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Proximate Composition, Mineral Profile and Trypsin-Inhibitory Activity of West African Leafy Vegetables: Influence of Urea Micro-Dosing and Harvest Time

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In order to ensure the nutritional quality of leafy vegetables produced under intensive cultivation systems, the proximate composition, mineral profile, and trypsin-inhibitory activity of three priority West African vegetable species (*Amaranthus cruentus*, *Ocimum gratissimum*, and *Solanum macrocarpon*) produced with urea micro-doses (20, 40, and 60 kg/ha) were compared with control leaves raised without urea. Plants were harvested three consecutive times to determine effects on nutrient and trypsin-inhibitory activity. Proximate and mineral compositions were mainly species-dependent (p<0.05) though the 60 kg/ha urea dose produced the significantly (p<0.05) highest moisture content in leaves. Calcium, copper, iron, and zinc contents were significantly (p<0.05) influenced by harvest time and its interaction with vegetable species. Furthermore, trypsin inhibition was significantly (p<0.05) higher for the second and third leaf harvests, which suggests that initial wounding from the first harvest enhanced synthesis of the inhibitor protein molecules.

INTRODUCTION

Plant foods play a key role in the maintenance of human life as they supply the needed nutritional elements that boost the immune system [Fonge *et al.,* 2016; Das *et al.,* 2017]. Vegetables are food resources that are considered as healthy foods worldwide [Souza *et al.,* 2016; Das *et al.,* 2017]. Among all vegetables, green leafy vegetables are available all year round, at relatively low cost, and are widely used as basic ingredients in traditional dishes and nutrition therapy [Fonge *et al.,* 2016; Souza *et al.,* 2016; Das *et al.,* 2017]. In the Republic of Benin, a biodiversity inventory revealed 187 plant species used as indigenous leafy vegetables with *Amaranthus cruentus* (*Amaranthaceae*), *Ocimum gratissimum* (*Lamiaceae*), and *Solanum macrocarpon* (*Solanaceae*) being among the priority species [Dansi *et al.,* 2008]. These species are essential in West African diets, because they are used in the preparation of sauces [Dansi *et al.,* 2008; Vodouhè *et al.,* 2012]. They are also rich in fibers, minerals, vitamins, and polyphenols, which are involved in the growth and maintenance of good health for all age groups [Nana *et al.,* 2012; Adewale *et al.,* 2014; Akinwunmi & Omotayo, 2016]. The extracts obtained from these leafy vegetables have antioxidant and enzyme inhibitory activities that provide evidence for sustaining their use in traditional medicine to prevent and manage several human ailments [Nana *et al.,* 2012; Adewale *et al.,* 2014; Djibril Moussa *et al.,* 2019a, b].

However, these leafy vegetables contain anti-nutrients such as protease inhibitors, which may have a negative impact on human health, by reducing protein digestibility and limiting the availability of essential amino acids [Patel & Zaveri, 2015]. Thus, protease inhibitors can limit the caloric value of diets, which could be useful in disease conditions where excessive catabolic activity (*e.g.*, pancreatitis) is undesirable [Jedinak *et al.,* 2010]. Smaller quantities

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of trypsin inhibitors induced satiety by promoting the secretion of cholecystokinin, a satietogenic hormone associated with limited food, energy, and weight intakes [Carvalho *et al.,* 2016]. Hence, consumption of leafy vegetable extracts having trypsin-inhibitory activities could be beneficial for the management of metabolic abnormalities resulting from high food and energy intakes. Moreover, anti-viral effects of trypsin inhibitors [Shahwar *et al.,* 2012] could extend their health benefits, especially in rural areas where diarrhea is a common occurrence. Otherwise, trypsin inhibitors are proteinaceous in nature, which means that they are heat sensitive and can be partially or completely denatured and inactivated at elevated temperatures (>60°C) [Allen *et al.,* 2012; Pesoti *et al.,* 2015].

The nutritional profile of leafy vegetables is affected by soil fertility [Fonge *et al.,* 2016; Souza *et al.,* 2016; Das *et al.,* 2017], while efficient use of fertilizers (mineral and/or organic) is of great interest because of consumer demand for healthier food sources [Tovihoudji *et al.,* 2017; Likpètè *et al.,* 2019]. A recent farming system known as fertilizer micro-dosing technology involves the application of small quantities of mineral fertilizer at an optimized time, depth, and distance to the target plant [Tovihoudji *et al.,* 2017; Likpètè *et al.,* 2019]. This technology gave promising prospects in terms of crops productivity although the combined application of mineral and organic fertilizers was recommended for its sustainability [Tovihoudji *et al.,* 2017; Likpètè *et al.,* 2019]. However, the effect of fertilizer micro-dosing on the nutritional properties of farming products, including leafy vegetables is far from being fully understood. Moreover, leaves maturity stage at harvest is an important factor that could influence their nutrient contents since plants' development stage affected nutrients uptake by plants [Sossa-Vihotogbé *et al.,* 2013; Bvenura & Afolayan, 2014a, b]. Therefore, this study was undertaken to assess proximate composition, mineral profile, and trypsin-inhibitory activity of *A. cruentus*, *O. gratissimum*, and *S. macrocarpon* leaves produced with urea micro-doses and harvested at different times.

MATERIALS AND METHODS

Materials

Plants (*A. cruentus*, *O. gratissimum*, and *S. macrocarpon*) were cultivated between January and June 2016 in an experimental farm at the Northern Center of National Agricultural Research Institute ("INRAB") in Republic of Benin (9°57' N, 2°43' E and 358 m a.s.l.; Ina village, Bembereke district). Seeds were bought from "INRAB" and grown in nurseries for four (*A. cruentus*) or six (*O. gratissimum* and *S. macrocarpon*) weeks. The experimental design was a randomized complete block, in four replicates for each vegetable species, with three rates of urea (20, 40, and 60 kg/ha) applied immediately after transplanting through micro-dosing technology [Likpètè *et al.,* 2019] while the control plots contained no urea (0 kg/ha). Cattle manure (5 t/ha) was applied a week before transplantation to all the experimental plot units (6 m × 1 m) as base fertilizer. Harvests were done, on the same plants by cutting the main stems at a height of 10 cm from soil level, excluding those of the border lines, at 4–6–8, 6–10–14, and 8–12–16 weeks after transplanting for *A. cruentus*, *S. macrocarpon*, and *O. gratissimum*, respectively. These harvest times correspond to late vegetative stage (before flower initiation) for each species. For the purpose of this study, samples of 2.5 kg of fresh leaves with stems were harvested from each experimental plot for each vegetable species at each harvest, then edible leaves were washed and oven-dried at 60°C using a Memmert UN450 drier (Memmert GmbH + Co. KG, Schwabach, Germany), for a minimum of 24 h or until a constant weight was achieved. Dried leaves were ground using a Cuisinart Grinder (Model DCG-12 BCC, Cuisinart, ON, Canada) and leaf powder was stored at -20°C.

Proximate composition analysis

Proximate analysis was carried out in duplicate using standard methods [AOAC, 2005; AOCS, 2009]. Moisture was determined by oven-drying (Heraeus T 5042 EK oven, Heraeus, Hanau, Germany) of fresh leaves at 105°C for 24 h [AOAC, 2005; method 930.15]. Protein content was determined as total nitrogen (× 6.25) using Kjeldahl method after H_2SO_4 (95%) digestion using Kjeldahl pastilles as a catalyst followed by distillation into 4% (*w/v*) H_3BO_3 and titration with 0.1 M HCl [AOAC, 2005; method 990.03]. Crude fat content was determined after petroleum ether extraction in an Ankom XT10 extraction system (ANKOM Technology, NY, USA) [AOCS, 2009; method Am 5–04]. Crude fiber content was estimated after digestion with H_2SO_4 (1.25%, *v/v*) and NaOH (0.3 M), using an Ankom 220 fibre analyzer [AOCS, 2009 method Ba6a-05]. Ash content was determined by the incineration method in a muffle furnace (Vulcan A-550, Dentsply Ceramco Inc, York, PA, USA) at 550°C overnight for 6 h [AOAC, 2005; method 923.03]. Except for moisture content, proximate composition was expressed on dry weight basis (dwb).

Mineral profile analysis

Mineral profile (dwb) was determined in duplicate by digesting white ashes in a mixture of HNO_3 and $HClO_4$ (4:1) for 24 h [AOAC, 2005; method 968.08D]. The supernatant was filtered using Whatman No. 42 filter paper and the filtrate was analyzed by atomic absorption spectroscopy (PerkinElmer AAnalyst 200, PerkinElmer, MA, USA) to assess calcium, copper, iron, magnesium, manganese, potassium, sodium, and zinc contents. Phosphorus content was determined by colorimetric method at 680 nm using a UV mini 1240 spectrophotometer (Shimadzu Corp., Kyoto, Japan) [AOAC, 2005; method 968.08D]. The nutritional interrelationships between mineral levels were evaluated by calculating the following content ratios: calcium/magnesium, calcium/phosphorus, calcium/potassium, iron/copper, sodium/magnesium, sodium/potassium, and zinc/copper [Watts, 2010].

Trypsin inhibition assay

Trypsin-inhibitory activity was assessed (in triplicate) by Patel & Zaveri [2015] method with slight modifications using aqueous extract of the leaf powder [Djibril Moussa *et al.,* 2019b]. Leaf powder (10 g) was transferred into double de-

ionized water (200 mL) and the mixture was stirred for 2 h at 60°C before centrifuging for 30 min (5000 × g at 4°C). The resultant supernatant was stored while the precipitate was redispersed in water (200 mL). The process above was repeated and both supernatants were pooled together, concentrated using a vacuum evaporator and freeze-dried. The dried extracts (0.16–250.00 μg/mL) and 4(-2-aminoethyl) benzenesulfonyl-fluoridehydrochloride (AEBSF, 0.78–25.00 μg/mL) were dissolved in 20 mM Tris-HCl buffer (pH 7.5). A 200 μL aliquot of the sample was mixed with 200 μL of trypsin (270 μg/mL) and incubated for 5 min at 37°C and 400 rpm using a thermomixer (Eppendorf AG, Hamburg, Germany). Thereafter, 500 μL of N-benzoyl-DL-arginine-p-nitroanilidehydrochloride (BAPNA, 0.9 mM) was added and agitated (400 rpm) for 10 min at 37°C. The addition of 100 μL of acetic acid (30%) stopped the reaction with continuous shaking (400 rpm) for 5 min at 37°C. The absorbance of the mixture was determined in comparison with that of a blank at 410 nm using a microplate reader Synergy H4 (Biotek Corp., Winooski, VT, USA) coupled with Gen5 2.04 data analysis software. Concentration of extracts required to inhibit 50% of trypsin activity was estimated using Prism 6 software (GraphPad Corp., La Jolla, USA).

Statistical analysis

Data were analyzed using R statistical software version 3.1.0 (The R Foundation for Statistical Computing, Vienna, Austria). Three-way analysis of variance on repeated measures was performed to investigate the main and interaction effects of vegetable species, urea micro-doses, and harvest times on proximate composition, mineral profile, and trypsin--inhibitory activity of the leafy vegetables. Significance was set at 5% and means were segregated using Duncan's multiple range test.

RESULTS AND DISCUSSION

Variation in proximate composition of the leafy vegetables

Moisture content was the most abundant component followed by protein, fiber, and fat. The range of moisture content of *S. macrocarpon* (86.0–90.1 g/100 g) was higher than those of *A. cruentus* (76.7–87.6 g/100 g) and *O. gratissimum* (79.3–87.4 g/100 g) which are similar to those reported for *Justicia tenella* and *Sesamum radiatum* (72.0–83.3 g/100 g) by Sossa-Vihotogbé *et al.* [2013]. High moisture content is an index of freshness and fast perishability due to the activity of water-soluble enzymes involved in metabolic processes and microbial growth [Vodouhè *et al.*, 2012; Adjatin *et al.*, 2013]. The range for protein and fiber contents of *S. macrocarpon* (27.6–34.1 g/100 g and 16.7–25.5 g/100 g) were higher respectively than those of *A. cruentus* (21.2–26.3 g/100 g and 9.8–12.7 g/100 g) and *O. gratissimum* (17.1–26.9 g/100 g and 13.1–16.7 g/100 g) which are comparable to those reported earlier [Sossa-Vihotogbé *et al.,* 2013; Akinwunmi & Omotayo, 2016] for *A. cruentus* (17.0 g/100 g and 10.4 g/100 g), *J. tenella* (17.2–33.8 g/100 g and 8.7–13.1 g/100 g), *O. gratissimum* (12.2 g/100 g and 10.5 g/100 g), and *S. radiatum* (18.8–27.8 g/100 g and 6.9–10.9 g/100 g). Protein contents in leaves (>12 g/100 g, dwb) suggest their potency as good

sources of proteins, which may act as anabolic agents and as alternative sources of energy through gluconeogenesis when carbohydrates metabolism is impaired [Vodouhè *et al.*, 2012; Adjatin *et al.*, 2013]. The potential preventive effects of high fiber contents against gastrointestinal disorders, absorption of excess cholesterol and colon cancer [Vodouhè *et al.*, 2012; Adjatin *et al.*, 2013] suggest that these three species are good candidates for healthy maintenance of the human body.

Fat contents in *A. cruentus* (1.0–4.0 g/100 g), *O. gratissimum* (2.7–5.4 g/100 g), and *S. macrocarpon* (2.6–4.6 g/100 g) were similar and are within the range previously reported [Sossa-Vihotogbé *et al.*, 2013; Akinwunmi & Omotayo, 2016] for *A. cruentus* (1.2 g/100 g), *S. macrocarpon* (3.0 g/100 g), *J. tenella*, and *S. radiatum* (1.9–7.3 g/100 g). Conversely, higher fat contents were reported for *O. gratissimum* (6.6 g/100 g), *T. occidentalis* (7.6 g/100 g), and *V. amygdalina* (6.0 g/100 g) by Akinwunmi & Omotayo [2016]. The range of ash recorded for *O. gratissimum* (14.6–17.8 g/100 g) were lower than those of *A. cruentus* (22.1–24.9 g/100 g) and *S. macrocarpon* (16.6–23.9 g/100 g). The ash contents recorded were higher than that reported [Sossa-Vihotogbé *et al.*, 2013; Akinwunmi & Omotayo, 2016] for *J. tenella* (10.4–17.0 g/100 g), *O. gratissimum* (5.7 g/100 g), *S. radiatum* (7.7–12.3 g/100 g), and *T. occidentalis* (11.2 g/100 g) but similar to the values reported for *A. cruentus*, *S. macrocarpon*, and *V. amygdalina* (15.5––23.1 g/100 g) by Akinwunmi & Omotayo [2016]. Ash content is an index of mineral levels in food products, and it indicates potential health benefits because of the key roles of minerals in various metabolic processes [Adjatin *et al.*, 2013; Bvenura & Afolayan, 2014a, b].

Proximate composition as linked to vegetable species, urea micro-doses, and harvest times

There was no significant effect of urea micro-doses (except on moisture content, p=0.03) and their interaction with vegetable species and/or harvest times on proximate composition of the leafy vegetables (Table 1). The significantly highest moisture contents were found with the application of 60 kg/ha of urea (85.1 g/100 g). Moreover, moisture (p=0.02) and ash (p=0.04) contents were significantly influenced by interaction of vegetable species and harvest times (Table 1) with the highest contents recorded at the second harvest of *S. macrocarpon* (Figure 1a) and at the first harvest of *A. cruentus* (Figure 1b). Vegetable species had a significant effect on moisture (p<0.01), fat (p=0.03), fibers (p=0.02), protein (p=0.02), and ash (p<0.01) contents while moisture content was significantly (p=0.01) affected by harvest times (Table 1). The highest fibers and protein contents were recorded with *S. macrocarpon* whereas *A. cruentus* and *O. gratissimum* had the highest ash and fat contents, respectively (Table 2). Genotype and its interaction with fertilization type and rate were reported to influence plant responsiveness to nutrient availability [Sossa-Vihotogbé *et al.*, 2013; Fonge *et al.*, 2016]. Thus, the proximate composition of the leafy vegetables was expected to vary with vegetable species, urea micro-doses, and harvest times.

The lack of significant effect of urea micro-doses on proximate composition can be explained by the fertilization method applied. Indeed, high protein contents were related

TABLE 1. Effect of vegetable species (VS), urea micro-doses (UMD), and harvest times (HT) on proximate composition and mineral profile of the vegetable leaf powders as well as on trypsin-inhibitory activity of vegetable leaf extracts.

Variables		Main factors			Interaction between factors			
		VS	UMD	HT	VSxUMD	VSxHT	UMDxHT	VSxUMDxHT
Proximate composition	Moisture	4696.2β**	487.1*	12702.9**	146.5	648.7*	53.1	61.2
	Fat	543.0*	26.3	27.4	33.8	40.9	18.6	22.4
	Fibers	1382.8*	23.6	30.9	21.7	770.3	73.5	11.3
	Protein	1371.6*	3.6	0.2	3.0	98.8	1.7	7.1
	Ash	5618.2**	2.9	122.9	30.9	303.4*	50.2	64.8
Major minerals and their ratios	Calcium	124.5	8.3	1596.7*	16.4	198.7*	2.8	7.1
	Magnesium	3089.7*	10.7	135.1	69.0	205.1	7.1	12.8
	Phosphorus	2107.9*	9.6	0.6	0.8	133.7	12.3	2.9
	Potassium	186.1*	28.6	11.0	11.4	160.9	3.3	10.5
	Sodium	710.7*	3.4	5.2	2.6	3.5	7.8	9.4
	Calcium/magnesium	217.2*	12.6	8.8	2.2	45.7	5.6	8.6
	Calcium/phosphorus	1427.1*	25.9	1584.9*	19.4	349.3*	34.9	13.9
	Calcium/potassium	504.5*	11.2	612.1*	5.0	412.9*	4.0	1.9
	Sodium/magnesium	687.2*	27.3	2.3	2.4	2.6	12.8	3.9
	Sodium/potassium	1389.6*	17.1	6.7	4.9	0.8	14.8	5.4
Trace minerals and their ratios	Copper	4675.1**	3.5	188.3*	0.9	493.3*	6.6	4.9
	Iron	6885.1**	49.5	564.9*	17.8	205.4*	8.1	17.1
	Manganese	2477.3*	28.8	382.5*	45.5	86.9	9.6	17.1
	Zinc	579.1*	179.5*	152.2*	42.7	567.5*	99.1	42.0
	Iron/copper	1609.8*	152.4	249.5*	281.8	266.3*	10.2	23.3
	Zinc/copper	2101.9*	71.3	274.0*	78.7	1535.3*	91.1	58.7
Trypsin inhibition		1692.5***	37.4*	4.2	13.4	15.9	165.2**	180.4**

VS is used for the three vegetable species (*A. cruentus*, *O. gratissimum* and *S. macrocarpon*), UMD correspond to the four urea micro-doses (0, 20, 40, and 60 kg/ha) and HT indicate the three successive harvests of *A. cruentus* (4, 6 and 8 weeks after transplanting, respectively), of *O. gratissimum* (8, 12, and 16 weeks after transplanting, respectively) and of *S. macrocarpon* (6, 10 and 14 weeks after transplanting, respectively). β: F-value. Significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

to sufficient nitrogen and moisture contents in the soil, which increase nutrient uptake by plants, plant growth, and synthesis of nitrogen-based compounds [Souza *et al.*, 2016]. Moreover, urea micro-dosing optimizes nitrogen application in order to avoid any nitrogen deficiency while ensuring adequate uptake by the root system [Tovihoudji *et al.*, 2017; Likpètè *et al.*, 2019]. Likewise, higher ash contents recorded when compared to those previously reported [Sossa-Vihotogbé *et al.*, 2013; Akinwunmi & Omotayo, 2016] suggest that urea micro-dosing has promoted plant nutrients uptake, despite the lack of direct relation between urea micro-doses and ash contents. Given that the application of organic fertilizer increases synthesis of carbon-based compounds including fibers [Fonge *et al.*, 2016; Souza *et al.*, 2016; Das *et al.*, 2017], the lack of effect of urea micro-doses on fiber and protein contents indicates that the based organic fertilizer contributed to a sufficient level of organic nitrogen [Souza *et al.*, 2016].

The significant effects of harvest times on proximate composition agreed with findings of Sossa-Vihotogbé *et al.* [2013] for *J. tenella* and *S. radiatum* leaves and could be linked to the occurrence of structural changes within plants over time [Sossa-Vihotogbé *et al.*, 2013].

Mineral profile and its potential effects on human micronutrient status

Potassium and calcium were the most abundant major minerals found in *A. cruentus* (4.2–6.8 g/100 g and 2.7–5.0 g/100 g), *O. gratissimum* (3.9–4.8 g/100 g and 2.1–2.9 g/100 g), and *S. macrocarpon* (5.1–8.2 g/100 g and 2.2–3.8 g/100 g) followed by magnesium (*A. cruentus*: 1.0–1.6 g/100 g, *O. gratissimum*: 0.4–0.6 g/100 g, *S. macrocarpon*: 0.5–1.0 g/100 g), phosphorus (*A. cruentus*: 0.3–0.4 g/100 g, *O. gratissimum*: 0.4–0.6 g/100 g, *S. macrocarpon*: 0.5–0.8 g/100 g), and sodium (<0.1 g/100 g). The levels of specific minerals in the vegetables are similar to

values reported for calcium (1.2–4.0 g/100 g), magnesium (0.3–0.9 g/100 g), phosphorus (0.2–0.8 g/100 g), potassium (3.9–7.0 g/100 g), and sodium (0.02–1.5 g/100 g) in *Solanum nigrum* [Bvenura & Afolayan, 2014a] whereas lower contents (<0.1 g/100 g) were reported for *A. cruentus*, *O. gratissimum*, and *S. macrocarpon* [Akinwunmi & Omotayo, 2016]. Iron and manganese were the most abundant trace minerals determined in *A. cruentus* (271.3–971.9 mg/kg and 501.2––976.4 mg/kg, respectively), *O. gratissimum* (132.6–442.2 mg/kg and 89.8–125.6 mg/kg, respectively), and *S. macrocarpon* (153.8–606.3 mg/kg and 109.6–213.9 mg/kg, respectively) followed by zinc (*A. cruentus*: 41.7–171.1 mg/kg, *O. gratissimum*: 34.5–78.5 mg/kg, *S. macrocarpon*: 41.9–661.6 mg/kg) and copper (*A. cruentus*: 7.2–11.2 mg/kg, *O. gratissimum*: 11.8––30.3 mg/kg, *S. macrocarpon*: 13.5–27.4 mg/kg). The ranges of iron and copper in the vegetables fit with values previously reported [Bvenura & Afolayan, 2014b; Akinwunmi & Omotayo, 2016] for *S. macrocarpon* (403.1 mg/kg and 12.8 mg/kg), *S. nigrum* (178.0–766.0 mg/kg and 7.2–23.5 mg/kg), and *V. amygdalina* (467.9 mg/kg and 25.5 mg/kg). Zinc contents are similar to those reported for *O. gratissimum* (44.7 mg/kg) and *S. nigrum* (19.0–78.0 mg/kg) but lower than those of *A. cruentus* (951.5 mg/kg) and *T. occidentalis* (1023.1 mg/kg) [Bvenura & Afolayan, 2014b; Akinwunmi & Omotayo, 2016]. Manganese contents in *A. cruentus* were higher than values reported [Akinwunmi & Omotayo 2016] for *O. gratissimum* (4.5 mg/kg) and *S. macrocarpon* (49.6 mg/kg).

Mean values for calcium, copper, iron, magnesium, phosphorus, potassium, and zinc are higher than their recommended daily intakes (RDI) for children and adults [Trumbo *et al.,* 2001; Sawka, 2005; Bergman *et al.,* 2009]. Thus, consumption of these vegetables could improve human micronutrient status although high quantities (100–300 g) of dried leaf powders must be consumed to meet the RDI. Conversely, our values for sodium and manganese contents are lower than the RDI for children and adults [Trumbo *et al.,* 2001; Sawka, 2005], suggesting that these vegetables are poor-sodium and -manganese sources. Considering that sauces that accompany starchy staple foods are currently the common consumption forms of these vegetables [Dansi *et al.,* 2008; Vodouhè *et al.,* 2012], it is likely that the quantities of the vegetables (either leaf powders or fresh leaves) required to meet these RDI cannot be easily consumed in a day. Thus, a diversification of vegetables consumption forms is needed to improve individual nutritional profile.
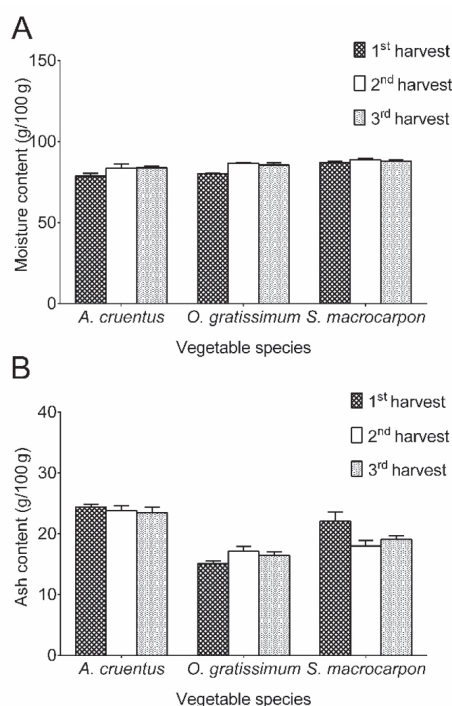

FIGURE 1. Variations in moisture (A) and ash (B) contents of the leafy vegetable species produced with four urea micro-doses (0, 20, 40, and 60 kg/ha) depending on harvest times.

Results are expressed on dry weight basis (except for moisture content). 1st, 2nd, and 3rd harvests correspond to 4, 6, and 8 weeks after transplanting, respectively for *A. cruentus*; 8, 12, and 16 weeks after transplanting, respectively for *O. gratissimum* and 6, 10, and 14 weeks after transplanting, respectively for *S. macrocarpon*.
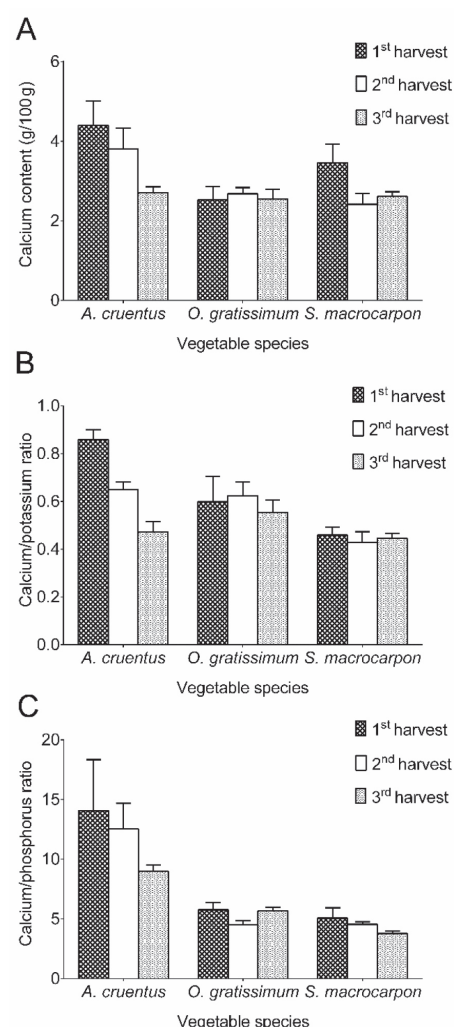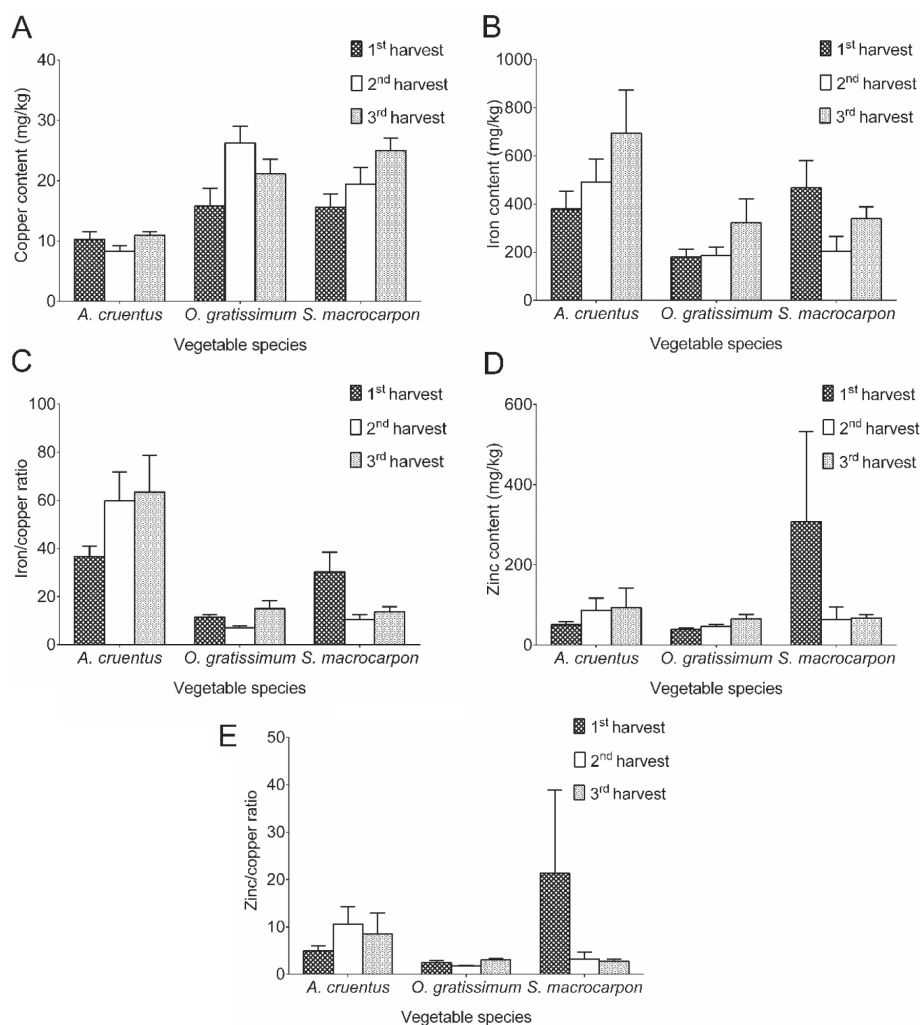

FIGURE 2. Variations in calcium content (A), calcium/potassium ratio (B) and calcium/phosphorus ratio (C) of the leafy vegetable species produced with four urea micro-doses (0, 20, 40, and 60 kg/ha) depending on harvest times.

Results are expressed on dry weight basis. 1st, 2nd and 3rd harvests correspond to 4, 6 and 8 weeks after transplanting, respectively for *A. cruentus*; 8, 12, and 16 weeks after transplanting, respectively for *O. gratissimum* and 6, 10, and 14 weeks after transplanting, respectively for *S. macrocarpon*.

FIGURE 3. Variations in iron (A), zinc (B) and copper (C) contents as well as in iron/copper (D) and zinc/copper (E) ratios of the leafy vegetable species produced with four urea micro-doses (0, 20, 40, and 60 kg/ha) depending on harvest times.

Results are expressed on dry weight basis. 1st, 2nd and 3rd harvests correspond to 4, 6, and 8 weeks after transplanting, respectively for *A. cruentus*; 8, 12, and 16 weeks after transplanting, respectively for *O. gratissimum* and 6, 10, and 14 weeks after transplanting, respectively for *S. macrocarpon*.

Mineral ratios and their potential effects on human health disorders

The calcium/magnesium and calcium/phosphorus ratios of *A. cruentus* (2.3–3.5 and 8.3–18.3, respectively), *O. gratissimum* (4.6–6.2 and 4.3–6.4, respectively), and *S. macrocarpon* (2.6–4.5 and 3.6–6.2, respectively) were higher than their calcium/potassium (*A. cruentus*: 0.5–0.9, *O. gratissimum*: 0.5––0.7 and *S. macrocarpon*: 0.4–0.5), sodium/magnesium (<0.1), and sodium/potassium (<0.1) ratios. The calcium/magnesium ratios are below (*A. cruentus*) or within (*O. gratissimum* and *S. macrocarpon*) the acceptable range of 3–11 [Watts, 2010], indicating potency for low or balanced glucose metabolism, which is inversely associated with the risk of type 2 diabetes [Watts, 2010]. Inversely, the calcium/phosphorus ratios (>1.8–3.6) of these vegetables may increase intestinal absorption of calcium, which is useful for the formation of strong bones and teeth [Watts, 2010; Adjatin *et al.,* 2013] but the calcium/potassium ratios (<2.2–6.2) could promote hyperthyroidism and its related complications [Watts, 2010]. The sodium/magnesium (<2–6) and sodium/potassium (<1.4–3.4) ratios associated with these vegetables could contribute to high blood pressure

lowering and anti-inflammatory activities [Watts, 2010; Adjatin *et al.,* 2013]. The iron/copper ratio of *A. cruentus* (31.7–87.9), and *O. gratissimum* (6.3–18.5) were higher than their zinc/copper ratio (*A. cruentus*: 4.0–15.5 and *O. gratissimum*: 1.7–3.3). A wide range was found for zinc/copper ratio of *S. macrocarpon* (2.3–49.0) when compared to its iron/copper ratio (8.3–42.9). The iron/copper (>0.2–1.6) and zinc/copper (>4–12) ratios observed may increase the risk of oxidative stress-related diseases because of the key role of these ratios in the maintenance of the oxidant/antioxidant balance [Watts, 2010]. The values of iron/copper ratio may also affect iron utilization by decreasing its incorporation into hemoglobin and promoting anemia [Watts, 2010]. These findings indicate that some adjustments (preferably by food-to-food fortification) are needed to avoid the adverse effects predicted for calcium/potassium, iron/copper, and zinc/copper ratios.

Mineral composition as affected by vegetable species, urea micro-doses and harvest times

The effect of urea micro-doses (except for zinc content, p=0.04) and their interaction with vegetable spe-

cies and/or harvest times were not significant (p>0.05) for the mineral contents and ratios (Table 1). Given that ash content is an index of mineral levels, the lack of significant influence of urea micro-doses and of their interaction with other factors on the mineral profile was expected due to the lack of direct relation between urea micro-doses and ash contents in this study. Vegetable species, harvest times, and their interaction had respectively significant effects on copper (p<0.01, p=0.04 and p=0.03), iron (p<0.01, p=0.03 and p=0.04), and zinc (p=0.03, p=0.04 and p=0.03) contents as well as on calcium/phosphorus (p=0.02, p=0.02 and p=0.03), calcium/potassium (p=0.03), iron/copper (p=0.02, p=0.04 and p=0.04), and zinc/copper (p=0.01, p=0.04 and p=0.02) ratios (Table 1). Harvest time and its interaction with vegetable species had significant effects on calcium content (p=0.02 and p=0.04, respectively) (Table 1). The interaction of vegetable species and harvest times revealed that the first harvest of *A. cruentus* had the highest calcium content (Figure 2a), calcium/potassium ratio (Figure 2b), and calcium/phosphorus ratio (Figure 2c). The highest copper content was recorded at the second harvest of *O. gratissimum* (Figure 3a) whereas the third harvest of *A. cruentus* had the highest iron content (Figure 3b) and iron/copper ratio (Figure 3c). Conversely, the highest zinc content (Figure 3d) and zinc/copper ratio (Figure 3e) were recorded at the first harvest of *S. macrocarpon*. Significant effect of harvest time was recorded for manganese content (p=0.03) (Table 1), with the highest content found at the first (339.8 mg/kg) and the second (330.5 mg/kg) harvests.

Vegetable species had a significant effect on magnesium (p=0.01), manganese (p=0.01), phosphorus (p=0.01), potassium (p=0.04), and sodium (p=0.02) contents as well as on calcium/magnesium (p=0.04), calcium/phosphorus (p=0.02), calcium/potassium (p=0.03), sodium/magnesium (p=0.02), and sodium/potassium (p=0.02) ratios (Table 1). The highest magnesium, manganese, and sodium contents as well as sodium/magnesium and sodium/potassium ratios were found in *A. cruentus* (Table 2). Phosphorus content was the highest in *S. macrocarpon* while *O. gratissimum* had the highest calcium/magnesium ratio (Table 2). Higher potassium contents were found in *A. cruentus* and *S. macrocarpon* (Table 2). Plant growth depends greatly on water and nutrient availability in the soil and their uptake by the root system depending on plants' developmental stage although genotype and its interaction with fertilization type and rate influence plant responsiveness to nutrient availability [Sossa-Vihotogbé *et al.,* 2013; Fonge *et al.,* 2016]. Mineral fertilization supplies plants in readily available nutrients unlike organic fertilization, which delays nutrients release and availability due to slow mineralization [Souza *et al.,* 2016; Das *et al.,* 2017]. Thus, variation in the rate of nutrients availability into the soil and their uptake by plants may explain the significant differences observed in calcium content and trace mineral profile according to harvest time. The decrease in calcium content with increase of harvest time could be linked to the immobility of this mineral once taken up into plants tissues, which stops its translocation from older to growing parts of the plants [Bvenura & Afolayan, 2014a, b] while that of zinc contents may be linked to zinc accumulation in young plants [Bvenura

TABLE 2. Effect of vegetable species on proximate and mineral compositions of the leafy vegetables produced with four urea micro-doses and harvested three successive times.

Variables		*Amaranthus cruentus*	*Ocimum gratissimum*	*Solanum macrocarpon*
Proximate composition[1]	Moisture	82.0±0.1 β[c]	84.1±0.0[b]	87.9±0.0[a]
	Fat	2.2±0.1[c]	4.2±0.0[a]	3.2±0.0[b]
	Fibers	11.4±0.2[c]	14.6±0.0[b]	20.4±0.0[a]
	Protein	23.6±0.1[b]	22.2±0.0[c]	30.3±0.2[a]
	Ash	23.9±0.0[a]	16.2±0.0[c]	19.7±0.1[b]
Major minerals[1] and their ratios	Calcium	3.6±0.0[a]	2.6±0.1[a]	2.8±0.0[a]
	Magnesium	1.2±0.0[a]	0.5±0.0[c]	0.8±0.0[b]
	Phosphorus	0.3±0.0[c]	0.5±0.0[b]	0.6±0.0[a]
	Potassium	5.6±0.0[a]	4.4±0.0[b]	6.3±0.1[a]
	Sodium	0.1±0.0[a]	0.0±0.0[b]	0.0±0.0[b]
	Calcium/ magnesium	3.0±0.0[c]	5.4±0.1[a]	3.6±0.0[b]
	Calcium/ phosphorus	11.9±0.1[a]	5.3±0.1[b]	4.5±0.0[b]
	Calcium/ potassium	0.7±0.0[a]	0.6±0.0[a]	0.4±0.0[b]
	Sodium/ magnesium	0.1±0.0[a]	0.0±0.0[b]	0.0±0.0[b]
	Sodium/ potassium	0.1±0.0[a]	0.0±0.0[b]	0.0±0.0[b]
Trace minerals[2] and their ratios	Copper	9.8±0.2[b]	21.1±0.2[a]	20.0±0.1[a]
	Iron	521.9±4.9[a]	229.7±8.4[c]	337.0±7.4[b]
	Manganese	694.7±11.5[a]	106.7±0.1[c]	154.2±0.2[b]
	Zinc	77.1±0.0[b]	50.2±0.9[c]	146.5±1.1[a]
	Iron/copper	53.3±0.7[a]	11.2±0.3[c]	18.1±0.4[b]
	Zinc/copper	8.1±0.3[b]	2.4±0.0[c]	9.1±0.2[a]

Units: [1] g/100 g; [2] mg/kg. Results are expressed on dry weight basis (except for moisture content). β: Mean values ± standard error. Mean values with different alphabets within the same row are significantly different (p<0.05).

& Afolayan, 2014b]. The increase in iron and copper contents with increase of harvest time could be related to high organic matter concentration into the soil that promotes a continuous release of easily absorbable minerals into the soil thereby, enhancing their uptake by plants and their contents in leaves as plants matured [Bvenura & Afolayan, 2014a, b].

Trypsin inhibition as related to vegetable species, urea micro-doses, and harvest times

The IC$_{50}$ value of AEBSF (3.7 μg/mL) was lower than that of *A. cruentus* (0.2–0.3 mg/mL), *O. gratissimum* (<0.1 mg/mL), and *S. macrocarpon* (0.0–4.8 mg/mL). Values recorded for trypsin inhibition from *Juglans regia* (42.6 μg/mL) and *Prunus spinosa* (970.2 μg/mL) by Jedinak *et al.* [2010] and for *Justicia gendarussa* (13.4 μg/mL) by Patel & Zaveri [2015] are within the range found for *S. macrocarpon* and *O. gratissimum*. There were significant (p<0.01) effects of the interaction of vegeta-
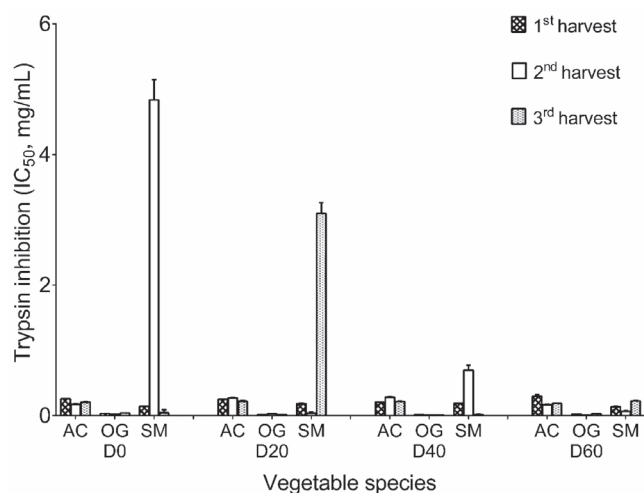
FIGURE 4. Trypsin-inhibitory activity of aqueous vegetable leaf extracts produced with four urea micro-doses and harvested at different times.

A. cruentus, O. gratissimum and *S. macrocarpon* were respectively coded AC, OG and SM; D0, D20, D40, and D60 indicate applied urea doses (0, 20, 40, and 60 kg/ha, respectively); 1st, 2nd, and 3rd harvests correspond to 4, 6, and 8 weeks after transplanting, respectively for *A. cruentus*; 8, 12, and 16 weeks after transplanting, respectively for *O. gratissimum* and 6, 10, and 14 weeks after transplanting, respectively for *S. macrocarpon*.

ble species, urea micro-doses, and harvest times on IC_{50} values for trypsin inhibition by aqueous leaf extracts (Table 1). The lowest IC_{50} for trypsin inhibition by *A. cruentus* and *O. gratissimum* extracts were obtained from the samples produced with 60 kg/ha of urea and harvested the second time while that of *S. macrocarpon* was from the sample cultivated with 40 kg/ha of urea and collected at the third harvest (Figure 4). Irrespective of vegetable species, urea micro-doses and harvest times, the lowest IC_{50} value was recorded for *O. gratissimum* (Figure 4). The interactive effect of vegetable species, urea micro-doses, and harvest times on the trypsin inhibitions was not expected considering that trypsin inhibitors are usually protein molecules and that there was no evidence of such an interactive effect on protein contents. The interactive effect confirmed the beneficial role of fertilization on the synthesis of nitrogen-based compounds [Souza *et al.,* 2016], including trypsin inhibitors. Plants synthesize protease inhibitors during their normal ontogeny and as a wound-activated response to mechanical wounding [Clemente *et al.,* 2019]. Thus, plant cutting (at harvest) activates stress of mechanical wounding and the up-regulation of genes encoding for synthesis of protease inhibitors [Clemente *et al.,* 2019], thereby leading to the lowest trypsin IC_{50} value at the third harvest (*S. macrocarpon*). The lowest IC_{50} found at the second harvest of *A. cruentus* and *O. gratissimum* could have resulted from a species-specific response to a high urea dose (60 kg/ha) and mechanical wounding at harvests. The first harvest has probably caused physiological stress that enhanced the synthesis of the trypsin-inhibitory protein molecules.

The activity of trypsin inhibitors of products from plant origin were reported to markedly increase after heat treatment at 60°C while a loss of activity occurred above 60°C and the decreasing rate depended on the type of the heat treatment applied (dry or moist) [Allen *et al.,* 2012; Pesoti *et al.,*

2015]. Indeed, a less decreasing rate was recorded for dry-heated products with an average loss of 10% of activity when temperature increase by 20°C whereas a rapid decreasing rate occurred for the moist-heated products (loss of 45% of activity from 60 to 80°C and of 20% of activity from 80 to 100°C) [Allen *et al.,* 2012]. Based on these findings, we can assert that oven-drying (at 60°C) of the studied leaves and the aqueous extraction of dry leaf powder (at 60°C) did not inactivate their trypsin inhibitors. Regardless of the applied heat treatment type, the trypsin inhibitors are rapidly inactivated by very high temperatures (>100°C) [Allen *et al.,* 2012; Pesoti *et al.,* 2015]. Given that the studied vegetables are usually consumed in sauce and that their cooking temperature is very high (>100°C), we may assume that trypsin inhibitors could be inactivated by the moist heat treatment, which has been shown to provide better inactivation of trypsin inhibitors than dry heat treatment [Allen *et al.,* 2012; Pesoti *et al.,* 2015]. However, to the best of knowledge, there is no scientific report on the effect of heat treatment on the status of the trypsin inhibitors in sauces made with the studied leafy vegetables. Considering that various ingredients are combined during cooking, there is a need for further studies that will explore the complexity of biochemical reactions occurring during cooking in order to provide clear answers regarding the inactivation of trypsin inhibitors in leafy vegetables during sauces preparation.

CONCLUSION

This study highlighted the nutritional properties of leafy vegetables produced using urea micro-doses and harvested at different times. Consumption of *A. cruentus* leaves could improve diets quality due to their high mineral contents while the richness of *S. macrocarpon* leaves in fibers could facilitate digestion and reduce the potential for colon carcinogenesis. The effects of vegetable species and harvest times were more marked than that of urea micro-doses and uphold the recommendation of early (the first two) harvests for the vegetable species in order to fully benefit from their high nutritional properties. The aqueous leaf extracts had potent trypsin inhibition; a useful nutraceutical property for the management of high energy intake diets and of disease conditions associated with excessive protein catabolism. Moreover, the trypsin-inhibitory activity may be helpful against virus-dependent diarrhea. However, the nutritional and functional properties exhibited by the studied vegetables were based on *in vitro* assays and should be confirmed by *in vivo* animal and human trials.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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