9×10^6	034	3.4×10^6	-	-
OSB1	110	1.10×10^6	039	3.9×10^4	026	2.6×10^4	-	-
OSB2	182	1.82×10^6	082	3.2×10^4	051	5.1×10^4	05	5.0×10^3
OSB3	119	1.19×10^6	041	4.1×10^4	013	1.3×10^4	-	-
OSB4	153	1.53×10^6	052	5.2×10^4	016	1.6×10^4	-	-
OSB5	129	1.29×10^6	062	6.2×10^6	029	2.9×10^6	-	-
OSB6	192	1.92×10^6	029	2.9×10^6	034	3.4×10^6	06	6.0×10^3
OOJ1	280	2.80×10^6	048	4.8×10^6	044	4.4×10^4	05	5.0×10^3
OOJ2	190	1.90×10^6	029	2.9×10^4	032	3.2×10^6	-	-
OOJ3	125	1.25×10^6	036	3.6×10^4	017	1.7×10^4	02	2.0×10^5
OOJ4	110	1.10×10^6	081	1.1×10^4	029	2.9×10^4	-	-
OOJ5	139	1.39×10^6	077	2.7×10^4	031	3.1×10^4	02	2.0×10^5
OOJ6	192	1.92×10^6	029	2.9×10^6	034	3.4×10^6	05	5.0×10^3

Source: Author

surfaces, raised elevation, circular shaped, mucoid colony, pigmented, translucent, opaque, shiny colony, large, medium colonies. Five of the isolates were Gram negative rod while the remaining 20 were Gram positive (4 Gram positive cocci and 21 Gram positive rod). Most of the isolates were indole negative, catalase positive and spore formers. The bacteria isolates were biochemically identified as *Bacillus cereus, Bacillus subtillis, Staphylococcus* spp., *Staphylococcus* spp. and *Salmonella* sp.

Figure 1 shows the percentage frequency of occurrence of bacteria isolated from *I. gabonensis*. Table 4 shows the colonial and morphological characteristics features of fungi isolates on PDA. A total of eighteen fungi were isolated. The fungal isolates showed different cultural appearances such as a velvety and flaky surface with grey to black coloration, White and green variants powdery surface growth, dust-like sporulating surface light brown with smooth border, abundant mycelium with pale brown and dark zonation, white air mycelium with quick differentiation, upper side white color with irregularly smooth and fringed. The fungal isolates were identified as *Aspergillus flavus, Aspergillus niger, Penicillium* sp., and yeast. The percentage frequency of occurrence of fungal isolated from *I. gabonensis* is shown in Figure 2. 17% of the fungal isolates were *A. flavus,* 33% were *A. niger,* 28% were *Penicillium* sp., while yeast had 22%.

Table 2. Macroscopic characteristics of bacteria isolates on Nutrient agar, eosine methylene blue and MacConkey agar.

S/N	Sample	Margin	Colour	Elevation	Texture	Shape
1	OOW1	Entire	Milky	Convex	Shiny	Round
2	OOW2	Entire	Milky	Flat	Shiny	Puntiform
3	OOW3	Lobate	Opaque	Flat	Mucoid	Irregular
4	OOW4	Lobate	Opaque	Flat	Moist	Irregular
5	OOW5	Undulate	Opaque	Flat	Mucoid	Irregular
6	OOW6	Lobate	Opaque	Flat	Mucoid	Filamentous
7	OAJ1	Filamentous	White	Flat	Dry	Filamentous
8	OAJ2	Lobate	White	Flat	Moist	Irregular
9	OAJ3	Undulate	Opaque	Flat	Moist	Irregular
10	OAJ4	Lobate	White	Obonate	Slimy	Irregular
11	OAJ5	Lobate	White	Flat	Moist	Irregular
12	OAK1	Smooth	Opaque	Flat	Moist	Irregular
13	OAK2	Entire	Entire	Milky	Convex	Shiny
14	OAK3	Entire	Milky	Flat	Shiny	Puntiform
15	OAK4	Filamentous	White	Flat	Dry	Filamentous
16	OAK5	Smooth	Opaque	Flat	Moist	Irregular
17	OAK6	Entire	Entire	Milky	Convex	Shiny
18	OSB2	Filamentous	White	Flat	Dry	Filamentous
19	OSB6	Undulate	Opaque	Flat	Mucoid	Irregular
20	OOJ1	Undulate	Opaque	Flat	Mucoid	Irregular
21	OOJ2	Smooth	Opaque	Flat	Moist	Irregular
22	OOJ3	Entire	Milky	Flat	Shiny	Puntiform
23	OOJ4	Smooth	Opaque	Flat	Moist	Irregular
24	OOJ5	Undulate	Opaque	Flat	Mucoid	Irregular
25	OOJ6	Undulate	Opaque	Flat	Mucoid	Irregular

Source: Author

Mineral composition of the *Irvingia gabonensis* Kernels

Mineral composition, proximate analysis and the detection of aflatoxin were determined using three samples of *I. gabonensis* Kernels due to their high microbial load and the presence of *A. flavus* in the karnel. The *I. gabonensis* Kernels without the growth of *A. flavus* (OAJ3) was used as control. Table 5 shows the mineral composition of *I. gabonensis* Kernels. The *I. gabonensis* Kernels without the growth of *A. flavus* (OAJ3) had the highest mineral composition as follows 7.5% sodium, 28.2 ppm vitamin C, 87.4 ppm potassium, 37.9 ppm calcium, 13.4 mg/100g iron, 49.9 mg/100g magnesium and 0.03 mg/100g zinc) compare to *I. gabonensis* Kernels with the growth of *A. flavus*. Sample OOW1 had the least mineral composition with 5.4% sodium, 20.0 ppm vitamin C, 53.6 ppm potassium, 29.4 ppm calcium, 0.9 mg/100g iron, 34.4 mg/100g magnesium and 0.0.2 mg/100g zinc. However, there are no heavy metals present in any of the samples analyzed.

Proximate composition of *I. Gabonensis* Kernels

Table 6 shows the mineral composition of *I. gabonensis* Kernels. The *I. gabonensis* kernel without the growth of *A. flavus* (OAJ3) had the 16.3% carbohydrate content, 5.5% moisture content, 7.8% crude protein, 58.9% crude fat, 10.7% crude fibre and 1.8% total ash, 47.1 kg/100g fatty acids with 2589.0 kg/100g metabolized energy. Sample OOW1 had the least mineral composition with 29.9% carbohydrate, 5.5% moisture content, 4.2% crude protein, 50.7% crude fat, 9.1% crude fibre, 1.5% total ash, 40.6 kg/100 g fatty acids and 2340.3 kg/100 g metabolized energy.

Presence of aflatoxin in the *I. gabonensis* kernels

Aflatoxin level of the *I. gabonensis* Kernels is shown in Table 7. The *I. gabonensis* Kernels without the growth of *A. flavus* (OAJ3) had the no aflatoxin, The *I. gabonensis* Kernels with the growth of *A. flavus* (OOW4, OOJ6 and OOW1) had aflatoxin level of 3.47, 3.69 and 5.10 ppb,

Table 3. Biochemical characterization of the bacterial isolates isolated from *Irvingia gabonensis* Kernels.

S/N	Isolate code	Gram reaction	Cellular morphology	Catalase test	Oxidase test	Indole test	Motility test	Endospore	Glucose	Sucrose	Lactose	Fructose	Maltose	Probable organism
1.	OOW1	+	Cocci	+	-	-	-	+	+	+	+	-	+	*Staphylococcus* spp.
2.	OOW2	+	Rod	+	-	_	+	+	+	+	-	+	+	*Bacillus cereus*
3.	OOW3	+	Rod	+	-	_	+	+	+	-	-	+	+	*Bacillus cereus*
4.	OOW4	+	Rod	+	+	-	+	+	+	+	-	-	+	*Bacillus subtilis*
5.	OOW5	+	Cocci	+	-	-	-	+	+	+	+	-	+	*Staphylococcus* spp.
6.	OOW6	+	Rod	+	-	_	+	+	+	+	-	+	+	*Bacillus cereus*
7	OAJ1	+	Cocci	-	+	_	-	+	+	+	+	+	+	*Streptococcus* spp
8.	OAJ2	-	Rod	+	-	_	_	+	+	+	-	+	+	*Salmonella* sp.
9.	OAJ3	+	Rod	+	+	-	+	+	+	+	-	-	+	*Bacillus subtilis*
10.	OAJ4	+	Cocci	+	-	-	-	+	+	+	+	-	+	*Staphylococcus* spp.
11.	OAJ5	+	Rod	+	-	_	+	+	+	+	-	+	+	*Bacillus cereus*
12.	OAK1	-	Rod	+	-	_	_	+	+	+	-	+	+	*Salmonella* sp.
13.	OAK2	+	Cocci	+	-	-	-	+	+	+	+	-	+	*Staphylococcus* spp.
14.	OAK3	+	Rod	+	-	_	+	+	+	+	-	+	+	*Bacillus cereus*
15.	OAK4	+	Rod	+	+	-	+	+	+	+	-	-	+	*Bacillus subtilis*
16.	OAK5	-	Rod	+	-	_	_	+	+	+	-	+	+	*Salmonella* sp.
17.	OAK6	+	Cocci	+	-	-	-	+	+	+	+	-	+	*Staphylococcus* spp.
18.	OSB2	+	Rod	+	-	_	+	+	+	-	-	+	+	*Bacillus cereus*
19.	OSB6	+	Rod	+	-	_	+	+	+	+	-	+	+	*Bacillus cereus*
20.	OOJ1	-	Rod	+	-	_	_	+	+	+	-	+	+	*Salmonella* sp.
21.	OOJ2	+	Rod	+	-	_	+	+	+	-	-	+	+	*Bacillus cereus*
22.	OOJ3	-	Rod	+	-	_	_	+	+	+	-	+	+	*Salmonella* sp.
23.	OOJ4	+	Rod	+	+	-	+	+	+	+	-	-	+	*Bacillus subtilis*
24.	OOJ5	+	Rod	+	-	_	+	+	+	+	-	+	+	*Bacillus cereus*
25.	OOJ6	+	Rod	+	-	_	+	+	+	+	-	+	+	*Bacillus cereus*

Source: Author

respectively.

DISCUSSION

The bacterial isolates obtained from this study is similar to the study of Adebayo-Tayo et al. *(*2006) and Adegbehingbe et al. (2014) who isolated similar bacteria from seeds of *I. gabonensis.* The fungal isolated from this research include *Aspergillus flavus*, *Aspergillus niger* and *Penicillum* sp. Which is in line with the work of Chibundu et al. (2016). Tersoo-Abiem et al. (2020) also isolated the similar fungal strains from Ogbono samples that were obtained from different markets in Benue states.

The source of microbial contamination in samples could be due to the environment in which

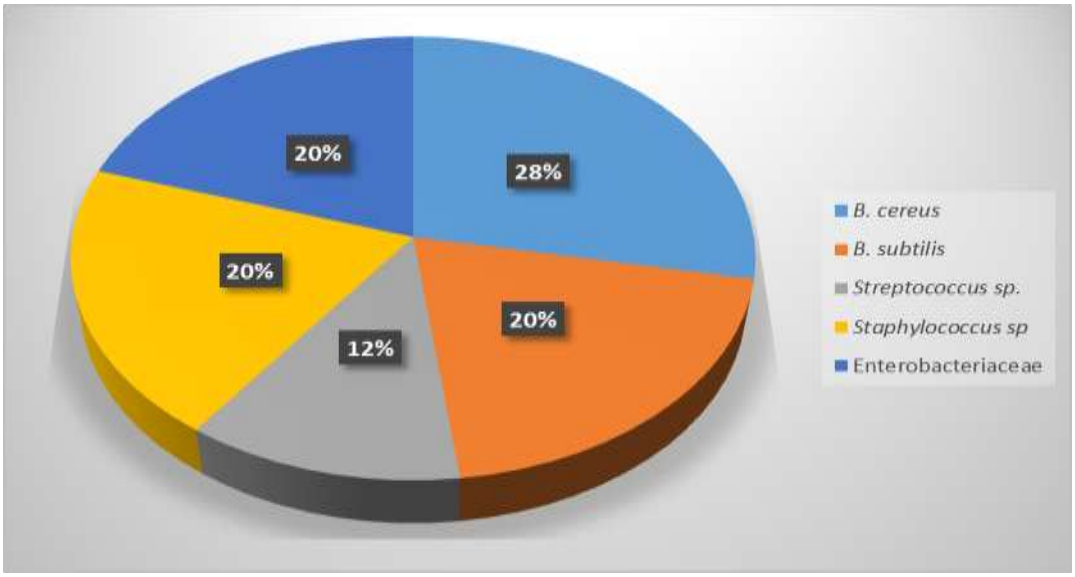

Figure 1. Percentage frequency of occurrence of the bacteria isolated from *Irvingia gabonensis* Kernels.
Source: Author

the Ogbono samples were sold. Ekundayo et al., (2003) isolated some pathogenic microorganisms from his research and he opined that some of the Ogbono samples could have been stored for a very long period before selling. The presence of Staphylococcus aureus and Bacillus sp. Indicate a potential risk and could be harmful to humans when ingested, due to their ability to produce toxins (Saliu, 2008)**.** *Bacillus* sp*.* is normally found in the soil and may have been transported via vegetables. The consumption of these organisms in the Ogbono samples in large numbers could lead to gastrointestinal illness.

The occurrence of *S. aureus* which are Gram positive cocci, catalase-positive, coagulase-positive,oxidase-negative and facultative anaerobes in most of the samples strongly indicated a high level of poor personal hygiene by the sellers. Although *S. aureus* is often associated with the skin and mucous glands (especially in the nose of healthy persons) as commensals (Ibrahim, 2017). Since the market is a busy place, particulate matter carrying microorganisms may have been deposited and unhealthy practices carried out in the market could put the unsuspecting public at massive potential risk with strong public health concern of food poisoning.

Mineral composition

The mineral composition of the ogbono samples without the growth of *Aspergillus flavus* (OAJ3) used as a control had high mineral composition compared to ogbono samples with the growth of *Aspergillus flavus* which is similar to the work of Oseni and Ekperigin (2007). This result is also line with the work of Ibrahim et al. (2017) and Mgbemena et al. (2019) who recorded higher amount of iron and calcium in Ogbono seeds. They also recommended that the seed of Ogbono seed should be consumed due to the high iron content. Mineral content in food is a measure of the amount of specific inorganic components present within the food. Minerals act as co-factors for enzyme reactions. Sodium, calcium and magnesium are required in major quantities. Sodium acts as charge carriers and is a major factor in extra cellular fluid. It also participates in the functioning of muscle nerve (Mgbemena et al*.,* 2016). Sodium and potassium are needed to help maintain the pH of the body so as to regulate muscles and nerves irritability as well as osmotic balance of the body fluids. Iron content in OAJ3 is higher. This is required for blood formation and normal functioning of the central nervous system. Vitamin C is higher in OAJ3 than other samples. It is a fat-soluble vitamin that serves as a good antioxidant, for healthy vision, skins and other tissues in the body (Onojah et al., 2018).

OOW4, OOJ6 and OOW1 had least mineral composition which could be due to the high microbial load, presence of enteric bacteria and toxin producing microorganisms which made it unfit for consumption (Ezekiel et al., 2016).

The proximate composition of *I. gabonensis* Kernels.

Most of the *I. gabonensis* Kernels samples with high microbial load were observed to have high moisture content. The carbohydrates and moisture content ranged from 15.3% to 30.0% and 4.5 to 5.5%. Sample OAJ3

Table 4. Colonial and morphological characteristics features of fungi isolated from *Irvingia gabonensis* Kernels

Isolate	Macroscopy	Microscopy	Probable organism
OOW1	Colonies appears greenish-yellow with white-like borders	Septate hypha with long conidiophores arranged in clusters supporting the phialides. The conidiophores have rough texture and spiny bellow the vesicle.	*Aspergillus flavus*
OOW2	Small to medium colony, round, raised, smooth and colony colour is white to cream	Oval cell shape, multilateral germination pattern, reproduces by budding.	Yeast
OOW3	Velvety and flaky surface due to marked sporulation with grey to green coloration.	Septate hyphae with borne laterally conidiophores and conidia borne in the chain on sterigma. Conidiophores smooth-walled.	*Aspergillus niger*
OOW4	Colonies appears greenish-yellow with white-like borders	Septate hypha with long conidiophores arranged in clusters supporting the phialides. The conidiophores have rough texture and spiny bellow the vesicle.	*Aspergillus flavus*
OOW5	Velvety and flaky surface due to marked sporulation with grey to green coloration.	Septate hyphae with borne laterally conidiophores and conidia borne in the chain on sterigma. Conidial heads are large, globose and dark brown with smooth-walled conidiophores.	*Aspergillus niger*
OOW6	Colonies appear granular, wool-like and yellow to brown	Conidia are rough, smooth or slightly rough and form long chains, conidiospores are long	*Penicillum* sp.
OAJ1	Colonies appear granular, White to yellow fluffy or wool-like.	Conidia are rough, smooth and form long chains, septate hyphae with simple conidiospores.	*Penicillum* sp
OAJ2	Velvety and flaky surface due to marked sporulation with grey to green coloration.	Septate hyphae with borne laterally conidiophores and conidia borne in the chain on sterigma. Conidial heads are large, globose and dark brown with smooth-walled conidiophores.	*Aspergillus niger*
OAJ4	Colonies appear granular, White to yellow fluffy or wool-like.	Conidia are rough, smooth and form long chains, septate hyphae with simple conidiospores.	*Penicillum* sp
OAJ5	Small to medium colony, round, raised, smooth and colony colour is white to cream	Oval cell shape, multilateral germination pattern, reproduces by budding.	Yeast
OAK2	Small to medium colony, round, raised, smooth and colony colour is white to cream	Oval cell shape, multilateral germination pattern, reproduces by budding.	Yeast
OAK3	Velvety and flaky surface due to marked sporulation with grey to green coloration.	Septate hyphae with borne laterally conidiophores and conidia borne in the chain on sterigma. Conidial heads are large, globose and dark brown with smooth-walled conidiophores.	*Aspergillus niger*
OAK4	Velvety and flaky surface due to marked sporulation with grey to green coloration.	Septate hyphae with borne laterally conidiophores and conidia borne in the chain on sterigma. Conidial heads are large, globose and dark brown with smooth-walled conidiophores.	*Aspergillus niger*
OSB2	Velvety and flaky surface due to marked sporulation with grey to green coloration.	Septate hyphae with borne laterally conidiophores and conidia borne in the chain on sterigma. Conidial heads are large, globose and dark brown with smooth-walled conidiophores.	*Aspergillus niger*

Table 4. Contd.

OSB6	Colonies appear granular, White to yellow fluffy or wool-like.	Conidia are rough, smooth and form long chains, septate hyphae with simple conidiospores.	*Penicillum* sp.
OOJ1	Colonies appear granular, White to yellow fluffy or wool-like.	Conidia are rough, smooth and form long chains, septate hyphae with simple conidiospores.	*Penicillum* sp.
OOJ3	Small to medium colony, round, raised, smooth and colony colour is white to cream	Oval cell shape, multilateral germination pattern, reproduces by budding.	Yeast
OOJ6	Colonies appears greenish-yellow with white-like borders	Septate hypha with long conidiophores arranged in clusters supporting the phialides. The conidiophores have rough texture and spiny bellow the vesicle.	*Aspergillus flavus*

Source: Author

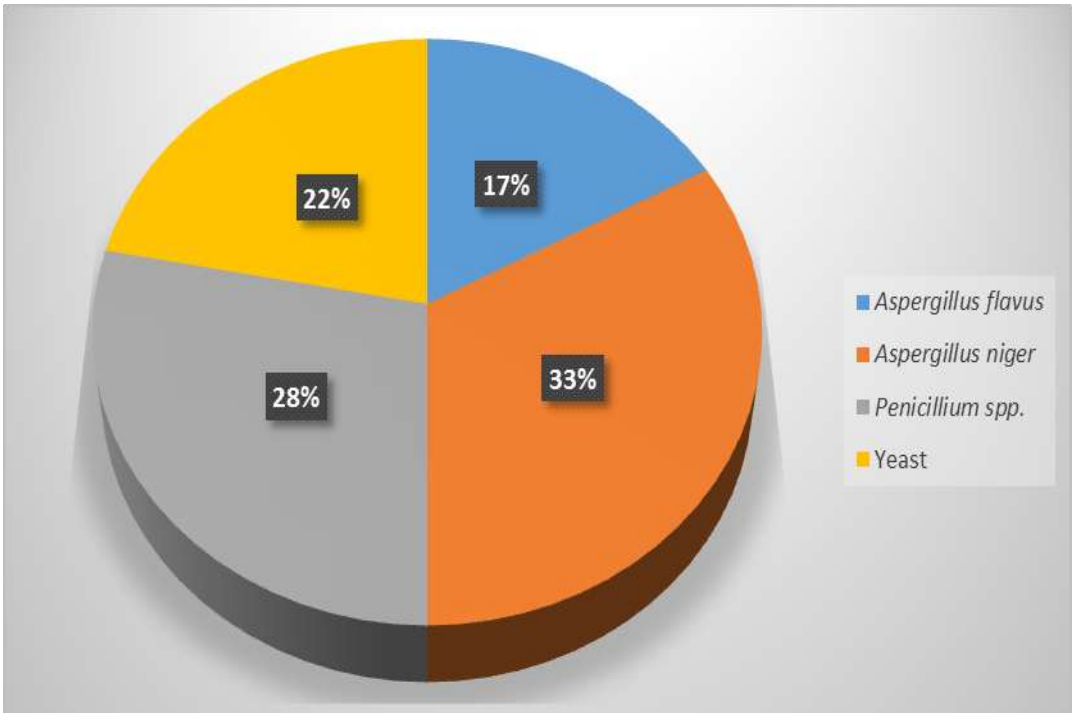

Figure 2. Percentage frequency of Occurrence of Fungi isolated from *Irvingia gabonensis* Kernels
Source: Author

which is the control has the high moisture content compare to OOJ6 and OOW1 which could be the reason why the microbial load is higher. The moisture content is however within the range value of most seeds and contamination.

The crude fibre percentage ranged from 10.7 to 9.1%. Sample OAJ3 had the highest crude fibre while OOW had the least, these results are similar to the report of legumes (Onojah et al., 2018). This is in line with the research of Brooker (2005) who suggested that high moisture content in fruits is an index of its water activity, measure of stability and susceptibility to microbial Onojah et al. (2018) who recorded 10.4% crude fibres from Ogbono samples. It is however lower to the result of Aremu et al. (2005) were 15.2% was reported from Bambara groundnut. The intake of dietary fibre can lower

Table 5. Mineral composition of *Irvingia gabonensis* Kernels.

Parameter	OAJ3	OOW4	OOJ6	OOW1
Sodium %	7.5	6.0	6.2	5.4
Vitamin C mg/kg	28.2	23.5	22.4	20.0
Potassium	87.4	78.1	72.5	53.6
Calcium mg/kg	37	33.4	30.2	29.4
Iron mg/kg	13.4	13.3	11.4	09.1
Magnesium mg/100g	49.9	41.7	40.8	34.4
Zinc mg/100g	0.03	0.03	0.02	0.05
Heavy metal (pb, As) ppm	NIL	NIL	NIL	NIL

Source: Author

Table 6. Proximate analysis of the ogbono sample.

Proximate composition	OAJ3	OOW4	OOJ6	OOW1
%Carbohydrate content	16.3±0.55	20.5±0.44	22.6±0.16	34.0±0.58
%Moisture content	4.5±0.51	5.2±0.15	4.8±0.10	5.5±0.25
%Crude protein	7.8±0.43	6.9±0.46	5.4±0.56	4.2±0.47
%Crude fat	58.9±0.55	55.3±0.37	55.9±0.25	45.7±0.184
%Crude fibre	10.7±0.26	10.4±0.22	9.8±0.39	9.1±0.10
%Total ash	1.8±0.61	1.7±0.14	1.5±0.13	1.5±0.52
Fatty acids (kg/100 g)	47.1±0.36	44.2±0.09	44.7±0.66	36.6±0.05
Metabolized energy (kg/100 g)	2589.0±0.38	2511.9±0.49	2544.3±0.42	2340.3±0.50

Source: Author

Table 7. Presence of aflatoxin in the *Irvingia gabonensis* Kernels.

Proximate composition	Level of aflatoxin (ppb)
OAJ3	1.05±0.64
OOW4	3.47±0.21
OOJ6	3.69±0.28
OOW1	5.10±0.07

Source: Author

cholesterol level, risk of coronary heart disease, diabetes and hypertension (Ramola and Raw, 2003).

The total ash content of *I. gabonensis* Kernels ranged from 1.5 to 1.8%. Sample OAJ3 was higher than OOW4, OOJ6 and OOW1. The result obtained is related to the work of Efosa et al. (2017). Ash content in food is the inorganic residue left after the removal of moisture and organic matter. It provides the measure of the total amount of minerals within a food. Crude fiber contains indigestible cellulose which helps to absorb water, provide roughage and better functioning of the alimentary system.

The crude fat content of I*. gabonensis* Kernels ranged from 58.9 to 45.7%. The fat in sample OAJ3 was higher than that of OOW1. This could be due to the infestation of microorganisms in the Kernels. The high value of the crude fat in OOJ3 suggests that the Kernels may be a source of vegetable oil for industrial uses.

The crude protein in *I. gabonensis* Kernels samples ranged from 4.2 to 7.8%. The crude protein value for Sample OAJ3 is higher than the ogbono samples obtained from OOW4, OOJ6 and OOW1. The crude protein value of OOW1 is low compared to some commonly consumed plant protein in Nigeria and this does not qualify the seed as a protein rich food. The lowvalue obtained could also be as a result of the long storage period before been purchased for this research (Onojah et al., 2018). Protein contents contribute positively to the requirement for biomolecules needed for repair and maintenance of the body tissues as well as

synthesis of vital hormones for the body (Soetan et al., 2010).

The calculated fatty acid value for *I. gabonensis* Kernels ranged from 47.1 to 36.6 kg/100g. The results suggest that oil gotten from samples OAJ3, OOW4 and OOJ6 are suitable as edible oil and can be used for industrial purposes. Sample OOW1 had lower fatty acid value due to the presence of some pathogenic microorganisms in the Kernels.

The calculated metabolized energy ranged from 2589.0 to 2340.3 kg/100g which shows that the sample have good concentration of energy.

The detection of aflatoxins in the *I. gabonensis* Kernels reveals the production of toxins by *A. flavus*. Aspergillus species such as *A. flavus* and *A. parasiticus, these doubles* as the most notorious fungi commonly isolated from *I. gabonensis* seed due to their high potential for producing aflatoxins (Osibona et al., 2018). Several factors such as moisture contents, high relative humidity, temperature, substrate composition and the presence of competing microorganisms influenced mold growth on the seeds (Adebayo-Tayo et al., 2006). The environmental conditions in some part of Nigeria favours the growth of fungi and aflatoxin production in foods. Some measures of precautions should be taken when handling and processing dry foods. The growth of molds on *I. gabonensis* seeds is a pointer to the potential health risk associated with its consumption (Osibona et al., 2018). In Nigeria, several foods including nuts, cereals, dry fish, spices, and melon seeds among other food substances, are susceptible to contamination with aflatoxins due to the critical conditions such as temperature and humidity which is known to favour the growth of aflatoxin-producing molds (Ubwa et al., 2014, Chigoziri and Ekefan, 2013).

Conclusion

This study revealed different microorganisms associated with *I. gabonensis* Kernels sold in different markets within Oyo Town, Oyo State, Nigeria. Microorganisms isolated include *Bacillus cereus, Bacillus subtillis, Staphylococcus* spp. *Staphylococcus* spp., Enterobacteriaceae, *Aspergillus flavus, Aspergillus niger, Penicillium* spp and yeast. The *I. gabonensis* seeds with the growth of *A. flavus* had low mineral composition and proximate value. Although, the total aflatoxin levels of the samples analysed were below the maximum acceptable limits specified by International Regulatory Agencies in food and agricultural products (20 ppb), frequent and prolonged intake of the *I. gabonensis* Kernels could result in health hazards and reduced the economic value of the food. This study showed that some of the *I. gabonensis* Kernels samples used had been stored for a longer period. Inadequate elimination of moisture and exposure to dirty environment (markets), made them lose some of their nutrients as well as minerals.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest

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Full Length Research Paper

Engineering of high production of salicylic acid in transgenic hairy roots of Marigold *Calendula officinalis* L. by *Agrobacterium rhizogenes* ATCC 13332

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This study is directed to engineering the high production of salicylic acid from both genetically transformed hairy roots and callus cultures of Marigold, *Calendula officinalis* L., by pRi-DNA plasmid of the wild type *Agrobacterium rhizogenes* ATCC 13332, via "Direct injection" and "Co-cultivation" techniques. High frequency of transformed hairy roots was recorded as leaves were inoculated by mixture of pRi-DNA and PEG. Also, incubation of Marigold leaves with pRi-DNA plasmid in the presence of PEG, enhanced hairy roots formation up to 60%. Genomic DNA was isolated from these hairy roots and the derived callus. Amplification of *rol* C gene was done by polymerase chain reaction (sPCR) carried on T-DNA of Ri plasmid. These genes were expected to be inserted in Marigold DNA. Electrophoresis results were a decisive proof that point out from the transfer of *rol* C genes. The latter separated from agarose coincide with molecular weights of the specific primers. Subsequently, HPLC data confirmed the availability of salicylic acid in both transformed hairy roots and callus that recorded 45 and 42-fold, respectively more than SA content in the natural field plants. This finding indicates that this increase was due to the incidence of transgenesis of these tissues.

Key words: Transgenic plant, *Agrobacterium rhizogenes, Calendula officinalis* L., salicylic acid.

INTRODUCTION

Transgenic tissues obtained from transformed plant cells, by *Agrobacterium rhizogenes*, can synthesize the natural products characteristic of native plants (Zhou et al*.,* 2011). *A. rhizogenes* was specified as a natural vector with Ri-plasmid DNA. This Ri (root-inducing) plasmid can be transferred from the bacterial vector to plant cells producing hairy roots (Chilton et al*.,* 1982; Gelvin, 2009). Bacterial vectors are considered successful systems for genetic transformation and the production of genetically transformed plants due to their ability to donate Ri plasmid or part of its "T-DNA" fragment that integrate with genome of plant cell producing genetically modified hairy roots (Al-Nema and Al-Mallah, 2018). Gene transfer process involves four sequential events, chemo-attraction, release of this single-stranded T-DNA region from the plasmid Ri and translocation to plant cells, followed by nuclear targeting of T-DNA, its integration into the plant genome, and finally expression of loaded genes. At sites of infection, in the host plant, which usually appears after 1 to 4 weeks, is an evidence of their altered phenotypes (Zhou and Wu, 2006). These hairy roots are considered a unique pattern in their genetic and

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biological stability, as well as their role in increasing industrial plant products that are useful in many pharmaceutical and food industries (Hom-utai, 2009). The success of genetic transformation in plant cells and tissues can be inferred by several methods represented by observing a group of phenotypic differences that appear either on genetically modified plants or in some molecular genetics tests (Davey and Anthony, 2010). This is to detect the inclusion of T-DNA genes in the host genome, indicating their transmission and expression (Daniell, 2004). The gene expression of T-DNA genes is phenotypically expressed by the formation of hairy roots from regions inoculated with the bacterial vector carrying the Ri plasmid (Sretenovic et al., 2006). This specific molecular evidence for detection of transgenesis and T-DNA conservation in the plant cell genome is polymerase chain reaction (PCR) test. The *rol* genes are settled in the LT-DNA of the Ri plasmid. Success of their expression in the genome of plant cells results in a change in some phenotypic traits in the plant, such as production of hairy roots (Palazón et al., 1998). The *rol* C genes may be responsible for changes in secondary metabolism and increase by-products in transgenic plant tissues (Shkryl et al., 2008). Some studies reported that the expression of *rol* C genes succeeded in crossing from the bacterial vector *A. rhizogenes* ATCC18534 to hairy roots formed in *Calendula officinalis* L. leaves, and their role in increasing the secondary metabolites (Alsoufi et al., 2021). Marigold, *C. officinalis* L., is a winter annual plant species (Asteraseae), including 20 species of medicinal and ornamental plants of dual use of a high economically importance (Dole and Wilkins, 2004). Its importance is due to the abundance and diversity of its secondary metabolites, such as tannins, coumarins, sterols, steroids, terpenes, a large group of vitamins, quinones (aromatic compounds), amino acids, resins, saponins, and essential oils (Andersen et al., 2010). Also, carotenoids and flavonoids are natural pigments, as well as phenolic acids, such as, para-hydroxybenzoic, vanillic, caffeic and salicylic acids (Rigane et al., 2013). Generally, phenolic compounds are of a great importance for human health (Martins et al., 2016). Salicylic acid (SA) compound is found in trace amounts in Marigold plants (Andersen et al., 2010). Moreover, salicylic acid (orthohydroxybenzoic acid) synthesized by plants, that possess an aromatic ring and a hydroxyl group (Klessig et al., 2018) probably present in all plants. SA is as a free phenolic acid and as a conjugate form in plants, which is an essential ingredient in the manufacture of aspirin drug and cosmetics (Andersen et al., 2010). To date, SA biosynthesis can be created from two freelance and compartmentalized pathways, (1) isochorismate (*IC*) pathway localized into the plastids, and (2) phenylalanine ammonia-lyase (*PAL*) pathway, that takes place in the cytosol. Generally, SA is produced in the chloroplast, and afterward exported to the cytosol (Maruri-López et al., 2019). The aim of this work is to investigate the role of *rol* C genes in engineering the high yield of salicylic acid in transgenic hairy roots and calli of *C. officinalis* L.

MATERIALS AND METHODS

Plant

Healthy and fully-expanded leaves of Marigold, *C. officinalis* L. (Hortus-Calendula Doppia IN MIXED, Italy) were excised from 12-weeks-old plants grown in the nurseries of the main campus of Mosul University. Leaves were soaked in solution consisting of distilled water and bleaching solution (FAS, Babylon Comp. for detergents, Baghdad) 3:1 v:v ratio for 25 min on rotary shaker. Then they were washed with sterilized water three times for 3 min each (Al-Abasi et al., 2018). Leaf explants were cultured on agar-solidified MS (Murashige and Skoog, 1962) medium provided with sucrose 32 gL^{-1} and supplemented with 0.1 mgL^{-1} 6-benzylaminopurine (BAP). Induced callus was sub-cultured on MS medium supplemented with 1.0 mgL^{-1} naphthaleneacetic acid (NAA) and 0.1 mgL^{-1} BAP for growth.

A. rhizogenes strain and pRi-DNA isolation

Wild type *A. rhizogenes* strain ATCC 13332, containing pRi-DNA was supplied by Leibniz Institute (DSMz- German Collection of Microorganisms and Cell Cultures, Germany). Single colony of the bacterial vector was grown in 25 ml of nutrient broth (NB) medium at 6.9 pH, in the dark at 28°C. The plasmid pRi-DNA was isolated (Brinboim and Doly, 1979), and then migrated on agarose layer to confirm its presence.

Induction of hairy roots

Fifteen microliters of isolated pRi-DNA suspension were mixed with 20% PEG of M.Wt 4000 sterile solution (PEG+). Similar volume of pRi-DNA suspension was not mixed with PEG solution (PEG-). Each mixture was used for inoculation sterilized leave segments 2.5 cm, by direct injection method, in which these segments were wounded at the midrib, using a sterile needle (Insulin Syringe U-100-29G1/2-0.33×13 mm 1CC), loaded with plasmid suspension (Christen, 2002). Also, co-cultivation method, where segments were incubated in 10 ml of plasmid suspension in 9.0 cm diameter plastic Petri dishes (tightly closed) for 30 min, with gentle shaking, in dark was also done. These two methods were applied once with plasmid suspension alone (Abou Rayya et al., 2010), and with the presence of 20% PEG solution at ratio 1:1 (V:V). Control segments were inoculated with sterile distilled water, and with PEG solution. After co-cultivation, explants were dried with sterile filter paper, as well as the control. All samples were transferred to the surface of agar-solidified WP free medium (Al-Mahdawe et al., 2013).

Hairy roots developed from leaf inoculated with pRi-DNA plasmid in the presence of 20% PEG solution were transferred to the surface of 25 ml of solidified MS medium supplemented with 0.1 mg L^{-1} BAP (Al-Abasi et al., 2018). Subsequently, callus formed from hairy roots was transferred to the MS medium supplemented with 1.0 mg L^{-1} BAP and 0.1 mg L^{-1} IBA. Cultures were maintained in culture room conditions.

Establishment of hairy roots cultures

As hairy roots formed at site of inoculation of Marigold leaves, 2 to 3 cm in length hairy roots tufts with a small part of the leaf midrib were excised and cultured on the surface of solidified WP medium

supplemented with 0.5 mg L^{-1} NAA in 9.0 cm diameter plastic. Petri dishes were kept in darkness at 22±2°C. Data was recorded after 5, 10, 15, 20 and 30 days of inoculation.

Extraction of DNA

DNA was extracted from each hairy roots, transformed callus, normal callus and leaf of field plant (negative control). pRi-DNA was isolated from the vector *A. rhizogenes* ATCC 13332, CTAB extraction solution was used to extract DNA (Weigand et al., 1993), 0.5 g of each sample was taken, placed in the (pre-cooled) mortar in the presence of 0.5 ml of the extraction solution containing 0.25 ml of 2-Mercaptoethanol. Each sample was ground, then transferred to 2.0 ml Eppendorf tube, all tubes were tightly closed, and placed in a water bath at 55°C for 1 h, 0.5 ml of the mixture consisting of chloroform/iso-amyl alcohol 1:24 v:v was added, centrifuged at 16000×*g* for 10 min, the supernatant was taken into a new 2.0 ml Eppendorf tube, 0.028 ml of ammonium acetate CH_3COO^- NH_4^+ was added, this was followed by addition of 0.204 ml of (cooled) isopropanol, the produced solutions was well mixed, and kept for an hour at -20°C. The mixture was centrifuged at 16,000 ×*g* for 5 min. The supernatant was discarded, and 0.7 ml of cooled 70% ethanol was added, sample was mixed, centrifuged at the same speed for 5 min. Then, the supernatant was discarded, and 0.7 ml of 95% cooled ethanol was added to the precipitate, mixed by hand. It was centrifuged at the same speed for 3 min. The entire supernatants were carefully taken and discarded. The tubes were left open for 10 min. As alcohol had evaporated, 0.03 ml of TE-buffer was added to the precipitate, and kept at -20°C until use.

sPCR analysis

Each sPCR reaction was carried out by using total volume of 9.0 µl premixed solution ($MgCl_2$ buffer 10X + KCl + Tris-HCl + dNTPs + Taq DNA polymerase), 4.0 µl of 50 ng $µl^{-1}$ Template DNA, and 10 µl $Bicamol^{-1}$ of each one of forward and reverse specific primer. Possible transfer and integration of *rol* C genes into genome of these tissues were detected the using specific primers for *rol* C gene, as positive control.

Determination of concentration and purity of the isolated DNA

The concentration and purity of each DNA were measured at wavelengths of 260 and 280 nm, respectively, 1.0 µl of DNA samples isolated from plant tissue and pRi-T-DNA isolated from *A. rhizogenes* ATCC 13332 were each placed on a nanodrop (Nanodrop, BioDrop-England) for measuring the absorbance, the concentration and purity of DNA (Dhahi et al., 2011).

Quantification of salicylic acid

This test involved three steps to obtain free salicylic acid (Al-Abasi et al., 2020), as the following.

Preparation of samples

The stock solution of standard SA was prepared carefully by weighing 100 mg of salicylic acid (BDH-England), dissolved in 100 mL of distilled water. Four hundred milligrams of each transformed hairy roots and callus were each transferred to a separate pre-cold pestle and mortar, as well as normal callus, field plant leaf as a control samples. These samples were ground in the presence of liquid nitrogen; the obtained powder was kept in 2.0 ml Eppendorf tube. To the latter 1.6 ml of 70% ethanol was added. Specimens were carefully vortexed for 1.0 min, centrifuged at 10000 ×*g* for 10 min at room temperature. Each supernatant was transferred to 15 ml falcon tubes. Again 1.6 ml of 90% methanol was added to the pellet, re-vortexed, for 1.0 min for re-extraction. The produced mixture was centrifuged again under the same conditions. The supernatant was added to the stock supernatant in 15 ml falcon tube. The pooled and clear supernatant solution contains free SA and conjugate SA (Allasia et al., 2018).

Free salicylic acid

Two milligrams of each supernatant were placed in 2.0 ml microcentrifuge tubes to evaporate EtOH and MeOH by air currents for 2.0 h. The remaining supernatants were transferred to 2.0 ml Eppendorf tubes and concentrated up to approximately 600 µl. To this aqueous solution, 65 µl of 20% of aqueous trichloroacetic acid (TCA, w/v) was added to each solution, and 650 µl of ethyl acetate and cyclohexane 1:1 v/v solution was added, vortexed for 30 s, centrifuged for 2.0 min at 10,000 ×*g* for phase separation. Transfer the upper organic phase to 2.0 ml eppendorf tube, re-extract the aqueous phase again with 650 µl of ethyl acetate-cyclohexane mixture. Then, centrifuged for 2.0 min at 10,000 ×*g* for phase separation. Evaporate the solvent to dryness for 30 to 45 min, solubilize the dry residue in 100 µl of 10% aqueous methanol (v/v) containing 0.1% aqueous trifluoracetic acid TFA (v/v), and vortex for 1 .0 min. The samples are ready to be assessed by HPLC.

Quantification of SA in plant tissue

Twenty microliters of each sample were separately injected in high-performance liquid chromatography (Sykman-2014-Germany). Separation conditions were carried out by C18 column (250 × 4.6 mm, 5 µm) at 30°C and flow rate (1.0 ml/min) of aqueous MeOH gradient from 10% (v/v) was used as linear fluorometric detection (excitation at 305 nm; emission at 407 nm). Salicylic acid concentrations were quantified by comparing peak area of the assessed samples with peak area of standard sample under the same conditions using the standard equation (Kimura and Amaya, 2002).

RESULTS

Production of hairy roots via direct injection

Data (Table 1) demonstrate efficient stimulation and development of hairy roots on Marigold leaf inoculated by different number of nicks with needle tip immersed in the mixture of plasmid suspension with 20% PEG solution. These hairy roots involved 10 days to emerge on leaves segments grown on WPM medium containing 5.0 mg L^{-1} NAA, with dense of root hairs compared to their numbers induced using plasmid suspension alone. They required 20 days to emerge.

Five replicates/treatment of hairy roots

Also, data refer to the superiority of injection method with plasmid-PEG mixture in induction of hairy roots, and its high density at the injection sites, as well as, a few of

Table 1. Induction of transformed hairy roots on *Calendula officinalis* L. leaf segments inoculated by direct injection with pRi-DNA and/or pRi-DNA-PEG mixture of *Agrobacterium rhizogenes* ATCC 13332.

No. of Nicks	PRi-DNA + PEG			PRi-DNA		
	Leaves response	Response (%)	No. of H.R	Leaves response	Response (%)	No. of H.R
1	26	14	53	12	24	17
2	38	76	83	23	46	52
3	40	80	86	23	46	38
5	**47**	**94**	**196**	34	68	73
Dist. Water(Cont.)	0.0	0.0	0.0	0.0	0.0	0.0
PEG(Cont.)	0.0	0.0	0.0	0.0	0.0	0.0

Source: Author

Figure 1. Tranformed hairy roots formation from leaves of *Calendula officinalis* L. inoculated with pRi-DNA plasmid of *A. rhizogenes* ATCC 13332 + PEG mixture grown on agar-solidified WP medium + 0.5 mg L^{-1} NAA. A: Hairy roots after 10 days of inoculation at the nicking sites, note two types of hairy roots type- rich with root hairs (double arrows), type- without root hairs (single arrows), and callus was formed (black arrows). B: Development of hairy roots in (A) after being transferred to agar-solidified WP medium, note the numerous roots and formation of callus. C: Development of hairy roots excised from (B) after 50 days of transferring to agar-solidified WP medium. The active growth of hairy roots and the formation of micro-clumps of callus.
Source: Author

these hairy roots were developed in the neighboring tissues. This was not observed with leaves segments inoculated with plasmid alone. The direct injection method by five Pricks with 20% PEG and plasmid mixture was more efficient in inducing hairy roots than single prick injections (Table 1).

Marigold leaves segments inoculated with the mixture of pRi-DNA plasmid and PEG stimulate formation of hairy roots at the midrib of leaves inoculated with 5 nicks. Transfer of inoculated leaves to solid WP medium led to the emergence of hairy roots after 10 days of inoculation (Figure 1A). The results observed two types of hairy roots, the first was rich with root hairs and the other was without root hairs. As callus primordia formed hairy roots were transferred to agar solidified WP medium supplemented with 0.5 mg L^{-1} NAA that sustained growth of hairy roots (Figure 1B), excised hairy roots that cultured forming hairy roots cultures of abundant branching (Figure 1C).

Induction of hairy roots by co-cultivation

Data show that incubating leaf segments of Marigold with pRi-DNA plasmids of *A. rhizogenes* ATCC 13332 with an equal volume of 20% PEG solution supported the formation of hairy roots on leaf at submerged sites, as compared with incubation with plasmid alone or with distilled water alone, as control treatments (Table 2).

Twenty replicates/treatment

Again, results of co-cultivation methods of Marigold leaf segments with pRi-DNA plasmid suspension in the presence of PEG solution for 30 min express a clear stimulation of hairy roots on the leaf bases after 20 days of culture on agar-solidified WP medium supplemented with 0.5 mg L^{-1} NAA. They were characterized by rapid emergence at the cutting ends (Figure 2A). Additionally, roots were elongated with frequent branching from the center of the tuft (Figure 2B).

Production of transgenic callus from hairy roots

The results indicate the ease of producing callus from transformed hairy roots. The latter was of compact

Table 2. Formation of transformed hairy roots by co-cultivation of *Calendula officinalis* L. leaves with pRi-DNA plasmid of *Agrobacterium rhizogenes* ATCC 13332 in presence or absence of 20 % (W/V) PEG solution.

Co-cultivation	Leaves response	No. of hairy roots	Response (%)
Leaves + pRi-DNA-PEG	**15**	**61**	**60**
Leaves + pRi-DNA	3	11	12
Leaves + PEG (Cont.)	0.0	0.0	0.0
Leaves + Dist. Water (Cont.)	0.0	0.0	0.0

Source: Author

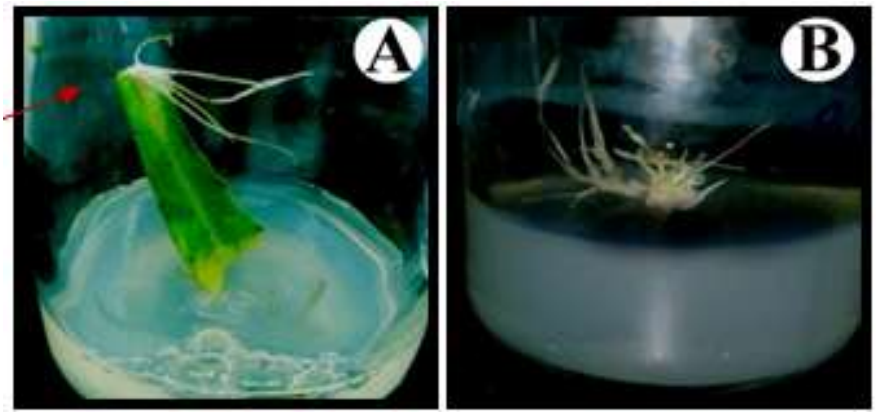

Figure 2. Induction of transformed hairy roots by co-cultivation of *Calendula officinalis* L. with *A. rhizogenes* leaves with pRi-DNA of *A. rhizogenes* ATCC 13332 in the presence of 20% PEG solustion, grown on agar-solidified WP medium + 0.5 mg L^{-1} NAA. A: Formation of transformed hairy roots after 20 days of incubating leaves with pRi-DNA + PEG, note the hairy roots at the basal sites of the leaf segments (arrowed). B: A tuft of transformed hairy roots excised from (A) 30 days old grown in fresh WP medium. Note the development of hairy roots from the center of the culture.
Source: Author

texture and was characterized by its yellowish-green color (Figure 3A). Callus also was observed at the sites of inoculation of leaves segments with pRi-DNA plasmids of *A. rhizogenes* ATCC 13332 in the presence of 20% PEG solution. This type of callus was distinguished by its friable appearance and creamy white color (Figure 3B).

sPCR of pRi-DNA plasmid of *A. rhizogenes* ATCC 13332

Specific polymerase chain reaction of each sample of DNA isolated from leaves of field plant, hairy roots, transgenic callus and normal callus, as well as the pRi-DNA isolated from the bacterial vector *A. rhizogenes* ATCC 13332. Electrophoresis results demonstrated the separation of bands from both DNA of hairy roots and callus formed at sites of inoculation with pRi-DNA, as well as pRi-DNA. The bands molecular weights matched with the molecular weights of the specialized primers was 545 bp of the *rol* C gene (Figure 4). This evidence confirms its genetic transformation conclusively as a result of the transfer of a piece of T-DNA from the plasmid and expression of *rol* C genes in the genomic DNA of plant cells. The absence of DNA bands from other control samples submits a strong evidence of the absence of these genes in their genomic DNA (Figure 4).

Presence of salicylic acid in transgenic Marigold tissues

HPLC data (Table 3) exhibit that leaves of Marigold field plants and their normal callus contained salicylic acid at low concentrations, whereas, each of the transformed hairy roots and transgenic calli samples recorded a high content of salicylic acid reaching about 45-fold.

In other words, data refers to the low levels of salicylic acid in the leaves of field plant, and conclusively revealed an increase of salicylic acid concentrations in transgenic hairy root samples. This data confirmed that transgenesis, achieved in these samples, supports the engineering of the high production of salicylic acid in transgenic calli and hairy roots.

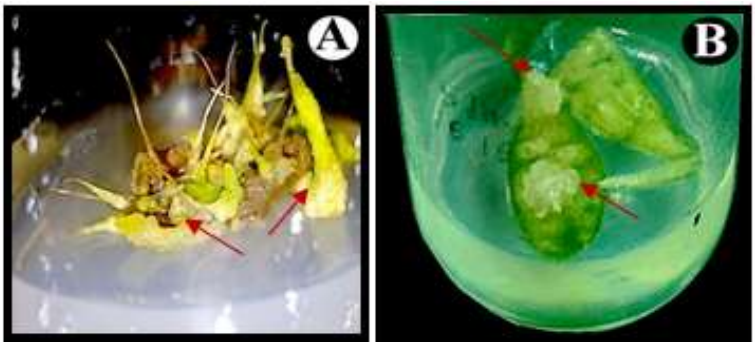

Figure 3. Transgenic callus induction from transformed hairy roots induced on leaf of *Calendula officinalis* L. inoculated with pRi-DNA plasmid of *A. rhizogenes* ATCC 13332 in the presence of PEG grown on agar-sloidified MS + 0.1 mg L^{-1} BA medium. A: Callus formation from hairy roots (arrows). Note the beginning of callus appearance at the sites where hairy roots in touch with medium. B: Genetically transformed callus formed at inoculation sites (arrowed) with pRi-DNA + PEG mixture.
Source: Author

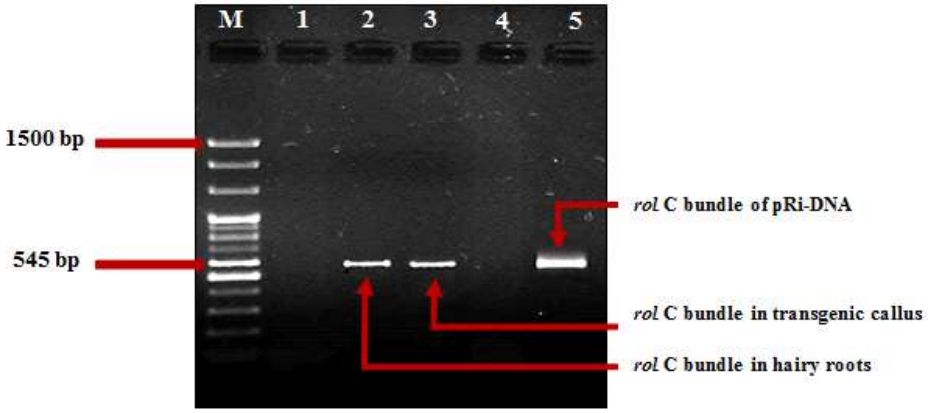

Figure 4. Amplified *rol* C gene transferred from plasmid DNA vector of *A. rhizogenes* ATCC13332 to genomic DNA of *Calendula officinalis* L. Lane M: Volumetric Directory (DNA Ladder). Lane 1: Amplified genomic DNA of field leaves. Lane 2: Amplified genomic DNA of transformed hairy roots. Lane 3: Amplified genomic DNA isolated from a transgenic callus. Lane 4: Amplified genomic DNA of a normal callus. Lane 5: Ri-Plasmid DNA isolated from the bacterial victor *A. rhizogenes* ATCC13332.
Source: Author

DISCUSSION

In this investigation, transgenic hairy roots production is attributed to the successful interaction between this plant species and the plasmid DNA of the bacterial vector *A. rhizogenes* (Patel and Krishnamurthy, 2013). Studies indicated that hairy roots are considered the first signs of genetic transformation (Zupan et al., 2000). Many reports confirmed that wounding plant tissues was an important step for the attachment of bacterial vector to cells and stimulating the formation of hairy roots. This explains the high response achieved by direct injection method compared to the co-cultivation method. The effect of PEG solution when interfering with the bacterial plasmid DNA in inoculation was expected to be due to the nature of PEG as a hydrophilic polymer, and to its carbonyl group (Al-Mallah et al., 1990). Development of hairy roots in infection locus of plasmid may be attributed to a group of physiological and morphological changes as a result of the gene expression of T-DNA fragment that carry *iaaH* and *iaaM* genes that encode to the biosynthesis of cytokinins and auxins, especially IAA (Takei et al., 2001). This causes a disturbance in the hormone balance that stimulates cell divisions to produce a unique pattern of hairy roots (Meyer et al., 2000). This may support the emergence of minute masses of callus besides hairy roots in their growth medium and their development into large biomass. The formation of hairy roots is due to the

Table 3. Salicylic acid Contents in transgenic tissues of *Calendula officinalis* L. genetically transformed by *Agrobacterium rhizogenes* ATCC13332.

Sample	Peak area	SA conc. (µg/g)
Standard SA (cont.)	7101.385	100
Transformed hairy roots	6751.079	**94.46**
Transgenic callus	6304.240	**88.21**
Normal callus	359.327	5.02
Field plant leaves	147.861	2.069

Source: Author

fact that the RiT-DNA genes contributed to directing cellular aggregates to hairy roots as a result of hormonal balance loss between auxin and cytokinin (Hom-utai, 2009), or perhaps due to the contribution of *vir*-genes in the formation of hairy roots (Fu et al., 2005). It can be said that co-cultivation technique is an efficient protocol in the processes of genetic transformation, specifically when using pRi-DNA as a substitute for bacteria. Also, the ease of implementation and detection of the interference of these plasmids with the genome of plant recipient cells (Fu et al., 2005), *Onco*-genes group represents the most important stable genes in the T-DNA segment carrying the *rol* A, *rol* B, *rol* C and *rol* D genes, which (all or some of them) are described as easily expressed in the plant genome (Bulgakov, 2008). Also, the have effect on cell growth and differentiation, as well as play role in synthesizing secondary metabolites in transgenic cells of plants (Bulgakov, 2008), in particular *rol* C genes (Tzfira and Citovsky, 2008). However, if *rol* A and *rol* B co-exist together, they stimulate the activity of hairy root cultures (Pavlova et al., 2014). In this study, hairy roots production or their transgenic callus mediated by the plasmid vector interfering with the Marigold plant, represents evidence that the genomic DNA of the transgenic tissues retains the *rol* C genes, according to the sPCR data. This gave an explanation and evidence for the expression of *rol* C in the plant genome (Zupan et al., 2000). The *rol* C gene provides a signal that activates secondary metabolic processes. It is likely that *rol* C may confer a wider spectrum of defense reactions in addition to secondary metabolite stimulation. Interestingly, *rol* C gene encodes cytokinin glucosidase and stimulates the production of many secondary compounds in various plants (Dilshad et al., 2015). Phenols accumulation in hairy roots was more than its concentration in field plants. This may be due to the effectiveness of hairy roots in increasing the content of secondary metabolites, perhaps because the high expression activity of *rol* C gene (Wang et al., 2006).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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